NONSENSUAL AMINO ACID METABOLISM IN HUMANS

by

Leah Cooper

B.Sc., The University of British Columbia, 2012

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(EXPERIMENTAL MEDICINE)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2015

© Leah Cooper, 2015
Abstract

Nutritionally, there is a dietary requirement for the essential amino acids (EAA) but also a requirement for nitrogen (N) intake for the de novo synthesis of the nonessential amino acids (NEAA). It has been suggested that some NEAA may be more metabolically important than others. The first study (Glutamate Requirement Study) aims to examine the application of the indicator amino acid oxidation (IAAO) technique to determine if a dietary requirement for glutamate exists in adult humans. The second study (NEAA Study) aims to determine the metabolic demand of nine of the NEAA (Ala, Arg, Asn, Asp, Gln, Glu, Gly, Pro, Ser) as an ideal N source using the IAAO technique.

Seven subjects were maintained on an adaptation diet for 2 days prior to each test day. Each subject participated in two or eleven test diet intakes, assigned randomly, in the glutamate study and the NEAA study, respectively. In the glutamate study, the diets corresponded to the amino acid pattern present in egg protein, in which all glutamate and glutamine was present as glutamate, or removed, with serine used to make the diets isonitrogenous. In the NEAA Study, one test intake was a base diet consisting of only the EAA provided at the recommended dietary allowance. All other test intakes involved the base diet with the addition of one NEAA to meet a 50:50 ratio of EAA: NEAA on a N basis. Each study day followed the IAAO protocol using L-[1-13C]-Phenylalanine as the indicator. Breath and urine samples were collected at baseline and isotopic steady state. Enrichments of 13C in breath were analyzed by isotope ratio mass spectrometry to calculate F13CO2.

In the glutamate study, a paired-samples t-test did not find a significant difference between the F13CO2 in response to the two glutamate intakes. In the NEAA study, repeated
measures ANOVA with post hoc multiple comparisons showed that seven of the nine NEAA decrease IAAO significantly. Thus the results suggest that in healthy adults, there is no dietary requirement for glutamate, and that most NEAA are good N sources, in the presence of adequate EAA.
Preface

The experimental protocols were previously developed and the study design was developed by myself, Leah Cooper, and by my supervisor Dr. Rajavel Elango. We discussed the experimental design with Dr. Ryosei Sakai, Dr. Ron Ball, and Dr. Paul Pencharz. I prepared all dietary intakes and conducted all laboratory analyses in the Elango Lab in the Child and Family Research Institute on the Children’s and Women’s Health Centre of British Columbia. I conducted all study days in the Clinical Research Evaluation Unit of the Child and Family Research Institute. I was responsible for all statistical analysis with the consultation of Boris Kuzeljevic, MA, and Dr. Leanne Currie.

Work from Chapter 3 was submitted in an abstract titled: Nonessential Amino Acids as Nitrogen Sources in Adult Men Examined Using the Indicator Amino Acid Oxidation Technique was submitted to the Experimental Biology 2015 Conference and will be published in the FASEB J 2015. This work will be presented in a poster during the conference and is co-authored by Leah Cooper B.Sc., Dr. Ryosei Sakai, Dr. Ron Ball, Dr. Paul Pencharz, and Dr. Rajavel Elango. I conducted the study and performed the analysis for this study. I was also responsible for writing the abstract and creating the poster for this conference.

The University of British Columbia – Children’s & Women’s Health Centre of BC Research Ethics Board granted approval for this study titled: Non-essential amino acid requirements and metabolism in humans on November 21st 2012 (CW12-0223 / H12-01683).
Table of Contents

Abstract.................................................................................................................. ii

Preface................................................................................................................... iv

Table of Contents .................................................................................................. v

List of Tables ......................................................................................................... ix

List of Figures ......................................................................................................... x

List of Equations .................................................................................................... xi

List of Abbreviations .............................................................................................. xii

Acknowledgements ................................................................................................. xiii

Dedication ................................................................................................................ xiv

Chapter 1: Introduction .......................................................................................... 1

1.1 Background ........................................................................................................ 3

1.1.1 Classification of Amino Acids ..................................................................... 3

1.1.2 Amino Acid Metabolism ............................................................................ 5

1.1.2.1 Nitrogen Excretion .............................................................................. 7

1.1.2.2 Carbon Skeleton .................................................................................. 10

1.1.2.3 Amino Acid Biosynthesis .................................................................. 12

1.1.3 Essential vs. Nonessential ......................................................................... 13

1.1.4 α-Amino Nitrogen Source ......................................................................... 14

1.1.5 Amino Acid Requirements ......................................................................... 17
Chapter 2: Glutamate Requirement Study ................................................................. 33

2.1 Glutamate ........................................................................................................ 33

2.2 Methods ............................................................................................................ 34
  2.2.1 Subjects ....................................................................................................... 34
  2.2.2 Dietary and Energy Intakes ......................................................................... 35
  2.2.3 Study Diets .................................................................................................. 36
  2.2.4 Amino Acid Composition .......................................................................... 37
  2.2.5 Tracer Protocol ............................................................................................ 38
  2.2.6 Sample Collection and Analysis ................................................................ 39
  2.2.7 Statistical Analysis ...................................................................................... 41

2.3 Results .............................................................................................................. 41
  2.3.1 Additional Test Diets ................................................................................ 43
  2.3.2 Additional Urinary Analysis ....................................................................... 44

2.4 Discussion ......................................................................................................... 44
Appendices

References

Chapter 3: Nonessential Amino Acid Metabolism ................................................................. 49

3.1 Nonessential Amino Acids ................................................................................................. 49

3.2 Methods ........................................................................................................................... 50

   3.2.1 Subjects .................................................................................................................... 50

   3.2.2 Dietary and Energy Intakes ....................................................................................... 50

   3.2.3 Study Diets ............................................................................................................... 51

   3.2.4 Amino Acid Composition ......................................................................................... 52

   3.2.5 Tracer Protocol ........................................................................................................ 54

   3.2.6 Sample Collection and Analysis .............................................................................. 54

   3.2.7 Statistical Analysis ................................................................................................... 54

3.3 Results ............................................................................................................................. 56

   3.3.1 Statistical Analysis ................................................................................................... 58

   3.3.2 Additional Urinary Analysis ..................................................................................... 59

3.4 Discussion ........................................................................................................................ 59

Chapter 4: Summary .............................................................................................................. 68

4.1 Conclusions ...................................................................................................................... 68

4.2 Future Directions ............................................................................................................ 70

References ............................................................................................................................. 73

Appendices ............................................................................................................................. 80

Appendix A Experimental Design of NEAA Study .............................................................. 80

Appendix B Protein Free Cookie Preparations ..................................................................... 81

   B.1 Cherry Shortbread Cookie .............................................................................................. 81

   B.2 Butterscotch Brownie ................................................................................................... 82
Appendix C Posters ..............................................................................................................................83
  C.1 Glutamate Study ............................................................................................................................83
  C.2 Nonessential Amino Acid Study ..................................................................................................83

Appendix D Forms ..............................................................................................................................85
  D.1 Subject Consent Form .................................................................................................................85
  D.2 Preliminary Study Day Form .......................................................................................................92
  D.3 Study Day Form ..........................................................................................................................94
  D.4 Subject ID Master List ...............................................................................................................95
List of Tables

Table 1: Early Classification of Amino Acids with Respect to their Dietary Role in Healthy Adult Males ............................................................................................................................................. 4
Table 2: Current Classification of Amino Acids with Respect to their Dietary Role in Healthy Adults ............................................................................................................................................. 5
Table 3: The Involvement of Amino Acids in Physiological and Metabolic Function .................. 19
Table 4: Functions of Glutamate ............................................................................................................. 22
Table 5: Comparison of the daily EAA Requirements from the Current DRI and the IAAO technique ............................................................................................................................................. 27
Table 6: Amino Acid Pattern in Egg Protein ......................................................................................... 38
Table 7: Glutamate Study Subject Characteristics ................................................................................. 42
Table 8: NEAA Study Subject Characteristics ....................................................................................... 56
Table 9: Functions of Glutamine ........................................................................................................... 60
Table 10: Functions of Proline ............................................................................................................... 65
List of Figures

Figure 1: The Transamination of Glutamate and Pyruvate to Form α-Ketoglutarate and Alanine. 7

Figure 2: The Relationships of Amino Acids in the TCA and Urea Cycles .............................9

Figure 3: Amino Acid Metabolism Summary including the Fate of Different Groups after
Catabolism ..................................................................................................................................................13

Figure 4: The Formation of an Imino Acid from Ammonia and an α-keto acid, Followed by a
Reduction to an Amino Acid ..................................................................................................................15

Figure 5: The Biochemical Reaction Catalyzed by GDH in Cells .......................................................16

Figure 6: The Rate of \(^{13}\text{CO}_2\) Appearance from Orally Administered L-[\(^{13}\text{C}\)]Phenylalanine in
Breath ..................................................................................................................................................26

Figure 7: IAAO Study Day Protocol ....................................................................................................39

Figure 8: \(^{13}\text{CO}_2\) for 0 and 113 mg Glutamate Intakes ...........................................................................43

Figure 9: Mean \(^{13}\text{CO}_2\) for Four Glutamate Intakes .................................................................................44

Figure 10 Mean \(^{13}\text{CO}_2\) for Eleven Test Intakes .......................................................................................57

Figure 11 Individual \(^{13}\text{CO}_2\) for Eleven Test Intakes ...............................................................................58

Figure 12: Interconnection of the TCA and Urea cycles ......................................................................67
List of Equations

Equation 1 The Rate of $^{13}$CO$_2$ Production

28
List of Abbreviations

5-OP 5-oxoproline
AAA Amino acid analyzer
APE Atom percent excess
BCAA Branched chain amino acids
BMI Body mass index
DRI Dietary reference intake
EAA Essential amino acids
EAR Estimated average requirement
GDH Glutamate dehydrogenase
HPLC High-performance liquid chromatography
IAAO Indicator amino acid oxidation
$K_m$ Michaelis constant
N Nitrogen
NEAA Nonessential amino acids
P5C Pyrroline-5-carboxylate
RDA Recommended dietary allowance
REE Resting energy expenditure
SD Standard deviation
Acknowledgements

It is with great gratitude that I acknowledge and thank my supervisor, Dr. Rajavel Elango, for his guidance, support, and for enlarging my vision of my studies throughout my masters program. His profound questioning and our insightful discussions have strengthened my understanding in amino acid metabolism and encouraged my curiosity in research. I would also like to express my appreciation for the time, commitment, and feedback from my committee members, Dr. Dan Rurak and Dr. James Thompson. It gives me pleasure to thank all the members of the Elango lab, notably Magdalene Payne, Abrar Turki, and Betina Rasmussen for all their collaborations, continuing support and motivation.

I consider it an honor to have worked with Dr. Ryosei Sakai and Ajinomoto Co. Inc. and am grateful for their research support and contributing to scientific discussions throughout my study design and execution. I would also like acknowledge the scientific discussions and insights from Dr. Ron Ball and Dr. Paul Pencharz in my study design. I am indebted to Boris Kuzeljevic MA, and Dr. Leanne Currie for all their time and efforts in coherently answering my endless statistical questions.

I would like to extend my gratitude to the faculty of medicine and staff of the experimental medicine program for their support and organization of networking and research events during my masters program. I would not have had as many opportunities to present and share my research without them.
Dedication

This thesis would not have become a reality without the immense encouragement from my parents, who have continued to support me throughout my many years of education, both emotionally and financially. The moral support from my parents and close friends has motivated me throughout all my studies and has led me to success in my masters program.
Chapter 1: Introduction

Nutrition is the utilization of food and nutrients, which affects development, performance, and the health of a person. Protein and amino acids in particular can impact human health, because proteins are the functional machinery of the cell, making up almost half of the solid content of most tissues in the body (Young and Pellett 1987, Gropper et al. 2009). Each protein is unique in its characteristics, structure, and amino acid sequence. Amino acids contain an amine group, a carboxylic acid group and a specific carbon skeleton side chain. They are covalently bonded together in strong chains that make up the distinctive primary structure of a protein. There are 20 unique amino acids necessary for mammalian protein synthesis, each characterized by its carbon side chain. The carbon side chain is what determines the essentiality of an amino acid, whether it can be synthesized de novo (nonessential/dispensable) or whether it must be ingested from a dietary source (essential/indispensable).

Currently, nine amino acids have been categorized as essential amino acids (EAA) and the remaining 11 are referred to as nonessential amino acids (NEAA) (Reeds 2000). It is important to note that there is a metabolic demand for all 20 amino acids in the appropriate proportions to ensure that sufficient protein synthesis can take place in the human body. Nutritionally, there is a dietary requirement for the EAA but also a requirement for nitrogen, such that the de novo synthesis of the NEAA can occur. Optimal growth and development in animals happens only when an equal ratio of EAA: NEAA are present in the diet (Hiramatsu et al. 1994, Reeds 2000).

What is not clear is whether all NEAA are, from a dietary source, similar in their ability to act as a nitrogen source to ensure adequate endogenous synthesis of all other NEAA and protein occurs. Recently it has been hypothesized that there is a need for a preformed α-amino
nitrogen source from specific “nonessential” amino acids (Katagiri and Nakamura 2002). Moreover in growth and development, Wu (2010, 2013) has claimed that the existence of biochemical pathways for NEAA synthesis does not necessarily mean that the body has the capacity to synthesize NEAA in sufficient quantities. Furthermore, there is increasing evidence that glutamate (a NEAA) promotes cell replications by providing nitrogen for purine and pyrimidine synthesis, as well as playing a role in the TCA cycle (Burrin and Stoll 2009), and in gluconeogenesis (Brosnan 2000). There is a strong correlation between glutamate concentration and the rate of protein synthesis in muscles (Millward et al. 1989). Thus, in addition to being a substrate for protein synthesis, glutamate has many essential metabolic and physiological roles in the body.

Therefore, the first goal of the current study is to determine whether there is a dietary requirement for glutamate in healthy adult males. Secondly, we are interested in determining whether there exist differences among the nine NEAA (Ala, Arg, Asn, Asp, Gln, Glu, Gly, Pro, and Ser) to act as an endogenous nitrogen source for protein synthesis. Our studies use the stable isotope (L-1-13C-Phenylalanine) based minimally invasive indicator amino acid oxidation (IAAO) technique. This method is based on the concept that when one amino acid is limiting, then all other amino acids, including the indicator amino acid, will be oxidized to a greater extent. As the limiting amino acid intake increases, the oxidation will decrease, indicating increased incorporation into proteins. The IAAO technique uses a stable isotope labeled EAA at the 1-carboxyl position (1-13C) as an indicator to reflect protein synthesis in vivo, by measuring it’s oxidation noninvasively in expired breath CO2 (Elango et al. 2009a, 2012).
1.1 Background

Amino acids are a crucial part of the human diet with many different roles in the body once absorbed or synthesized. They help maintain nitrogen balance in the body, synthesize proteins, and act as substrates for many metabolic pathways (Wu 2009). The carbon skeleton of amino acids can be used to produce numerous products, such as creatine as well as glucose through oxaloacetate and gluconeogenesis (Voet et al. 2008, Wu 2009, Wu et al. 2013). Amino acid carbon skeletons can be used to produce ketone bodies, cholesterol, hormones, as well as act as neurotransmitters. Amino acids can also be converted to fatty acids through acetyl CoA production (Gropper et al. 2009). Epinephrine, norepinephrine, dopamine, serotonin, γ-aminobutyric acid (GABA), and histamine are neurotransmitters and/or hormones derived from amino acids. Tryptophan and glutamate are the precursors to serotonin (a precursor of melatonin) and the mammalian inhibitory neurotransmitter GABA, respectively (Fonnum 1984). The amino acid glycine, is very important in heme biosynthesis, an essential component of myoglobin, hemoglobin, and the cytochromes (Voet et al. 2008). Amino acids, which participate in cell signaling pathways through protein kinases, are involved in the regulation of gene expression, and the synthesis of gaseous signaling molecules (such as nitric oxide, carbon monoxide, and hydrogen sulfide) (Wu 2010, Wu et al. 2013) Therefore, understanding the metabolic demand for each amino acid has key implications for nutritional research and human health.

1.1.1 Classification of Amino Acids

Rose et al. (1949, 1954) were the first to study and categorize the amino acids as seen in Table 1 below. Since this early ground breaking work by William Rose (1949, 1954), dietary requirements of the EAA have been extensively studied, mostly in adult men. It should, however, be noted that this earlier work was all done by the classic nitrogen balance method, which has
several limitations. This technique is highly invasive as it involves the collection of all nitrogen loses, including: urine, feces, fingernails, and sweat. It can be unethical as it includes a minimum of 3 day and up to 7 day adaptation to a test intake of an amino acid, which could be limiting or given in excess. The nitrogen balance technique results in considerable error as it tends to overestimate nitrogen intakes and underestimate nitrogen loses, resulting in a false positive nitrogen balance and thus an underestimation of the amino acid requirement. These classifications are based on the body’s capacity to balance nitrogen intake versus excretion and fail to consider the metabolic needs for all amino acids in not only protein synthesis but also in the formation of other physiologically important biomolecules.

Table 1: Early Classification of Amino Acids with Respect to their Dietary Role in Healthy Adult Males

<table>
<thead>
<tr>
<th>Essential</th>
<th>Nonessential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valine</td>
<td>Glycine</td>
</tr>
<tr>
<td>Leucine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Serine</td>
</tr>
<tr>
<td>Threonine</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Methionine</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Lysine</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Proline</td>
</tr>
<tr>
<td></td>
<td>Hydroxyproline</td>
</tr>
<tr>
<td></td>
<td>Histidine</td>
</tr>
<tr>
<td></td>
<td>Citrulline</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
</tr>
</tbody>
</table>

Footnote – The final classification of amino acids with respect to their dietary role in the maintenance of nitrogen equilibrium in normal adult males. Table adapted from Rose et al. 1954.

Subsequent studies between 1950-1980’s revealed that a re-classification of amino acids was necessary. Reeds (2000) published a revision of the classification and currently the amino acids are divided into three groups: essential, nonessential, and conditionally essential (Table 2). Since some NEAA can become limiting in metabolic processes during specific physiological
conditions (infancy, childhood) or disease and thus may become nutritionally essential, they are referred to as “conditionally essential” (Reeds 2000). Histidine, which was previously classified as a NEAA is now considered an EAA due to its rather limited ability to undergo transamination reactions (Gropper et al. 2009).

