The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them. ~William Lawrence Bragg

**University of Alberta**

# Determination of the Limiting Amino Acids in a Total Parenteral Nutrition Solution fed to Neonatal Piglets using the Indicator Amino Acid Oxidation Method

by

Christine Pendlebury

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

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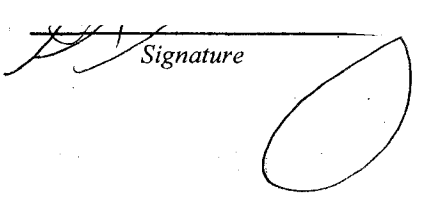

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Determination of the Limiting Amino Acids in a Total Parenteral Nutrition Solution fed to Neonatal Piglets using the Indicator Amino Acid Oxidation Method** submitted by **Christine Pendlebury** in partial fulfillment of the requirements for the degree of **Master of Science**.

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**ABSTRACT**

The main objective was to combine the indispensable amino acid requirements, determined using indicator amino acid oxidation, into a single total parenteral nutrition solution, the B/P profile, and compare the performance of parenterally-fed neonatal piglets receiving the B/P profile to piglets receiving a different parenteral amino acid pattern. The first experiment demonstrated that the B/P profile contained at least one severely limiting amino acid. Consequently, lysine, leucine, methionine, threonine and/or proline were added to the B/P profile and $^{14}C$ phenylalanine oxidation was measured to determine which of these amino acids was first limiting. Plasma lysine concentrations increased significantly ($p<0.05$) when the B/P pattern was supplemented with lysine. In combination with previous research, it was determined that proline may be indispensable to the parenterally-fed piglet. No other amino acid supplementation significantly ($p>0.05$) reduced oxidation. Therefore, we were unable conclude if any of these amino acids were limiting in the B/P profile.

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## LIST OF ABBREVIATIONS

TPN- Total parenteral nutrition
IV- intravenous
B/P- Ball/Pencharz
IAAO- Indicator amino acid oxidation
DAAO- Direct amino acid oxidation
IAAB- Indicator amino acid balance
$CO_2$- Carbon dioxide
C- Carbon
MT- methionine and threonine
ML- methionine and leucine
TL- threonine and leucine
MLT- methionine and leucine and threonine

# 1.0 LITERATURE REVIEW

This literature review will cover material relevant to the use of a total parenteral nutrition (TPN) piglet model to study amino acid metabolism and the development of a new neonatal TPN profile. The nutrient requirements and the common morbidities associated with the currently available TPN solutions will also be addressed. This review will also examine the previous research done to determine the optimal requirements for the indispensable and conditionally indispensable amino acids being fed to TPN piglets; with particular emphasis on the different methods available to quantify amino acid requirements.

## 1.1 Introduction

Morbidity and/or mortality are common in premature infants. It is essential that premature infants and infants afflicted with intestinal abnormalities, such as short bowel syndrome or necrotizing enterocolitis (NEC), meet their requirements for growth and maintenance. Low birth weight infants suffering from these morbidities are unable to meet their nutrient and energy requirement by enteral feeding only; therefore, nourishment must be supplied in another form (Ball et al, 1996). TPN is the intravenous administration of the required vitamins, minerals, lipids, glucose, and amino acids to sustain viability.

At this time, the commercially available TPN diets are the best option but not the optimal solution. Currently amino acid patterns in TPN solutions are plagued with over and under-estimations of what is required parenterally by the human neonate (Brunton et al, 2000). Failure to accurately meet the parenteral requirement of the neonate can result in an amino acid deficiency and/or toxicity. This in turn will place additional challenges

in the form of metabolism and excretion and may limit growth in already health compromised infants.

Commercially-available TPN amino acid profiles are based on various naturally occurring reference proteins, such as cord blood, egg protein, and breast milk (**Table 1.1**). Although these patterns, specifically breast milk and egg protein, may be appropriate when the neonate is enterally-fed, there is evidence that suggests that the requirements for indispensable amino acids in parenterally-fed neonates are very different from those of enterally-fed neonates (House et al., 1997, House et al., 1998, Bertolo et al., 1998, Elango et al., 2002, Shoveller et al., 2003, Cvitkovic et al, 2004). The enteral requirements for many indispensable amino acids, determined by growth rate and nitrogen balance, are significantly higher than the parenteral requirements, likely due to the fact that the gastrointestinal tract (and specifically the small intestine), utilizes nearly 30% of amino acids that are supplied orally (Stoll et al, 1998). Certain conditionally indispensable amino acids, such as proline and arginine, are primarily synthesized by the small intestine and therefore the necessary amounts may be underestimated in profiles primarily designed for enterally-fed neonates (Bertolo et al, 2003). As a result, many parenterally fed infants may be receiving an insufficient or excess amount of specific amino acids.

When an amino acid pattern is deficient in one or more amino acids, the rate of protein synthesis will be determined by the first limiting amino acid. The first limiting amino acid is defined as the indispensable amino acid that is the most below its requirement for protein synthesis and limits net protein metabolism. The first limiting amino acid can restrict neonatal growth, protein utilization and overall amino acid

deposition (Wykes et al, 1994). When a limiting amino acid is present, all other amino acids are in excess and therefore must be degraded and the excess nitrogen excreted, potentially resulting in hyperammonemia, increased plasma urea and altered protein metabolism (Brunton et al, 2000). The optimal parenteral amino acid profile will ultimately improve neonatal health, growth and ultimately survival of the human neonate.

Over the years, the requirements of many indispensable and conditionally indispensable amino acids during TPN have been determined using the piglet model (**Table 1.2**) and the indicator amino acid oxidation (IAAO) technique. However, at this time these determined requirements have not been combined into a single complete amino acid profile for a TPN solution. Determining an optimal amino acid profile for the TPN fed neonate would eliminate most of the potential consequences associated with the current TPN solution profiles. In this thesis, a complete amino acid profile was developed based on previously determined requirements (Ball/Pencharz (BP) Profile) and its profile will be compared to that of a currently available TPN solution using the IAAO technique.

Due to the ethical restrictions and other practical constraints, such as a heterogeneous population, research with premature and term infants is difficult to conduct. The development of the appropriate animal models has been used to combat the previously stated constraints. An animal model allows the researcher greater control over the experimental conditions, and allows for surgical and other modifications to effectively create the condition, such as parenteral feeding, of research interest. The piglet and human neonate have similar anatomy, metabolism, and gastrointestinal physiology (Book et al, 1974). When examining the digestive tract of the premature

infant and its function, the neonatal piglet is an excellent model due to similarities in the maturation and development of the gastrointestinal system (Ball et al., 1996). Within the past decades the piglet model has become the gold standard for examining digestive and metabolic functions of the human neonate (Baracos, 2004).

There are many different methods of measuring the various nutrient requirements of the piglet. The indicator amino acid oxidation method (IAAO) is one such method that can indirectly examine the amino acid requirements of a neonatal piglet. IAAO is the best method available because it allows the researcher to quickly and accurately determine a specific amino acid requirement or verify a limiting amino acid (Pencharz and Ball, 2003). Therefore the neonatal piglet model in combination with the IAAO is the ideal method to assess the appropriateness of the parenteral amino acid profile that combines the indispensable amino acid requirements previously determined by the Ball/Pencharz group. These requirements are invaluable in their contribution to neonatal nutrition research and may be translated to parenteral nutrition in the human neonate.

**Table 1.1:** Amino acid content in parenteral solutions, sow and human milk, and the requirement of the piglet and human neonate

| | Vamin (Pharmacia &Upjohn)[1] | Vaminolact (Pharmacia &Upjohn)[1] | Primene (Baxter)[1] | TrophAmine (McGraw)[1] | Sow milk[2] | Human milk[2] | Pig tissue[4] | Human tissue[3] | Piglet Requirement for adequate intake[5,6] | Human Requirement for adequate intake[5,7] |
|---|---|---|---|---|---|---|---|---|---|---|
| Isoleucine | 56 | 55 | 67 | 82 | 36 | 59 | 38 | 35 | 32 | 32 |
| Leucine | 75 | 108 | 99 | 140 | 88 | 108 | 72 | 75 | 42 | 73 |
| Valine | 61 | 55 | 76 | 78 | 50 | 61 | 52 | 47 | 34 | 42 |
| Lysine | 55 | 86 | 109 | 82 | 73 | 68 | 75 | 72 | 60 | 47 |
| Methionine | 27 | 20 | 24 | 34 | 16 | 16 | 20 | 20 | 29 | 26 |
| Cysteine | 20 | 15 | 19 | 1 | NR[7] | NR | NR | NR | INC[10] | INC |
| Phenylalanine | 79 | 42 | 42 | 48 | 42 | 41 | 42 | 41 | 47 | 57 |
| Tyrosine | 7 | 8 | 9 | 23 [9] | 49 | 44 | 32 | 29 | INC | INC |
| Threonine | 43 | 55 | 37 | 42 | 48 | 49 | 37 | 41 | 34 | 40 |
| Tryptophan | 14 | 22 | 20 | 20 | NR | NR | NR | NR | 9 | 7 |
| Histidine | 35 | 32 | 38 | 48 | 33 | 25 | 28 | 26 | 15 | 13 |
| Arginine | 47 | 63 | 84 | 122 | 50 | 40 | 69 | 77 | 25 | NR |
| Glycine | 30 | 32 | 40 | 36 | 34 | 27 | 91 | 118 | NR | NR |
| Alanine | 43 | 97 | 79 | 54 | 40 | 42 | 72 | 72 | NR | NR |
| Aspartate | 59 | 63 | 60 | 32 | 79 | 91 | 117 | 90 | NR | NR |
| Glutamate | 129 | 109 | 99 | 50 | 193 | 184 | 134 | 130 | NR | NR |
| Proline | 116 | 86 | 30 | 68 | 113 | 95 | 60 | 84 | NR | NR |
| Serine | 107 | 58 | 40 | 38 | 55 | 49 | 48 | 44 | NR | NR |
| Taurine | 0 | 0.5 | 6 | 2 | -- | -- | -- | -- | -- | -- |
| Ornithine | 0 | 0 | 22 | 0 | -- | -- | -- | -- | -- | -- |

[1]Values are modified from Brunton et al, 2000. Values were originally presented as % of total amino acids by weight. Values have been converted to mg/g total amino acids for comparative purposes.
[2]Values are means of human milk determined in Davis et al,1994 and expressed as mg/g total amino acids.
[3]Means of values from Widdowson et al (1979) in 160-180 d, 180-200 d, 200-220 d, 220-240 d, 240-260 d, and 260-280 d 6f age human fetus (mg/g total amino acids) as reported in Davis et al (1993).
[4]Means (mg/g total amino acids) as reported in Davis et al (1993).
[5] Values are modified from Ball et al, 1996. Values were orginally presented as g/100g protein. Values have been converted to mg/g total amino acids for comparative purposes
[6] Calculated from NRC 1998 for swine weighing between 3 to 5kg
[7] Calculated from FAO/WHO 1990 for infants between 3 to 4 months of age
[8]NR, not reported.
[9]Supplied as L-tyrosine (0.7%) and N-acetyl-tyrosine (1.6%)
[10] INC, included in previous requirement

**Table 1.2:** Estimated enteral and parenteral requirements of indispensable and conditionally indispensable amino acids in 1-3kg piglets

| | Parenteral Requirement | Enteral Requirement |
|---|---|---|
| | g/kg*d | |
| Isoleucine[2,3] | 0.51 | 0.66 |
| Leucine[2,3] | 0.51 | 1.188 |
| Valine[2,3] | 0.51 | 0.792 |
| Lysine [5] | 0.84 | 1.17 |
| Total Sulfur Amino Acids [1] | 0.26 | 0.44 |
| Methionine[8] | 0.18 | 0.25 |
| Cysteine[9] | 0.08 | 0.19 |
| Phenylalanine | 0.48 [4] | ND |
| Tyrosine | 0.41 [4] | ND |
| Threonine [6] | 0.21 | 0.51 |
| Tryptophan [7] | 0.185 | 0.164 |
| Histidine | ND | 0.4 [10] |
| Arginine | 1.2[11] | ND |
| Glycine | ND | ND |
| Glutamate | ND | ND |
| Proline | ND | 0.78 [12] |

[1] Values obtained from Shoveller et al, 2003a. Requirement includes the sulfur amino acids inclusively
[2] Values obtained from Elango et al, 2002. Requirement includes the branched-chain amino acids inclusively
[3] Values obtained from Elango et al 2004
[4] Values obtained from House et al, 1997
[5] Values obtained from House et al, 1998
[6] Values obtained from Bertolo et al, 1998
[7] Values obtained from Cvitkovic et al, 2004
[8] Values obtained from Shoveller et al, 2003b
[9] Values calculated from Shoveller et al, 2003a and Shoveller et al, 2003b
[10] Values calculated from Kim et al., 1983
[11] Value obtained from Brunton et al., 2003
[12] Value calculated from Ball et al., 1986
ND Not yet determined

## 1.2 History of TPN in Human Neonates

Nutritional status has been found to play an important role in morbidity and survival, especially in infants because of their increased nutrient needs for growth and development (Dudrick, 2003). Low birth weight infants have difficulty maintaining a healthy nutritional status, and meeting growth and development requirements, which sometimes results in surgical and medical interventions. Recently, surgeons have found that a good nutritional status decreases surgical complications, improves wound healing, and significantly decreases morbidity and mortality (Lugli et al., 2007). Supplying adequate nutrition to those patients who are plagued with co-morbidities that hindered digestion, metabolism and absorption became the primary focus of much research. Over the past couple of centuries it had been determined that nourishment could be supplied via the intravenous circulation thus laying the foundation for parenteral nutrition (Vinnars and Wilmore, 2003).

In the early 17$^{th}$ century, Claude Bernard, the pioneer of total parenteral nutrition (TPN), examined the utilization of infused carbohydrates in animals. He determined that infused glucose was taken up by the body's cells and utilized and was not excreted in the urine as previously thought (as cited by Vinnars and Wilmore, 2003). Bernard's finding led to the utilization of constant-infused glucose in humans by Woodyatt and colleagues who found that glucose could be administered intravenously in large amounts without the risk of glucosuria (as cited by Wretlind, 1992). This early research demonstrated that the potential existed to satisfy energy requirements by intravenous infusions of glucose.

Investigators then turned to protein metabolism and the effects of intravenous (IV) administration of protein hydrolysates of amino acids. At the turn of the 20$^{th}$ century,

Henrique and Anderson infused hydrolyzed beef protein into a goat and reported a positive nitrogen balance (as cited by Wretlind, 1992). In response to this finding Robert Elman infused a fibrinogen hydrolysate into a human and observed a positive nitrogen balance. The use of pure amino acids soon followed which allowed for the tailoring of a specific amino acid profile that could be easily replicated (as cited by Wretlind, 1992). These experiments showed that protein supplied via an intravenous hydrolysate or amino acid solution could be used as an alternative source to the oral intake of protein, and could adequately meet the protein needs of adult humans who were unable to consume adequate oral nutrition.

In the early days, glucose was the only available source of non-protein energy. However it was unable to meet total energy needs without adverse consequences such as essential fatty acid deficiency and fatty liver (Vinnars and Wilmore, 2003). As a result, fat emulsions of varying composition were used to help meet the total energy requirement of the individual. Many of these lipid emulsions also produced adverse reactions, such as fever, liver damage and vomiting, due to the pharmacological and toxic agents present in either the fat or the emulsifier (Wretlind, 1992). Schuberth and Wretlind (1961) derived a solution called Intralipid®, a combination of soybean oil (fat) and egg yolk phospholipids (emulsifier), which was found to have minimal adverse reactions and was able to meet the non-protein energy requirement during TPN feeding (as cited in Wretlind, 1983). With advances in emulsification techniques and agents, several fat emulsions are now available and proven to be effective.