Table 2: Current Classification of Amino Acids with Respect to their Dietary Role in Healthy Adults

<table>
<thead>
<tr>
<th>Essential</th>
<th>Conditionally Essential</th>
<th>Nonessential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>Arginine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Cysteine</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Leucine</td>
<td>Glutamine</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Lysine</td>
<td>Glycine</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Methionine</td>
<td>Proline</td>
<td>Serine</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Tyrosine</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Footnote – Current classification of the standard 20 amino acids necessary for protein synthesis in the human body. Classification is based on dietary protein and nutritional importance.

1.1.2 Amino Acid Metabolism

The amount to which amino acids are metabolized by a first pass mechanism, specifically by the gut and by the liver, determine their availability to the peripheral tissues in the body (Stoll et al. 1998). The liver is the primary organ for the uptake of amino acids after a dietary protein has been digested, and absorbed or transported into the bloodstream (Thompson et al. 2010). The liver modifies the rate of amino acid metabolism according to the requirements of the body by adjusting amino acid catabolism and protein synthesis rates (Gropper et al. 2009). It has been shown in piglets that 64% of the intake of protein appeared in the portal vein as free amino acids. However there were varying differences in amounts of individual amino acids that are absorbed by the intestine and released into the portal vein (Stoll et al. 1998, Mariotti et al. 2000). It has
been well established that glutamate, glutamine, and aspartate are almost completely oxidized by the enterocytes and serve as an essential source of energy for the integrity of the gut mucosa (Mariotti et al. 2000, Wu 2010). Furthermore, studies by Stoll et al. (1998, 1999) showed significant extraction of EAA by the intestine, with only 12-21% used for protein synthesis, the remainder was catabolized completely. Thus, some amino acids are metabolized first by enterocytes along the intestinal tract and others are transported directly into portal blood. Jungas et al. (1992) performed quantitative studies in humans to show that most amino acids entering the liver are not completely oxidized to CO$_2$; instead many are converted to glucose and ketones, usually through a glutamate intermediate. The pathways in which amino acids are metabolized appear to be regulated by the gut due to its sensitivity to nutritional status and hormonal secretions (Mariotti et al. 2000). However it does appear the glutamate plays a central role of amino acid metabolism.

Amino acid metabolism usually involves the removal or transfer of the amino group, known as deamination and transamination, respectively. The deamination of an amino acid produces a $\alpha$-keto acid and ammonia/ammonium, which can be used for urea synthesis. Transamination involves the transfer of the amino group from either an EAA or NEAA to generate a new NEAA from its $\alpha$-keto acid. A predominant acceptor of the amino group is $\alpha$-ketoglutarate, producing glutamate (Voet et al. 2008). In turn, glutamate can be transaminated with oxaloacetate to create aspartate, or with pyruvate to create alanine (Figure 1) (Gropper et al. 2009). It has been discussed by Wu (2009, 2010) that the catabolism of glutamine, glutamate, and aspartate is essential to maintain whole-body homeostasis and prevent elevated plasma levels of these amino acids, which could become neurotoxic. Therefore it is clear that these amino acids are involved in many transamination reactions for their disposal and for the synthesis of other
NEAA. Transamination is a reversible process that is used by the body to maintain sufficient amounts of each amino acid, with the exception of lysine and threonine, which cannot undergo transamination reactions (Voet et al. 2008).

![Diagram of transamination](image)

**Figure 1: The Transamination of Glutamate and Pyruvate to Form α-Ketoglutarate and Alanine.**
Figure modified from Gropper et al. 2009.

### 1.1.2.1 Nitrogen Excretion

Excess ammonia in the body can cause brain failure, thus its removal from the portal blood is crucial. Ammonia that is formed during chemical reactions in the body or that is absorbed from food is used in ureagenesis, performed by the periportal cells in the liver (Gropper et al. 2009). Ammonia in the perivenous hepatocytes is used in glutamine synthesis by glutamine synthetase. These cells are also responsible for most amino acid catabolism in the body, thus ammonia is abundant and glutamine acts as an ammonia transporter to the kidneys where ammonia is excreted after glutamine is hydrolyzed by glutaminase. Excess ammonia in the liver is excreted from the body by the kidney as urea in the urine (Wu 2009, Voet et al. 2008). As described above, the intestine is responsible for a large amount of amino acid catabolism, thus the enterocytes do have some capacity to synthesize urea as well to help prevent ammonia toxicity in the body (Morris 2002, Wu 2009). Liver enzymes for the urea cycle are present early in fetal development and their levels are regulated by a variety of factors such as glucocorticoid...
levels and dietary protein intake (Wu and Morris 1998). Thus, the presence of these enzymes in early life suggests the importance of the urea cycle in the prevention of toxic ammonia build up in the body.

The urea cycle begins with ammonia which reacts with bicarbonate when it enters the liver to produce carbamoyl phosphate by carbamoyl phosphate synthetase, with the utilization of 2 ATP (Figure 2). The following step of the urea cycle is the formation of citrulline by ornithine transcarbamoylase, using ornithine and carbomyol phosphate as the reactants. Aspartate then enters the urea cycle as the second nitrogen source and reacts with citrulline in a rate-limiting step, forming argininosuccinate. Argininosuccinate is cleaved to produce arginine and fumarate, which is a TCA cycle intermediate (Morris 2002). Arginine in the liver has been described as essential in the ammonia detoxification process through its involvement in the final reaction of the urea cycle (Wu and Morris 1998, Wu 2010). The cleavage of arginine completes the cycle with the production of urea and the reformation of ornithine to participate in another round of the cycle (Morris 2002, Gropper et al. 2009). The urea cycle is regulated by substrate availability. When amino acid catabolism increases, glutamate concentrations increase due to transamination reactions, which stimulate \( N \)-acetylglutamate synthase to produce \( N \)-acetylglutamate from glutamate and acetyl-CoA. This is important because \( N \)-acetylglutamate allosterically activates carbamoyl phosphate synthetase, the first step of the urea cycle, thus effectively removing excess nitrogen from the body (Morris 2002, Wu and Morris 1998, Voet et al. 2008).
Figure 2: The Relationships of Amino Acids in the TCA and Urea Cycles
The fate of amino acid carbon skeletons and incorporation into the TCA cycle after deamination. Ketogenic amino acids: lysine and leucine. Partially ketogenic and glucogenic amino acids: phenylalanine, isoleucine, threonine, tryptophan, tyrosine. Glucogenic amino acids: alanine, glycine, cysteine, serine, aspartic acid, asparagine, glutamic acid, glutamine, arginine, methionine, valine, histidine, proline. Amino acids are categorized as essential, conditionally essential, and nonessential, shown in blue, green, and red, respectively. Image modified from Gropper et al. 2009.
1.1.2.2 Carbon Skeleton

When there is inadequate energy in the diet, amino acids can be completely oxidized to generate ATP, ammonia and carbon dioxide (Figure 3, page 13). After an amino acid has been transaminated or deaminated, it is known as the carbon skeleton. Amino acids are metabolized to water and carbon dioxide, or used for gluconeogenesis. The production of glucose from amino acids depends on the glucagon: insulin ratio in the body, which is increased when the body is not receiving sufficient energy or carbohydrates (Gropper et al. 2009). There are seven metabolic intermediates that the standard 20 amino acids in protein can be degraded into, and they are categorized as glucogenic or ketogenic (Figure 2, page 9) (Gropper et al. 2009). Jugas et al. (1992) estimated that one half of the liver’s oxygen is used for the oxidative conversion of amino acids to glucose and nearly two-thirds of the glucose is released into the peripheral circulation. Due to the large role the liver plays in amino acid catabolism, it is unnecessary for many peripheral tissues to express all the enzymes necessary for these pathways. Many carbon skeletons can be broken down in a number of pathways and their fate is dependent on different dietary and physiological factors (Mariotti et al. 2000)

The ketogenic intermediates include acetyl-CoA, and acetoacetate, which can be converted into ketone bodies or fatty acids. Some amino acids (phenylalanine, isoleucine, threonine, tryptophan, tyrosine) have the capacity to produce ketone body intermediates as well as glucose intermediates, and are thus ketogenic and glucogenic. Phenylalanine is always hydroxylated to form tyrosine, which can be further degraded to either acetoacetate, a ketone body, or fumarate, another TCA cycle intermediate; thus making tyrosine glucogenic as well (Voet et al. 2008). Aspartate is also broken down to fumarate during the urea cycle (Gropper et al. 2009). The amino acids lysine and leucine, which are degraded to only acetyl-CoA and/or
acetoacetate, are solely ketogenic and no carbohydrate synthesis is possible. Whereas, alanine, cysteine, glycine, serine, threonine, and tryptophan (converted to alanine), are all precursors of pyruvate, which can be broken down to acetyl-CoA and fuel the TCA cycle or converted to fatty acids. Pyruvate can also be converted to glucose through gluconeogenesis. Isoleucine, threonine, and tryptophan can also all be degraded to acetyl-CoA. Additionally, isoleucine, methionine, threonine, and valine are all degraded into succinyl-CoA. The carbon skeletons are first converted to acetyl-CoA and then completely oxidized to carbon dioxide and water in the TCA cycle to produce ATP (Wu 2009).

The glucogenic intermediates include pyruvate, α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate. Arginine, glutamate, glutamine, histidine, and proline are a part of the glutamate family and are thus precursors of α-ketoglutarate, through the conversion to glutamate (Brosnan 2000). α-ketoglutarate can then be used in the TCA cycle to generate ATP or in transamination reactions with other amino acids. Asparagine is converted to aspartate, which is degraded to oxaloacetate in the TCA cycle. Oxaloacetate is decarboxylated and phosphorylated by phosphoenolpyruvate (PEP) carboxykinase to produce PEP, which then enters the glucogenic pathway in the cell. It is important that all intermediates of the TCA cycle are replenished to keep the cycle functioning. However the ATP production from amino acid oxidation is less efficient than from fat or glucose, when compared on a molar basis (Wu 2009). The use of amino acids to generate ATP usually involves transamination or deamination reactions and the resulting ammonia must be removed from the body. Ammonia enters the urea cycle and requires 4 high-energy bonds to produce urea, which can then be excreted by the kidneys (Gropper et al. 2009). Thus amino acids result in less net ATP production when compared to other nutrients.
1.1.2.3 Amino Acid Biosynthesis

Many NEAA are synthesized in the body through transamination reactions, involving preformed α-amino nitrogen, usually from glutamate, and their respective α-keto acids (Wu 2010, Katagiri and Nakamura 2002, 2003). All NEAA, except for tyrosine (synthesized from phenylalanine hydroxylation), are formed from one of the metabolic intermediates: pyruvate, oxaloacetate, α-ketoglutarate, and 3-phosphoglycerate. Pyruvate, oxaloacetate, and α-ketoglutarate, are the α-keto acids of alanine, aspartate, and glutamate, respectively, which can be transaminated to form their respective amino acids. 3-phosphoglycerate, a glycolytic intermediate, is the precursor for serine, cysteine, and glycine. Aspartate and glutamate can synthesize asparagine and glutamine through ATP-dependent amidation. Glutamate is also the precursor for proline through multiple reduction reactions, as well as arginine through conversion to ornithine and urea (Wu 2009, Voet et al. 2008). It has been demonstrated that glutamate clearly plays a central role in the synthesis and metabolism of all NEAA. Thus animals have the ability to synthesize the NEAA but lack the enzymes required to synthesize the EAA (Wu 2010, Katagiri and Nakamura 2002, 2003).

The EAA are synthesized from similar metabolic precursors as the NEAA, except these processes take place in plants and microorganisms. Humans are unable to synthesize the EAA which must therefore be sufficiently ingested from the diet (Wu 2010, Wu et al. 2013).
There are three main perspectives regarding the essentiality of amino acids: dietary, metabolic, and functional. Focusing on the dietary or nutritional perspective, which mostly relates to growth and protein deposition, an amino acid is considered essential when mammalian enzymes cannot catalyze the de novo synthesis of a structural feature of that amino acid at an appropriate speed to support normal growth (Reeds 2000, Wu 2010). It is important to note that the ability to synthesize these structural features was lost early in evolution and has been conserved in eukaryotic organisms, not just mammals (Reeds 2000). However, some EAA may be synthesized from structurally similar precursors (Gropper et al. 2009). This applies to many nutritionally EAA, such as the branched-chain amino acids, which can be synthesized from their α-keto acids in transamination reactions, however these α-keto acids aren’t readily available to the cells, making these amino acids still nutritionally essential. In particular circumstances some amino acids cannot be synthesized at an appropriate rate and they can become limiting in the
cell. Conditionally essential amino acids are those that have limitations to their synthesis, and when that limit is reached they become essential in the diet (Reeds 2000, Wu 2010, Wu et al. 2013). The limitations to the rate of an amino acid synthesis include, the availability of its precursors, including nitrogen, the lack of enzymatic activity, the route of nutrition, as well as special circumstances such as stress or injury; which can elevate the demands for particular amino acids (Wu 2010, Burrin and Stoll 2009, Barbul, 2008). Amino acids are considered nonessential when de novo synthesis is possible from a source of utilizable nitrogen. Reeds (2000) has suggested that in a strict sense only glutamic acid and serine are nonessential, as they are synthesized easily from a nitrogen source and are the primary precursors to all other NEAA. The current categorization of the amino acids used in protein synthesis can be seen above in Table 2 (page 5).

1.1.4 α-Amino Nitrogen Source

The essentiality of amino acids should also take into consideration the origin of the α-amino nitrogen, and not solely depend on the de novo synthesis of the carbon skeleton (Katagiri and Nakamura 2002, 2003). Originally, it was believed that animals could synthesize some amino acids using dietary protein as the main nitrogen source through the reaction of ammonia and α-keto acids to form imino acids, which are further reduced to amino acids, (Figure 4) (Foster et al. 1938). Foster et al. (1938) described early experiments by Embden and by Schmitz that showed the formation of amino acids after organs were infused with ammonia and their respective α-keto acids. However this does not prove that the amino nitrogen was derived from the added ammonia. Foster et al. (1938) performed their own experiments using isotopic ammonia in adult rats. They found the incorporation of $^{15}$N in protein nitrogen after rats were fed low protein diets. Six individually tested amino acids contained excess $^{15}$N, except lysine, which
had normal isotope concentration. The highest levels of isotope concentration were found in glutamic acid and aspartic acid. Interestingly, the dicarboxylic acids appear to be primary intermediates in the formation of other amino acids.

![Image](image.png)

**Figure 4: The Formation of an Imino Acid from Ammonia and an \( \alpha \)-keto acid, Followed by a Reduction to an Amino Acid.**

Image modified from Foster et al. 1938.

However, many experiments designed to demonstrate the utilization of ammonia as a nitrogen source have yielded no concrete proof. Foster et al. 1938 described studies performed by Herbst and by Braunstein and Kritzmann suggested a direct shift of the amino nitrogen from one amino acid to a \( \alpha \)-keto acid, forming its corresponding amino acid, with a Schiff base intermediate. This reaction does not include the incorporation of ammonia during amino acid synthesis (Foster et al. 1938). Katagiri and Nakamura (2002) disagree with the isotopic ammonia experiment conclusions of Foster et al. (1938) due to the use of high toxic concentrations of \(^{15}\)N-ammonium citrate in the rats and the uncertain interpretation of the metabolic processes during the resultant weight loss in rats. Instead, they suggest that the incorporation of isotopic nitrogen into amino acids may have been due to the reactions catalyzed by glutamate dehydrogenase (GDH), or nitrogen exchanges by aminotransferases (Katagiri and Nakamura 2002). In animals the kinetics of GDH, which plays a central role in glutamate metabolism (Figure 5), suggest that the operating direction of the enzyme is toward glutamate catabolism (Manchester 2001). This reaction is further driven to the right by the supply of ammonia to carbamoylphosphate synthetase in the synthesis of urea, thereby decreasing its availability. Thus it seems highly
unlikely that ammonia is used as a nitrogen source in the formation of NEAA and that this nitrogen requirement is acquired elsewhere.

\[
\text{Glutamate}^- + \text{NAD(P)}^+ + \text{H}_2\text{O} \xrightarrow{\text{GDH}} \text{Oxoglutarate}^{2-} + \text{NAD(P)H} + \text{NH}_4^+ + \text{H}^+ 
\]

**Figure 5: The Biochemical Reaction Catalyzed by GDH in Cells.**

There was speculation that ammonia or urea could be salvaged as a nitrogen source in animals for amino acid synthesis. However studies performed by Levenson et al. (1959) used germ-free rats and excluded the possibility of direct urea recycling and attributed the salvage to the resident bacteria that contain the enzyme urease. It has been shown in rats and pigs that bacteria can synthesis EAA from urea, but there remains no evidence of this in humans (Mariotti et al. 2000) Microorganisms can synthesize glutamate from high concentrations of ammonium salts using an NADPH-specific GDH (Katagiri and Nakamura 2002, 2003). They are also capable of forming glutamine from ammonia, glutamate, and ATP. However, Manchester (2001) argues that the reverse reaction towards glutamate anabolism is not plausible in plants due to the high \( K_m \) of GDH towards ammonia. Instead, Manchester (2001) supports the concept of nitrogen assimilation through the coupling of glutamine synthase and glutamate synthase. The \( \alpha \)-amino nitrogen in animals may be retained either through bacterially derived \( \text{NH}_3 \) or absorption of bacterially derived amino acids across the colon (Jackson 1995). This leads to the conclusion that animals rely on microorganisms and higher plants to create \( \alpha \)-amino nitrogen using the enzyme glutamate synthase. Katagiri and Nakamura (2002, 2003) furthermore state that there could be a ‘requirement’ for preformed \( \alpha \)-amino nitrogen in the form of some NEAA, such as glutamate, alanine, or aspartate.
1.1.5 **Amino Acid Requirements**

For protein synthesis to occur, all EAA must be available to the cell for their incorporation (Thompson et al. 2010). Amino acids are not only the constituents of proteins but also play many other metabolic roles in the body and are involved in pathways that could limit their availability for protein synthesis. The EAA requirements in humans for net protein deposition are a very small portion of the total amino acid requirement, more than 90% of the requirement is for the maintenance of body protein stores (Reeds 2000). The minimum needs for EAA for protein deposition is remarkably similar among mammals, specifically humans, rats, pigs, sheep, and calves, differing only due to the differences in rates of protein deposition. Nutritional studies in humans have shown that a low protein diet containing only EAA can be improved in nutritional value by improving nitrogen balance when NEAA are added to the diet (Hiramatsu et al. 1994). There are clearly dietary requirements for a nitrogen source in addition to the EAA in the human diet.