Historically vitamin and mineral requirements have been adapted from adult requirements and therefore may be inadequate in meeting the growth and metabolic

demands of the preterm infant (Hay Jr., 1986). By the 1990s the nutrient ranges for vitamins and minerals were determined for the human preterm infant receiving TPN (Krug, 2000). While investigating the effective and safe administration of micronutrients via TPN, scientists inadvertently discovered the parenteral need for many trace minerals and electrolytes. The protein source in TPN had changed from protein hydrolysates to crystalline amino acid mixtures resulting in a TPN solution devoid of trace elements (Shils, 1984). Current parenteral solutions now contain trace elements and trace element deficiency is rare in neonates receiving TPN except when TPN is fed very long-term. When fed TPN long-term, supplementation of manganese, molybdenum, iodide, zinc, copper, chromium, and selenium may be required (Krug, 2000). Experiments with parenteral nutrition have now demonstrated that all macro and micronutrients could be supplied in a clinically appropriate and safe IV solution.

Although TPN has been administered for the past three to four decades, the nutritional needs of a growing human neonate receiving TPN require additional investigation. Further research is required to determine the amino acid, lipid and carbohydrate requirements of the human infant during parenteral feeding.

## 1.3 **The effect of parenteral feeding on metabolism**

The amino acid profiles of TPN solutions are currently based on the profiles of various reference proteins, such as cord blood, egg protein and breast milk. The first oversight with many TPN solutions is the fact that the reference protein used to develop the profile is generally consumed enterally and not administered parenterally (Wykes et al., 1994). The liver and gut have high nutrient requirements (Duffy and Pencharz,

1986); yet in the parenterally fed infant nutrients enter the systemic circulation directly (Riedijk and van Goudoever, 2007). It has been shown that the splanchnic tissues account for up to 35% of the whole body protein turnover (Stoll and Burrin, 2006). First-pass splanchnic metabolism, metabolism that occurs within the liver, stomach, intestines, pancreas and spleen, is bypassed during TPN feeding; therefore the amino acid profiles derived from proteins generally consumed enterally are likely inappropriate during TPN feeding (Burrin and Davis, 2004).

It has been demonstrated that when infants are parenterally fed, liver apoptosis and gut atrophy are induced (Wang et al., 2006), and these changes significantly affect protein metabolism. Liver apoptosis is thought to be induced due to a reduction in gut hormone secretion and bile flow (Wang et al., 2006); whereas gut atrophy is induced due to reduced intestinal blood flow and decreased cell proliferation when compared to enterally fed subjects (Niinikoski et al., 2004). As a result studies have shown that methionine, cysteine, threonine and arginine requirements may be inaccurate due to the lack of intestinal metabolism. These inaccuracies lead to deficiencies and toxicities of many of the indispensable amino acids (Brunton et al., 2000).

## 1.4 **Amino acid patterns in current TPN solutions**

Amino acid metabolism is different in neonates than in adults (Poindexter et al., 2001). Yet the problem exists that some TPN solutions that are used in a pediatric setting have nutrient profiles based on adult requirements (Brunton et al., 2000). Two specific examples of amino acids where the composition of a TPN solution designed for adults may be inappropriate for neonates are the aromatic and sulfur amino acids. Due to the

immaturity of the liver, the hydroxylation of phenylalanine to tyrosine is limiting in the human neonate, making tyrosine an indispensable amino acid (Brunton et al., 2000). However, due to the solubility problems of tyrosine, most commercial TPN solutions contain less than 1% of the total amino acids as tyrosine (**Table 1.1**) which is inadequate to meet the metabolic needs of the neonate (Brunton et al., 2000). To account for poor tyrosine solubility, some parenteral solutions increase the phenylalanine concentration which leads to the problem of hyperphenylalaninanemia (Brunton et al., 2000). The aromatic amino acid requirements needed to be determined together and individually to fully understand phenylalanine and tyrosine metabolism in the parenterally fed neonate.

Another potentially conditionally indispensable amino acid in premature human neonates is the sulfur amino acid cysteine. Cysteine, another sulfur amino acid, is synthesized from methionine in the liver by the enzyme cysthionase. However, due to the immaturity of the liver in premature infants a dietary source of cysteine is required (reviewed by Pencharz et al., 1996). Recent work from our laboratory (Shoveller et al., 2003) and in human neonates (Courtney-Martin et al., 2008) provide evidence that cysteine is not conditionally indispensable. Like tyrosine, solubility is also an issue with cysteine and parenterally solutions either contain little or no cysteine (**Table 1.1**) (Brunton et al., 2000). On the other hand we have shown that cysteine can spare around 40% of the methionine requirement (when methionine is fed as the only source of sulphur amino acids); while eliminating the chance of methionine toxicity (Shoveller et al., 2003b). Methionine and cysteine requirements needed to be established to optimize growth and health of the parenterally fed neonate.

In current commercial solutions, arginine has the largest intake variation of any of the indispensable amino acids (**Table 1.1**)(Brunton et al., 2000). It has been demonstrated that when gut function is compromised, as it is in the TPN-fed infant, de novo arginine synthesis is negatively affected and adequate dietary arginine is required (Brunton et al., 1999). Thus arginine is yet another amino acid for which a parenteral requirement needs to be determined to eliminate the risk of hyperammonaemia when feeding low intakes present in many commercial solutions (Brunton et al., 2000).

Due to the inadequacy of current TPN solutions meeting metabolic demands of the parenterally fed infant a new amino acid profile needs to be derived using a more accurate method of individually determining requirements.

## 1.5 METHODS FOR DETERMINING AMINO ACID REQUIREMENTS

### 1.5.1 Classical Methods: Growth Rate and Nitrogen Balance

The most traditional method of determining amino acid requirements is examining growth rate in response to amino acid quantity. With this method graded levels of intake of the specific amino acid is used and the rates of growth are measured. It is assumed that once the amino acid requirement has been met there will be no further growth as a result (Pencharz and Ball, 2003). Measuring growth rate in relation to the test amino acid level is a relatively simple method which does not use any invasive techniques or require a prolonged period of time. The simplicity of measuring growth rate has its benefits, but this less invasive technique does not give any indications for the biological mechanisms behind the empirical amino acids requirements.

Though growth rate has been traditionally used to determine protein requirements, these results may be misleading. Water retention and increased fat deposition are characteristic of low birth weight infants and may account for some of the growth recorded, leading to an inaccurate interpretation of protein requirements for this population (Yudoff et al., 1986). The major downfall of growth rate is that it is only applicable when the experimental animal is young and rapidly growing. Therefore this method is ineffective when investigating amino acid requirements of any adult population (Pencharz and Ball, 2003).

Unlike growth rate, nitrogen balance can been used to determine amino acid requirements in subjects both during periods of rapid growth and during maturity. As with growth rate, the test amino acid must be fed at varying increments while measuring nitrogen intake and the nitrogen excreted in the feces and urine (Young, 1986). Nitrogen retention will increase until the requirement for the test amino acid is reached, after which the nitrogen retention will be maintained (Pencharz and Ball, 2003). However, when determining amino acid requirements in children, nitrogen balance is defined as a positive nitrogen balance to account for adequate growth (FAO/WHO/UNO, 1985).

When using nitrogen balance, the body urea pool may need up to ten days to adapt to the varying intake levels consequently prolonging the experimental period (Rand et al., 1976). Because nitrogen balance must be determined over a range of protein and/or amino acid intakes, this means long periods of feeding potentially deficient intakes of protein/ amino acids. Therefore, the nitrogen balance approach to determining amino acid requirements is not appropriate in premature infants and an alternative method is required. One of the assumptions made in some nitrogen balance studies is that one-tenth

of the nitrogen intake is excreted in stool. This assumption is invalid in the parenterally-fed infant population where fecal losses are minimal and/or non-existent (Yudkoff et al., 1986). Endogenous nitrogen loss, and thus requirement, can be significantly affected by climate, age, sex and dietary protein source (Rand et al., 2003). The magnitude of endogenous nitrogen losses may also account for the discrepancy seen within the literature. A single estimated allowance for additional nitrogen losses, such as sweat, has been applied over the years. Yet further research has shown that this correction factor may not be applicable to all conditions and specific populations (Young, 1986). Another shortcoming of nitrogen balance studies is that the mechanisms behind the nitrogen balance are unaccounted for (Zello et al., 2003). Nitrogen balance can be achieved via protein synthesis, protein breakdown and/or oxidation of an indispensable amino acid (Young, 1986). Nitrogen balance alone does not permit any insight into the absolute values pertaining to protein synthesis and breakdown.

Both nitrogen balance and growth rate are indirect and consequently may be insensitive and inaccurate in determining the actual amino acid and/or protein requirements of the neonatal infant. The combination of all of these inaccuracies may lead to a serious overestimation of amino acid requirements in the infant population (Pencharz and Ball, 2003). Overestimation of the protein requirement of premature human neonates can lead to hyperammonemia, neurotoxicity, and mental retardation as a result of accumulation of protein (Yudkoff et al., 1986). Consequently, an alternative method for determining amino acid requirements in the premature infant population is required.

### 1.5.2 Carbon Oxidation Methods: Direct Amino Acid Oxidation (DAAO), 24 Hour Indicator Amino Acid Balance (24h IAAB) and Indicator Amino Acid Oxidation (IAAO)

DAAO measures the oxidation rate of an amino acid when the subject is fed graded intakes of that same amino acid (Zello et al., 1995). All of the carbon oxidation methods rely on graded intakes of amino acids and the range should include at least three points below the estimated requirements and three above the requirement (Baker, 1986). DAAO is based on the principle that amino acid catabolism increases as the supply of amino acid increases above the requirement for protein synthesis (Zello et al., 1995). When the amino acid is fed at varying increments below the requirement, oxidation will remain low because most of the amino acid will be used for protein synthesis. When represented graphically by plotting oxidation rate against level of amino acid intake, an inflection will occur, representative of the physiological mean requirement of the amino acid being tested (**Figure 1.1**)(Zello et al., 1995). The benefit of the DAAO method is that it is sensitive in detecting the change in oxidation and has a short adaptation period (as cited in Pencharz and Ball, 2003). However, the biggest limitation of this method is that it can only be used to determine the requirements of amino acids that lose their carboxyl group to the bicarbonate pool so that can be measured in breath samples (Pencharz and Ball, 2003). Amino acids such as threonine and methionine are not appropriate for tracers due to their complex degradative pathways (Zello et al., 1995); therefore the requirement of these two amino acids can not be determined using DAAO.

Another criticism of DAAO is that the requirement is determined in the fed state and this may not be representative of the daily needs. To combat the issue of fed state versus a non-fed state 24 hour (24h) DAAO was introduced. 24h DAAO uses the same

methodology of DAAO but over a period of 24 hours not 2-4 hours as previously described. El-Khoury and colleagues re-examined leucine requirements using tracer techniques and found that there was a negligible difference between the measured and predicted estimates of daily leucine oxidation and balance (El-Khoury et al., 1994). This study validated the assumption made by Ball, Pencharz, and colleagues that the 4h fed state study accurately represents the 24h amino acid requirement; therefore eliminating the need for the long experimental period, and large amounts of isotope utilization, associated with 24h DAAO.

The greatest flaw of the DAAO technique is the methodological argument that requirements determined may be an over-estimation due the dilution of the tracer (Millward et al., 1989). Dilution occurs because the precursor pool size of the test amino acid, from which the oxidation is presumed to take place, simultaneously increases with the dietary intake level (Pencharz and Ball, 2003); therefore the requirement is confounded and may be overestimated. This methodological flaw cannot be overcome without validation from more expensive amino acid tracer techniques. A sensitive, inexpensive method that maintains the minimal adaptation period of the DAAO technique while making up for its theoretical constraints is required.

The initial assumption of the IAAO technique is that amino acids in excess of what is required for protein synthesis are not stored, but are oxidized (Zello et al., 1995). Therefore, if one amino acid is limiting in the diet, then all other amino acids, including the indicator, are in excess and must be oxidized (Zello et al., 1995). Increasing the dietary intake of the limiting amino acid from deficient to adequate will improve the use of all the other amino acids for protein synthesis, resulting in a decrease in indicator

amino acid oxidation until the requirement is met. Once the requirement for the test amino acid is met the level of oxidation increases (Zello et al., 1995). The IAAO technique uses diets providing a constant intake level, greater than the estimated requirement, of the indicator amino acid and graded intakes of the amino acid of interest (test amino acid). Then, the indicator amino acid that has been isotopically labelled at the 1-C position (which can be stable or radioactive) is infused, over a predetermined period of time, to determine oxidation of the indicator by the isotopic enrichment (specific radioactivity) of the $CO_2$ in expired breath samples (Ball and Bayley, 1984). These measurements, taken from the varying intakes, are then plotted on an oxidation curve that is representative of the physiological range of the test amino acid intake (**Figure 1.1**). Once the requirement level has been reached higher dietary intakes will have no effect on protein synthesis or oxidation of the other amino acids thus resulting in an inflection (break point) on the curve and representing the requirement for 50% of the population (Zello et al., 1995). This method has proven to be successful in determining the amino acid requirements of rats, pigs, and humans through varying stages of life (Zello et al., 1995).

Currently, isotopically labelled phenylalanine (with excess tyrosine), leucine and lysine have been used to determine amino acid requirements with the IAAO technique (Zello et al., 1995). The initial dilemma of the IAAO technique is to determine which indicator, phenylalanine, methionine, lysine or leucine, would the best to use when determining amino acid requirements (Pencharz et al 2003.) Phenylalanine (in the presence of excess tyrosine), methionine, lysine and the branched chain amino acids all irreversibly oxidize their carboxyl carbon atom (Zello et al., 1995); thus allowing for the

possibility of these amino acids being indicators. Methyl labelled methionine was found to be an inappropriate indicator since the methyl group has many different fates and can be incorporated into many reactions (Zello et al., 1995). Leucine is the most inexpensive indicator amino acid however it is inappropriate due to the large and variable pool size, complex pathway and the complex relationship between the other two branched chained amino acids (Hsu et al., 2006). Lysine is not an optimal indicator amino acid due to its large pool size and long pathway; resulting in increased variability and decreased sensitivity to subtle diet changes (Ball and Bayley, 1984). Currently labelled phenylalanine (in the presence of excess tyrosine) is the most commonly used indicator amino acid due to the small whole body pool size, concise pathway and rapid turnover (Pencharz and Ball, 2003).

Using the IAAO eliminates some of the major criticisms of the DAAO technique. The methodological issue of tracer dilution is not a problem with the IAAO technique because phenylalanine concentration was kept at a constant. The ratio of specific radioactivity of the indicator amino acid protein bound compared to that which is in the liver as the total free amino acid pool did not change relative to the intake of the test amino acid (Ball and Bayley, 1986); therefore the pool from which the oxidation of the indicator amino acid takes place will remain the same throughout the various intakes of the test amino acid.

Another benefit of the IAAO technique is greater flexibility in which amino acids can be examined (Zello et al., 1995). No other technique has the ability to test the requirement of both indispensable and conditionally indispensable amino acids. The limitation of DAAO is that the carboxyl-labeled carbon must be released exclusively in

the breath ($CO_2$), and CO2 formation must be due to catabolism of the amino acid and not due to metabolism to other intermediates, in order to be able to quantity a requirement. With the IAAO technique this is not the case since it is the oxidation of the indicator amino acid that is being measured.

When using the IAAO technique the adaptation period is critical. If the adaptation period is too long it will deplete the whole body pool of the test amino acid. This may cause further metabolic changes that could affect the estimate of the requirement. Unlike the nitrogen balance studies which require a long adaptation period (~7-10 days) to stabilize body urea pool, a direct response of the aminoacyl t-RNA levels to the changes in dietary amino acid intake takes place in less than 4 hours (Crim and Munro, 1994). In human adults the oxidation of an indicator amino acid stabilizes within 4 hours of the initial change of the test amino acid (Zello et al, 1995, Pencharz and Ball, 2003). The rapid response in oxidation is due to the lack of storage for free amino acids and the short half life of the plasma free amino acid pool (Pencharz and Ball, 2003). The short adaptation period and minimal invasiveness allows scientists to study the full range of intakes and to experiment on fragile populations, such as infants and adults in various disease states (Pencharz and Ball, 2003).

Besides determining amino acid requirements, one of the many applications of the IAAO technique is determining limiting amino acids in various amino acid profiles. Brunton et al., (2007) successfully determined the limiting amino acids in two amino acid profiles by using the IAAO method. The IAAO technique allowed for rapid and accurate assessment of the amino acids within these profiles which were limiting to protein synthesis (Brunton et al., 2007).

Similar to the DAAO technique, one issue regarding the IAAO technique is whether or not oxidation studies can be conducted only in the fed state or whether both fed and fasted periods need to be measured. Although amino acid oxidation is higher in the fed state, calculation of the lysine requirement in human adults was not different when 24h fed and fasted was compared to fed only data (Kurpad et al, 2001); therefore, it is appropriate to determine amino acid requirements in either the fed or fed plus fasted states.

24h indicator amino acid balance combines the 24h direct amino acid oxidation with the technique of the indicator amino acid oxidation. 24h IAAB measures the oxidation of a stable or radiolabeled amino acid during a 12 hour fasted state and a 12 hour fed state (Bos et al., 2002). The major benefit of this technique is the fact that protein metabolism is measured over a 24h period, giving a more of a complete understanding of the metabolic demands throughout the day.

The major limitations of 24h IAAB is the adaptation period. The subject is fed a graded intake of the test amino acid for approximately 5-7 days. The long adaptation period eliminates the ability of this technique to measure amino acid requirements of young subjects and those in disease states (Pencharz and Ball, 2003).

When determining the requirements for a neonatal infant IAAO is the best technique currently available. Experimentation on human premature infants is hindered by countless ethical and practical restrictions; therefore, to determine the amino acid requirements using the IAAO technique using an appropriate animal model is useful.

## 1.6 The piglet as a model for the human pre-term infant

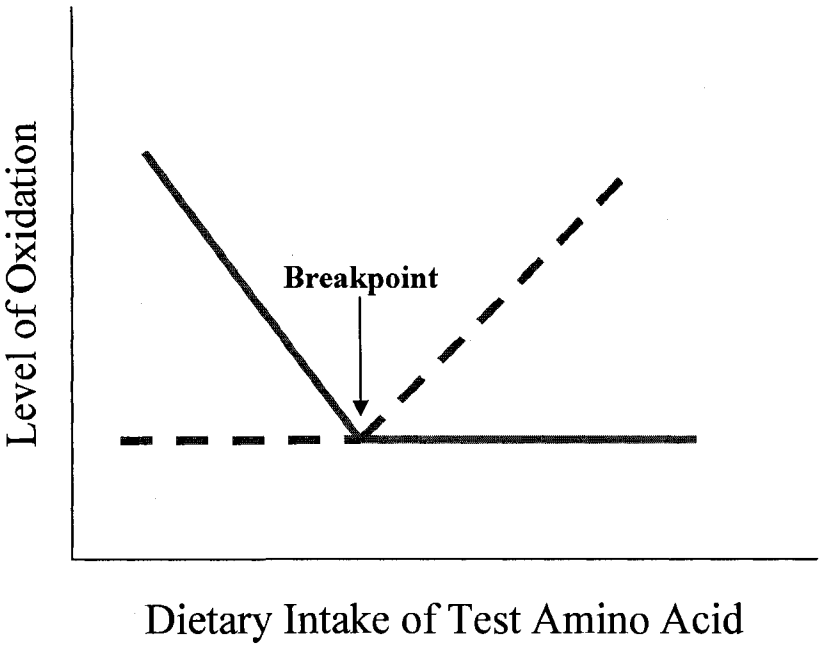
Premature infants are frequently inflicted with various conditions and are at a higher risk for mortality than their full-term counterparts. The immaturity of the respiratory and gastrointestinal system in the premature infant often prevents the administration of sufficient nutrients via enteral feeding; therefore an appropriate nutritional intervention is required to sustain viability (Brunton et al., 2000). Parenteral feeding is a commonly used nutritional intervention that allows the infant to receive the necessary nutritional support required to promote growth and development by introducing nutrients directly into the venous circulation. The optimal TPN nutrient balance, particularly for amino acids, needs to be determined. Yet due to the ethical and practical complications, experimentation with human infant subjects is severely limited.

The premature infant population is heterogeneous due to the variety of illnesses and complications associated with prematurity, resulting in varying metabolic demands/stresses among individuals (Wykes et al., 1993). These complications may have a significant effect on experimental results and their interpretation (Ball et al., 1996). Ethical constraints hinder the design and application of the experimentation required to determine amino acid requirements of premature human neonates. Invasive methods and extensive sampling are unethical in an already comprised neonate and clinically approved methods only allow limited access to blood, urine and breath which affects the level to which one can monitor the nutritional intervention (Ball et al., 1996). Together these limitations make it difficult to elucidate the effects of small, but important changes in the metabolism of dietary nutrients without relying on large sampling populations, which are often not available (Ball et al, 1996). To determine the effects of

various parenteral nutrition interventions, specifically those examining protein metabolism, a more invasive and sensitive method using a homogenous population was required. For these reasons, the development of an animal model was required to determine empirically the nutrient requirements and nutrient metabolism of the premature infant.

Generally an animal model, such as the piglet, comes from a readily available, homogenous population which allows for more sensitive and invasive techniques to be applied. As a result, the researcher has the ability to detect small but important changes in metabolism with relatively low numbers of subjects required. The neonatal piglet is a good model for the premature human infant due to the physiological and anatomical similarities between the two species (Ball et al, 1996). The piglet and a pre-term human infant are also similar in body composition due their low fat reserves, low birth weight, susceptibility to hypoglycaemia (Mount et al, 1971) and high metabolic rate (Book et al, 1974). The piglet model is optimal when investigating various aspects pertaining to protein and amino acid metabolism due to the similar requirements, amino acid tissue concentrations and amino acid composition of milk between the piglet and human (**Table 1.1**). When compared to the premature infant, the piglet has a faster growth rate; therefore, small differences in metabolism may be detected over a relatively short period of time (Ball et al, 1996). Use of the piglet model effectively eliminates the concerns and problems associated with human experimentation while maintaining the integrity of the research being conducted; allowing researchers to empirically determine the nutrient requirements and nutrient metabolism of the premature infant.

**Figure 1.1:** Oxidation curves of amino acids when determining amino acid requirements using oxidation. *As illustrated the solid line represents the oxidation response of other amino acids when using IAAO whereas the dotted line represents the response of the test amino acid (direct oxidation). Breakpoint is representative of the requirement of the individual or the mean requirement of the population. Adapted from Zello et al., 1995.*



## 1.7 Determination of amino acid requirements using IAAO in the parenterally – fed piglet

To determine parenteral amino acid requirements using the IAAO technique at least three graded dietary levels are required above and below the requirement. Six or more levels are generally preferred as it more clearly defines the shape of the oxidation response allowing for a more accurate determination of the requirement break point of the test nutrient/amino acid. Changes in amino acid pool will occur rapidly, however the protein content of the diet should be standardized for a minimum of 24 hours prior to initiation of test diets. The statistical analysis should then define the mean requirement and upper 95% confidence interval for safe intake for the test nutrient. The variability between subjects can be affected by body composition, sex and breed differences; and can significantly affect the estimate of an amino acid requirement. Therefore, by selecting subjects of the same sex from a homogenous population one can more accurately estimate the requirement of the test amino acid. Ultimately, by properly designing requirement studies, any bias can be removed and with proper analysis of the data, an objective estimate of the requirement can be made (Zello et al., 1995).

The neonatal piglet model, in conjunction with the IAAO technique, has successfully determined the parenteral phenylalanine (House et al., 1997), lysine (House et al., 1998), threonine (Bertolo et al., 1998), branched chain amino acid (Elango et al., 2002), sulfur amino acid (Shoveller et al., 2003) and tryptophan (Cvitkovic et al, 2004) requirements. Within these experiments the parenteral ratio of the branched chain amino acids was found to be 1:1:1 (isoleucine/leucine/valine) not 1:1.8:1.2 as previously thought (Elango et al., 2002). It was also found that cysteine has a sparing effect on methionine

requirement (Shoveller et al., 2003b). With the exception of histidine, all of the indispensable amino acid requirements for parenterally fed piglets have been determined by the Ball/Pencharz group (**Table 1.2**). However, the parenteral amino acid profile containing all of the IAAO determined amino acid requirements has never been constructed or tested in vivo.

This review of the literature showed that there is a need to test a complete amino acid profile, the Ball/Pencharz profile (B/P profile), using the experimently determined amino acid requirements and to compare it to a modified commercial TPN solution that is currently being fed to infants throughout North America. The IAAO technique can determine if protein synthesis is greater when animals are fed the B/P profile compared to the commercially available amino acid solution. The neonatal piglet model, in combination with the IAAO technique could be used to determine the possible first limiting amino acid in the B/P profile. In combination the findings of this thesis will contribute and help better understand protein metabolism in piglets and human neonates.

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## 2.0 RATIONALE AND OBJECTIVES

### 2.1 Scope of thesis

The aims of the research presented in this thesis are to determine if the previously determined parenteral amino acid requirements (Ball/Pencharz profile) were adequate in meeting the metabolic demands of a growing parenterally-fed neonatal piglet when fed as a complete diet, and to elucidate which indispensable amino acid was the first limiting in the Ball/Pencharz profile.

### 2.2 Rationale

Previous research has determined requirements for most of the indispensable amino acids administered in a parenteral solution. Yet a complete amino acid profile, which is optimal in meeting the metabolic requirements of a neonatal piglet, remains elusive. Since the neonatal piglet is a model for the preterm human infant, this optimal profile can be initially examined without compromising the health of an already fragile infant. The first limiting amino acid is the indispensable/conditionally indispensable amino acid that is provided in the smallest quantity relative to its requirement. Feeding a diet with a limiting amino acid may have detrimental effects as it is below the requirement and will significantly effect protein utilization. The first limiting amino acid will limit whole-body protein synthesis, dietary amino acid utilization and could result in excessive amino acid break down resulting in metabolic consequences such as hyperammonemia. Evaluation of an amino acid profile based on the previously estimated requirements can be undertaken using the IAAO technique. This can determine whether the combination of previously-determined requirements are adequate in meeting the metabolic requirements of a growing parenterally-fed piglet and identify which amino

acids are limiting to piglet protein synthesis and optimal health. These findings will ultimately aid in eliminating the morbidities and mortalities observed in parenterally-fed human neonates, resulting from over- and underestimations of the parenteral amino acid requirements.

## 2.3 Specific hypotheses and objectives

**Hypothesis 1:** The Ball/Pencharz (B/P) profile is adequate in meeting the protein requirements of the parenterally-fed neonatal piglet.

The first study in this thesis examined whether or not the previously determined amino acid requirements, the B/P profile, were able to meet the metabolic needs of parenterally-fed neonatal piglets. This diet will be compared to a second dietary treatment, a slightly modified version of a commercially available amino acid profile. A third dietary treatment, the isonitrogenous profile, was isonitrogenous to the commercial diet but contained the same ratio of amino acids to the B/P diet. The isonitrogenous profile will be used to compare the adequacy of the amino acid requirements in the B/P profile with identical nitrogen intake as the commercial profile. Comparison of phenylalanine flux, using the indicator amino acid technique, will be used to determine which is best in accurately meeting the metabolic demands of a neonatal piglet.

**Hypothesis 2**: Either lysine, leucine, methionine, or threonine is the first limiting amino acid in the Ball/Pencharz profile

The B/P amino acid profile did not meet the metabolic demands of the neonatal infant. Plasma concentrations of leucine, methionine and threonine were low when fed

the B/P profile, compared to the commercial profile and the sow-fed reference range. Typically in grain based swine diets lysine is the first limiting amino acid (Moehn et al., 2005) and in the first trial (hypothesis 1 research) the lysine intake was lower in the B/P amino acid profile than the commercial profile. In the second study of this thesis, lysine, leucine, methionine or threonine, were fed above the previously determined requirement level and phenylalanine oxidation and flux were used to determine which of these amino acids was the first limiting in the B/P amino acid profile (Brunton et al. 2007).

**Hypotheses 3:** Proline is an indispensable amino acid in parenterally fed neonatal piglets.

Plasma concentrations of proline in the parenterally fed piglet were low across all treatments when compared to that of a healthy piglet. Proline content of the solutions used in the previous experiments was lower than most other commercially available solutions (Brunton et al., 2000)). It is known that parenterally fed piglets experience varying degrees of gut atrophy and because the gut was the primary location for endogenous proline synthesis (Bertolo et al, 2000), it was hypothesized that there may be a dietary proline requirement that was greater than that provided by the B/P and commercial TPN solutions. In the third study of this thesis additional proline was added to the commercial profile. Phenylalanine oxidation in the commercial profile elemental diet and the commercial profile with additional proline were measured using the indicator amino acid technique. Phenylalanine flux was then calculated to determine if proline is an indispensable amino acid and supplementation increased protein synthesis in the parenterally fed piglet.

**Hypotheses 4:** Leucine, methionine, and/or threonine are co-limiting amino acids in the BP profile.

A limiting amino acid could not be determined in the second study (hypothesis 2), suggesting that two or more of the amino acids studied may have been co-limiting in the B/P profile. Because proline was found to be a limiting amino acid in the commercial amino acid profile (hypothesis 3), and the B/P diet proline intake in the first studies (hypothesis 1 and 2) was based on the commercial profile, additional proline was added to the B/P profile for this study. In the second study the plasma concentrations of leucine, threonine and methionine were still quite low when fed the B/P amino acid profile and thought to be limiting. Lysine was not believed to be limiting because when supplemented the plasma amino acid values were well above the sow fed reference range (Wykes et al., 1994). Therefore in the final study of this thesis the B/P profile was supplemented with various combinations of the suspected limiting amino acids. The B/P profile was supplemented with the following amino acid concentrations, fed above the previously determined requirements, leucine/threonine or leucine/methionine, or threonine/methionine, or leucine/threonine/methionine. Possible limiting amino acids were fed in combinations to examine the possibility of co-limiting amino acids. Phenylalanine flux was again calculated in all treatments to determine which amino acid is most likely limiting in the B/P amino acid profile.

## 2.4 **Literature Cited**

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# 3.0 EXPERIMENTAL SECTION

## 3.1 THE COMPLETE BALL/PENCHARZ AMINO ACID PROFILE (B/P PROFILE) IS INADEQUATE IN SUSTAINING PARENTERALLY-FED NEONATAL PIGLETS

### 3.1.1 Introduction

The currently available parenteral amino acid solutions may not provide the appropriate profile of amino acids needed to support health, growth and development in human neonates. The amino acid patterns of these TPN solutions are generally based on an enterally administered reference protein profile such as breast-milk or egg protein (Brunton et al., 2000), which may not be appropriate when first-pass splanchnic metabolism is bypassed. Previously it was assumed that when feeding a human neonate there was no difference between enteral and parenteral requirements. However it has been shown that several amino acids, including threonine and the branched chain and sulfur amino acids are metabolized during first-pass intestinal metabolism, resulting in lower parenteral versus enteral amino acid requirements (Bertolo et al., 1998, Elango et al., 2002, Shoveller et al., 2003a, Stoll and Burrin, 2006).