Decreased availability of nitrogen could limit the synthesis of many NEAA. The de novo synthesis of NEAA is crucial for survival because they are the precursors for many metabolites and peptides, required for maintaining physiological homeostasis (Table 3, page 19) (Gibson et al. 2002). A portion of amino acid requirements is due to the inefficiency of the recycling of amino acids after they are released from tissue protein. When there is less than optimal synthesis of the NEAA due to a lack of nitrogen, these amino acids will become limiting in metabolic functions (Reeds 2000). There are many factors which affect the metabolism and de novo synthesis of NEAA, including exercise, energy intake, and total protein intake (Yu et al. 1985). All of these factors can be influenced by a person’s age, sex, activity level, and BMI (Devlin 2011).
When protein or nitrogen is limiting, the synthesis of NEAA is of the upmost importance to prevent the use of EAA in their place. Rose (1949) suggested that at low protein intakes, the requirements for the EAA can be lowered when NEAA are added to the diet because they have the ability to spare the EAA. However Hiramatsu et al. (1994) later questioned these assumptions given the limitations of the nitrogen balance technique. They were unable to replicate this sparing effect of the NEAA with tracer studies in adult men. Early studies in rats by Bressani and Mertz (1957) demonstrated that the total protein intake influences the needs for specific amino acids. The lysine requirement, as a percentage of crude protein, actually rapidly increased as the protein level increased in the diet until remaining constant after 16% protein diets (Bressani and Mertz 1957). Reeds (2000) showed growth responses in rats and pigs due to deficiencies of NEAA in the diet, indicating their inclusion in the diet is necessary to maintain normal growth. Furthermore, there appears to be an upper limit to the de novo synthesis of NEAA, suggesting NEAA could become metabolically limiting driving a dietary demand (Jackson 1995, Yu et al. 1985). Studies in adult humans demonstrated limited NEAA synthesis due to the availability of nitrogen during periods of low protein intake. Whole body protein turnover was decreased and net protein catabolism and nitrogen excretion were reduced, indicating the conservation of nitrogen for the synthesis of NEAA during marginal protein intakes. The synthesis of NEAA is necessary for survival and these pathways have been conserved in animals throughout evolution because of their key metabolic roles in the body.

It is interesting to note that many of the necessary precursors in functional and metabolic roles of the body, including in the immune system, the skeletal musculature system, and the central nervous system, are NEAA, as seen in Table 3 (Reeds 2000). It is important that the body has the precursors, including available nitrogen, for the immediate synthesis of NEAA to
support a healthy metabolism in animals. Furthermore, there is an increased requirement for amino acids under stressful stimuli, such as a host immunologic response (Gibson et al. 2002). A large metabolic demand for many NEAA underlines the importance of their availability, and thus de novo synthesis, in the body as they play key roles in many pathways, including the TCA cycle and gluconeogenesis. To look further into this concept, we have highlighted some important metabolic and physiologic roles of two key NEAA, glutamate and glycine (Chapter 2: Glutamate Requirement Study, page 33).

Table 3: The Involvement of Amino Acids in Physiological and Metabolic Functions.

<table>
<thead>
<tr>
<th>System</th>
<th>Function</th>
<th>Product</th>
<th>Precursor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>Energy generation</td>
<td>ATP</td>
<td>Glu, Asp, Glutamine</td>
</tr>
<tr>
<td></td>
<td>Proliferation</td>
<td>Nucleic acids</td>
<td>Glutamine, Gly, Asp</td>
</tr>
<tr>
<td></td>
<td>Protection</td>
<td>Glutathione</td>
<td>Cys, Glu, Gly, Arg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitric oxide</td>
<td>Thr, Cys, Ser, Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mucins</td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>Energy generation</td>
<td>Creatine</td>
<td>Gly, Arg, Met</td>
</tr>
<tr>
<td></td>
<td>Peroxidative</td>
<td>Taurine</td>
<td>Cys</td>
</tr>
<tr>
<td></td>
<td>protection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nervous System</td>
<td>Transmitter</td>
<td>Adrenergic</td>
<td>Phe</td>
</tr>
<tr>
<td></td>
<td>synthesis</td>
<td>Serotinergic</td>
<td>Try</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycinergic</td>
<td>Gly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutaminergic</td>
<td>Glu</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitric oxide</td>
<td>Arg</td>
</tr>
<tr>
<td></td>
<td>Peroxidative</td>
<td>Taurine</td>
<td>Cys</td>
</tr>
<tr>
<td></td>
<td>protection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune system</td>
<td>Lymphocyte</td>
<td>Glutathione</td>
<td>Glutamine, Arg, Asp</td>
</tr>
<tr>
<td></td>
<td>proliferation</td>
<td></td>
<td>Cys, Glu, Gly</td>
</tr>
<tr>
<td></td>
<td>Peroxidative</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>protection</td>
<td>Nitric oxide</td>
<td>Arg</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Blood pressure</td>
<td></td>
<td>Cys, Glu, Gly</td>
</tr>
<tr>
<td></td>
<td>regulation</td>
<td>Nitric oxide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peroxidative</td>
<td>Red cell glutathione</td>
<td>Arg</td>
</tr>
<tr>
<td></td>
<td>protection</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Footnote - Table modified from Reeds 2000.
1.1.6 Glutamate

Glutamate is a NEAA, which has a high abundance in nature relative to other amino acids, consisting of 11-22% by weight of animal proteins and up to 40% in plant proteins. Members of the glutamate family (glutamate, glutamine, proline, histidine, and arginine) make up one quarter of dietary amino acid intake and are catabolized through the conversion to glutamate (Brosnan 2000). Glutamate is a key component to many pathways and cycles in the body and has many functions (Table 5, page 27) (Young and Ajami 2000). Structurally, glutamate contributes to the tight packing of α-helices in the secondary structure of proteins due to hydration from the “pocket” that is formed between the oxygen molecules. It appears to play a role in the cytoskeleton, as polyglutamylation of tubulin regulates the interactions with other proteins (Young and Ajami 2000). Glutamate is an important amino acid for fuel and integrity of the gut. The splanchnic tissues extract almost all glutamate and 81-85% of it is oxidized on first pass. Glutamate can also be converted to proline via pyrroline-5-carboxylate (P5C) in the gut; this proline is an important precursor for citrulline in the urea cycle (Bertolo and Burrin 2008, Burrin and Stoll 2009). There is increasing evidence that glutamate promotes cell replications by providing nitrogen for purine and pyrimidine synthesis, as well as playing a role in the TCA cycle (Figure 2, page 9) and in gluconeogenesis (Millward et al. 1989). Thus glutamate acts as an intermediate between the urea and TCA cycles, as it is needed for both cycles to proceed (Burrin and Stoll 2009, Brosnan 2000). There is a strong correlation of glutamate concentration and the rate of protein synthesis in muscles, as it donates nitrogen, affecting the whole-body nitrogen balance (Millward et al. 1989). Thus, in addition to being a substrate for protein synthesis, glutamate has many essential metabolic and physiological roles in the body. It is
important that the body is able to synthesize glutamate in sufficient amounts to fulfill the metabolic demand of each pathway.

Many NEAA and nitrogen containing biomolecules can be derived from glutamate, indicating its central role in amino acid nutrition (Katagiri and Nakamura 2002). Glutamate is maintained at high intercellular concentrations in almost all cells compared to the extracellular fluid concentrations, indicating its important roles in all tissues (Brosnan 2000). Glutamate is especially abundant in the liver, as it is the principal location of amino acid catabolism. Many amino acids undergo oxidative conversion to glutamate, which acts as the link to gluconeogenesis, providing glucose for the peripheral tissues (Burrin and Stoll 2009, Katagiri and Nakamura 2003). Glutamate can be oxidatively deaminated by the enzyme glutamate dehydrogenase, producing $\alpha$-ketoglutarate and ammonia. As stated before, $\alpha$-ketoglutarate is a principal amine acceptor in transamination reactions and is thus important for the synthesis of other NEAA, such as alanine (Figure 1, page 7) (Voet et al. 2008). All common amino acids, except for lysine, are able to participate in transamination reactions with glutamate (Brosnan 2000). Glutamate is a good candidate for amino transfers due to the longer half-life and stability of its Shiff base (imine form) intermediate compared to its homolog, aspartate (Young and Ajami 2000). These pathways have been conserved throughout evolution and demonstrate the need for glutamate during metabolic processes as well as for nitrogen transport throughout the body.

Glutamate has also shown growth responses in experiments by Rose et al. (1948), which showed increased growth in rats when glutamate was added to the exclusively EAA diet. More recent studies in rats and pigs also indicated a statistically significant small decrease in growth rates when fed diets devoid of glutamate (Reeds 2000). However, glutamate was still classified as a NEAA, ignoring the stimulatory effects. It was classified as nonessential because its removal
from the diet only showed a small effect (Katagiri and Nakamura 2002). It appears that some NEAA, such as glutamate, might be limiting in cellular processes and when the metabolic demand is high, which may be creating a dietary requirement.

**Table 4: Functions of Glutamate**

- Substrate for protein synthesis
- Precursor of glutamine
- N transport (muscle-glutamine; brain)
- Neurotransmitter (and γ-aminobutyrate)
- Polyglutamate and cell signaling
- δ-Carboxylation of glutamate
- Substrate for glutathione production
- Precursor of N-acetylglutamate
- Active sites of enzymes
- Inhibitor of glutaminase reaction
- Precursor of TCA cycle intermediates
- Energy source for some tissues (mucosa)

Footnote - Table modified from Young and Ajami 2000.

1.1.7 Glycine

Glycine is an important NEAA, as it is the precursor for many metabolic intermediates, most of which do not return glycine as an end product to the metabolic pool (Jackson 1995). Studies by Snyderman et al. (1962) have shown that glycine might act as a limiting nutrient in infants and growing children. Millward et al. 1989 described studies that showed when glycine is added to a low protein diet, weight gain and nitrogen balance were promoted in infants and growing children. Glycine plays an important role in infants as it accounts for 30% of collagen residues and it accumulates faster than any other amino acid. However, breast milk satisfies less than 20% of the glycine requirement in infants and is thus limiting for growth (Millward et al. 1989). Jackson et al. showed the increase in excretion of 5-oxoproline (5-OP) in urine is a response to limiting glycine in the metabolic pool of developing fetuses, infants, and children. 5-OP has been found in the developing fetus, infants, children, as well as pregnancy (Millward et
al. 1989). Glycine is also very important for wound repair and for burn victims (Yu et al. 1985). The de novo synthesis of glycine in adult humans decreases with the removal of NEAA from the diet, especially at low protein intakes (Yu et al. 1985). It appears that NEAA like glycine can become limiting and affect body processes, especially during the developing and growing years of life.

1.1.8 Essential Amino Acids

There have been many studies conducted to determine the minimum EAA requirements, since the original work by Rose et al. (1949), and have all been used by the Dietary Reference Intake (DRI) (2005) as well as summarized recently by Elango et al. (2008) (Table 5, page 27). However the minimum dietary requirements of amino acids are still a subject of debate and many have argued the current DRI recommendations are too low, mostly due to the limitations of the nitrogen balance technique and the improper regression models used (Humayun et al. 2007).

1.1.9 Traditional Method to Define Essential Amino Acid Requirement

Nitrogen balance is the relation of nitrogen intake, principally in the form of protein, to nitrogen excretion, mostly in the form of urea/NH₃ in the urine and as undigested protein in the feces (Devlin 2011). The nitrogen balance technique has significant limitations because the intakes tend to be overestimated, and the loses tend to be underestimated, resulting in a falsely positive nitrogen balance and therefore an underestimate of the amino acid requirement (Humayun et al. 2007). Nitrogen balance requires a long adaptation of the subjects to the test amino acid intake, which can be up to 7 days, making it a difficult and unethical method to apply to a human population (Rand et al. 1976, Pencharz and Ball 2003). This method excludes vulnerable populations, such as children and pregnant women, due to the lengthy adaptation stage on possibly insufficient protein intakes. Furthermore, an analytical problem arises with the
nitrogen balance technique because the physiologic response between nitrogen intake and balance is not linear but curvilinear due to decreased efficiency of protein utilization as it approaches zero balance (Humayun et al. 2007). Therefore, newer methods based on stable isotope labeled amino acids have been developed.

1.1.10 Indicator Amino Acid Oxidation (IAAO)

The Indicator Amino Acid Oxidation technique, unlike the nitrogen balance technique, can be used to noninvasively determine the dietary amino acid requirements in healthy populations as well as those with disease or in vulnerable populations. This technique has been used to determine the daily EAA requirements in humans (Table 5, page 27) (Elango et al. 2012). The main concept of this technique is that when one amino acid is deficient in the diet, then all other amino acids, including the indicator amino acid, will be oxidized (Elango et al. 2008). When the limiting amino acid is increased, the oxidation of the indicator will decrease linearly, indicating increased incorporation into protein. The dietary requirement for the limiting amino acid is determined to be the point at which there is no change in the oxidation of the indicator amino acid, known as the breakpoint (Figure 6, page 26) (Humayun et al. 2007).

Recent studies have shown that the tyrosine breakpoint measured with the IAAO technique was no different than the breakpoint for phenylalanine hydroxylation measured using apo B-100, a hepatic export protein synthesized from intrahepatocyte amino acids (Rafii et al. 2008). This represents the intracellular incorporation of phenylalanine during protein synthesis in the liver. Thus, study supports the inverse relationship of phenylalanine oxidation and the change in liver protein synthesis (Elango et al. 2012).

IAAO uses a stable isotope carbon labeled amino acid as an indicator to determine individual amino acid requirements in vivo noninvasively (Elango et al. 2009a). An isotope is an
atom of a chemical element, such as carbon, with the same atomic number and virtually the same chemical behavior but has different physical properties and a different atomic mass (Abrams and Wong 2003). Stable isotopes of elements are commonly found in nature, unlike the radioactive and unstable isotopes, which breakdown spontaneously (Abrams and Wong 2003). Amino acids that are labeled with stable isotopes function the same metabolically as unlabeled amino acids, and can therefore be measured and studied in vivo.

Phenylalanine is chosen as the indicator amino acid in our studies because it is not synthesized within the body (essential) so its flux in a fasted state can be used as an insight to whole body proteolysis (Gibson et al. 2002). This gives an overall indication of protein turnover in the body. There are several reasons for the careful selection of phenylalanine as an indicator; firstly phenylalanine is oxidized in the liver, which is altered by the patterns of amino acids entering the liver after a meal. Secondly, tyrosine is only formed from phenylalanine in the liver and is degraded before equilibrating with the plasma tyrosine. Thirdly, the carboxyl carbon of phenylalanine is lost as CO₂ early during the formation of metabolic precursors, except for thyroid hormones and melanin precursors (Ball and Bayley 1984). After phenylalanine has lost its carboxyl carbon it does not participate in any further reactions. It is also important to ensure there is excess of tyrosine in the diet, to partition the carboxyl carbon of phenylalanine between incorporation into protein or oxidation and inhibit the reaction of phenylalanine into tyrosine (Elango et al. 2011). Tyrosine has a sparing effect of up to 75% on the phenylalanine requirement, due to the irreversible formation of tyrosine from phenylalanine by the enzyme phenylalanine hydroxylase (Rose and Wixom 1955a). Tyrosine is an important NEAA, as it is the precursor for many neurotransmitters such as dopamine and epinephrine (Gibson et al. 2002).
Therefore it is necessary to supply excess tyrosine in the diet to prevent its formation from phenylalanine.

Figure 6: The Rate of $^{13}$CO$_2$ Appearance from Orally Administered L-[1-$^{13}$C]Phenylalanine in Breath
The relation between various protein intakes and the rate of appearance of orally administered L-[1-$^{13}$C]phenylalanine as breath $^{13}$CO$_2$ in eight healthy men (left). As the limiting amino acid is increased, the oxidation of the indicator amino acid decreases until there is no further change, which is equal to the dietary requirement or breakpoint (right). Images taken from Humayun et al. 2007 (left) and Elango et al. 2008 (right).
**Table 5: Comparison of the daily EAA Requirements from the Current Dietary Reference Intake and the IAAO technique**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>DRI Estimated Average Requirement (g/kg)</th>
<th>DRI Recommended Dietary Allowance (mg/kg)</th>
<th>IAAO EAR (g/kg)</th>
<th>IAAO RDA (g/kg)</th>
<th>Base Diet (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>0.011</td>
<td>0.014</td>
<td>N/A</td>
<td>N/A</td>
<td>0.014</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.015</td>
<td>0.019</td>
<td>0.042</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.034</td>
<td>0.042</td>
<td>0.055</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.031</td>
<td>0.038</td>
<td>0.035</td>
<td>0.052</td>
<td>0.052</td>
</tr>
<tr>
<td>Cysteine</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0.0109</td>
<td>0.0109</td>
</tr>
<tr>
<td>Methionine*</td>
<td>0.015</td>
<td>0.019</td>
<td>0.0045</td>
<td>0.0101</td>
<td>0.0101</td>
</tr>
<tr>
<td>Tyrosine**</td>
<td>N/A</td>
<td>N/A</td>
<td>0.006</td>
<td>0.007</td>
<td>0.0407</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.027</td>
<td>0.033</td>
<td>0.0091</td>
<td>0.014</td>
<td>0.0305</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.016</td>
<td>0.02</td>
<td>0.019</td>
<td>0.0262</td>
<td>0.0262</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.004</td>
<td>0.005</td>
<td>0.004</td>
<td>0.00502</td>
<td>0.00502</td>
</tr>
<tr>
<td>Valine</td>
<td>0.019</td>
<td>0.024</td>
<td>0.047</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>BCAA***</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0.21</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>0.172</strong></td>
<td><strong>0.214</strong></td>
<td><strong>0.2326</strong></td>
<td><strong>0.34922</strong></td>
<td><strong>0.39942</strong></td>
</tr>
</tbody>
</table>

N/A = Data not available

*When Cysteine is fed in excess of 0.021 g/kg. The mean requirement and population safe intake for total sulfur amino acid is 12.6 and 21 mg/kg respectively

** Tyrosine requirement was determined when Phenylalanine intake was adequate (9mg/kg)

*** The Branched Chain Amino Acids (Isoleucine, Leucine, Valine) have a total RDA (g/kg) = 0.21

Footnote - These requirements were determined in the absence of cysteine and tyrosine. It is important to note that there is only one RDA for the total branched chain amino acids, represented under isoleucine, with N/A in place for leucine and valine. IAAO values were determined by Elango et al. 2012.