The IAAO technique can be used to assess the adequacy of an amino acid profile as a measure of protein synthesis. As previously described, the IAAO technique assumes that amino acids are not stored. Therefore if an amino acid is limiting, all other amino acids are in excess and are oxidized (Brunton et al., 2007). Oxidation is then measured and the lowest oxidation of the indicator amino acid suggests an optimization of protein synthesis. This technique has been used to determine the parenteral requirement for all of

the indispensable amino acids except for histidine (House et al., 1997, House et al., 1998, Bertolo et al., 1998, Elango et al., 2002, Shoveller et al., 2003 and Cvitkovic et al, 2004). When the amino acid profile was constructed, using the previously determined indispensable amino acid requirements, there were many differences between the new profile and solutions that are currently being administered in hospitals (Brunton et al., 2000). The IAAO technique can be used to determine if oxidation is lower (and hence protein synthesis is optimized) when administering the new amino acid profile compared to the commercially available alternatives. The premature piglet is a well validated model for this type of research and some of the results from this model has been replicated in subsequent human research (Bross et al., 2000 and Mager et al., 2003).

In the present study, the objective was to determine whether a new parenteral amino acid profile (B/P profile) based on the previously determined amino acid requirements in a parenterally-fed piglet model (House et al., 1997, House et al., 1998, Bertolo et al., 1998, Elango et al., 2002, Shoveller et al., 2003 and Cvitkovic et al, 2004), would be better at supporting piglet growth, health and protein synthesis than a commonly used commercial parenteral solution (commercial profile). A parenterally-fed piglet model was used and protein synthesis was assessed using the indicator amino acid oxidation technique. We hypothesize that the Ball/Pencharz amino acid profile will be adequate in meeting the metabolic demands of the parenterally-fed neonatal piglet.

### 3.1.2 **Materials and Methods**

#### 3.1.2.1 ***Animals and study protocol***

The Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta approved all procedures used in this study. Nine Duroc-Landrace/Large White cross piglets were obtained from the Swine Research and Technology Centre at the University of Alberta Research Farm (Edmonton, Alberta, Canada) at 1-2 days of age and ~1.5-2.0 kg body weight. All animals were pre-medicated prior to surgery with atropine sulfate (0.05 mg/kg; MTC Pharmaceuticals, Cambridge ON, Canada), ampicillin sodium (10mg/kg, Ampicillin®, Novopharm, Toronto, ON) and meloxicam (0.2mg/kg, Metacam®, Boehringer Ingelheim (Canada), Burlington, ON). Atropine sulfate and ampicillan sodium were administered intramuscularly whereas the meloxicam was administered subcutaneously. Piglets were intubated and anaesthetized with 1% halothane and maintained throughout surgery on 1-2% isoflurane. Piglets were then fitted with two venous catheters using a modified surgical technique previously established by Wykes et al. (1993). A catheter for purpose of infusions and injections was implanted into the left jugular vein of the piglet and advanced to the superior vena cava; the second catheter for the purpose of sampling was implanted into the left femoral vein and advanced to inferior vena cava. Immediately after surgery, piglets were fitted in a cotton jacket, weighed and attached to a swivel/port system (Alice King Chatham Medical Arts, Los Angeles, California) for diet infusion. The swivel/port system allowed the piglets to move freely throughout their housing unit (described below) without compromising the patency of the individual catheters and allowing for continuous infusion of diet. Piglets were then injected intravenously with an

analgesic (0.1 mg/kg, Buprenex; Buprenorphrine HCl, Reckitt and Colman Pharmaceutical Inc., Richmond, VA) and the incision sites were treated with a topical antibiotic cream (Hibitane® Veterinary Ointment: Ayerst Laboratories, Montreal, PQ). Analgesic was given again 8 and 16 h post-surgery. Piglets were also treated intravenously with anti-bacterial (0.5mL/kg, Borgal®, Intervet (Canada), Whitby, ON) and antibiotic injections (10mg/kg, Ampicillan®, Novopharm, Toronto, ON) for 24 hours post-surgically.

3.1.2.2 ***Animal Housing***

Animals were housed individually in circular cages, each 75cm in diameter. One heat lamp was supplied per two cages, which allowed the piglet to move away from the heat source based on individual need. Cages were outfitted with toys for environmental enrichment. The temperature of the room was maintained between 23-27°C (degrees °F). Lighting was on a twelve hour light:dark cycle.

3.1.2.3 ***Daily Care***

Piglets were monitored and recorded daily for signs of distress and morbidity such as heavy, laboured breathing and increased temperature. Daily care consisted of weighing the piglet, a temperature reading and treatment of incision sites with a topical antibiotic cream (Hibitane® Veterinary Ointment: Ayerst Laboratories, Montreal, PQ). Piglets exhibiting signs of hyperammonemia, such as laboured breathing, rapid increase in weight and decrease in motor function, were immediately removed from the trial and were euthanized with pentobarbital. Twenty four hour urine samples were collected in a

1L plastic Erlenmeyer flask for 24h periods ending on days 4, 5, 6, and 7. Each flask contained 5 mL of orthophosphoric acid (85% v/v) to maintain the urine at a pH of approximately 1, to prevent the degradation of nitrogen during the collection and storage period. Total urine was recorded on a weight basis and then a 5 mL sub-sample was obtained and stored at –20°C until nitrogen analysis was completed. Diet bags were also weighed at this time, which allowed for diet intake to be determined.

### 3.1.2.4 ***Blood Sampling***

Blood samples (1 mL) were taken every 24 hours from the femoral vein catheter beginning on the morning of day 1 until the end of the trial on day 7 or day of euthanasia. Blood was obtained using a 3ml syringe and 18 gauge needle and transferred into a 4 mL vacutainer containing lithium heparin. The blood was then centrifuged at 3000 rpm (Technospin, Sorvall® Centrifuge, Mississauga, ON) for 10 min. The daily blood samples were stored in the –20°C freezer and later used to determine both plasma ammonia and urea concentrations. Additional blood samples were taken on d 7 as described under the *$^{14}C$ Phenylalanine Infusion, $^{14}CO_2$ and blood collection, and analytical procedures* section.

### 3.1.2.5 ***Diet Regimen***

Immediately following surgery piglets were randomly assigned to one of three treatment groups: commercial (n = 3), Ball/Pencharz (n = 3) profile (B/P) or isonitrogenous Ball/Pencharz (n = 3) profile (isonitrogenous), and remained on the same diet throughout the entire trial. Treatment groups were originally equal with 4 piglets per

treatment; however due to early morbidities only 3 piglets were used in the B/P and isonitrogenous treatments. Diets were then infused into the jugular vein catheter using a pressure-sensitive infusion pump. Actual intake of piglets receiving the B/P, isonitrogenous, and commercial profiles were approximately 12, 14 and 15 g amino acids $kg^{-1} \cdot d^{-1}$ respectively, and 1.1 MJ metabolizable energy $kg^{-1} \cdot d^{-1}$ with glucose and lipid (Intralipid 20%, PharmaciaUpjohn, Stockholm, Sweden) each supplying 50% of non-protein energy intake.

The amino acid profile of the commercial diet (Primene®, Baxter Healthcare, Mississauga, Canada) was based on cord blood content and is described in **Table 3.1.1**. Phenylalanine and tyrosine concentrations in this diet were modified to meet the estimated safe levels of intake previously determined (House et al. 1997a, House et al. 1997b), and to ensure that the intakes were the same across all treatments on a g/kg*d basis. Previously determined parenteral requirements of lysine, threonine, the sulfur amino acids, the branched chain amino acids, the aromatic amino acids (House et al. 1994, House et al. 1997a, Bertolo et al. 1998, House et al. 1998, Brunton et al. 1999, Elango et al. 2002, Shoveller et al. 2003a, Shoveller et al. 2003b, Elango et al. 2004, and Cvitkovic et al. 2004) were used to make up the amino acid profile for the B/P diet. For the B/P profile, the parenteral ratio of branched chain amino acids, determined by Elango et al. (2004), of 1:1:1 (isoleucine/leucine/valine) was used. The B/P amino acid profile also contained cysteine at 40% of the total sulfur amino acid intake based on the findings of Shoveller et al. (2003). The non-essential amino acids profile and histidine were supplied in the Ball/Pencharz diet at identical levels as found in the commercial diet, on a g/kg*d basis. The commercial profile and the B/P profile provided different amounts of

nitrogen. Because this could have an effect on protein synthesis, the isonitrogenous profile was created by increasing all amino acids (except Phenylalanine and Tyrosine), from the B/P profile, by a constant ratio to make a third treatment that would be isonitrogenous to the commercial diet. The third treatment, the isonitrogenous profile, would have the same profile as the B/P diet to determine if the increased nitrogen intake would improve N-retention/protein synthesis relative to the B/P and commercial diets. The amino acid profiles for the Ball/Pencharz and isonitrogenous Ball/Pencharz are described in **Table 3.1.2 and 3.1.3** respectively. In all diets, tyrosine was provided as a dipeptide, glycyl-tyrosine (House et al. 1997b), because of the low solubility of free-based tyrosine. Cysteine was added as a free base and was carefully monitored during dissolution to make sure that it was not being oxidized or forming the insoluble dimer cystine. Lysine-HCl and ornithine-HCl were also used to increase solubility. Diets were made under a nitrogen blanket to prevent amino acid oxidation. Once amino acids were in solution, glucose (90 g/L) was dissolved into the amino acid solution. The macro minerals, calcium, phosphorus, potassium, sodium, chloride, magnesium, manganese and were added to the solution. Diets were then filtered through 0.22 μm sterile filter into pyrogen-free sterile TPN bags and stored at 4 C until use.

Immediately before diets were fed, vitamins and mineral premixes were added. Vitamins, both fat and water-soluble, were supplied by a prefabricated commercial solution (Multi-12/$K_1$ Pediatric; Sabex Inc., Boucherville PQ, Canada) and provided 115% of the estimated NRC (1998) requirement for piglets 3-5 kg. The vitamin solution was added to the elemental diet with iron sulfate (Ferroforte; Bimeda-MTC, Cambridge ON, Canada) and a mineral solution. The micro-mineral solution contained zinc sulfate,

copper sulfate, manganese sulfate, chromium sulfate, selenium sulfate and sodium iodide and provided 200% of the NRC (1998) recommendation for piglets 3-5 kg. 20% Intralipid was then added to the diet in a 5:1 amino acid/glucose premix: lipid ratio.

Immediately following surgery all piglets received diet parenterally at 50% of the total infusion rate (6.75 mL/kg*d). 12 hours following surgery the infusion rate was increased to 75% of the total infusion rate (10.125 mL/kg*d). On the morning of day 1 all piglets received the diets at 13.5 mL/kg*d and continued at that rate for the remainder of the study.

#### 3.1.2.6 *$^{14}C$ Phenylalanine Infusion, $^{14}CO_2$ collection in breath sample and blood sample collection, and analytical procedures*

On day 7, those piglets that were still alive underwent a primed (7 μCi/kg), constant (3.5 μCi/kg) infusion of L-[1-$^{14}$C]phenylalanine, infused via the jugular vein catheter, to determine phenylalanine oxidation. The duration of constant infusion was 4h to ensure a plateau was reached in both breath and blood labelling. Blood samples (1 mL) were taken at time 0, 120, 150, 180, 210 and 240 minutes; whereas breath samples were collected every 30 minutes for the duration of the isotope infusion. Details of infusion protocol, $^{14}CO_2$ collection and blood collection procedures have been described previously (House et al, 1997a). Following the infusion on d 7, piglets were killed by injection of 1000 mg of sodium pentobarbital into a venous catheter.

The radioactivity of the breath samples collected during the [1-$^{14}$C]phenylalanine infusion was determined by combining a 1 mL aliquot of the collected breath sample with 6 mL of scintillant (Atomlight; PerkinElmer Life and Analytical Sciences, Boston

MA, USA), and counting the samples on a scintillation counter (Tri-Carb 4000 series, Canberra Packard, Canada). Phenylalanine oxidation was then calculated using the series of formulas from House et al. (1997a).

Plasma amino acids were determined using reverse-phase high performance liquid chromatography with the use of phenylisothiocyanate derivatives used to measure plasma amino acids, as previously described (Bidlingmeyer et al., 1984 and House et al., 1994). Plasma concentrations are represented as the mean concentration.

Urea nitrogen (Sigma Procedure No. 640; Sigma Diagnostics, St. Louis MO, USA) concentrations were determined every 24 h during the trial (d 3 – d 7) using spectrophotometric assays.

Total urinary excretion was collected within a 100 mL erlenmeyer flask containing 7mL orthophosphoric acid (85% v/v) for preservation. A 5mL sub sample was taken every 24 hours and stored at –20°C for future analysis.

Urinary nitrogen was determined using a macro-Kjeldahl method based on method 976.05 (AOAC, 1990). Nitrogen retention and balance were calculated using formulas previously described (Bertolo et al., 1999).

3.1.2.8 ***Statistical Analysis***

All data were analyzed using SAS Version 9.1 (2003 SAS Institute, Cary NC, USA), and data were considered statistically significant if $p < 0.05$. The dependant variable, plasma amino acid concentration, was analyzed using proc GLM where the class variable was diet. The serum urea and nitrogen intake, output and retention were analyzed using the mixed model where the fixed effect was diet and the random variable

was piglet. When the effects were significant ($p < 0.05$), least squares means were separated using the pdiff option.

**Table 3.1.1:** Amino acid concentration of the modified commercial parenteral solution administered to parenterally-fed neonatal piglets (commercial treatment group) from d0 to d7

| **Amino Acids** | Concentration (g/100g) | Concentration (g $L^{-1}$) |
|---|---|---|
| L-Alanine | 7.90 | 4.1489 |
| L-Arginine | 8.40 | 4.4114 |
| L-Aspartate | 6.00 | 3.1511 |
| L-Glutamate | 9.90 | 5.1993 |
| Glycine | 4.00 | 1.6544 |
| L-Histidine | 3.80 | 1.9957 |
| L-Isoleucine | 6.70 | 3.5187 |
| L-Leucine | 9.90 | 5.1993 |
| L-Lysine[1] | 10.90 | 7.1522 |
| L-Methionine | 2.40 | 1.2604 |
| L-Phenylalanine | 4.20 | 2.2058 |
| L-Proline | 3.00 | 1.5756 |
| L-Serine | 4.00 | 2.1007 |
| Taurine | 0.60 | 0.3151 |
| L-Threonine | 3.70 | 1.9432 |
| L-Tryptophan | 2.00 | 1.0504 |
| L-Tyrosine | 0.90 | 0.4412 |
| L-Valine | 7.60 | 3.9914 |
| Glycyl-Tyrosine[3] | 2.67 | 1.5780 |
| L-Cysteine | 1.90 | 0.9978 |
| Ornithine[2] | 2.20 | 1.4739 |

[1] Lysine was supplied as Lysine-HCl to provide the concentration shown.
[2] Ornithine was supplied as Ornithine-HCL to provide the concentration shown.
[3] Glycyl tyrosine supplying 0.32 g $L^{-1}$ of glycine and 1.19 g $L^{-1}$ tyrosine.

**Table 3.1.2:** Amino acid concentration of Ball/Pencharz amino acid pattern administered to parenterally-fed neonatal piglets (B/P treatment group) from d0 to d7

| **Amino Acids** | Concentration (g/100g) | Concentration (g $L^{-1}$) |
|---|---|---|
| L-Alanine | 9.58 | 4.149 |
| L-Arginine | 10.19 | 4.4118 |
| L-Aspartate | 7.28 | 3.1511 |
| L-Glutamate | 12.01 | 5.1993 |
| Glycine | 4.85 | 1.6544 |
| L-Histidine | 4.61 | 1.9957 |
| L-Isoleucine | 5.63 | 2.4386 |
| L-Leucine | 5.63 | 2.4386 |
| L-Lysine[1] | 7.13 | 3.8585 |
| L-Methionine | 2.29 | 0.9926 |
| L-Phenylalanine | 5.10 | 2.2059 |
| L-Proline | 3.64 | 1.5756 |
| L-Serine | 4.85 | 2.1007 |
| Taurine | 0.73 | 0.3151 |
| L-Threonine | 1.78 | 0.7721 |
| L-Tryptophan | 1.53 | 0.6618 |
| L-Tyrosine | 3.57 | 0.4412 |
| L-Valine | 5.63 | 2.4386 |
| Glycyl-Tyrosine[3] | 3.40 | 1.5074 |
| L-Cysteine | 1.27 | 0.5515 |
| Ornithine[2] | 2.67 | 1.4739 |

[1] Lysine was supplied as Lysine-HCl to provide the concentration shown.
[2] Ornithine was supplied as Ornithine-HCL to provide the concentration shown.
[3] Glycyl tyrosine supplying 0.32 g $L^{-1}$ of glycine and 1.19 g $L^{-1}$ tyrosine.

**Table 3.1.3:** Amino acid concentration of B/P amino acid pattern that is isonitrogenous to the modified commercial parenteral solution administered to parenterally-fed neonatal piglets (isonitrogenous treatment group) from d0 to d7

| **Amino Acids** | Concentration (g/100g) | Concentration (g $L^{-1}$) |
|---|---|---|
| L-Alanine | 9.58 | 5.0056 |
| L-Arginine | 10.19 | 5.3228 |
| L-Aspartate | 7.28 | 3.8018 |
| L-Glutamate | 13.31 | 6.934 |
| Glycine | 4.85 | 2.0871 |
| L-Histidine | 4.61 | 2.4077 |
| L-Isoleucine | 5.63 | 2.9422 |
| L-Leucine | 5.63 | 2.9422 |
| L-Lysine[1] | 7.13 | 4.6552 |
| L-Methionine | 2.29 | 1.1976 |
| L-Phenylalanine | 4.23 | 2.2059 |
| L-Proline | 3.64 | 1.9011 |
| L-Serine | 4.85 | 2.5345 |
| Taurine | 0.73 | 0.3801 |
| L-Threonine | 1.78 | 0.9315 |
| L-Tryptophan | 1.53 | 0.7984 |
| L-Tyrosine | 2.96 | 0.4412 |
| L-Valine | 5.63 | 2.9422 |
| Glycyl-Tyrosine[3] | 2.73 | 1.5074 |
| L-Cysteine | 1.27 | 0.6653 |
| Ornithine[2] | 2.67 | 1.7783 |

[1] Lysine was supplied as Lysine-HCl to provide the concentration shown.
[2] Ornithine was supplied as Ornithine-HCL to provide the concentration shown.
[3] Glycyl tyrosine supplying 0.32 g $L^{-1}$ of glycine and 1.19 g $L^{-1}$ tyrosine.

### 3.1.3 **Results**

#### 3.1.3.1 ***Piglet performance***

Piglets were all healthy in the 1 – 2 days following surgery and recovered fully from surgery without complication. Beginning on days 3-5 piglets receiving the B/P and isonitrogenous treatments started to experience rapid weight gain, laboured breathing, and loss of motor function (**Table 3.1.4**). Sick piglets were immediately removed from the trial and humanely euthanized.

#### 3.1.3.2 ***Plasma urea concentrations***

The average plasma urea concentrations for the B/P profile, isonitrogenous profile and the profile based on Primene were 7.28, 5.36 and 0.87 μmol/L respectively (**Table 3.1.5**). The plasma urea concentrations of the Ball/Pencharz and isonitrogenous profiles differed significantly ($p<0.05$) from the commercial amino acid profile. The plasma urea reference range for sow-raised piglets is 0.7 to 4.9 μmol/L (Wykes et al., 1993). The only serum concentration values that were within this reference range were the piglets fed the profile based on Primene; piglets fed the B/P profile and the isonitrogenous profile had serum concentrations well above this reference range.

#### 3.1.3.3 ***Urine nitrogen concentrations***

The nitrogen intakes of the B/P, isonitrogenous and commercial profiles were 3.76, 4.38, and 4.30 g/kg*d respectively and were not different between diets (**Table 3.1.5**). Had the number of piglets in each treatment group been larger a significant difference would likely appear between the B/P profile and the isonitrogenous or

commercial profiles due to the different nitrogen content of the diets Nitrogen output in urine in piglets receiving the B/P and isonitrogenous profiles were greater than in those piglets receiving the commercial profile. As a result, the percent nitrogen retained was 42.69%, 48.48% and 91.54% for the B/P profile, the isonitrogenous profile and the commercial profile, respectively. The percent nitrogen retained in the piglets receiving B/P and isonitrogenous profiles were significantly lower than in those piglets receiving the commercial profile, and on a g/kg*d basis all treatment groups were significantly different from each other (**Table 3.1.5**).

3.1.3.4 ***Plasma amino acid concentrations***

For the dispensable amino acids, diet had a significant effect on the plasma concentrations of alanine, aspartate and asparagine. In piglets fed the B/P and the isonitrogenous diets plasma concentrations of alanine, aspartate and asparagine increased significantly ($p<0.05$) when compared to the commercial profile. Of the conditionally indispensable amino acids, glutamine and proline were significantly affected by diet ($p<0.05$, **Table 3.1.6**) and were higher when fed the B/P and isonitrogenous profiles. Of the indispensable amino acids, diet only had an effect on histidine and isoleucine concentrations ($p< 0.05$, **Table 3.1.6**). The concentrations of histidine and isoleucine for the isonitrogenous diet were significantly different ($p<0.05$, **Table 3.1.6**) from the commercial profile.

In piglets receiving the commercial profile diet, alanine, glutamate and aspartate plasma amino acid concentrations were below the sow-fed reference range; whereas when fed either the B/P amino acid profile or the isonitrogenous profile only glutamate

was below the reference range (**Table 3.1.6**). When animals were fed the isonitrogenous diet, the plasma concentration of glycine was higher than the enterally-fed reference range. Proline plasma concentrations were lower than the enterally-fed reference range for all dietary treatments. In piglets fed the B/P Profile and isonitrogenous profile, histidine and isoleucine concentrations were above the sow-fed reference range. Phenylalanine concentrations were also above the reference range due to the imbalanced amino acid profile suggesting that the phenylalanine in the diet could not be used for protein synthesis and there was likely a greater rate of protein breakdown. Methionine, threonine and leucine plasma concentrations in piglets fed the B/P profile or the isonitrogenous profile were below the orally-fed reference range (**Table 3.1.6**). Threonine and methionine were also below the reference range when fed the commercial profile.

When comparing the plasma concentrations taken on D1 to those prior to death or end of trial, plasma alanine concentration, for the piglets fed the B/P profile, increased significantly ($p<0.05$) when compared to the profile based on Primene (**Table 3.1.7**). When comparing plasma concentrations of the conditionally indispensable amino acids no significant differences ($p>0.05$) were found across all treatment groups (**Table 3.1.7**). The piglets receiving the B/P profile and isonitrogenous profiles had an increased plasma concentrations of glutamine, glycine, and tyrosine; whereas only glycine increased when piglets were fed the profile based on Primene. For the indispensable amino acids, the only significant difference was for valine. Plasma valine concentrations were significantly lower ($p<0.05$) in piglets receiving the isonitrogenous profile when compared to the B/P profile (**Table 3.1.7**). Of the indispensable amino acids, histidine,

lysine and phenylalanine increased when fed the B/P profile and the isonitrogenous profile. When fed the isonitrogenous profile, isoleucine, leucine and valine concentrations in plasma all were lower suggesting a failure to meet branched-chain amino acid requirements.

#### 3.1.3.5 ***Phenylalanine oxidation***

Piglets receiving the commercial amino acid profile all lived to day 7 and the percent of dose oxidized of $^{14}C$ phenylalanine was found to be 6.62% (**Table 3.1.4**). Piglets receiving the other 2 diets, the B/P and isonitrogenous profiles, did not live until d7 and so there is no oxidation data available for these groups.

**Table 3.1.4**: Overall piglet performance

| Piglet | Diet | Body Weight day 0 (kg) | Body Weight day 3 (kg) | Daily gain (kg/day) | Percent Dose Oxidized (%) | Days on Trial |
|---|---|---|---|---|---|---|
| 1 | Isonitrogenous | 1.84 | 2.17 | 110.00 | --- | 6 |
| 2 | B/P | 1.86 | 1.96 | 33.33 | --- | 6 |
| 3 | Isonitrogenous | 1.71 | 2.15 | 146.67 | --- | 5 |
| 4 | Commercial | 1.87 | 2.07 | 66.67 | 6.58 | 7 |
| 5 | B/P | 1.45 | 1.61 (d2) | 80.00 | --- | 2 |
| 6 | Isonitrogenous | 1.47 | 1.8 | 110.00 | --- | 5 |
| 7 | B/P | 1.11 | 1.55 | 146.67 | --- | 3 |
| 8 | Commercial | 1.4 | 1.66 | 86.67 | 6.12 | 7 |
| 9 | Commercial | 1.72 | 2.04 | 106.67 | 7.17 | 7 |

--- Piglet did not live until d 7; therefore no oxidation data available for these animals

**Table 3.1.5:** Nitrogen balance and retention on day 4 in neonatal piglets receiving total parenteral nutrition containing different amino acid profiles[1]

| | Ball/Pencharz Profile (n=3) | SE | Isonitrogenous (n=3) | SE | Commercial (n=3) | SE | p Value |
|---|---|---|---|---|---|---|---|
| Plasma Urea (umol/L) | 7.28[a] | 1.55 | 5.36[a] | 0.41 | 0.87[b] | 0.07 | <.0001 |
| Nitrogen Intake (g/kg*d) | 3.76[a] | 0.10 | 4.38[a] | 0.21 | 4.30[a] | 0.05 | 0.6274 |
| Nitrogen Output (g/kg*d) | 2.14[a] | 0.34 | 2.28[a] | 0.28 | 0.36[b] | 0.01 | <.0001 |
| Nitrogen Retained (g/kg*d) | 1.62 | 0.08 | 2.13 | 0.06 | 3.94 | 0.01 | 0.0004 |
| Nitrogen Retention (%) | 42.69[a] | 3.79 | 48.48[a] | 3.24 | 91.54[b] | 0.21 | <.0001 |

Significant difference at $p<0.05$.
[abc]Values sharing a superscript are not significantly different ($p<0.05$)

**Table 3.1.6:** Plasma amino acid concentrations of piglets receiving different amino acid profiles after 4 days of adaptation to test diets[1]

| | Plasma Amino Acid Concentrations[2] umol/L Diet | | | | | | |
|---|---|---|---|---|---|---|---|
| | B/P Profile | SE | Isonitrogenous Profile | SE | Commercial Profile | SE | Orally-Fed Reference Range[3] |
| **Dispensable Amino Acids** | | | | | | | |
| Alanine | 564.24[a] | 101.38 | 726.69[a] | 151.37 | *192.57[b]* | 40.23 | 413-1015 |
| Aspartate | *13.48[ab]* | 4.04 | *19.23[a]* | 3.7 | *5.33[b]* | 1.79 | 22-63 |
| Asparagine | 33.98[a] | 11.69 | 38.33[a] | 4.42 | 8.65[b] | 1 | |
| Glutamate | *46.25* | 10.56 | *65.13* | 7.68 | *48.91* | 15.21 | 92-217 |
| Serine | 238.94 | 70.68 | 365.83 | 83.07 | 218.56 | 53.39 | 137-442 |
| **Conditionally Indispensable Amino Acids** | | | | | | | |
| Arginine | 66.31 | 4.51 | 104.03 | 14.3 | 74.94 | 24.62 | 50-267 |
| Cysteine | 49.42 | 18.99 | 68.37 | 19.18 | 41.63 | 14.83 | |
| Glutamine | 215.64[a] | 58.05 | 224.58[a] | 22.38 | 48.51[b] | 17.62 | |
| Glycine | 975.64 | 77.72 | **1507.67** | 368.59 | 759.04 | 142.72 | 450-976 |
| Proline | *190.23[a]* | 20.59 | *234.49[a]* | 27.32 | *76.26[b]* | 11.59 | 304-890 |
| Tyrosine | *81.7* | 21.92 | 150 | 58.29 | *39.12* | 12.74 | 88-231 |
| **Indispensable Amino Acids** | | | | | | | |
| Histidine | **151.34[ab]** | 80.71 | **339.56[a]** | 120.08 | *32.92[b]* | 22.9 | 47-121 |
| Isoleucine | **190.58[ab]** | 24.54 | **225.24[a]** | 40.5 | 97.69[b] | 26.62 | 81-143 |
| Leucine | *79.5* | 9.48 | *85.33* | 14.52 | 160.78 | 51.48 | 102-175 |
| Lysine | 203.04 | 59.6 | 289.05 | 64.2 | **398.44** | 96.46 | 77-317 |
| Methionine | *17.12* | 7.56 | 68.44 | 31.14 | *21.96* | 8.44 | 33-74 |
| Phenylalanine | **167.94** | 56.7 | **268.54** | 115.67 | 62.99 | 8.54 | 34-86 |
| Threonine | *20.44* | 3.45 | *21.34* | 9.54 | *27.51* | 10.11 | 214-500 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Tryptophan | --- | --- | 10.02 | 6.36 | 13.79 | --- | |
| Valine | 280.43 | 19.18 | **337.47** | 51.04 | 229.56 | 64.94 | 175-318 |
| **Other Amino Acids** | | | | | | | |
| Taurine | 170.61[a] | 50.98 | 167.03[a] | 12.85 | 69.81[b] | 7.57 | |
| Ornithine | 103.54[ab] | 17.37 | 128.60[a] | 19.59 | *59.77[b]* | 18.42 | 79-195 |
| hydroxy-Proline | 62.97 | 22.25 | 66.59 | 4.94 | 39.91 | 10 | |
| Citrulline | *52.05[a]* | 10.7 | *46.51[a]* | 8.07 | *17.67[b]* | 4.87 | 73-151 |

[1] Values are means of N=3 piglets.
[2] Significant difference at $p<0.05$. Values sharing a superscript are not significantly different ($p<0.05$)
[3] Wykes et al. (1993). **Bold value** indicates a value above orally fed reference range. *Italic value* indicates a value below orally fed reference range.