Diets prior to the study day play an important role. Thorpe et al. (1999) demonstrated that the amount of protein consumed 2 days prior had an affect on phenylalanine flux and oxidation due to the regulation of oxidizing enzymes (Gibson et al. 2002). Therefore subjects must maintain a standardized daily protein intake of 1.0 g protein per kg and 1.7 × their resting energy expenditure (REE) for the 2 days prior to the study day. Earlier studies in animals and humans
have shown that the IAAO method does not require an adaption to the level of the test amino acid (Elango et al. 2009b). This is an additional advantage that the IAAO method has over the nitrogen balance method and is the reason it can be applied in vulnerable populations, such as children and pregnant women.

1.1.11 Tracer Kinetics

Isotopic steady state in the tracer enrichment at baseline and plateau will be represented by unchanging values of L-[1-13C]phenylalanine in 13CO2 (atoms percent) in breath. At plateau, the atom percent excess (APE) will be calculated by subtracting the mean breath 13CO2 enrichments (atoms percent) of the three baseline samples from the six plateau samples (Humayun et al. 2007). Baseline 13CO2 in breath must be measured to account for the large background presence of the naturally occurring isotope of 13C (~1.1%) (Schoeller et al. 1980).

F13CO2 represents the rate of 13CO2 released by phenylalanine tracer oxidation (µmol/kg/hour), which is calculated by the following equation:

Equation 1 The Rate of 13CO2 Production

\[
F_{13CO_2} = \left[ \frac{FCO_2 \times ECO_2}{W} \right] \times \left[ \frac{44.6 \times 60}{0.82 \times 100} \right]
\]

Where FCO2 is the CO2 production rate (mL/min); ECO2 is the 13CO2 enrichment in expired breath at isotopic steady state (APE); and W is the weight (kg) of the subject. The constants 44.6 (µmol/mL) and 60 (min/h) were used to convert FCO2 to µmol/h. The factor 0.82 is the correction for CO2 retained in the bicarbonate pool of the body in the fed state. A factor of 100 changes the APE to a fraction (Humayun et al. 2007).

1.1.12 Protein and Total Nitrogen Requirements

Using the IAAO method, Humayun et al. (2007) found the dietary requirement for total protein in adult men to be 0.93 g/kg. Additional IAAO studies have determined the requirements...
of the individual EAA mean requirements, except histamine (Elango et al. 2008, 2012). The summation of all the EAA mean requirements determined using the IAAO method, along with the histidine requirement from the DRI equate to 0.2326 g/kg for adults (Table 5, page 27) (Elango et al. 2012). This is considerably less than the total protein requirement, indicating the need for additional amino acids (presumably NEAA) to fulfill the total nitrogen requirement in order for protein synthesis to occur in the body. Thus protein nutrition depends on not only the individual amino acid dietary requirements but also the total nitrogen intake.

Early studies by Rose and Wixom (1955b) in young men found the dietary requirement of nitrogen to permit the de novo synthesis of NEAA was 2.55 grams daily, which is a very small quantity. Their diet was based from the original classification of eight EAA, with double their requirement supplemented with glycine as a nitrogen source. The total nitrogen intake was then progressively decreased with an assessment of nitrogen balance. They observed that relatively small decreases in total nitrogen intake led to fluctuations in nitrogen balance from positive to negative (Rose and Wixom 1955b). Thus the additional nitrogen required on top of the EAA has to be supplied by NEAA, such as glycine. Whether this is true or not has not been validated since the 1955 study, and whether other NEAA may satisfy this demand is unknown.

Rechcigl et al. (1957) performed studies in 21-day-old rats, where they supplemented an EAA base diet with nonspecific nitrogen sources and found there was a hierarchy in nitrogen utilization effectiveness. These nitrogen sources were the NEAA: glutamic acid, glycine, glutamine, aspartic acid, asparagine, alanine, serine, and proline (as well as OH-proline). They also tested urea, diammonium citrate, and biuret as nitrogen sources. They looked at growth responses, food efficiency, as well as net nitrogen utilization. They found that addition of glutamic acid gave the best growth response, closely followed by glutamine, asparagine, aspartic
acid, and alanine. Glycine and serine gave the poorest growth responses, and there was even weight loss with the addition of OH-proline to the base diet (Rechcigl et al. 1957). They concluded that the nitrogen required for NEAA may be derived from a variety of nitrogen sources, including the NEAA, however they may not be equally effective. To the best of our knowledge, whether different efficiencies as a source of nitrogen among the NEAA in humans exist has not been tested systematically thus far.

1.2 Rationale

There is a metabolic demand for all 20 of the standard amino acids that make up protein in humans. However, dietary recommendations are made only for the EAA, due to the inability of the human body to synthesize them de novo. The NEAA do not have a dietary recommendation, since the body has biochemical pathways necessary to synthesize them, provided an adequate source of nitrogen is present. Recently Katagiri and Nakamura (2002) have argued that there could be a requirement for pre-formed α- amino nitrogen in the form of specific NEAA, such as glutamate, alanine, and aspartate, due to their central involvement in almost all other amino acid metabolic pathways. Furthermore, Wu (2013) has also suggested that the existence of the pathways does not necessarily mean that the ‘capacity to synthesize’ sufficient quantities of all NEAA exist under all conditions of growth, development and physiological states.

Earlier studies in growing rats by Recheigl et al. (1957) showed that addition of various nitrogen sources (urea, diammonium citrate, glutamate, glycine, glutamine, aspartate, asparagine, proline, serine, hydroxyproline) had different effects on growth. Addition of glutamate had the most significant effect on growth response and net nitrogen utilization, followed by alanine, aspartate, asparagine, proline, glycine, and serine. Such controlled studies are lacking in humans.
Furthermore, in humans growth might be a good marker during infancy, but not during other life-stages. The nitrogen balance method, which has several flaws, is also not a very sensitive and robust technique.

Recently, using the IAAO technique in adult men, the total protein requirement was determined to be 0.93 g/kg (Humayun et al. 2007). Interestingly the summation of all the dietary EAA requirements only equals 0.2326 g/kg (Elango et al. 2012). This suggests that a significant portion of the demand for total nitrogen is met by the NEAA even in adult men. Whether some NEAA might be nutritionally important to be provided in the diet, and whether some are more efficient in meeting the demand for total nitrogen needs to be determined.

1.3 Hypotheses

First, we hypothesize that in addition to the dietary requirements for all EAA, there exists a dietary demand for pre-formed  α-amino nitrogen from the NEAA. Furthermore, we hypothesize this demand may be fulfilled by glutamate, a metabolically important NEAA. There may be a dietary requirement for glutamate in healthy adult males.

Secondly, we hypothesize that there exists different efficiencies as a nitrogen source among nine of the NEAA to satisfy the metabolic demand for total nitrogen for the de novo synthesis of other NEAA and for protein synthesis in healthy adult males.

1.4 Objectives

The first objective (Glutamate Requirement Study) is to determine whether there is a dietary requirement for glutamate in adult humans, using L-1\(^{13}\)C-Phenylalanine as the indicator amino acid in the minimally invasive IAAO model.

The second objective (Nonessential Amino Acid Metabolism study) is to determine whether some of NEAA (Ala, Arg, Asn, Asp, Gln, Glu, Gly, Pro, Ser) are more efficient as
nitrogen sources to satisfy protein synthesis in healthy adult males, using L-1\textsuperscript{13}C-Phenylalanine as the indicator amino acid in the minimally invasive IAAO model.
Chapter 2: Glutamate Requirement Study

2.1 Glutamate

Glutamate is a NEAA that plays an important role in many different processes in the human body and is the most abundant amino acid in liver, kidney, skeletal muscle, and brain (Brosnan 2000) (Table 4, page 22). It is believed that glutamate is the most important fuel for the cells of the gastrointestinal tract (Burrin and Stoll 2009). The splanchnic tissues extract almost all glutamate and a majority of glutamate is oxidized during first pass metabolism. Glutamate is so central in amino acid metabolism that it can be used to synthesize many other NEAA, mainly through transamination reactions. Thus, glutamate plays a major role is transferring nitrogen throughout the body for building many biomolecules as well as for protein synthesis.

Furthermore, glutamate plays a key role in cultural cuisine, as it has been proposed as a fifth basic taste (UMAMI), through its role as a taste receptor (Kurihara 2009). Monosodium glutamate triggers this savory “umami” taste and is commonly used by the food industry to enhance flavor, for example in meat broths (Chandrashekar et al. 2006). Glutamate has the highest proportion (35-49%) of the free amino acids found in human milk, exposing infants to the umami flavor and indicating its nutritional importance during development (Rassin et al. 1978). Whether this key importance for glutamate makes it necessary as a dietary source is unknown.

As previously discussed, glutamate has shown positive growth responses in growing rat experiments by Rose et al. (1948), and by Recheigl et al. (1957). On the other hand, studies performed in rats fed diets devoid in glutamate showed negative growth rates (Reeds 2000). It has been suggested that because glutamate has a principal role in metabolism and because of the stimulatory effects in rats, there may be a dietary requirement in adult humans. Inadequate
glutamate in the diet could be limiting in cellular processes and when the metabolic demand is high, there may be a dietary requirement. The IAAO method is a robust method to determine the essentiality of an amino acid, as it is relatively quick and easy to apply. Therefore the primary goal of this experiment was to identify if there was a dietary requirement for glutamate in adult men.

2.2 Methods

2.2.1 Subjects

Seven healthy adult men (Table 7, page 42) were chosen as subjects. All subjects were selected after completing a preliminary study day, where medical history, dietary habits, as well as activity levels were collected (Appendix D.2 Preliminary Study Day Form, page 92). Subjects were all nonsmokers and were free of chronic diseases or illnesses (Bross et al. 1998). A previous study by Kriengsinyos et al. (2004) supports our decision to exclude women from this study because the different phases of the menstrual cycle appear to affect phenylalanine oxidation, which resulted in different lysine requirements, having higher dietary lysine requirements during the luteal phase. Subject’s anthropometric measurements, body composition and energy intakes were all measured and recorded on the preliminary study day (Bross et al. 1998). During the preliminary study day, subject body composition was measured by bioelectrical impedance analysis (model 101A; RJL Systems, Detroit, MI). Similarly, subjects resting energy expenditure (REE) was measured by continuous, open-circuit indirect calorimetry (Deltatrac II Metabolic Monitor; SensorMedics, Yorba Linda, CA). Informed written consent was provided by all subjects before participating in the study (Appendix D.1 Subject Consent Form, page 85). A wall-mounted stadiometer was used to measure standing height without shoes in the morning of each study day. Subjects’ weights were also recorded on the morning of
each study day (Bross et al. 1998). Subjects were offered financial compensation for their time at the end of each study day.

2.2.2 Dietary and Energy Intakes

Subjects were provided an adaptation diet containing a daily protein intake of 1.0 g/kg/d and $1.7 \times \text{REE}$ for the 2 days prior to the test day, which is equal to the amount of dietary protein that would be required by most adult males (Zello et al. 1990a). It was shown previously by Zello et al. (1990b) that neither mean phenylalanine flux nor phenylalanine oxidation were affected by prior adaptation level, or length of adaptation. A factor of 1.7 takes in account the daily energy needs for adults of moderate activity, this includes the energy needed for basal metabolism, diet induced thermogenesis, as well as activity (Zello et al. 1990a). Throughout the adaptation period, subjects were asked to maintain their habitual level of physical activity. Before the start of each study day, subjects were instructed to fast for 10-12 hours prior to their first study day meal (Bross et al. 1998). All seven subjects participated in 2 study days, assigned in random order, with two corresponding study day test diets, for a total of fourteen study days. All amino nitrogen in the test diets was from free amino acid mixtures made in the laboratory. The study day test diets corresponded to the amino acid pattern present in egg protein, in which all glutamate and glutamine was present as glutamate (113 mg/kg/d), or removed (0 mg/kg/d), with serine used to make the diets isonitrogenous. The study diets were given as eight isocaloric hourly meals, with each meal representing one-twelfth of the subject’s totally daily energy requirement (Appendix D.3 Study Day Form, page 94). These meals provided an energy intake of $1.5 \times \text{REE}$ for each subject and supplied 1.0 g/kg/d protein. Subjects were not allowed to consume anything other than water throughout the study day. All study days were separated by at
least 1 week for each subject and all studies were completed within three months (Roberts et al. 2001).

2.2.3 Study Diets

The standardized diet 2 days prior to the study day was provided in the form of six milkshakes (three milkshakes per day) (Scandishake; Scandipharm, Birmingham, AL), which were weighed in daily portions for each subject and supplemented with additional protein (BeneProtein; Nestle HealthCare Nutrition, Inc., Florham Park, NJ) to make it up to 1.0 g/kg/d, and energy (Polycose; Abbott Laboratories, Abbott Park, IL), depending on each subject’s requirement (1.7 x REE), as determined on the preliminary study day. Subjects were instructed to add a predetermined volume (measuring cup provided) of homogenized milk (containing 3.25% fat) to their dry daily portion of milk-shakes and to drink the milk shakes at regular times throughout the day (Humayun et al. 2007). Similarly to the study day, subjects were instructed not to consume anything in addition to their milkshakes, with the exception of water, black tea or black coffee.

The study day diets were developed by Zello et al. (1990a) during amino acid kinetic studies. The study day diet consisted of a liquid formula containing protein-free powder (PFDI; Mead Johnson, Evansville, IN), flavoring crystals (Tang and Kool-Aid; Kraft, Don Mills, Canada), corn oil, the crystalline amino acid mixture, and protein-free cookies (Appendix B 81). The crystalline amino acid mixture provided the only amino nitrogen in the diet and was based on the amino acid composition of egg protein. The amino acid content (g amino acid mixture/kg body weight) of each study day was calculated individually for each subject to ensure equal amounts of nitrogen per kg of body weight (Zello et al. 1990a). The carbohydrate content of each meal was adjusted according to the subject’s protein intakes to give isocaloric diets. The
study diet provided energy at 1.5 x REE with 35-36% of dietary energy from fat, 50-52% from carbohydrates, and 11-12% from protein. Subjects also consumed a daily multivitamin supplement for the duration of the study, however they were instructed not to take the supplement on either of the two study days. Subjects were encouraged to consume water between meals to ensure production of urine for study day samples. All diets were prepared in a metabolic kitchen and portioned into isoenergetic, isonitrogenous meals (Humayun et al. 2007).

2.2.4 Amino Acid Composition

The crystalline amino acid mixture was based on the pattern present in egg protein (Table 6) and provided at 1.0 g/kg/d. One study day test diet corresponded to the amino acid pattern present in egg protein, in which the glutamate and glutamine amounts were present as glutamate (113 mg/kg/d). The second study day test diet corresponded to the amino acid pattern in egg protein with all glutamate and glutamine removed (0 mg/kg/d), and with serine added in their place to make the study day diets isonitrogenous (1.0 g/kg/d). Serine was added to the study day test diet on a grams of nitrogen basis and not on a milligrams of protein basis.
### Table 6: Amino Acid Pattern in Egg Protein

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Reference Protein</th>
<th>0.3 g/kg protein</th>
<th>0.9 g/kg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>61.4</td>
<td>18.4</td>
<td>55.3</td>
</tr>
<tr>
<td>L-Arginine-HCL</td>
<td>75.1</td>
<td>22.5</td>
<td>67.6</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>33.3</td>
<td>10.0</td>
<td>30.0</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>33.3</td>
<td>10.0</td>
<td>30.0</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>22.1</td>
<td>6.6</td>
<td>19.9</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>56.5</td>
<td>17.0</td>
<td>51.0</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>56.6</td>
<td>17.0</td>
<td>51.0</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>33.3</td>
<td>10.0</td>
<td>30.0</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>22.7</td>
<td>6.8</td>
<td>20.4</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>62.8</td>
<td>18.9</td>
<td>56.6</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>83.3</td>
<td>25.0</td>
<td>75.0</td>
</tr>
<tr>
<td>L-Lysine-HCL</td>
<td>75.7</td>
<td>22.7</td>
<td>68.1</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>29.6</td>
<td>8.9</td>
<td>26.7</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>54.7</td>
<td>30.5</td>
<td>30.5</td>
</tr>
<tr>
<td>L-Proline</td>
<td>41.9</td>
<td>12.6</td>
<td>37.7</td>
</tr>
<tr>
<td>L-Serine</td>
<td>83.9</td>
<td>25.2</td>
<td>75.5</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>47.1</td>
<td>14.1</td>
<td>42.4</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>15.6</td>
<td>4.7</td>
<td>14.0</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>40.7</td>
<td>40.7</td>
<td>40.7</td>
</tr>
<tr>
<td>L-Valine</td>
<td>70.3</td>
<td>21.1</td>
<td>63.2</td>
</tr>
</tbody>
</table>

1. Represents egg protein composition.
2. Actual concentration of amino acids in HCl form in amino acid mixture: arginine, 62.1 mg/g, and lysine, 60.6 mg/g.
3. L-Phenylalanine intake was kept constant at 30.5 mg/kg/day at all protein intakes.
4. L-Tyrosine intake was kept constant at 40.7 mg/kg/day at all protein intakes.

Footnote – Table modified from Humayun et al. 2007.

#### 2.2.5 Tracer Protocol

The study day test diet was given as eight hourly meals, each isocaloric, isonitrogenous and representing one-twelfth of each subject’s daily requirements (based on a 12 hour fasted – 12 hour fed cycle). In each study day the subjects consumed four hourly meals before the start of the oral tracer infusion protocol and four hourly meals after the start (Figure 7) (Bross et al. 1998).

The fifth meal of the study day commenced the tracer protocol with an oral priming dose of 0.176 mg/kg NaH$^{13}$CO$_3$ (99 APE; Cambridge Isotope Laboratories) given simultaneously with 0.66 mg/kg L-[1-$^{13}$C]phenylalanine [99 atom% excess (APE); Cambridge Isotope laboratories,
Woburn, MA]. The priming and infusion doses of isotope were directly added to the liquid meals and thus the isotope was ingested orally. The tubes containing the isotope were thoroughly rinsed with water three times, with the washes added to the meals. The remaining three meals contained the same isotope dose of 1.2 mg/kg L-[1-\textsuperscript{13}C]phenylalanine. The amount of L-[1-\textsuperscript{13}C]phenylalanine given during the isotope tracer protocol in the afternoon of the study day was subtracted from the dietary provision of phenylalanine to give a total daily intake (30.5 mg/kg/day), with an excess amount of tyrosine intake (40 mg/kg/day) (Bross et al. 1998). Isotope solutions were prepared with deionized water and stored in a 4°C fridge.