**Table 3.1.7:** Differences in plasma amino acid concentrations of piglets receiving different amino acid profiles from beginning of trial to time of death

| Difference in Plasma Amino Acid Concentration (D1 to time of death)[1] µmol/L Diet | | | | | | |
|---|---|---|---|---|---|---|
| | B/P Profile | SE | Isonitrogenous Profile | SE | Commercial Profile | SE |
| **Dispensable Amino Acids** | | | | | | |
| Alanine | 921.20[a] | 366.34 | 610.30[ab] | 174.24 | -5.26[b] | 26.88 |
| Aspartate | 28.40 | 24.75 | 22.29 | 24.23 | 0.31 | 2.50 |
| Asparagine | 21.68 | 15.83 | -12.57 | 14.62 | -5.33 | 2.45 |
| Glutamate | 4.24 | 32.26 | 12.83 | 44.64 | -28.47 | 16.36 |
| Serine | 419.02 | 176.27 | 363.92 | 118.36 | 147.22 | 12.59 |
| **Conditionally Indispensable Amino Acids** | | | | | | |
| Arginine | 89.90 | 66.59 | -50.22 | 33.77 | 20.66 | 10.55 |
| Cysteine | 100.45 | 86.98 | 42.11 | 28.41 | 5.79 | 10.32 |
| Glutamine | 250.47[a] | 51.00 | 44.11[a] | 62.02 | -8.6[b] | 25.88 |
| Glycine | 1804.36 | 627.86 | 1779.71 | 439.92 | 439.19 | 161.14 |
| Proline | 190.36 | 65.14 | 71.76 | 107.28 | -1.14 | 14.81 |
| Tyrosine | 303.58[a] | 135.70 | 163.84[ab] | 70.43 | 21.95[b] | 34.40 |
| **Indispensable Amino Acids** | | | | | | |
| Histidine | 501.83[a] | 205.48 | 514.71[a] | 97.73 | 48.53[b] | 12.47 |
| Isoleucine | 22.75 | 71.92 | -91.63 | 38.31 | -22.74 | 22.49 |
| Leucine | 65.91[a] | 170.81 | -68.94[b] | 21.46 | -6.34[ab] | 59.77 |
| Lysine | 315.45 | 31.86 | 82.99 | 79.23 | 29.70 | 53.46 |
| Methionine | 33.87 | 29.73 | 66.96 | 63.88 | 0.14 | 12.72 |
| Phenylalanine | 949.76 | 478.47 | 442.54 | 210.47 | -12.43 | 9.00 |
| Threonine | 43.93 | 18.93 | 11.83 | 19.85 | 4.76 | 13.89 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Tryptophan | 8.55 | 8.55 | 2.86 | 3.74 | -0.98 | 5.09 |
| Valine | 116.43[a] | 83.21 | -238.33[b] | 147.29 | -55.45[ab] | 36.83 |
| **Other Amino Acids** | | | | | | |
| Taurine | 107.76 | 66.12 | 77.13 | 13.06 | -8.85 | 8.84 |
| Ornithine | 58.51 | 56.74 | -47.73 | 32.77 | -0.51 | 4.71 |
| hydroxy-Proline | 23.00 | 6.76 | 25.17 | 13.98 | -8.55 | 11.74 |
| Citrulline | 65.69[a] | 9.53 | 33.99[b] | 10.52 | -0.31[c] | 4.74 |

[1]Significant difference at $p<0.05$. Values sharing a superscript are not significantly different ($p<0.05$)

### 3.1.4 **Discussion**

This study marks the first time that independently measured parenteral amino acid requirements have been used together to formulate a new, complete TPN solution. When using the indicator amino acid technique oxidation to assess one profile compared to another, oxidation should be lower in the profile which is closest to meeting all indispensable amino acid requirements, indicating a greater rate of protein synthesis and fewer excess amino acids. The piglet's health also should be maintained throughout the trial and should be comparable to or better than in those piglets receiving the commercial profile. Unfortunately we were unable assess the effect of the newly developed profile on amino acid oxidation as all piglets receiving the experimental diets required euthanasia by d 3 – 4 of the study due to signs of multi-organ failure, secondary to protein malnutrition. This study clearly demonstrated that the newly developed TPN profile was unable to sustain a growing TPN-fed neonatal piglet, suggesting an imbalance of the amino acids in the profile.

Nitrogen balance is a traditional "Gold standard" indication of dietary protein utilization in the body. A positive nitrogen balance indicates protein requirements are being met and muscle growth is occurring, with a higher balance indicating a greater amount of nitrogen accretion. A negative nitrogen balance, on the other hand, is associated with protein breakdown in excess of protein accretion and poor utilization of dietary protein (House et al., 1994). In this study, only the isonitrogenous and commercial profiles provided the same nitrogen intake, on a grams of N per kg per day basis; however, there was no statistical difference between diets for nitrogen intake (**Table 3.1.5**). This finding may be a result of the small sample sizes for B/P and

isonitrogenous diets due to the fact that piglets on this diet became rapidly ill and the trial was terminated prematurely. Therefore, there was not a lot of statistical power; and large absolute differences would be required in order to detect a statistical difference. Regardless of diet, piglets all continued to gain weight, however only the commercial treatment group was in a positive nitrogen balance (**Table 3.1.5**). Therefore, the gain of weight observed in the other treatments was not protein gain. However, even with similar nitrogen intakes, piglets fed the commercial profile had lower urea concentrations, and lower nitrogen excretion. Piglets fed the commercial profile retained 91.5% of nitrogen intake compared to approximately 42% and 48% for the B/P profile and the isonitrogenous profile, respectively. These data indicate that the commercial profile supported greater piglet growth due to a better amino acid balance. High rates of protein degradation have been associated with low nitrogen balance that can result in decreased motor function and increased water retention, as exhibited by the piglets fed the B/P and isonitrogenous profiles (Thivierge et al., 2005).

The percent dose oxidized for the piglets fed the commercial profile was approximately 6.62% (**Table 3.1.4**). Piglets fed the B/P and isonitrogenous profile did not survive until day 7; therefore no data was collected. IAAO technique assumes that amino acids are not stored. Therefore if an amino acid is limiting, all other amino acids are in excess and are oxidized (Brunton et al., 2007). Due to the amino acid imbalance in the B/P and isonitrogenous profile, oxidation rates were expected to be higher than the commercial profile.

Plasma urea concentration was used as an indirect measure of protein synthesis and whole-body amino acid catabolism, since flux could not be calculated for the B/P and

isonitrogenous treatment groups. When protein synthesis was lower, due to a possible amino acid imbalance that was limiting to protein synthesis, more of the other dietary amino acids would have been in excess, resulting in a higher amount of amino acid catabolism and a higher need for urea synthesis. Piglets fed the B/P and isonitrogenous profiles had to dispose of more ammonia than the animals fed the commercial profile resulting in an increased generation of urea and glutamine; suggesting the B/P and isonitrogenous profiles contain a deficient amino acid which is limiting to protein synthesis.

Of the dispensable and conditionally indispensable amino acids the plasma amino acid data is consistent with the nitrogen balance data and suggests that there is an amino acid imbalance. When the piglets were fed the B/P profile or the isonitrogenous profile plasma concentrations of glutamine, glycine and serine increased (**Table 3.1.7**). Glutamine is a nitrogen carrier and typically increases in concentration when there is an excess of dietary nitrogen in the body (Wilkinson et al., 2003). Glycine and serine are also known to increase with excess nitrogen intake and low utilization (Iapichino et al., 1988). The increase in concentration of these amino acids indicates that there was either an excessive intake of nitrogen or poor utilization of dietary nitrogen for protein synthesis. Plasma concentrations of most indispensable amino acids were generally higher in piglets fed the B/P and isonitrogenous diets than in the commercial diet, which suggests a lower rate of protein synthesis and a higher rate of protein breakdown.

The plasma amino acid concentration data indicates that leucine, methionine, threonine and/or lysine may be limiting in the B/P and isonitrogenous profile. Elango et al (2002) found that the parenteral branched chain amino acid requirement was 1.99

g/kg*d with the ratio being 1:1:1. From this information, the requirements of each of leucine, isoleucine and valine were extrapolated to 0.66 g/kg*d. To determine the optimal parenteral ratio of branched chain amino acids, a basal diet containing 75% of the mean branched chain amino requirement was supplemented with either isoleucine, valine or leucine to reach the total mean requirement of 1.53 g/kg*d (Elango et al., 2004). By looking at improvements in the rate of indicator amino acid oxidation relative to the basal diet, it was determined, using the indicator amino acid technique that isoleucine was the first limiting followed by valine and then leucine and concluded that the optimal branched chain ratio for parenteral feeding may be near 1:1:1. However, the ratio was never tested in direct comparison to the 1:1.8:1.2 (isoleucine/leucine/valine) at the requirement level (1.99 g/kg/d) to determine if this new ratio was in fact better than the previously assumed ratio. Because the mean requirement was used to determine the ratio versus the safe intake level used in the current experiment, it is possible that up to half of the piglets on the previous trial were not receiving their total branched chain amino acid requirement. If the optimal ratio for BCAA is different in piglets below versus at or above requirement then the 1:1:1 ratio may not have been the appropriate choice for this study. Leucine and the other branched chain amino acids play an important role in stimulating protein synthesis in skeletal muscle while inhibiting degradation (Garlick, 2005); therefore, if leucine in particular was below requirement, it is possible that there was inadequate stimulation for protein synthesis, possibly contributing to the observed loss of motor function and muscle strength observed in the piglets fed the B/P profile and the isonitrogenous profile. The plasma concentration of leucine was the only branched-chain amino acid that was lower in the B/P and isonitrogenous piglets than in the piglets

receiving the commercial profile, suggesting that leucine was the most likely of the three BCAA to be limiting in the new diet profile (**Table 3.1.6**). Elango *et al.* (2002) stated that elevated plasma concentrations of isoleucine and valine are indicative of a possible leucine deficiency. The total parenteral branched chain amino acid requirement may be correct but the ratio of 1:1:1 and the individual requirements of isoleucine, valine and leucine need to be validated directly to accurately achieve the optimal parenteral amino acid profile.

The sulfur amino acids methionine and cysteine and have a complex relationship in which cysteine has been shown to have a sparing effect on methionine (Ball et al., 2006). Shoveller et al., (2003a) found that the parenteral methionine requirement in the absence of dietary cysteine was 0.42 g/kg*d. When excess cysteine was provided in the diet, this methionine requirement could be reduced to 0.27 g/kg/d due to the ability of cysteine to spare a portion of the methionine requirement (Shoveller et al. 2003b). This study also concluded that cysteine was not an indispensable amino acid for neonatal piglets based on the fact that basal oxidation when the methionine requirement was met was not affected by cysteine addition to the diet (Shoveller et al. 2003b). The plasma amino acid concentrations for all diets were well below the sow fed reference range which indicates that the methionine requirement is not being met (**Table 3.1.6**). Cysteine was concluded to be a dispensable amino acid in the requirement paper (Shoveller et al. 2003b) yet in the neonatal piglet the enzyme, cystathionase, is slow to mature (Gaull et al., 1972). Cysteine has many functions other than protein synthesis and so although the requirement may have been met for protein synthesis, it may not have been provided in high enough amounts to meet requirements for the other metabolic fates such as GSH

(Turner et al., 2006). As a result both the cysteine and methionine requirements may be underestimated in the neonatal piglet resulting in methionine, the indispensable amino acid, being limiting in the B/P profile.

Bertolo et al., (1998) found that the parenteral threonine requirement was approximately 45% lower than the mean enteral requirement. The parenteral mean requirement was determined to be 0.19 g/kg*d; whereas the safe level (95% confidence interval) for parenteral threonine intake was found to be 0.21 g/kg*d (Bertolo et al., 1998). This amount was fed within the B/P profile, whereas for the profile based on Primene the amount of threonine fed on a g/kg*d basis was approximately 0.53. The plasma concentrations for threonine were low across all treatments suggesting that threonine may be limiting in all the amino acid profiles (**Table 3.1.6**). Compared to other commercial amino acid profiles the commercial profile used had the lowest threonine intake on a percent amino acid by weight basis (Brunton et al., 2000). However, the intake of the B/P profile was lower still at 1.78 g/100g as compared to the commercial profile of 3.7g /100g (**Table 3.1.1 and 3.1.2**); therefore both intakes may not be sufficient in meeting the threonine requirement. Plasma threonine concentrations may also be low due the conversion into glycine. Glycine plasma concentrations were higher in the B/P and isonitrogenous profiles and were also higher than the orally fed reference range (Wykes et al., 1993). This may be due to the ability of glycine to be a nitrogen carrier and remove excess nitrogen present when fed the B/P or isonitrogenous profile or because the dietary glycine couldn't be used due to other amino acid deficiencies (**Table 3.1.6**). The threonine requirement may be limiting in the B/P profile due to an incorrect assessment of the metabolic demands of growing piglets and needs to be re-determined.

Lysine is a common limiting amino acid in many swine diets (Moehen et al., 2005). House et al., (1998) found that when lysine was limiting plasma amino acid concentrations of histidine, valine, isoleucine and phenylalanine increased. When the piglets were fed either the B/P or isonitrogenous diet increases in plasma amino acid concentrations of histidine, valine, isoleucine and phenylalanine, relative to the commercial profile, were also observed (**Table 3.1.6**); suggesting that lysine may be limiting in these profiles. Although plasma lysine concentrations were within the orally fed reference range, the determined lysine requirement, 0.84 g/kg*d (House et al., 1998), was nearly half of amount being fed from the commercial profile, 1.56 g/kg*d. Therefore lysine should be considered a possible limiting amino acid in the B/P profile due to the large variation between dietary treatments.

One important flaw in the experiment may be due to the fact that all the amino acid requirements were determined using a different breed of piglet than the breed used to test the requirements. Breed differences do exist within the pig population which may result in varying metabolic needs and requirements (Rivera-Ferre et al., 2006). Such breed difference may be in the response of mucosal protein production in the atrophied gut. Threonine utilization is high in the intestine with its primary function being mucosal protein production (Schaart et al., 2005). The low plasma threonine concentration may also be due to a greater metabolic demand for threonine in the Duroc piglets than the Yorkshire piglets. Branched chain amino acids are primarily metabolized by the extrahepatic tissue and used for muscle growth (Harper et al., 1984). Low plasma concentrations of all the branched chain amino acids, especially leucine, were found in piglets fed the B/P profile (**Table 3.1.6**). This maybe due to the greater muscle growth

commonly seen in the Duroc piglets; resulting in the determined requirement being inadequate in meeting the higher demand of the Duroc breed. The rationale of breed difference in protein metabolism can be applied to all the amino acids that may be limiting in the B/P profile.

The newly developed amino acid profile does not meet the metabolic demands of the parenterally-fed neonatal Duroc piglet as evidenced by the piglets fed the B/P and isonitrogenous profile being unable to survive more than 6 days (**Table 3.1.4**). Additional research is needed to determine which indispensable amino acid(s), leucine, methionine, lysine or threonine, are limiting in the B/P amino acid profile.

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## 3.2 DETERMINATION OF THE FIRST LIMITING AMINO ACID IN THE BALL/PENCHARZ AMINO ACID PROFILE

### 3.2.1 Introduction

The indicator amino acid oxidation (IAAO) method was initially used by Bayley and colleagues (1983) to determine individual amino acid requirements of growing piglets. Using this method Drs. Ball and Pencharz were able to define the parenteral amino acid requirements for lysine, threonine, tryptophan, the aromatic amino acids, branched chain amino acids, and the sulfur amino acids (House et al, 1998, Bertolo et al, 1998, Cvitkovic et al., 2004, House et al, 1997a, Elango et al, 2002, and Shoveller et al., 2003) in ~ 1 wk old piglets. These requirement values presented the opportunity to develop a new amino acid profile. To complete the profile, referred to as the B/P amino acid profile, the amino acids where the requirements had not been previously determined (dispensable amino acids, conditionally indispensable amino acids and histidine), values were taken from a commercial formulation (Primene®). In the previous trial (Chapter 3.1), piglets were fed this new TPN solution and a common commercial amino acid profile to compare protein synthesis rates using the IAAO technique. As previously described (Chapter 3.1), the piglets fed the B/P profile were unable to meet their metabolic demands and exhibited symptoms consistent with hyperammonaemia and were immediately euthanized. The conclusion reached from this experiment was that the B/P amino acid profile did not meet the metabolic demands of the neonatal piglet and at least one amino acid was a severely limiting.

The first limiting amino acid is defined as the indispensable amino acid whose intake is the most below its requirement and therefore limits net protein metabolism. The first limiting amino acid can restrict neonatal growth, protein utilization and overall amino acid deposition (Wykes et al, 1994). When piglets were fed the B/P profile (Chapter 3.1), the plasma concentrations of leucine, threonine and methionine were lower than the plasma concentrations reported by Wykes et al. (1994) in sow-suckled piglets. Additionally, in grain based swine diets, lysine is typically the first limiting amino acid for growing piglets (Mavromichalis et al., 1998), and in the first experiment (Chapter 3.1), the lysine intake was lower in the B/P amino acid profile than the comparative commercial diet. From results of the previous experiment, we hypothesized that either leucine, threonine, methionine or lysine was limiting in the B/P amino acid profile.