![Figure 7: IAAO Study Day Protocol](image)

**Figure 7: IAAO Study Day Protocol**

### 2.2.6 Sample Collection and Analysis

Baseline samples of breath carbon dioxide were collected 45, 30, and 15 minutes before the isotope protocol began (Figure 7). Baseline urine samples were collected 45 and 15 minutes before the isotope protocol began. A background isotopic steady state was achieved in all subjects within 4 hours of the commencement of feeding (Bross et al. 1998). After the subjects
had reached isotopic steady state, additional breath carbon dioxide samples were collected 2.5 hours and 3 hours after the tracer protocol began. The remaining four breath samples were collected every 15 minutes, to give a total of six samples during isotopic steady state, and nine breath samples throughout the study day. Isotopic steady state has been previously achieved with this sampling protocol (Bross et al. 1998, Kriengsinyos et al. 2002). We have achieved a similar pattern in the current study (Figure 8), neither the slope of the plateau enrichment in breath nor the baseline enrichment in breath were statistically different from zero. The four urine samples were collected at 30-minute intervals during isotopic steady state, starting 2.5 hours after isotope administration began, to give a total of six urine samples throughout the study day. Breath samples were collected in triplicate in disposable Exetainers (Labco Limited, U.K.), which use a collection mechanism that allows the removal of dead-air space. Carbon dioxide production was measured during each study day for 20 minutes with a variable-flow indirect calorimeter (Vmax 29n; Sensormedics). Breath samples were stored at room temperature until they were analyzed. Each urine sample was mixed with 200 µL of 30% HCl as a preservative and stored at -80°C until it was analyzed.

**Figure 8 Breath $^{13}$CO$_2$ Enrichment**
Breath $^{13}$CO$_2$ enrichment in atoms percent (APC $^{13}$C) of nine timed samples from a typical subject. Baseline and isotopic steady state plateaus were established on the basis of no significant differences among timed samples and confirmed with regression analysis.
Enrichment of $^{13}$C in breath was analyzed by multi-flow continuous-flow isotope ratio mass spectrometry (IsoPrime Limited; United Kingdom). All analyses were performed in triplicate. Enrichments were expressed as atoms % excess (APE) compared with a reference standard of compressed carbon dioxide gas. Isotopic enrichment was expressed as molecule % excess and was calculated from peak area ratios at isotopic steady state at plateau and baseline (Bross et al. 1998). The rate of $^{13}$CO$_2$ released by phenylalanine tracer oxidation in breath samples was then calculated for each study day using the tracer kinetics Equation 1 (page 28).

2.2.7 Statistical Analysis

A paired-samples t-test was conducted (SPSS version 22) to compare L-[1-$^{13}$C]-phenylalanine oxidation as F$^{13}$CO$_2$ in breath samples in the two glutamate intakes. Statistical significance was set at 5% level of significance (P<0.05).

2.3 Results

Seven subjects (Table 7) completed both study days with corresponding study day diets. There were no statistical tests done on the additional glutamate test intakes due to the small and variable sample sizes. The mean F$^{13}$CO$_2$ values were calculated using Equation 1 (page 28) for the two test diet intakes and statistical tests were done. A Shapiro-Wilk test for normality was conducted for 0 mg/kg/d glutamate (p = 0.890) and 113 mg/kg/d glutamate (p = 0.596). The F$^{13}$CO$_2$ results are consistent with a Gaussian distribution (p>0.05). There was not a significant difference in F$^{13}$CO$_2$ for 0 mg/kg/d (Mean = 0.4296, Standard Deviation (SD) = 0.0775) and 113 mg/kg/d (Mean = 0.3763, SD = 0.0736) intakes; t(6) = 1.257, p = 0.255 (Figure 9, page 43).

The individual responses in IAAO due to the removal of glutamate (and glutamine) were mixed; two subjects had a numerically higher IAAO (Glu-03, Glu-06), two subjects had
numerically lower IAAO (Glu-04, Glu-07) and three subjects had no change in IAAO (Glu-01, Glu-05, Glu-09) (Figure 9).

Table 7: Glutamate Study Subject Characteristics

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Age (year)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>BMI (kg/m²)</th>
<th>REE (kcal/day)</th>
<th>REE x 1.7 (kcal/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-01</td>
<td>22</td>
<td>95.1</td>
<td>188</td>
<td>26.9</td>
<td>2033</td>
<td>3456</td>
</tr>
<tr>
<td>Glu-03</td>
<td>20</td>
<td>75.3</td>
<td>176</td>
<td>24.2</td>
<td>1772</td>
<td>3012</td>
</tr>
<tr>
<td>Glu-04</td>
<td>20</td>
<td>65.2</td>
<td>172</td>
<td>22.0</td>
<td>1498</td>
<td>2547</td>
</tr>
<tr>
<td>Glu-05</td>
<td>23</td>
<td>75.6</td>
<td>181</td>
<td>23.1</td>
<td>1640</td>
<td>2788</td>
</tr>
<tr>
<td>Glu-06</td>
<td>22</td>
<td>85.8</td>
<td>183</td>
<td>25.6</td>
<td>1695</td>
<td>2882</td>
</tr>
<tr>
<td>Glu-07</td>
<td>23</td>
<td>85.5</td>
<td>190</td>
<td>23.7</td>
<td>1786</td>
<td>3036</td>
</tr>
<tr>
<td>Glu-09</td>
<td>21</td>
<td>75.0</td>
<td>173</td>
<td>25.5</td>
<td>1669</td>
<td>2837</td>
</tr>
<tr>
<td>Mean</td>
<td>22</td>
<td>79.6</td>
<td>181</td>
<td>24.4</td>
<td>1728</td>
<td>2937</td>
</tr>
<tr>
<td>SD</td>
<td>1.27</td>
<td>9.80</td>
<td>7.09</td>
<td>1.68</td>
<td>165.09</td>
<td>280.66</td>
</tr>
</tbody>
</table>
Figure 9: F$^{13}$CO$_2$ for 0 and 113 mg Glutamate Intakes

n = 7; F$^{13}$CO$_2$ data for two test intakes of 0 mg/kg/day Glx and 113 mg/kg/day Glu. Where Glx = glutamate + glutamine. 113 mg/kg of glutamate represents the amount of Glx present in egg protein. Serine was used to make the diets isonitrogenous; protein = 1 g/kg/day; energy = 1.5 x REE. There was not a significant difference in F$^{13}$CO$_2$ for 0 mg/kg/d and 113 mg/kg/d (p=0.255).

2.3.1 Additional Test Diets

After the completion of 14 study days, we added two additional glutamate intakes of 25 and 75 mg/kg/d for three subjects (Glu-03, Glu-06, Glu-09) who showed the largest increases in IAAO due to the removal of glutamate (and glutamine). We included additional intakes to determine if a full IAAO breakpoint analysis would be possible for the NEAA, glutamate. Traditionally, removal of an EAA from the diet would increase IAAO significantly. That was not the case in the current study. Three subjects completed the 25 mg/kg/d test diet and two subjects completed the 75 mg/kg/d test diet, for a total of 19 study days. The mean F$^{13}$CO$_2$ of all study day test diets are plotted Figure 10. The study day test diets were completely randomized for
each subject. The mean $F^{13}\text{CO}_2$ values were calculated for the two additional test diet intakes but statistical analysis was not done due to the very small sample sizes.

![Figure 10: Mean $F^{13}\text{CO}_2$ for Four Glutamate Intakes](image)

Mean values ± Standard Deviation for four test intakes of glutamate. Glu= glutamate. n=7 (navy) for 0 and 113 mg/kg/d Glu; n=3 (red) for 25 mg/kg/d Glu; n=2 (green) for 75 mg/kg/d Glu.

### 2.3.2 Additional Urinary Analysis

Analysis of the urine samples from each study day is currently underway by Erin Gilbert, a research assistant in the Elango Lab. Urinary concentration of amino acids and related metabolites (such as urea, hydroxyproline, etc.) will be analyzed, expressing the results in nmol, with an amino acid analyzer (AAA) (Hitachi L-8900; Tokyo, Japan). Normalization of the AAA data to the creatinine concentration (µmol /g creatinine) will be done with a high-performance liquid chromatography (HPLC) (Hitachi Chromaster; Tokyo, Japan).

### 2.4 Discussion

With removal of glutamate (and glutamine) from the diet the oxidation of the indicator amino acid did not go up significantly in the current study. If we had seen high oxidation in
response to the removal of glutamate and glutamine, this would have indicated low protein synthesis and a deficiency in the diet. A significant increase in the rate of $^{13}$CO$_2$ released by phenylalanine tracer oxidation is commonly seen due to removal of an EAA, such as lysine. For example, in a similar study design to the current study, the mean ± SD $F^{13}$CO$_2$ of seven males fed a lysine intake of 30 and 5 mg/kg were 0.328 ± 0.022 and 0.526 ± 0.052 µmol/kg/h, respectively (Zello et al. 1993). This is a much greater difference in $F^{13}$CO$_2$ compared to the current study data at 113 and 0 mg glutamate, mean ± SD equal to 0.376 ± 0.073 and 0.429 ± 0.077 µmol/kg/h, respectively. The previous study had a mean difference in $F^{13}$CO$_2$ of 0.198 µmol/kg/h, and the current study only had a mean difference of 0.053 µmol/kg/h. There was no significant increase in the rate of $^{13}$CO$_2$ released by phenylalanine tracer oxidation when glutamate was removed from the diet, thus the body was capable of sufficient de novo synthesis of glutamate, even in the absence of glutamine, for protein synthesis to occur in healthy adult males. A study in adult men by Matthews and Campbell (1992) showed an inverse relationship between glutamate (and glutamine) plasma concentrations and protein intake, and an inverse relationship to $^{15}$N-glutamine flux and protein intake. They attributed these changes to changes in de novo synthesis of the amino acids in response to protein intake. This study supports our conclusion that when a dietary source of protein and glutamate is low, the body will increase the de novo synthesis to fulfill the demand.

Previous studies comparing growth effects in growing rats showed that glutamate gave the largest effect on growth when supplemented to a base diet of only the EAA. (Recheigl et al. 1957). This is especially interesting, because glutamate as a nitrogen source had larger growth effects than solely a diet of EAA, indicating the need for NEAA as nitrogen sources in the diet. These results further confirmed earlier studies by Rose et al. (1948), which showed increased
growth in rats when glutamate was added to the exclusively EAA diet. From the opposite perspective, more recent studies in rats and pigs indicated small decreases in growth rates when animals were fed diets devoid of glutamate (Reeds 2000). The effectiveness of nitrogenous compounds, such as glutamate, to supply nitrogen for the de novo synthesis of NEAA depends on its ability to supply an amino group. The central role of glutamate and its α-keto acid derivative, α-ketoglutarate, may explain its efficiency as a nitrogen source (Figure 1, page 7). Thus in actively growing animals, glutamate might support additional growth; but in adult men, it is possible that glutamate is not necessary through diet.

It is commonly known that glutamate is not only an important precursor for other biological molecules, but also one of the main oxidative fuels for the gut. Many studies have shown that glutamate is extensively and almost completely metabolized during first pass in the gut mucosa, specifically in the enterocytes, with carbon dioxide as the major product (Burrin et al. 2009). The splanchnic bed gets a majority of its energy from the catabolism of amino acids, specifically from glutamine, glutamate, and aspartate (Windmueller and Spaeth 1980). Studies in pigs fed high protein diets showed > 95% of glutamate is metabolized by the gastrointestinal tract mucosa and 50% of it is oxidized to carbon dioxide (Reeds et al. 2000). Additionally, it has been shown in piglets that even when glutamate is fed 3-to-4 fold higher than normal, the majority is oxidized in the gut (Janeczko et al. 2007). It has also been suggested by Burrin et al. (2009) that the stomach also has glutamate transport and absorption capacity, which may have implications for infants and children fed free amino acid based diets. The diets in the present glutamate study were in the form of free amino acid mixtures. Thus it appears likely that endogenous glutamate would have to be supplied to the entire gastrointestinal tract to be used as an energy source when dietary glutamate levels are low. All other tissues in the body must rely
on the endogenous synthesis of glutamate because of the extensive first pass metabolism of all dietary glutamate by the gut. Thus, glutamate might be crucial as a dietary source in growing animals and humans, but under our study conditions which had sufficient calories from carbohydrates and fat, and sufficient nitrogen from other amino acids, endogenous synthesis would have been sufficient.

Glutamate is synthesized in the body in multiple ways. Glutamate dehydrogenase and aminotransferases are capable of endogenous synthesis of glutamate from α-ketoglutarate. Glutamate can also be synthesized from amino acids of the “glutamate family,” specifically glutamine, arginine, ornithine, proline, and histidine (Brosnan and Brosnan 2013). An important point is that all these enzymes are reversible in the liver, which enables a tight control on glutamate catabolism and anabolism to meet the needs of the TCA cycle as well as the urea cycle (Brosnan and Brosnan 2009). A study in perfused rat livers by Haussinger and Gerok (1983) showed synthesis of α-ketoglutarate from pyruvate when glutamate was not provided. It is presumed that this reaction is performed by phosphoenolpyruvate carboxykinase and that the α-ketoglutarate is later aminated into glutamate (Brosnan and Brosnan 2009). Thus, in the absence of dietary glutamate, the liver is capable of synthesizing glutamate from many different compounds, including other amino acids, which is possibly why we did not see an effect due to removal of glutamate as a dietary source in adult men.

In summary, the IAAO technique did not show a significant (P>0.05) difference in tracer L-[1-13C]-phenylalanine oxidation between the two glutamate intakes. On the basis of these results, we predict that a glutamate requirement from a dietary perspective cannot be determined in adult men under conditions of adequate nitrogen supply. We suggest that because glutamate plays a central role in metabolism, the body has maintained pathways for sufficient and adequate
de novo synthesis of this very important “nonessential amino acid.” Therefore we proceeded to the second study, the Nonessential Amino Acid study, which focuses on comparing the nine NEAA as an adequate source of nitrogen for the de novo synthesis of other NEAA as well as protein synthesis in healthy adult males.
Chapter 3: Nonessential Amino Acid Metabolism

3.1 Nonessential Amino Acids

All 20 amino acids found in body protein in humans have a ‘metabolic demand.’ Although only the EAA have been extensively studied because the Dietary Reference Intakes (DRI) sets recommendations, including the EAR and RDA, for the general population. However, Katagiri and Nakamura (2002, 2003) recently put forth the argument that there could be a dietary requirement for pre-formed α-amino nitrogen in the form of specific NEAA. Wu et al. (2013) note that all NEAA are essential for growth, lactation, development and health of all animals. They also voiced the opinion that there could be a ‘nutritional’ need for NEAA in growth and development. Decreased protein or nitrogen intakes can lead to the conversion of EAA to NEAA through pathways, which have been conserved throughout evolution, indicating the vital functions NEAA have in the body and that this can be influenced by a person’s age, sex, activity level, and BMI (Devlin 2011). NEAA are the precursors for many metabolites and peptides, many of which are required for maintaining physiological homeostasis (Table 3, page 19) (Gibson et al. 2002). Whether all NEAA are similar in their potential to act as a nitrogen source for endogenous synthesis of other NEAA still remains unknown.

The total protein requirement in adult men was measured using the IAAO technique, and was determined to be 0.93 g/kg/day (Humayun et al. 2007). Interestingly the summation of all the dietary EAA requirements, determined previously using the IAAO method (Elango et al. 2012), only equals 0.2326 g/kg/day. This suggests that a significant portion of the demand for total protein/nitrogen is met by the NEAA. Studies in growing rats by Recheigl et al. (1957) showed that addition of various nitrogen sources had different effects on growth; with some NEAA showing more positive growth response than others. Whether some NEAA are more
efficient in meeting this demand for total nitrogen needs to be determined in humans. Since
growth and the classic nitrogen balance studies are impractical in humans, alternative methods
need to be used. We decided to use the minimally invasive IAAO methods in adult men to test
the hypothesis that some NEAA may be a preferred source of nitrogen for protein synthesis.

3.2 Methods

3.2.1 Subjects

Seven healthy adult men (Table 8, page 56) were chosen as subjects. All subjects were
selected after collecting medical history, dietary habits, as well as activity levels (Bross et al.
1998). Subject body compositions, and REEs were measured in the same way as in the previous
Glutamate Study (Appendix D.2 Preliminary Study Day Form, page 92). Similarly, informed
written consent was required from all subjects before participating in the study (Appendix D.1
Subject Consent Form, page 85). Subjects were offered financial compensation for their time at
the end of each study day.

3.2.2 Dietary and Energy Intakes

The same adaptation protocol as used in the Glutamate Study was followed in the
Nonessential Amino Acid Study with diets containing a daily protein intake of 1.0 g/kg/d and 1.7
× REE for the 2 days prior to the test day. Throughout the adaptation period, subjects were asked
to maintain their habitual level of physical activity. Before the start of each study day, subjects
were instructed to fast for 10-12 hours prior to their first study day meal (Bross et al. 1998). All
seven subjects participated in ten study days, assigned in random order, corresponding to
different study day test diets. Five of the subjects completed an additional eleventh study day, for
a total of 75 study days. All amino nitrogen in the test diets was from free amino acid mixtures
made in the laboratory. One study day corresponded to a base diet, consisting of only the EAA
provided at the recommended dietary allowance (RDA), previously determined by the IAAO method as seen in Table 5 (page 27). On the remaining study days, each subject received a test diet containing the Base diet with the addition of a single NEAA (one of Ala, Arg, Asn, Asp, Gln, Glu, Gly, Pro, Ser). The additional eleventh study day completed by five subjects had a test diet containing the Base diet with the addition of a mix of all nine NEAA (NEAA Mix) following the pattern in egg protein. Each diet was calculated on a gram nitrogen basis to ensure all diets were isonitrogenous. In total there were eleven different study day test diets: EAA only at RDA (Base), EAA+Ala, EAA+Arg, EAA+Asn, EAA+Asp, EAA+Gln, EAA+Glu, EAA+Gly, EAA+Pro, EAA+Ser, and EAA+NEAA Mix (Appendix A Experimental Design, page 80).

The study diets were given as eight isocaloric hourly meals, with each meal representing one-twelfth of the subject’s totally daily energy requirement (Appendix D.3 Study Day Form, page 94). These meals consisted of different crystalline amino acid compositions, as outlined above, and an energy intake of 1.5 × REE. Subjects were not allowed to consume anything other than water throughout the study day. All study days were separated by at least 1 week for each subject and all studies were completed within 4 months (Roberts et al. 2001).