The basis of the IAAO method is that when one indispensable amino acid is deficient/limiting to protein synthesis, then all the other amino acids cannot be maximally used for protein synthesis and will be oxidized (Pencharz and Ball, 2003). Oxidation of the indicator amino acid is inversely related to the intake of the limiting amino acid. As the intake of the limiting amino acid increases, the oxidation of the indicator amino acid will decrease; thus indicating in an increase in protein synthesis (Elango et al., 2008). Once the first-limiting amino acid is provided at a high enough level of intake, oxidation will remain stable, despite any further addition of that amino acid to the diet. Brunton et al., (2007) successfully applied the IAAO technique to determine the limiting amino acids in two TPN amino acid solutions. This study also demonstrated the ability of the IAAO technique to respond rapidly, within 18 hours, to short-term changes to amino acid intakes (Brunton et al., 2007), proving that the IAAO technique can be applied

successfully in determining the first limiting amino acid in the B/P amino acid profile over a relatively short time frame.

We hypothesized that supplementing the B/P profile with one of the four amino acids hypothesized to be limiting would reduce the oxidation of the indicator amino acid, phenylalanine, indicating an increase protein synthesis and a profile that better meets the metabolic demands of the parenterally-fed neonatal piglet.

### 3.2.2 **Materials and Methods**

#### *3.2.2.1Animals and study protocol*

The Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta approved all procedures performed within this study. A total of 12 Duroc-Landrace/Large White cross piglets were used within the study. Piglets were obtained from the Swine Research and Technology Centre at the University of Alberta Research Farm (Edmonton, Alberta, Canada) at the ages of 1-2 days and weights of 1.5-2.0 kg. No changes were made to the animal and surgical protocols previously described in section 3.1.2.1.

#### *3.2.2.2 Animal Housing*

The animal housing in this experiment was as described in section 3.1.2.2.

#### *3.2.2.3 Daily Care*

Piglets were monitored constantly throughout the day and night. General health was observed and recorded. Daily care consisted of weighing the piglet, temperature

reading, treatment of incision sites and ensuring symptoms associated with diet-induced hyperammonemia were not present.

Diet bags were also weighed at this time to calculate pump efficiency to allow for an accurate calculation of the amount of diet received on a mL/kg*day basis.

*3.2.2.4 Blood Sampling*

Beginning on day 1, blood samples (approx 1 mL) were taken every 24 hours via the femoral vein catheter. Blood collection was as described in section 3.1.2.4. Additional blood samples were taken on day 5, 6 and 7 as described under $^{14}C$ Phenylalanine Infusion, $^{14}CO_2$ and blood collection, and analytical procedures section.

3.2.2.5 *Diet Regimen*

Following surgery, piglets were fed a complete diet with the amino acid profile similar to the TPN solution, Primene. All diets were infused into the jugular vein catheter using a pressure-sensitive infusion pump. Piglets received 15g amino acids/kg*d and 1.1 MJ metabolizable energy/kg*d with glucose and lipid (Intralipid 20%, PharmaciaUpjohn, Stockholm, Sweden) each supplying 50% of non-protein energy intake. Vitamins, oil and water-soluble, were supplied by a prefabricated commercial solution (Multi-12/$K_1$ Pediatric; Sabex Inc., Boucherville PQ, Canada) and provided 115% of the estimated NRC (1998) requirement for piglets 3-5 kg. The vitamin solution, iron sulfate (Ferroforte; Bimeda-MTC, Cambridge ON, Canada) and a micromineral solution were added to the elemental diet immediately prior to feeding. The micromineral solution contained zinc sulfate, copper sulfate, manganese sulfate, chromium sulfate, selenium

sulfate and sodium iodide and provided 200% of the NRC (1998) recommendation for piglets 3-5 kg. Immediately following surgery, all piglets received diet parenterally at 50% of the targeted infusion rate (6.75 mL/kg*d). 12 hours following surgery the infusion rate was increased to 75% of the targeted infusion rate (10.125 mL/kg*d). On the morning of day 1, all piglets received the diets at 13.5 mL/kg*d and continued at that rate for the remainder of the study.

At noon of day 4 all diets were switched and piglets were fed the B/P profile diet for a period of 24 hours (n = 12). Following the oxidation study on d 5, piglets were randomly allocated to receive 1 of the four test diets for a period of 24 hours. Following the oxidation study on d 6, piglets were allocated to receive a second test diet for the last 24 hours of the study. This protocol was used previously by Brunton *et al.* (2007) and was shown to be sensitive to the intake of the limiting amino acid (Brunton et al., 2007). The four test diets were the B/P amino acid profile supplemented with either leucine (n = 6), methionine (n = 6), threonine (n = 6) or lysine (n = 6) at one and a half times the previously determined requirement (Brunton et al., 2007). The composition of the test diets are presented in **Table 3.2.1**.

**Table 3.2.1:** Amino acid concentrations of the B/P Profile, and the B/P Profile plus the addition of individual leucine, methionine, threonine and lysine administered to parenterally-fed neonatal piglets

| | Concentration g $L^{-1}$ [1] | | | | |
|---|---|---|---|---|---|
| **Amino Acids** | B/P Profile | Leucine | Methionine | Threonine | Lysine |
| L-Alanine[2] | 12.93 | 12.10 | 12.63 | 12.64 | 11.05 |
| L-Arginine | 4.41 | 4.41 | 4.41 | 4.41 | 4.41 |
| L-Aspartate | 3.15 | 3.15 | 3.15 | 3.15 | 3.15 |
| L-Glutamate | 5.20 | 5.20 | 5.20 | 5.20 | 5.20 |
| Glycine | 1.65 | 1.65 | 1.65 | 1.65 | 1.65 |
| L-Histidine | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| L-Isoleucine | 2.44 | 2.44 | 2.44 | 2.44 | 2.44 |
| L-Leucine | 2.44 | **3.66** | 2.44 | 2.44 | 2.44 |
| L-Lysine HCl | 3.86 | 3.86 | 3.86 | 3.86 | **5.79** |
| L-Methionine | 0.99 | 0.99 | **1.49** | 0.99 | 0.99 |
| L-Phenylalanine | 2.21 | 2.21 | 2.21 | 2.21 | 2.21 |
| L-Proline | 1.58 | 1.58 | 1.58 | 1.58 | 1.58 |
| L-Serine | 2.10 | 2.10 | 2.10 | 2.10 | 2.10 |
| Taurine | 0.32 | 0.32 | 0.32 | 0.32 | 0.32 |
| L-Threonine | 0.77 | 0.77 | 0.77 | **1.16** | 0.77 |
| L-Tryptophan | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 |
| L-Tyrosine | 0.44 | 0.44 | 0.44 | 0.44 | 0.44 |
| L-Valine | 2.44 | 2.44 | 2.44 | 2.44 | 2.44 |
| Glycyl-Tyrosine[3] | 1.58 | 1.58 | 1.58 | 1.58 | 1.58 |
| L-Cysteine | 0.55 | 0.55 | 0.55 | 0.55 | 0.55 |
| Ornithine HCl | 1.47 | 1.47 | 1.47 | 1.47 | 1.47 |

[1] This solution was provided to the piglets at a rate of 272 mL/kg/d
[2] Alanine concentrations vary to allow for all treatments to be isonitrogenous
[3] Glycyl tyrosine supplying 0.32 g $L^{-1}$ of glycine and 1.19 g $L^{-1}$ tyrosine
Lysine and Ornithine were supplied as Lysine-HCl and Ornithine-HCL to provide the concentration shown.

*3.2.2.6 $^{14}C$ Phenylalanine Infusion, $^{14}CO_2$ and blood collection, and analytical procedures*

On days 5, 6 and 7 piglets were given a primed (7 μCi/kg), constant (3.5 μCi/kg) intravenous infusion of L-[1-$^{14}$C]phenylalanine via the jugular vein catheter to determine phenylalanine flux and oxidation. To reach plateau in both breath and blood labelling, the duration of constant infusion was 4h and blood samples (1 mL) were taken at time 0, 120, 150, 180, 210 and 240 minutes. On day 6 and 7, additional blood samples were taken one hour (–60 minutes) and half an hour (-30 minutes) prior to the start of isotope infusion to correct for the background specific radioactivity of phenylalanine in the blood, and breath samples were collected at -30 and 0 minutes to correct for any residual labelling of the breath $CO_2$. Details of infusion protocol, $^{14}CO_2$ collection and blood collection procedures have been described previously (House et al, 1997a). Following the infusion on d 7, piglets were killed by injection of 1000 mg of sodium pentobarbital into a venous catheter.

Plasma amino acids were determined using reverse-phase high performance liquid chromatography with the use of phenylisothiocyanate derivatives as previously described (Bidlingmeyer et al., 1984 and House et al., 1994). The internal standards norleucine and L-[4,5-$^{3}$H]leucine (1920 GBq/mmol; Amersham Pharmacia Biotech, St. Louis MO, USA) were added to each 300 μL plasma sample. Post-column radioactive derivatives of phenylalanine and tyrosine were collected in 2 mL fractions corresponding to the respective peaks, 14 mL of scintillant (Biodegradable Counting Scintillant; Amersham Canada, Ltd., Oakville ON, Canada) was added, and samples were counted on a scintillation counter.

*3.2.2.7 Calculations*

The formulas and procedures used to calculate intake, oxidation, flux, non-oxidative disposal, release from protein breakdown, balance and percent dose oxidized were previously described by House et al. (1997). Protein breakdown and non-oxidative loss were calculated using the Stochastic model of amino acid metabolism [Q (Flux) = S (Non-oxidative loss) + E (Oxidation) = B (Protein breakdown) + I (Intake)] (Waterlow et al. 1978).

*3.2.2.8 Statistical analysis*

All data were analyzed using SAS Version 9.1 (2003 SAS Institute, Cary NC, USA), and data were considered statistically significant if $p < 0.05$.

The dependent variables plasma amino acid concentration, phenylalanine flux, oxidation, non-oxidative disposal and release from protein, were analyzed using proc mixed where the fixed effect was diet (B/P profile, B/P profile plus leucine, B/P profile plus threonine, B/P profile plus methionine and B/P profile plus lysine) and the random variable was piglet. Carry over effect was also tested to determine if the previous diet had a significant effect on the phenylalanine oxidation. When the effects were significant ($p < 0.05$), least squares means were separated using the pdiff option.

### 3.2.3 **Results**

*3.2.3.1 Piglet performance*

Most piglets remained healthy and active during the course of the study. Three piglets exhibited distress symptoms similar to in the previous experiment and were

immediately removed from the trial. Piglet weight upon arrival (2 d, 1.72 kg, pooled SD= 0.17) and weight at the end of the study (7 d, 2.3 kg, pooled SD= 0.27) did not differ between dietary treatments.

*3.2.3.2 Phenylalanine Intake, Oxidation, Flux, Non-oxidative disposal, Release from Protein Breakdown, and Percent Dose Oxidized*

No significance differences were found between treatments when comparing phenylalanine flux, oxidation, non-oxidative disposal and release from protein ($p>0.05$, **Table 3.2.2**). Although no significant effect to the original B/P profile was found with the addition of either leucine, threonine, methionine, or lysine, threonine supplementation resulted in greater phenylalanine release due to protein breakdown and non-oxidative phenylalanine disposal. Piglets fed the B/P profile supplemented with methionine had the lowest amount of protein breakdown and synthesis but also had the greatest amount of variation. When the diets were supplemented with leucine and threonine percent dose oxidized decreased by 21.1% and 11.6% respectively from the B/P profile; whereas lysine and methionine supplementation increased percent dose oxidized by 14.4% and 3.6% respectively (**Table 3.2.2**). However, none of these differences were significant.

*3.2.3.3 Plasma amino acid concentrations*

Of the indispensable amino acids, histidine, isoleucine, leucine, lysine, threonine, tryptophan and valine, were affected by diet ($p<0.05$, **Table 3.2.3**). Piglets receiving the B/P profile had lower plasma concentrations of histidine compared to those receiving the B/P profile supplemented with either leucine, threonine or lysine. Isoleucine

concentrations were significantly greater in piglets receiving the B/P profile supplemented with threonine than in piglets receiving the other diets. When the B/P profile was supplemented with leucine, the plasma concentrations of histidine, leucine, and lysine were significantly greater than original the B/P profile ($p<0.05$, **Table 3.2.3**). Plasma threonine concentrations were significantly greater when piglets were fed the B/P profile supplemented with leucine, methionine and lysine ($p<0.05$, **Table 3.2.3**), compared to the B/P and B/P plus threonine diet. Valine plasma concentrations were significantly lower in the leucine and methionine treatments, compared to when piglets received the B/P profile. When the piglets were fed the B/P profile supplemented with either leucine, lysine, methionine or threonine, the plasma histidine concentrations increased to a level above the sow-fed reference range. With regards to leucine plasma concentrations, the B/P profile supplemented with leucine was the only treatment group to fall within the enterally fed reference range. Plasma lysine concentrations were above the reference range when the diet was supplemented with lysine and within the sow-fed reference range for all other treatments. Threonine and methionine plasma concentrations were still well below the enterally fed reference range regardless of treatment (**Table 3.2.3**).

For the conditionally indispensable amino acids, glutamine and glycine were significantly affected by diet ($p<0.05$, **Table 3.2.3**). When piglets were fed the B/P profile and the B/P profile supplemented with leucine, glutamine concentrations were significantly greater than in piglets fed the B/P profile supplemented with threonine. When piglets were fed the B/P diet supplemented with leucine, glycine concentrations were greater than when piglets were fed the original B/P profile ($p<0.05$, **Table 3.2.3**).

Plasma glycine concentrations were above the sow fed reference range in piglets receiving all of the test diets. Plasma proline concentrations were lower than the sow fed reference range for all treatment groups (**Table 3.2.3**).

For the dispensable amino acids, diet had a significant effect on the plasma concentrations of aspartate and glutamate. Plasma concentrations of aspartate and glutamate were significantly higher in piglets fed the B/P amino acid profile ($p<0.05$, **Table 3.2.3**). The plasma concentration of aspartate was higher than the sow fed reference when piglets were fed the B/P profile but were lower than the reference range when piglets received all other treatments.

These findings suggest that there still may be a limiting amino acid present in all treatment groups and similarities in dietary intakes may need to be evaluated.