### 3.2.3 Study Diets

The standardized diet 2 days prior to the study day was provided in the form of six milk shakes (three milkshakes per day) (Breakfast Essentials; Nestlé Canada Inc., North York, ON), which were weighed in daily portions for each subject and supplemented with additional protein (BeneProtein; Nestle HealthCare Nutrition, Inc., Florham Park, NJ) and energy (Polycose; Abbott Laboratories, Abbott Park, IL), depending on each subject’s requirement (1.7 x REE), as determined on the preliminary study day (Humayun et al. 2007). Subjects were instructed to add a predetermined volume (measuring cup provided) of homogenized milk (containing 3.25% fat)
to their dry daily portion of milk shakes and to drink the milk shakes at regular times throughout the day (Humayun et al. 2007). Similarly to the study day, subjects were instructed not to consume anything in addition to their milkshakes, with the exception of water, black tea or black coffee.

The same study day diets developed by Zello et al. (1990a), as detailed in the Glutamate Study (Chapter 2: Section 2.2.3, page 36), were used in this study. The study day diet consisted of a liquid formula containing protein-free powder (PFDI; Mead Johnson, Evansville, IN), flavoring crystals (Tang and Kool-Aid; Kraft, Don Mills, Canada), corn oil, the crystalline amino acid mixture, and protein-free cookies (Appendix B 81). The carbohydrate content of each meal was adjusted according to the subject’s protein intakes to give isocaloric diets. The Base study diet provided energy at 1.5 x REE with 39-40% of dietary energy from fat, 54-55% from carbohydrates, and 5-6% from protein. The Base plus one NEAA study diets provided energy at 1.5 x REE with 37-38% of dietary energy from fat, 51-52% from carbohydrates, and 9-11% from protein. The Base plus the NEAA Mix study diet provided energy at 1.5 x REE with 38-39% of dietary energy from fat, 53-54% from carbohydrates, and 6-8% from protein. Subjects also consumed a daily multivitamin supplement for the duration of the study, except on the actual study days. Subjects were encouraged to consume water between meals to ensure production of urine for study day samples. All diets were prepared in a metabolic kitchen and portioned into isoenergetic, isonitrogenous meals (Humayun et al. 2007).

3.2.4 Amino Acid Composition

The crystalline amino acid mixture provided the only amino nitrogen in the diet. The Base diet followed the RDA of the EAA as determined with the IAAO method (Table 5, page 27) (Elango et al. 2012). The crystalline amino acid mixture of the NEAA Mix followed the
amino acid composition of egg protein (Table 6, page 38). The amino acid content (g amino acid mixture/kg body weight) of each study day was calculated individually for each subject to ensure equal amounts of nitrogen per kg of body weight (Zello et al. 1990a). The crystalline amino acid mixture for the base diet corresponded to a total protein intake of 0.39942 g/kg body weight. The remaining study day diets contained the base diet with the addition of one NEAA or the NEAA Mix. However the amount of phenylalanine and tyrosine were kept constant to give a total daily intake of 30.5 mg/kg/d and 40 mg/kg/d, respectively, for each subject throughout all study days. The amount of individual NEAA or NEAA Mix added to the Base diet equated 50:50 ratio of EAA: NEAA and gave a daily protein intake of 0.73 g/kg body weight for each subject. This diet design ensured there was a sufficient amount of the EAA provided but the total nitrogen content of the diet was low.

There were two reasons for selecting diets low in total nitrogen, 0.73 g/kg is lower than the EAR for healthy adult men of 0.93 g/kg. The primary reason was to ensure the safety of the subjects participating in the study. We did not want to add a very large amount of individual NEAA to each test diets, especially since the upper limit for many of these NEAA has not been established. The second rationale for choosing a nitrogen intake lower than the EAR was to ensure we measured the $^{13}$CO$_2$ in breath during the linear decline phase of the IAAO curve (Figure 6, page 26), where the total nitrogen requirement has not been met for most adult males. After the nitrogen requirement is met the $^{13}$CO$_2$ data begin to plateau and there can be a lot of noise or variation within the plateau. We decided that for this study design we wanted to measure $^{13}$CO$_2$ where we knew the requirement had not yet been met and we could feel confident that the decrease in $^{13}$CO$_2$ was due to the addition of a NEAA in the diet.
The study day protocol from the Glutamate Study was followed (Figure 7, page 39). The studies had similar design in the timing of eight hourly meals and sample collections during baseline in the morning as well as during isotopic steady state in the afternoon. The only difference was more study days (10) for each subject in the NEAA Study.

3.2.5 Tracer Protocol

The same tracer protocol as in the Glutamate Requirement Study was followed (Section 2.2.5, page 38). A prime of NaH\(^{13}\)CO\(_3\) (99 APE; Cambridge Isotope Laboratories) was given simultaneously as a primed and continuous infusion of L-[1-\(^{13}\)C]phenylalanine [99 APE; Cambridge Isotope Laboratories, Woburn, MA].

3.2.6 Sample Collection and Analysis

The same sample collection and analysis as in the Glutamate Requirement Study was followed (Section 2.2.6, page 39). Baseline samples of breath carbon dioxide were collected 45, 30, and 15 minutes before the isotope protocol began (Figure 7, page 39). Baseline urine samples were collected 45 and 15 minutes before the isotope protocol began. After the subjects had reached isotopic steady state, additional breath carbon dioxide and urine samples were collected.

3.2.7 Statistical Analyses

A Shapiro-Wilk test for normality was conducted before the analysis. Prior to conducting the analysis to determine if differences in the mean F\(^{13}\)CO\(^2\) exist between the different test intakes, a Mauchly’s test was run to test the hypothesis that the variances of the differences between the different test diets are equal. A one way repeated measures ANOVA (GraphPad Prism version 6.0F) was chosen for the analysis to detect overall differences in the mean F\(^{13}\)CO\(^2\) for different test intakes. Repeated measures analysis was chosen because the results are not
independent; subjects participated all ten study days, thus their results from each day are related and a repeated measures design helps to control subject variation.

It is important to note that the NEAA Mix intake was excluded from statistical analysis, however the data remains plotted in Figure 11 and Figure 12 (pages 57 and 58). The NEAA Mix was excluded from the analysis due to the unequal sample size, which a repeated measures ANOVA would be sensitive to. The NEAA Mix intake was originally planned to be a positive control but after analysis of the study diet macronutrients, it was found that the % protein in the diet was lower than the other NEAA addition intakes (9-11% compared to 6-8% protein). This was due to the calculation of test intakes on a grams nitrogen basis and not on a grams protein basis in the diets. These were sufficient reasons to omit the NEAA Mix intake from the statistical analysis in this study.

A Post-hoc Dunnett’s test was chosen because it is used in special multiple comparisons studies to compare treatment groups, i.e. different NEAA intakes, to a control group, i.e. the Base diet. Dunnett’s test is designed to control type 1 error to below $\alpha = 0.05$ when making multiple comparisons of the treatment groups to the control group (Paulson 2003). Both the Dunnett’s test and the Bonferroni T tests are based on inequalities of the form family wise error $\leq 0.05$, however but Dunnett’s test is more powerful when comparing treatments to a control because it uses a sharper inequality. Therefore the Dunnett’s test needs a smaller difference in the means of the treatments and the control to reject the null hypothesis of no difference (Howell 2013). Additionally, the validity of the Bonferroni t rests on the assumption of homogeneity of variance because it calculates the error term by pooling the variance over all conditions (Elder 2011). This would not be applicable to this study’s results and thus Dunnett’s test is a more appropriate choice for a post-hoc analysis.
3.3 Results

Seven subjects (Table 8) completed ten study days and five of the seven subjects completed an additional eleventh study day, containing the NEAA Mix test intake. The $F^{13}CO_2$ was calculated using Equation 1 (page 28) for each study day and the values (mean ± SD) of the 11 test intakes are plotted in Figure 11. Overall, there was a visible decrease in IAAO from the Base diet (EAA) to the addition of each NEAA or NEAA Mix (Figure 12, page 58). Most NEAA had significant decreases in IAAO compared to others, notably alanine, arginine, aspartic acid, asparagine glutamic acid, glycine, and serine. Only glutamine and proline were not significant as nitrogen sources for protein synthesis in healthy adult males.

Table 8: NEAA Study Subject Characteristics

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Age (year)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>BMI (kg/m²)</th>
<th>REE (kcal/day)</th>
<th>REE x 1.7 (kcal/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEAA-01</td>
<td>22</td>
<td>72.4</td>
<td>170</td>
<td>25.1</td>
<td>1547</td>
<td>2630</td>
</tr>
<tr>
<td>NEAA-02</td>
<td>20</td>
<td>66.8</td>
<td>177</td>
<td>21.3</td>
<td>1397</td>
<td>2375</td>
</tr>
<tr>
<td>NEAA-03</td>
<td>21</td>
<td>70.9</td>
<td>184</td>
<td>20.9</td>
<td>1741</td>
<td>2960</td>
</tr>
<tr>
<td>NEAA-04</td>
<td>21</td>
<td>60.0</td>
<td>167</td>
<td>21.5</td>
<td>1451</td>
<td>2467</td>
</tr>
<tr>
<td>NEAA-07</td>
<td>24</td>
<td>79.0</td>
<td>186</td>
<td>22.8</td>
<td>1850</td>
<td>3145</td>
</tr>
<tr>
<td>NEAA-08</td>
<td>20</td>
<td>77.7</td>
<td>173</td>
<td>26.0</td>
<td>1669</td>
<td>2837</td>
</tr>
<tr>
<td>NEAA-14</td>
<td>20</td>
<td>60.5</td>
<td>173</td>
<td>20.2</td>
<td>1503</td>
<td>2555</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>21</strong></td>
<td><strong>69.6</strong></td>
<td><strong>176</strong></td>
<td><strong>22.5</strong></td>
<td><strong>1594</strong></td>
<td><strong>2710</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>1.46</td>
<td>7.60</td>
<td>7.06</td>
<td>2.21</td>
<td>164.58</td>
<td>279.75</td>
</tr>
</tbody>
</table>
Figure 11 Mean $\text{F}^{13}\text{CO}_2$ for Eleven Test Intakes

Mean $\text{F}^{13}\text{CO}_2 \pm 1$ SD ($\mu\text{mol/kg/h}$) responses to 11 NEAA diets ($n=7$; except +NEAA Mix where $n=5$). The base EAA diet (red) consisted of only the EAA at the recommended dietary allowance (0.39 g/kg/d) as previously determined by the IAAO method. Additional study diets (blue) consisted of the EAA diet + one NEAA (0.73 g/kg/d). The NEAA Mix (green) consisted of EAA + all NEAA (0.73 g/kg/d) following the pattern in egg protein. The NEAA Mix was omitted from statistical analysis. There was a significant difference in mean $\text{F}^{13}\text{CO}_2$ for ALA, ARG, ASP, ASN, GLU, GLY, and SER when compared to the EAA only diet, $p$-values $<0.05$. 
Figure 12 Individual $^{13}$CO$_2$ for Eleven Test Intakes
Individual and Mean $^{13}$CO$_2$ ($\mu$mol/kg/h) responses to 11 NEAA diets (n=7; except NEAA Mix where n=5). The base EAA diet consisted of only the EAA at the recommended dietary allowance (0.39 g/kg/d) as previously determined by the IAAO method. Additional study diets consisted of the EAA diet + one NEAA (0.73 g/kg/d). The NEAA Mix consisted of EAA + all NEAA (0.73 g/kg/d) following the pattern in egg protein.

3.3.1 Statistical Analyses

A Shapiro-Wilk test for normality was conducted and all test intake $^{13}$CO$_2$ results were consistent with a Gaussian distribution (p > 0.05). The assumption of sphericity had been violated due to the repeated measures design of the experiment; therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.3324$).

A repeated measures ANOVA with a Greenhouse-Geisser correction determined that the mean $^{13}$CO$_2$ differed statistically significantly between test amino acid intakes intakes F (2.991, 17.95) = 4.877, p= 0.0119. Post hoc tests using Dunnett’s revealed that alanine (p = 0.0218), arginine (p = 0.0221), aspartic acid (p = 0.0017), asparagine (p = 0.0263), glutamic acid (p = 0.0381), glycine (p = 0.0454), and serine (p = 0.0250) were all significantly lower than the Base.
diet. However, glutamine and proline were also lower than the Base but not significantly different (p = 0.4297 and p = 0.1021, respectively). Therefore we can conclude that alanine, aspartate, asparagine, arginine, glutamate, glycine, and serine are more available nitrogen sources compared to glutamine and proline for protein synthesis in adult males.

3.3.2 Additional Urinary Analysis

Analysis of the urine samples from each study day is currently underway following the same protocol as detailed in the Glutamate Study (Section 2.3.2, page 44). Urinary concentrations will be analyzed with an AAA (Hitachi L-8900; Tokyo, Japan) and then normalized to creatinine with a HPLC (Hitachi Chromaster; Tokyo, Japan).

3.4 Discussion

Using the IAAO technique in healthy adult men, there is a significant difference in the rate of tracer L-[1-13C]-phenylalanine oxidation between alanine, arginine, aspartate, asparagine, glutamate, glycine, and serine test intakes when compared to the Base diet composed of only the EAA. On the basis of these results, we suggest that these seven NEAA supply nitrogen for protein synthesis better than glutamine and proline. Additionally, we predict that these seven NEAA may be more important than the remaining two NEAA from a dietary perspective in healthy adult males under conditions of adequate EAA as well as adequate nitrogen supply.

It is interesting to discover that glutamine is not an efficient nitrogen supplier in adult males when all EAA are provided in the diet. Glutamine plays many important roles in amino acid metabolism and is easily converted to glutamate (Table 9, page 60). Thus, because of its crucial roles in the body (Young and Ajami 2001), one would expect a large decrease in tracer oxidation using the IAAO technique, however the opposite appears to be true and protein synthesis was limited in the body. In the current study, the high rate of tracer oxidation due to the
addition of glutamine to the base diet, indicating low protein synthesis, may have occurred because there was less glutamine provided on a gram basis in the diet compared to other NEAA and thus glutamine itself was limiting protein synthesis. All diets were calculated to be isonitrogenous and because glutamine (C$_5$H$_{10}$N$_2$O$_3$) has an additional nitrogen group, less glutamine in grams was added to the study day diets, compared other NEAA such as glutamate (C$_5$H$_9$NO$_4$). For example, the amount of glutamine added to each meal for NE-01 (the first subject) was 1.817 grams, whereas the amount of glutamate (as they provide carbon skeletons as well) added to each meal for NE-01 was 3.6277 grams. There simply may not have been enough glutamine added to the diet to be sufficient to synthesize all other NEAA needed for protein synthesis in addition to providing enough glutamine for other metabolic processes in the body.

Table 9: Functions of Glutamine

<table>
<thead>
<tr>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate for protein synthesis</td>
</tr>
<tr>
<td>Anabolic/trophic substrate for muscle; intestine</td>
</tr>
<tr>
<td>Controls acid-base balance (renal ammoniagenesis)</td>
</tr>
<tr>
<td>Substrate for hepatic ureagenesis</td>
</tr>
<tr>
<td>Fuel for intestinal enterocytes</td>
</tr>
<tr>
<td>Fuel and nucleic acid precursor and important for generation of cytotoxic products in immunocompetent cells</td>
</tr>
<tr>
<td>Ammonia scavenger</td>
</tr>
<tr>
<td>Substrate for citrulline and arginine synthesis</td>
</tr>
<tr>
<td>Nitrogen donor (nucleotides, amino sugars, coenzymes)</td>
</tr>
<tr>
<td>Nitrogen transport</td>
</tr>
<tr>
<td>Shuttle for glutamate (central nervous system)</td>
</tr>
<tr>
<td>Stimulates glycogen synthesis</td>
</tr>
</tbody>
</table>

Footnote - Table modified from Young and Ajami 2001.

Additionally, all amino acids were provided orally and thus are subject to first pass metabolism. It has been shown that the majority of glutamine, 40-90%, is completely used by
enterocytes and immune cells present along the upper jejunum during first pass metabolism (Burrin and Stoll 2009). Therefore only a portion of enteral glutamine is able to enter the portal blood, of which the liver utilizes another fraction, before the rest enters the systemic circulation (Wernerman 2011). Although glutamate is also extracted by the small intestine, glutamine has been shown to be utilized in the large intestine, where a large amount is taken up from arterial blood. Thus overall glutamine utilization is greater and provides much of the ATP needed for gut integrity (Wu et al. 2011). There must be sufficient glutamine in the diet or formed by de novo synthesis or proteolysis to supply the gut mucosa as well as enter the peripheral circulation to supply other tissues in the body for adequate protein synthesis to occur. Perhaps in the current study there was inadequate glutamine in the diet on a gram basis to fulfill the metabolic demand of the intestine in order to reach other tissues at adequate levels, consequently limiting protein synthesis, as indicated by a no significant decrease in tracer oxidation.

Earlier, Ziegler et al. (1990) performed studies in adult males to test the safety of L-glutamine administration. They supplemented glutamine orally in adult males to study dose responses (0 g/kg, 0.1 g/kg, and 0.3 g/kg) on plasma levels of glutamine. They found glutamine blood concentrations rose proportionally to oral doses; glutamate and ammonia levels also tended to rise with dose but were not significantly different than baseline. Their work is consistent with the study by Matthews and Campbell (1992) describing the relationship of total protein intake and plasma glutamine levels. Interestingly, Ziegler et al. (1990) found that the branched chain amino acids (BCAA; isoleucine, leucine and valine) plasma concentrations fell; with increasing glutamine doses. The authors attributed it to the increased insulin release, which stimulated BCAA uptake in the skeletal muscle for protein synthesis. Conceivably similar pathways were being activated in our current study, which had a similar glutamine intake to the
highest dose in the Ziegler et al. (1990) study. However we did not collect plasma samples in this study and thus are not able to explore whether plasma concentrations of BCAA were affected.

Darmaun et al. (1994) performed similar glutamine supplementation studies in adult men and found a 29% increase in the rate of appearance of plasma glutamine, when compared to a fasted state. The enteral feeding diet contained 1.5 g/kg/day amino acids or peptides, of which 16.3% was glutamine + glutamate. They calculated a 176% rise from baseline in the fraction of glutamine appearing from sources other than proteolysis, i.e. the fraction from de novo synthesis and absorption from the diet. However the methodology lacked the ability to differentiate between the latter two sources. The researchers ascribed the high rates of glutamine appearance in plasma to the important role of glutamine as an interorgan nitrogen carrier in the body. Young and Ajami (2001) have also described the unique and energy efficient role of glutamine as an interorgan nitrogen carrier. It is important to note that compared to our current study, Darmaun et al. (1994) provided a two-fold higher protein intake. In the current study we may have not fed sufficient protein (as we only added 0.73 g/kg/d) or glutamine (on a gram basis compared to the other NEAA) to have excess glutamine entering peripheral circulation after first pass metabolism. Furthermore, we would have needed to test plasma concentrations of glutamine to prove this mechanism, which unfortunately we did not.

During critical illness or after trauma it has been observed that plasma glutamine levels fall due to increased demand and increased glutamine catabolism (Cynober and De Bandt 2014). Glutamine is not only a substrate for protein synthesis, but has many additional functions, which may become activated and increase the metabolic demand after injury (Table 9, page 60) (Young and Ajami 2001). It has become standard practice to supplement glutamine to patients in intensive care when receiving parenteral nutrition (Singer et al. 2009, Wernerman 2011).
However, there is still disagreement on the benefits of glutamine supplementation in the critically ill (Cynober and De Bandt 2014). A recent Cochrane Library review of randomized controlled trials found no beneficial effects of glutamine supplementation in patients after major surgery or with critical illnesses (Tao et al. 2014). However, there is mediocre evidence of reduced infection rates in patients supplemented with glutamine compared to those who were not. But it is possible that there may be some publication bias as well as heterogeneity within studies that impaired the strength of this evidence (Tao et al. 2014). Furthermore, there is a lack of conclusive evidence for recommendations on glutamine supplementation orally or entally for critically ill patients (Wernerman 2011). Hiramatsu et al. (1994) performed nutritional studies in adult men to determine whether glutamine supplementation improved amino acid balance but found no difference from its removal from the diet. As discussed earlier, since most of the glutamine is utilized in first pass, perhaps there is no added benefit for glutamine supplementation. Further knowledge on endogenous glutamine synthesis as well as on control/feedback mechanisms is needed before guidelines for glutamine supplementation can be made (Wernerman 2014).

Earlier studies in growing piglets showed that glutamine supplementation by parenteral nutrition increases live weight gain (House et al. 1994). However, it was discovered in the same study that the gain in weight was due to extracellular fluid expansion and not from differences in the body composition of the piglets. There were no significant differences in the final weights of protein, fat, or ash between the glutamine supplementation group and the control group (House et al. 1994). Because there is debate on whether there are benefits in glutamine supplementation, it seems reasonable to conclude that there is no benefit in the addition of oral glutamine to the diet of a healthy population, provided that there is sufficient EAA and nitrogen (protein) in the diet.
In the current study proline was also not an efficient source of nitrogen for protein synthesis in healthy adult males fed a daily protein intake of 0.73 g/kg/day that supplied the EAA at their RDA. Our results showed no increase in protein synthesis when proline was the source of additional nitrogen added to the base diet of only EAA, as shown by a non significant decrease in tracer oxidation (Figure 11, page 57; Figure 12, page 58). Proline has been suggested to be a functional amino acid in humans and contributes to different physiological and metabolic pathways (Table 10) (Wu et al. 2011). Recently Phang et al. (2013) have described proline as a bridge between metabolism and epigenetics. Furthermore, proline is essential for the physical structure of the human body, accounting for one third of the composition of the abundant collagen proteins found in extracellular connective tissue. It has been suggested that this could lead to proline having the highest requirement of all amino acids in order for protein synthesis to occur (Wu et al. 2011, Phang et al. 2013). During wound healing, proline plasma levels are high suggesting its important role in scar formation, however studies in rats by Barbul (2008) supplemented proline to the diet but saw no differences in wound strength or collagen deposition. When considering these results it is important to note that this study was performed in a stressed and injured population. Thus proline plays many important roles in the body, however it is not as effective as a nitrogen source when compared to other NEAA.

Wu et al. (2011) describe studies performed by Kirchgessner in young pigs supplemented with increasing amounts of proline to a 0.48% arginine and 2% glutamine diet and found dose responses in weight gain. Similarly, Wu et al. (2011) found that a 1% supplement of proline to corn and soy based meals increased small intestine weight and overall growth in piglets. However, Chung and Baker (1993) found no effect of adding proline to a diet when glutamine was absent. This indicates that amino acid supplementation not only depends on the dose but also
on the composition of the diet. Samuels et al. (1989) found decreased plasma proline concentrations in piglets fed proline deficient diets, indicating the necessity of dietary proline for growing piglets that are unable to synthesize it in sufficient quantities. Growing piglets’ proline synthesis enzymes, specifically pyrroline-5-carboxylate (P5C) reductase appear to be unaffected by the amount of proline in the diet, resulting in their inability to up regulate the de novo synthesis of proline when it is limiting in the diet. Therefore proline is an essential nutrient for neonatal pigs. It appears that proline has different metabolic demands depending on the species as well as the physiological stage. The current study was conducted in an adult population which is not growing as seen in the piglet studies, and found that proline was not an adequate nitrogen source for optimal protein synthesis.

**Table 10: Functions of Proline**

- Substrate for protein synthesis
- Regulation of gene expression and cell differentiation
- Proline signaling via pyrroline-5-carboxylate, superoxide anion (a free radical), and cellular redox reactions
- Hydroxyproline generation
- Arginine synthesis in mammals
- Scavenging oxidants

Footnote - Table modified from Wu et al. 2011.

Proline is also a very important substrate for the synthesis of arginine via the proline oxidase and ornithine aminotransferase enzymes, which are mainly present in the small intestine. In addition to being a substrate for protein synthesis, it has been well documented that arginine is important for the synthesis of creatine, nitric oxide, and urea (Wu and Morris 1998). It is possible that much of the proline fed in the current study diet was used for the synthesis of arginine, which has a large metabolic demand in the body. Similar to the proline supplementation studies in piglets, Rezaei et al. (2013) have described several studies in which supplemental
arginine increases weight gain and immune functions. Another metabolic demand for arginine comes from the final reaction of the urea cycle, where it is hydrolyzed by arginase to form ornithine and urea. The formation of urea is essential as it acts as an important nitrogen waste product and enables ammonia detoxification of the body. Urea is excreted in the urine to prevent the accumulation of excess nitrogen in the body after amino acid catabolism (Morris 2002, Voet et al. 2008). Burrin and Stoll (2009) suggest that much of the nitrogen from proline and arginine is converted to urea in the liver. Proline is also catabolized to P5C by P5C synthetase and then by P5C dehydrogenase to form glutamate, whose important and central roles have been outlined in Chapter 2 (page 33) (Watford 2008). This pathway would also limit the amount of dietary proline available for protein synthesis. Figure 13 shows the interconnection of the TCA and urea cycles, both of which will have high metabolic demands on proline when their substrates, glutamate and arginine, are absent from the diet. Furthermore, Brosnan (2000) suggests the level of carbamoylphosphate synthetase-I may regulate the urea cycle. This urea cycle enzyme requires glutamate derived N-acetylglutamate as an obligatory activator, and its synthesis is regulated by arginine. The synthesis of carbamoyl phosphate from ammonia is critical for the formation of urea in animals (Katagiri and Nakamura 2003). The metabolic demands for arginine and glutamate most likely activate the pathways for their synthesis from proline, thus limiting the amount of proline available for the conversion to other NEAA and for protein synthesis.
Figure 13: Interconnection of the TCA and Urea cycles
Image taken from Burrin and Stoll 2009.
Chapter 4: Summary

4.1 Conclusions

The first objective in the current study was to determine whether there existed a dietary requirement for $\alpha$-amino nitrogen in the form of L-glutamate in healthy adult men. It was hypothesized, based on earlier studies that there may be a dietary requirement because of its stimulatory effects on growth in rats and the decrease in growth rate when it was removed from the diet. Glutamate is also essential for the energy supply and integrity of the gut mucosa, where a majority of glutamate is absorbed and metabolized during first pass. Additionally, glutamate is central in amino acid metabolism and can be easily transaminated to form many of the other NEAA. The study design was to remove glutamate from the diet, while adding serine to keep the diet isonitrogeous, and determine the effect on protein synthesis as indicated by changes in $F^{13}\text{CO}_2$. The IAAO technique did not find a significant ($P>0.05$) difference in tracer L-$[1-^{13}\text{C}]$-phenylalanine oxidation ($F^{13}\text{CO}_2$) between the test glutamate intakes (0 and 113 mg/kg/day), suggesting that net protein synthesis was not affected due to the absence of glutamate in the diet.

Therefore we concluded that there was in fact no dietary requirement for glutamate in healthy adult men. It appears pathways for the de novo synthesis of endogenous glutamate are stimulated when glutamate is not supplied in the diet; one possible pathway is the synthesis of $\alpha$-ketoglutarate from pyruvate, which can then be transaminated to form glutamate; although the mechanism is unknown. We have shown in a healthy population consuming a diet lacking in glutamate, that sufficient endogenous glutamate synthesis can occur when the diet has adequate EAA and a source of nitrogen.

Our second study, the Nonessential Amino Acid Study, focused on comparing the nine NEAA as nitrogen sources for the de novo synthesis of other NEAA as well as for protein
synthesis in healthy adult males. It has been suggested that there is a dietary requirement for pre-
formed $\alpha$-amino nitrogen and that some NEAA may be more efficient in supplying nitrogen for
protein synthesis. Dietary essentiality has primarily focused on the ability to synthesize the
carbon skeleton of amino acids, and ignored the important nitrogen component. A significant
portion of the demand for total nitrogen is met by NEAA. We hypothesized that there would be
different efficiencies among the nine NEAA, specifically Ala, Arg, Asn, Asp, Gln, Glu, Gly, Pro,
and Ser, in their ability to supply nitrogen for protein synthesis. We tested this by supplying all
EAA at their RDA and then adding one NEAA at a time to meet a 50:50 ratio of EAA: NEAA
and compared the effects in F$^{13}$CO$_2$.

Using the IAAO technique we found a significant difference in the F$^{13}$CO$_2$ between Ala,
Arg, Asp, Asn, Glu, Gly, and Ser test intakes when compared to the base diet of only the EAA.
On the basis of these results, we suggest that these seven NEAA supply nitrogen for protein
synthesis better than the other NEAA, specifically Gln and Pro. We predict that these seven
NEAA may be metabolically more important from a dietary perspective in healthy adult males
under conditions of adequate EAA and nitrogen supply. It is possible that glutamine was not
adequate as a nitrogen source because diets were calculated on a gram of nitrogen basis and
because it contains two amino groups, less glutamine on a g/kg body weight was supplied, thus
limiting its availability for protein synthesis. Additionally, glutamine has the highest rate of
utilization in the small and large intestine where a majority is completely metabolized during
first pass, further limiting its availability to the peripheral tissues. We suspect that proline was
unable to supply enough nitrogen for optimal protein synthesis because the demands of other
amino acids involved in the urea cycle, such as arginine for use in the production of nitrous oxide
and urea, as well as for glutamate and glutamine, were so high that not enough proline was available for protein synthesis.

To our knowledge, this study is the first to look at the metabolic demand of different NEAA in healthy adult males. We have shown that all NEAA increase protein synthesis when added to an exclusively EAA diet, as shown by decreases in $\text{F}^{13}\text{CO}_2$ using the IAAO technique. All twenty amino acids are required in the appropriate proportions for protein synthesis to occur in the body, thus there is a metabolic demand for EAA as well as NEAA. Amino acids, both EAA and NEAA, play many biologically essential roles in the body as the precursors for numerous nitrogen containing molecules. The traditionally used definition of dietary essential or nonessential amino acid has limitations in protein nutrition because it only focuses on the ability to synthesize the carbon skeleton. Many of the NEAA have high metabolic demands that can limit protein synthesis in a healthy adult population.

In conclusion, our studies have shown that in healthy adult males when diets are lacking glutamate, endogenous glutamate synthesis can occur to satisfy the demands for protein synthesis, and there is no dietary requirement for glutamate, under conditions of adequate EAA and nitrogen. Furthermore, addition of all NEAA individually (except Gln and Pro) can act as an adequate nitrogen source in healthy adult males, for the endogenous synthesis of all other NEAA to satisfy demands for protein synthesis, when all EAA at their RDA are provided in the diet.

4.2 Future Directions

Amino acid metabolic demands vary depending on the species as well as the physiological state. Factors such as age, sex, and environment can all modulate the metabolic demands for different amino acids and thus their availability for protein synthesis. In the current study it was possible for a healthy adult male population to adapt to individual NEAA test
intakes over a short period of time (8 hour study day), and stimulate protein synthesis. However, whether more vulnerable populations such as growing children, pregnant women or neonates, will be able to adapt and stimulate protein synthesis to these individual NEAA intakes is unknown.

NEAA have been hypothesized to play key metabolic roles throughout pregnancy and may have a significant impact on growth and development of the fetus. As previously described, studies have shown that glycine is limiting in breast milk and could be essential for the growth of infants (Millward et al. 1989, Korslund 1974). Burrin and Stoll (2009) proposed a therapeutic effect on the gastroduodenel motor activity of feeding supplementary glutamate to premature infants. It was shown that proline supplementation also promoted growth and small intestine integrity in piglets, thus becoming essential in their diet (Samuels et al. 1989). Thus there may be dietary requirements for some NEAA under physiological conditions such as growth or pregnancy, and needs to be determined.

Recent work from our laboratory has focused on the protein requirements in a healthy pregnant population during early and late stages of gestation using the IAAO technique. It was shown that the protein requirement in a healthy pregnant population is considerably higher than the current DRI EAR of 0.88 g/kg. Specifically early and late gestation requirements were determined to be 1.22 and 1.52 g /kg/day, respectively (Stephens et al. 2015). These results have huge implications for the development and growth of the fetus and will change dietary recommendations for pregnant populations. Thus there could be increased requirements for some NEAA in a pregnant population, where the demand for protein synthesis is increased. It will be important to follow up on the metabolic demands of the NEAA in pregnant populations as well as during the active growth stages of children. These physiological states may have increased
requirements for NEAA, which could be limiting protein synthesis in the body.

Although human nutrition is in the form of food and not as calculated mixtures of amino acids, it is important to study the metabolic demands and thus dietary requirements to make recommendations to ensure optimal growth and health are obtained. Further research on the roles and mechanisms of NEAA is surely warranted in growth and development to better understand amino acid nutrition and help optimize health.
References


protocols of the indicator amino acid oxidation method provide the same estimate of lysine requirement in healthy men. *J Nutr* 132: 2251-2257.


Appendices

Appendix A  Experimental Design of NEAA Study

Subjects
• n=7. Healthy adult men selected, excluding women due to the effects of the menstrual cycle on phenylalanine oxidation (Kriengsinyos et al. 2004)

Preliminary Study Day
• Body composition, characteristics, and resting energy expenditure measured. Diet and medical history collected and informed written consent obtained.

Standardized Diet
• Standardized protein intake of 1.0 g/kg/day and 1.7 x resting energy expenditure (REE) for 2 days prior to each study day.
• 2-day protein intake adaptation in the form of milkshakes supplemented with carbohydrate, protein, and homogenized milk to meet the subject’s energy requirements.

Study Day 1
• Base diet containing the essential amino acids, (0.39 g/kg protein intake) with 1.5 x the REE.
• 8-hourly meals consist of crystalline amino acid mixture in liquid formula (protein free powder, flavoring crystals, corn oil) and protein free cookies.
• The fifth meal commences the tracer protocol with an oral priming dose of 0.176 mg/kg NaH\(^{13}\)CO\(_3\) and 0.66 mg/kg L-[\(^1\)\(^{13}\)C]phenylalanine. The next 3 hours and meals will contain 1.2 mg/kg/day L-[\(^1\)\(^{13}\)C]phenylalanine.
• Breath and urine samples collected. Open-circuit indirect calorimetry performed for 20 minutes to measure the rate of carbon dioxide production (\(\text{VCO}_2\)).

Study Days 2-11
• Test diet containing the essential amino acids plus any one nonessential amino acid (Ala, Arg, Asn, Asp, Gln, Glu, Gly, Pro, Ser, Mix of NEAA) (0.7 g/kg protein intake) with 1.5 x the REE.
• Tracer protocols and sampling will be followed as in Study day 1.
• There will be \(\geq 7\) days between study days.
Appendix B  Protein Free Cookie Preparations
B.1  Cherry Shortbread Cookie

Ingredients:

- Wheat starch 306 g (2.448 cups = 2 ¼ cups + 3 tbsp + ½ tsp)
- Margarine 178 g (12.714 tbsp = ¾ cup + ¼ tbsp)
- White sugar 100 g (0.5 cup)
- Corn syrup (light) 10.5 g (1.537 tsp)
- Liquid whip topping (whipping cream 33% MF) (10 g (0.667 tbsp = 2/3 tbsp)
- Salt 0.5 ml (0.102 tsp = ~1/8 tsp)
- Almond extract 5 ml = 1 tsp
- Maraschino cherries 100 g (~20 cherries + juice, finely chopped)

Apparatus and Equipment:

- Scale
- Measuring spoons
- Measuring cups
- Baking tray
- Bowls
- Electrical mixer
- Weighing trays

Cooking Procedure:

- Wash hands thoroughly with warm water and soap and put on gloves.
- Weigh margarine and sugar in a weighing tray and add to the bowl. Cream the two together using an electrical mixer.
- Measure corn syrup and almond extract using measuring spoons – add to creamed mixture. Weigh the whipping cream in a weighing tray and to the creamed mixture. Mix thoroughly.
- Cut cherries into fine pieces using a blender or chop finely using a knife. Add the finely chopped cherries to the mixture.
- Weigh the wheat starch using a weighing tray and measure salt using a measuring spoon and add both to the creamed mixture. Blend well using mixer, press batter into blades using a spatula until dough appears homogenous.
- Spoon small dough balls onto baking sheet flatten slightly.
- Bake in a preheated oven at 180°C (356°F) for 15 minutes.
- Let cool
B.2 Butterscotch Brownie

Ingredients:

- Vegetable shortening 89 g (0.434 cups = ¼ cup + 3 tbsp)
- Margarine 59 g (4.184 tbsp = 4 tbsp + ½ tsp)
- White sugar 25 g (1/8 cup)
- Brown sugar 41 g (0.186 cup packed = 2 tbsp + 3 tsp packed)
- Butterscotch Pudding mix 113 g (0.904 g = ¾ cup + 2.5 tbsp)
- Wheat starch 240 g (1.92 cups = 1 ¾ cups + 2.75 tbsp)
- Egg replacer 6 g (2 ¼ tsp)
- Salt 2 ml (0.137 tbsp = ~ 1/8 tbsp)
- Water 95 ml
- Vanilla extract 7.5 ml (1.571 tsp)
- Baking powder 7.5 ml

Apparatus and Equipment:

- Scale
- Measuring spoons
- Measuring cups
- Baking pan
- Bowls
- Electrical mixer
- Weighing trays

Cooking Procedure:

- Wash hands thoroughly with warm water and soap and put on gloves.
- Weigh the vegetable shortening and margarine in a weighing tray and add to the bowl. Cream the two together using an electrical mixer.
- Weigh the white and brown sugar, pudding mix, egg replacer, vanilla extract and water in weighing trays and add to the margarine/shortening mixture. Mix well.
- Weigh wheat starch, salt and baking powder in a weighing tray and mix well a separate bowl using a spoon. The dry ingredients are then added to the creamed mixture and beat in well. The dough will be quite firm.
- Pour the dough onto lightly-greased cookie sheet (9”x13”)
- Bake in a preheated oven at 200°C (392°F) for 15 minutes
- Let cool
Appendix C Posters

C.1 Glutamate Study

RESEARCH STUDY: GLUTAMATE REQUIREMENT IN HUMANS

There are 20 amino acids which make up protein in humans, and they are divided into two groups: essential and non-essential, indicating their necessity in the diet. This classification was based on studies using older techniques. Recent studies show that non-essential amino acids play important roles in our body's health. Glutamate, a non-essential, is important for providing energy to our small intestine (or gut). We are interested in determining if glutamate is needed more in our diet.

We are looking for healthy adult men, aged 20-40 years.

This study involves an initial assessment followed by 2 separate visits to the BC Children’s Hospital, each lasting 8 hours. Each visit involves a special diet, collection of breath and urine, and measurements of body size, and muscle mass.

Compensation will be offered to participants.

If you would like more information about this study, please contact us today!

Principal Investigator: Dr. Rajavel Elango
Primary Contact: Leah Cooper
There are 20 amino acids which make up protein in humans, and they are divided into two groups: essential and nonessential, indicating their necessity in the diet. This classification was based on studies using older techniques. Recent studies show that nonessential amino acids play important roles in our body's health. We are interested in determining if our bodies have a preference for certain nonessential amino acids found in our diet.

**We are looking for healthy adult men, aged 20-40 years.**
Comensation will be offered to participants.

**Study Details:**
- An initial assessment (1 hour)
- 9 separate visits to the BC Children's Hospital (8 hours each)
- Each visit involves a special diet, collection of breath and urine, and measurements of body composition.

If you would like more information about this study, please contact us today!

**Primary Contact:** Leah Cooper
**Principal Investigator:** Dr. Rajavel Elango
Appendix D Forms

D.1 Subject Consent Form

SUBJECT INFORMATION AND CONSENT FORM

Department of Pediatrics

950 West 28th Avenue, Room 170A
Vancouver, BC, V5Z 4H4

Non-essential amino acid requirements and metabolism in humans

Principal Investigator: Dr. Rajavel Elango, PhD
Department of Pediatrics
Faculty of Medicine
The University of British Columbia

Primary Contact: Leah Cooper, M.Sc candidate
Department of Pediatrics
Faculty of Medicine
The University of British Columbia

Sponsors: Ajinomoto Co Inc. Japan

Emergency Phone Number: Rajavel Elango
Leah Cooper
available 24 hours per day and seven days per week

Site: Oak Street Campus, UBC
Child & Family Research Institute

1. INVITATION

You are being invited to take part in this research study because there is currently little information regarding non-essential amino acid needs in healthy humans. Amino acids are the building blocks of protein, which are used to build muscle, body tissue, and to support the immune system. Among the 20 amino acids that make protein in our bodies, 9 are called essential because our bodies cannot make them. The remaining 11 amino acids are usually called non-essential, as it is assumed that our bodies can make enough of them. However, this assumption is based on earlier studies, using outdated methods. For this reason, it is very important to know whether some of these 11 amino acids are more needed in our diet.
2. YOUR PARTICIPATION IS VOLUNTARY

Your participation is voluntary. You have the right to refuse to participate in this study. If you decide to participate, you may still choose to withdraw from the study at any time without any negative consequences to the medical care, education, or other services to which you are entitled or are presently receiving. Before you decide, it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done, what will happen to you during the study, as well as the possible benefits, risks, or discomforts. If you wish to participate in this study, you will be asked to sign this form within 7 days. Please take time to read the following information carefully and to discuss it with your family, friends, and doctor before you decide.

3. WHO IS CONDUCTING THE STUDY?

The Principal Investigator, Dr. Rajavel Elango, and the Nutrition and Metabolism Research Program of the Child and Family Research Institute. The University of British Columbia has received financial compensation from the sponsor Ajinomoto Co. Inc for the work required in doing this clinical research. Financial compensation to researchers for conducting the research is associated with obligations defined in a signed contractual agreement between the researchers and the sponsor. Researchers must serve the interests of the subject and also abide by their contractual obligations. Ajinomoto Co Inc. will receive summarized data reports from the study results as part of this contractual agreement. For some, the payment of financial compensation to the researchers can raise the possibility of a conflict of interest. You are entitled to request any details concerning this compensation from the Principal Investigator.

4. BACKGROUND

The nutritional importance of protein is due to their amino acids. The 20 amino acids that are part of protein are divided based on how important they are in our diet, either essential amino acids or non-essential amino acids. Recent studies have suggested that these so-called non-essential amino acids are important in our body’s health. For example, glutamate, a non-essential amino acid, provides energy to our small intestine (or gut). To gain a better understanding of how this non-essential amino acids is needed in our body, we plan to study healthy men from all ethnic backgrounds, aged 20-40 years, using a modern technique called the indicator amino acid oxidation (IAAO) technique. This technique involves the consumption of protein shakes composed of specific amounts of glutamate mixed with a stable isotope tracer. The stable isotope tracer is a labeled amino acid, which is colourless, odourless, tasteless, and is completely safe; they are present in the air we breathe, water we drink, and food we eat. Amino acids are made of mostly $^{12}\text{C}$, a kind of carbon, however the isotope tracer contains $^{13}\text{C}$, a different kind of carbon. The tracer can be detected in breath and urine samples with special equipment because it looks different than the rest of the amino acids in the body. This allows us to measure if you are eating enough of the amino acid for protein synthesis to take place in your body. This technique has been used previously in healthy babies, children, and pregnant women.

5. WHAT IS THE PURPOSE OF THE STUDY?
The purpose of the study is to determine whether some of the non-essential amino acids in our diets are more important than other non-essential amino acids. The results from this study being conducted in adult men may allow us to do similar studies in other important age groups, such as children and during pregnancy.

6. WHO CAN PARTICIPATE IN THE STUDY?

You may be able to participate in this study if:
- You are 20 to 40 years-of-age
- Male
- Men who are classified as normal body weight and BMI (18.5-25 kg/m²)
- Free of any concurrent illness (cold, flu, vomiting etc.)

7. WHO SHOULD NOT PARTICIPATE IN THE STUDY?

- Men not in good health or have a metabolic, neurological, genetic, or immune disorder, including diabetes and hypertension
- Men who are classified as underweight (<18.5 kg/m²), overweight (25-30 kg/m²), or obese (>30 kg/m²) using the BMI classification
- Men who are allergic to milk, eggs and egg protein

8. WHAT DOES THE STUDY INVOLVE?

Overview of the Study

This study will be conducted at the Oak Street Campus of UBC at the Child and Family Research Institute (CFRI). If you agree to participate in this study, then you will be asked to complete the procedures described below. Following a Preliminary study day to ensure your eligibility, you will participate in nine separate study days. Each of these study days will be 8 hours in length and will involve hourly meals (protein shakes and cookies) and non-invasive breath and urine sampling.

If You Decide to Join This Study: Specific Procedures

a. Preliminary Study Day Procedures:
- The preliminary assessment is done to collect basic information about you, make sure you are informed about the study details, and to collect information about you to design the study diet specifically to meet your body needs.
- The preliminary assessment will be conducted at the Clinical Research Evaluation Unit (CREU) at the CFRI located in BC Children’s Hospital. You will be asked to come at approximately 8AM after having fasted overnight (10-12h). We are measuring the metabolic rate during a resting state, thus eating breakfast or drinking coffee/tea would have an affect on the metabolic rate. The whole procedure will take 1 hour to complete.
- During the preliminary assessment, a Research Assistant (L. Cooper) will measure your weight, height, body fat, muscle mass, and resting metabolic rate, which tells us how
much energy your body needs. Body fat will be measured using skin-fold thickness measured from the arm and shoulder using a caliper (a handheld instrument that gently pinches your skin between two moving arms). Body muscle will be measured using bioelectrical impedance which measures the passage of a small, safe amount of current (that cannot be felt) between four electrodes on the arms and legs while you lay still for a few minutes. The body fat and muscle measurements are completely safe and do not cause any discomfort or harm. Metabolic rate is measured using an indirect calorimetry machine, which consists of a clear hood that is placed over your head while you lay on a bed, breathing normally. You can see everything through the hood and breathe normally without any discomfort. This measurement takes about 20 minutes to complete.

☐ You will also be asked health related questions to assess your medical history. If you are not taking vitamins, we will provide you with some at this time.

☐ During the preliminary assessment we will evaluate your normal dietary protein intake. Based on these measurements, we will provide protein milk shakes for the two days prior to each study day.

☐ The 2 days prior to each study day, you will consume the provided diet in the form of protein milk shakes (your choice of vanilla, strawberry and chocolate flavor). This is required to adapt each person participating in the study to a standardized diet before the study day. You can only consume the protein shakes, water, coffee or tea with no added milk, cream, or sugar, for the two days prior to each study day.

b. Study Day Procedures:

☐ The study day will be conducted in the Clinical Research Evaluation Unit (CREU) at the Child & Family Research Institute located in BC Children’s Hospital. You will be asked to come at 8AM after having fasted overnight (10-12h).

☐ Only water may be consumed prior to and during the study day. The study day test diet as described below will provide your daily energy and nutritional needs. At the end of the study day, you are free to resume your normal food intake.

☐ On the study day a Research Assistant will again measure your weight and height. The Research Assistant will also measure the rate at which you are breathing out carbon dioxide (VCO₂) using the same indirect calorimetry machine that was used to determine metabolic rate in the preliminary assessment.

☐ You will eat the test liquid diet as eight small hourly meals on the study day. Each meal is made up of 1) a mixture of amino acids, 2) an amino acid-free flavored liquid and amino acid-free cookies that provide energy and other nutrients, and 3) the labeled amino acid is added to the last four meals. The test meals will meet all your daily energy, vitamin and mineral needs, as they were determined during the preliminary assessment.
☐ To measure how your body responds to the test diet we will collect your breath sample 9 times and urine sample 6 times during the study day. To collect breath you will have to breathe into a container - just like blowing through a straw into a bag. To collect urine you will have to pass urine into a urine sample hat in the privacy of the washroom. When we are not collecting samples, you can watch television, listen to music, read, or bring computer related work to complete.

☐ In total, you can expect to dedicate approximately 8 hours per study day you participate in. You are invited to participate in 11 studies over the course of 2-3 months. If you choose to participate in all 11 studies, you will be asked to dedicate approximately 72 hours to the study day projects. There will be one preliminary study requiring an hour of your time.

9. WHAT ARE THE POSSIBLE HARMs AND DISCOMFORTS?

There are no known risks involved with participating in this research. Some men may find having nothing to eat other than the milk based protein shakes to be challenging during the two days prior to each study day. We recognize that the length of the study day, and travel to BC Children’s Hospital might pose an inconvenience for you.

10. WHAT ARE THE POTENTIAL BENEFITS OF PARTICIPATING?

There are no direct benefits to you from taking part in this study. However, we hope that the information learned from this study can be used in the future to improve amino acid nutrition, especially in other age groups such as children and during pregnancy.

11. WHAT HAPPENS IF I DECIDE TO WITHDRAW MY CONSENT TO PARTICIPATE?

You may withdraw from this study at any time without giving reasons. A decision to withdraw will not have any negative ramifications to your health care at any hospital, research centre or physician’s office. If you choose to enter the study and then decide to withdraw at a later time, all data collected about you during the enrolment part of the study will be retained for analysis, after which the study information may be shredded.

12. CAN I BE ASKED TO LEAVE THE STUDY?

If you are not able to follow the requirements of the study or for any other reason, the principal investigator may withdraw you from the study. If the principal investigator considers withdrawal to be in your best interest to ensure your health (e.g. in the case of an acute illness), you will be withdrawn from the study without your consent.

13. WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?
Your confidentiality will be respected. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law. However, for the purpose of monitoring the research, research records and medical records identifying you may be inspected in the presence of the Investigator or his/her designate by representatives of Health Canada and the UBC Research Ethics Boards.

You will be assigned a unique study number as a subject in this study. Only this number will be used on any research-related information collected about you during the course of this study, so that your identity [i.e. your name or any other information that could identify you] as a subject in this study will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique identifier that is used on your research-related information will not be removed or released without your consent unless required by law. No information that identifies you will be allowed to leave the study center or be used in any reports or publications about the study.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to insure that your privacy is respected and also give you the right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study investigator.

14. WHAT WILL THE STUDY COST ME?

Participation in the study will not cost you anything. In appreciation of the time that it takes to complete this study you will receive $100 upon each study day completion to a maximum of $1100 for 11 study days.

15. WHO DO I CONTACT IF I HAVE QUESTIONS OR CONCERNS ABOUT MY RIGHTS AS A SUBJECT?

This study will be fully explained to you, and you will be given the opportunity to ask questions. If you have questions or want more information about the study procedures before or during participation, you may contact Dr. Rajavel Elango or Leah Cooper at any time.

If you have any concerns or complaints about your rights as a research subject and/or your experiences while participating in this study, contact the Research Subject Information Line in the University of British Columbia Office of Research Services by e-mail at RSIL@ors.ubc.ca or by phone at 604-822-8598 (Toll Free: 1-877-822-8598).
19. SUBJECT CONSENT

My signature on this consent form means:

☐ I have read and understood the subject information and consent form
☐ I have had this study explained to me, read this form and understand the information concerning this study.
☐ I have had sufficient time to consider the information provided and to ask for advice if necessary.
☐ I have had the opportunity to ask questions and have had satisfactory responses to my questions.
☐ I understand that all of the information collected will be kept confidential and that the results will only be used for scientific objectives.
☐ I understand that my participation in this study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without giving any reason(s) and my decision to withdraw will not change in any way the quality of care that I receive.
☐ I agree that the study investigators may re-contact me to participate in follow up studies, which will arise from the current study results.
☐ I understand that signing this consent form in no way limits my legal rights against the sponsor, investigators or anyone else.
☐ I understand that there is no guarantee that this study will provide any benefits to myself.
☐ I understand that if I have any further questions or desire further information I should contact Dr. Rajavel Elango.
☐ I understand that if I have any concerns about my rights as a research subject or my experiences while participating in this study, I may contact the toll free Research Subject Information Line at any time at 1-877-822-8598 or via e-mail to RSIL@ors.ubc.ca.

I will receive a signed copy of this consent form for my own records.

I, ___________________________________ voluntarily give consent for my participation in the

(Subject. Please print your name)

research study entitled:

Non-essential amino acid requirements and metabolism in humans

__________________________________  ______________________  ________________
Signature of Subject                  Printed Name                  Date

__________________________________  ______________________  __________________
Signature of person obtaining consent  Printed Name                  Study Role                  Date
D.2 Preliminary Study Day Form

Pre-Study Form
Non-Essential Amino Acid Requirements and Metabolism in Humans

Principal Investigator: Dr. Rajavel Elango
Student Investigator: Leah Cooper

PRELIMINARY ASSESSMENT

Subject ID: ______________________ Date: ______________________

Birthday (mo/yr): _______/______ Age (yr): __________

Height (cm): ___________ Weight (kg): _________ BMI: ________________

Bioelectrical Impedance Analysis

BIA:R ___________________________ XC ___________________________
(resistance) (reactance)

Impedance: ______________________

Body Composition Profile

Lean body mass (BIA): ______________ % Body fat (BIA): ______________

Indirect Calorimetry

Measured REE (kcal/day): __________
Estimated REE (kcal/day): __________

Daily energy requirement (kcal/day): ______________________

Medical History

Are you currently taking any medications? Yes_______ No_______

If yes, list of medications: __________________________________________

Do you have any preexisting health condition(s)? Yes_______ No_______

If yes, details of health condition(s) ___________________________________
Have you ever been diagnosed with any of the following health conditions?

Diabetes □  Hypertension □

**Nutritional Supplement Intake**

Are you currently taking vitamins? Yes ______  No ______

If yes, how long have you been taking them? _______________________

Are you taking any other vitamins/ nutritional supplements? Yes ______  No ______

If yes, please list all vitamin/nutritional supplements:

1. __________________________________________________________
2. __________________________________________________________
3. __________________________________________________________
4. __________________________________________________________

**Activity Level**

Daily exercise (minutes) _______________________

Sedentary ______  Moderate ______  High ______

**Availability for 11 studies of different nonessential amino acid intakes**

Yes ______  No ______

**Comments:**
### Study Day Form

**Non-Essential Amino Acid Requirements and Metabolism in Humans**

<table>
<thead>
<tr>
<th>Time</th>
<th>Sample Collection/Anthropometry</th>
<th>Meals and isotope tracer</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:00</td>
<td></td>
<td>Meal #1</td>
<td></td>
</tr>
<tr>
<td>9:00</td>
<td></td>
<td>Meal #2</td>
<td></td>
</tr>
<tr>
<td>10:00</td>
<td></td>
<td>Meal #3</td>
<td></td>
</tr>
<tr>
<td>11:00</td>
<td></td>
<td>Meal #4</td>
<td></td>
</tr>
<tr>
<td>11:15</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:30</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:45</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:00</td>
<td>VCO&lt;sub&gt;2&lt;/sub&gt; measurement</td>
<td>Meal #5 – primer dose and 1&lt;sup&gt;st&lt;/sup&gt; oral dose</td>
<td></td>
</tr>
<tr>
<td>13:00</td>
<td></td>
<td>Meal #6 – 2&lt;sup&gt;nd&lt;/sup&gt; oral dose</td>
<td></td>
</tr>
<tr>
<td>14:00</td>
<td></td>
<td>Meal #7 – 3&lt;sup&gt;rd&lt;/sup&gt; oral dose</td>
<td></td>
</tr>
<tr>
<td>14:30</td>
<td>4&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15:00</td>
<td>5&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4&lt;sup&gt;th&lt;/sup&gt; urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15:15</td>
<td>6&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15:30</td>
<td>7&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5&lt;sup&gt;th&lt;/sup&gt; urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15:45</td>
<td>8&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:00</td>
<td>9&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6&lt;sup&gt;th&lt;/sup&gt; urine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Subject Code Master List

**Non-essential amino acid requirements and metabolism in humans**

<table>
<thead>
<tr>
<th>Subject Name</th>
<th>Code (Alpha-Numeric)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>