**Table 3.2.2:** Phenylalanine kinetics (mmol $kg^{-1}$ $h^{-1}$) of piglets receiving total parenteral nutrition with the B/P profile, and B/P profile supplement with either leucine, threonine, methionine or lysine

| Diet ($mmol \cdot kg^{-1} \cdot h^{-1}$) | B/P Profile | SE | B/P Profile + Leucine | SE | B/P Profile + Threonine | SE | B/P Profile + Methionine | SE | B/P Profile + Lysine | SE | P value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| n | 12 | | 6 | | 6 | | 6 | | 6 | | |
| Flux (Q) | 299.12 | 17.22 | 282.45 | 14.07 | 308.54 | 19.83 | 266.25 | 24.57 | 277.01 | 17.97 | NS |
| Intake (I) | 147.29 | 0.76 | 153.79 | 2.74 | 152.97 | 1.59 | 147.73 | 1.13 | 148.07 | 0.78 | NS |
| Oxidation (E) | 36.47 | 3.28 | 34.07 | 4.50 | 37.70 | 2.53 | 32.18 | 9.00 | 42.21 | 2.07 | NS |
| Non-Oxidative Disposal (S) | 262.75 | 16.25 | 248.38 | 15.91 | 270.84 | 17.97 | 234.07 | 19.47 | 234.8 | 16.81 | NS |
| Release from protein (B) | 147.78 | 17.22 | 131.11 | 14.07 | 157.20 | 19.83 | 114.91 | 24.57 | 125.67 | 17.97 | NS |

Significant difference at $p<0.05$.
[abc]Values sharing a superscript are not significantly different ($p<0.05$)

**Table 3.2.3:** Mean plasma amino acid concentrations of piglets receiving the B/P profile, and B/P profile supplement with either leucine, threonine, methionine or lysine

| | Plasma Amino Acid Concentrations[1] µmol/L Diet | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | B/P Profile | SE | B/P + Leucine | SE | B/P + Threonine | SE | B/P + Methionine | SE | B/P + Lysine | SE | Orally-Fed Reference Range[2] |
| **Dispensable Amino Acids** | | | | | | | | | | | |
| Alanine | 955.15 | 68.70 | 934.23 | 95.38 | 818.10 | 95.38 | 820.36 | 106.25 | 704.21 | 106.25 | 413-1015 |
| Aspartate | 76.46$^{a}$ | 7.22 | 14.99$^{b}$ | 10.15 | 14.15$^{b}$ | 10.15 | 18.42$^{b}$ | 11.34 | 14.61$^{b}$ | 11.34 | 22-63 |
| Asparagine | 40.54 | 2.44 | -- | -- | 27.85 | 2.44 | 37.52 | 2.44 | 31.52 | 2.44 | |
| Glutamate | 315.79$^{a}$ | 20.75 | 87.60$^{b}$ | 28.60 | 117.92$^{b}$ | 28.60 | 117.35$^{b}$ | 31.81 | 95.31$^{b}$ | 31.81 | 92-217 |
| Serine | 336.61 | 34.32 | 459.33 | 46.73 | 366.55 | 46.73 | 431.92 | 51.84 | 411.95 | 51.84 | 137-442 |
| **Conditionally Indispensable Amino Acids** | | | | | | | | | | | |
| Arginine | 111.30 | 12.68 | 113.43 | 17.15 | 77.92 | 17.15 | 121.07 | 18.99 | 108.66 | 18.99 | 50-267 |
| Cysteine | 21.35 | 6.74 | 44.58 | 8.36 | 33.84 | 8.33 | 31.53 | 9.00 | 39.56 | 10.09 | |
| Glutamine | 488.15$^{a}$ | 36.18 | 531.34$^{a}$ | 51.17 | 288.61$^{b}$ | 51.17 | 349.47$^{ab}$ | 57.21 | 432.39$^{ab}$ | 57.21 | |
| Glycine | 766.03$^{a}$ | 112.55 | 1629.48$^{b}$ | 159.12 | 978.60$^{ab}$ | 159.12 | 1276.042$^{ab}$ | 177.89 | 1170.66$^{ab}$ | 177.89 | 450-976 |
| Proline | 269.15 | 13.85 | 305.18 | 19.32 | 223.58 | 19.32 | 264.40 | 21.54 | 247.32 | 21.54 | 304-890 |
| Tyrosine | 391.29 | 77.20 | 272.17 | 94.97 | 216.30 | 94.97 | 501.20 | 102.69 | 175.42 | 102.69 | 88-231 |
| **Indispensable Amino Acids** | | | | | | | | | | | |
| Histidine | 114.13$^{a}$ | 34.12 | 338.31$^{b}$ | 48.24 | 257.77$^{b}$ | 48.24 | 180.13$^{ab}$ | 53.93 | 309.69$^{b}$ | 53.93 | 47-121 |
| Isoleucine | 156.54$^{a}$ | 17.57 | 111.61$^{a}$ | 23.81 | 259.72$^{b}$ | 23.81 | 127.82$^{a}$ | 26.40 | 166.62$^{a}$ | 26.40 | 81-143 |
| Leucine | 69.28$^{a}$ | 7.54 | 107.10$^{b}$ | 10.67 | 47.97$^{a}$ | 10.67 | 55.50$^{a}$ | 11.93 | 73.41$^{ab}$ | 11.93 | 102-175 |
| Lysine | 220.81$^{a}$ | 34.30 | 250.79$^{ab}$ | 43.48 | 196.56$^{b}$ | 43.48 | 190.91$^{ab}$ | 47.41 | 456.02$^{c}$ | 47.41 | 77-317 |
| Methionine | 5.50 | 3.79 | 6.11 | 4.64 | 3.46 | 6.57 | 16.41 | 4.64 | 8.70 | 9.29 | 33-74 |
| Phenylalanine | 144.59 | 20.08 | 167.55 | 27.74 | 148.04 | 27.74 | 165.54 | 30.88 | 129.07 | 30.88 | 34-86 |
| Threonine | 6.88$^{a}$ | 28.33 | 66.27$^{ab}$ | 34.28 | 6.26$^{a}$ | 34.90 | 65.18$^{ab}$ | 44.72 | 173.88$^{b}$ | 44.96 | 214-500 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Tryptophan | 27.00[a] | 3.49 | 4.88[b] | 4.29 | 9.15[b] | 5.48 | 9.10[b] | 4.29 | 10.63[ab] | 4.74 | |
| Valine | 320.42[a] | 22.57 | 200.43[c] | 31.68 | 297.88[ab] | 31.68 | 231.77[bc] | 35.37 | 259.72[abc] | 35.37 | 175-318 |
| **Other Amino Acids** | | | | | | | | | | | |
| Taurine | 187.74[a] | 16.79 | 237.33[ab] | 22.87 | 209.43[ab] | 22.87 | 228.44[ab] | 25.38 | 265.33[b] | 25.38 | |
| Ornithine | 84.54 | 9.35 | 73.76 | 12.85 | 111.50 | 12.85 | 77.09 | 14.29 | 92.13 | 14.29 | 79-195 |
| hydroxy-Proline | 41.37[a] | 4.01 | 61.91[b] | 5.61 | 60.25[b] | 5.61 | 57.32[ab] | 6.27 | 57.33[ab] | 6.27 | |
| Citrulline | 94.87 | 9.45 | 72.64 | 13.19 | 91.67 | 13.19 | 127.35 | 14.71 | 114.46 | 14.71 | 73-151 |

[1] Significant difference at $p<0.05$. Values sharing a superscript are not significantly different $p<0.05$)
[2] Wykes et al. (1994)

### 3.2.4 **Discussion**

Presently, the amino acid profile of most parenteral solutions is based upon reference proteins such as egg protein or human breast milk (Brunton et al, 2000). These may not be accurate reference standards given that parenteral feeding bypasses first-pass splanchnic nutrient metabolism and generally results in an atrophied gut and a lower metabolic demand (Brunton et al, 2000). In neonatal piglets, most of the indispensable amino acids requirements were previously determined using the IAAO technique and were combined to form what is referred to as the B/P amino acid profile. From the previous experiment (Chapter 3.1) it was apparent that the B/P profile had at least one limiting amino acid which resulted in hyperammonemia and mortality.

The oxidation rate of the B/P profile was approximately 36.5 $\mu mol \cdot kg^{-1} \cdot hr^{-1}$. The piglets fed the diets supplemented with threonine and lysine had a higher oxidation than the B/P profile; whereas the piglets fed the diets supplemented with leucine and methionine were lower, however none of these were significantly different. The original B/P profile resulted in the protein synthesis as a percent of phenylalanine flux of 87.65%. Protein synthesis as a percent of phenylalanine flux for threonine, leucine, methionine and lysine supplementation were 89%, 89.44%, 88.98% and 85.47%, respectively. Supplementing the B/P profile with threonine, leucine and methionine resulted in an increase in protein synthesis of 1 to 2%. Because no significant differences were found due to supplementation of individual amino acids, this result could indicate the possibility of co-limiting amino acids.

Supplementing the B/P profile with leucine or threonine decreased the percent dose oxidized of phenylalanine by 21.1% and 11.6% respectively. This finding suggests

an improved efficiency of utilization of the dietary amino acids in the B/P profile when the diet was supplemented with leucine or threonine. When the B/P profile was supplemented with lysine and methionine the percent dose oxidized increased from baseline (B/P Profile) by 14.4% and 3.6% respectively (**Table 3.2.4**). No significance was found when comparing percent dose oxidized and percent change due to the variance of the means being so great. This variance may be a result of TPN-induced liver dysfunction (Wang et al., 2006). Wang and colleagues (2006) found that when piglets are fed TPN for a period longer than 7 days liver damage occurs. Since the liver is a primary site of metabolism for many amino acids, including being the primary site of amino acid oxidation, oxidation and plasma values may not be reflective of dietary intake due to the level of liver dysfunction.

The plasma amino acid concentrations of leucine, methionine and threonine (**Table 3.1.6**) were lower than the sow reference range (Wykes et al, 1994); suggesting that these requirements may have been underestimated or these amino acids were deficient when combined in a complete profile. Compared to the commercial profile the isonitrogenous B/P profile, on a g/100g basis, is lower in threonine, leucine, valine, and lysine resulting in a higher nitrogen output (**Table 3.1.5**). This finding again reiterates the theory that there is a limiting amino acid in the B/P profile. From these data it was hypothesized that either leucine, threonine, methionine or lysine were the first limiting amino acid in the B/P profile.

For the indispensable amino acids the plasma amino acid concentrations for leucine and lysine increased significantly when the diets were supplemented respectively (**Table 3.2.3**). This finding suggests leucine and lysine supplementation may be adequate

in meeting the metabolic demands of the piglet. Lysine supplementation increased the lysine concentration to a value above the sow-fed reference range, suggesting that lysine may actually be in excess with the B/P profile was supplemented. Methionine and threonine concentrations did not increase or decrease significantly when supplemented implying that methionine and threonine still may be limiting. Even though some dietary treatments resulted in an increase in plasma concentrations, no supplementation had a significant decrease in oxidation suggesting that a limiting amino acid may still be present in the treatment profiles.

For the dispensable and conditionally indispensable amino acids the plasma amino acid data from the present study was consistent with the data from the previous experiment and suggests that there was an amino acid imbalance in the B/P profile. When the piglets were fed the B/P profile, plasma concentrations of glutamate and aspartate were significantly higher than for the supplemented treatment groups (**Table 3.2.3**). As stated previously, glutamine and aspartate are nitrogen carriers and typically increase in concentration when there is an excess of dietary nitrogen in the body. The increase in concentration of these amino acids indicates that there was either an excessive intake of nitrogen or poor utilization of dietary nitrogen for protein synthesis when fed the B/P profile. The decrease in glutamate and aspartate concentrations when piglets were fed the supplemented treatment groups indicates better amino acid utilization for protein synthesis and thus decreased excess nitrogen. Of the conditionally indispensable amino acid data, the supplemented treatment groups had a significant effect on glycine concentrations (**Table 3.2.3**). Glycine is known to increase when nitrogen intake is in excess versus utilization (Iapichino et al., 1988). The higher concentration of these

amino acids, in piglets fed the B/P profile, indicates that there was either an excessive intake of nitrogen or poor utilization of dietary nitrogen for protein synthesis. Because dietary treatments were isonitrogenous excessive nitrogen intake is not applicable to this situation and therefore when the diets were supplemented there appeared to be better utilization of nitrogen for protein synthesis.

Brunton and colleagues (2007) found that supplementing TPN diets with limiting amino acids methionine or phenylalanine resulted in a significant decrease of lysine oxidation. No significant differences were found when comparing oxidation rates, suggesting that either they were not the limiting amino acid or they were co-limiting.

The B/P profile was comprised of the IAAO determined amino acid requirements and the remainder of amino acid intakes were taken from a commercial TPN profile. Examination of the amino acid profile of the commercial TPN solution revealed that proline concentrations were significantly lower than for other commercial TPN solutions (**Table 1.1.1**). The proline concentration of the commercial profile is also much lower than the proline concentration available in sow's milk (**Table 1.1.1**). Finally, plasma proline concentrations were below the sow-fed reference range. These data suggest that proline could be a limiting in the commercial amino acid profile and this needs to be assessed.

In conclusion, we were unable to conclusively identify a single a limiting amino acid in the B/P profile in this experiment. This could be due to the presence of another limiting amino acid, such as proline, or co-limiting amino acids.

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## 3.3 PROLINE IN A COMMERCIAL TOTAL PARENTERAL NUTRITION (TPN) SOLUTION MAY BE INADEQUATE IN MEETING THE METABOLIC REQUIREMENTS OF THE PARENTERALLY-FED NEONATAL PIGLET

### 3.3.1 Introduction

An indispensable amino acid is defined as an amino acid that cannot be synthesized de novo in high enough quantities to meet metabolic requirements; whereas a conditionally indispensable amino acid is one that is indispensable only in certain populations/physiological states. Proline is described by the NRC requirements for swine (1998) as a conditionally indispensable amino acid and has several critical functions such as maintaining intestinal health and de novo synthesis of arginine, a vital component to the urea cycle (Bertolo et al., 2003). However, previous studies have shown that proline may actually be an indispensable amino acid in the neonatal piglet (Ball et al., 1986, Bertolo et al., 2003).

Davis and colleagues found that mammalian milk contains large amounts of proline (7.3 to 10.6 g/100g total amino acid); yet some TPN solutions contain less proline than milk would provide (Davis et al., 1994). Proline concentrations in current TPN solutions vary from 3.0 g/100g to 11.6g/100g total amino acid (Brunton et al., 2000), demonstrating the need to evaluate the metabolic requirement of proline in the parenterally fed neonate. TPN solutions are based on various different nutritional sources such as egg protein, cord blood and breast milk resulting a diverse composition of amino acid profiles (Brunton et al., 2000). These solutions do not appear to have been specifically tested for proline adequacy in vivo. The results from a previous study

(**Chapter 3.2**) suggested that a TPN solution modelled on a commercial product might not contain sufficient proline (3.0g/100g, 0.4286g/kg*d), despite apparently adequate arginine, to meet the metabolic needs of a growing neonatal piglet.

The empirical value for the parenteral requirement of proline has yet to be determined. Previous studies have shown that TPN feeding results in reduced gastrointestinal function and metabolism associated to gut atrophy (Bertolo et al., 1999). Proline is primarily synthesized within the small intestine; however, it has been shown that intestinal mucosa concentrations of proline are generally lower in a atrophied gut suggesting that whole body synthesis is inadequate in maintaining metabolic needs when parenterally fed (Bertolo et al., 2000).

By using the IAAO technique, we hypothesized that supplementing the commercial profile with proline would reduce the oxidation of phenylalanine, the indicator amino acid; thus indicating an increase in protein synthesis.

### 3.3.2 Materials and Methods

*3.3.2.1Animals and study protocol*

The Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta approved all procedures performed in this study. Five intact male Landrace/ Large White piglets (Hypor, Regina SK, Canada) (1.5 – 2.0 kg) were obtained from the University of Alberta Swine Research and Technology Centre at 1-2 days of age. No changes were made to the animal and surgical protocol from what was previously described in section 3.1.2.1.

*3.3.2.2 Animal Housing*

The animal housing within this experiment is identical to what was described in section 3.1.2.2.

*3.3.2.3 Daily Care*

Piglets were monitored constantly throughout the day and night. General health was observed and recorded. Daily care consisted of weighing the piglet, temperature reading, treatment of incision sites and ensuring symptoms associated with diet-induced hyperammonia, such as loss of motor function, increased weight gain and respiratory distress, were not present.

Diet bags were also weighed at this time to calculate and account for diet infusion pump efficiency and to determine accurately the amount of diet received on a mL/kg*day basis.

*3.3.2.4 Blood Sampling*

Beginning on day 1, blood samples (approximately 1 mL) were taken every 24 hours via the femoral vein catheter. Blood collection was identical to the methods previously described in section 3.1.2.4. Additional blood samples were taken on day 6 and 7 as described under $^{14}C$ Phenylalanine Infusion, $^{14}CO_2$ and blood collection, and analytical procedures section.

*3.3.2.5 Diet Regimen*

Following surgery, piglets were fed a complete diet with the amino acid profile similar to the TPN solution, Primene®. All diets were infused into the jugular vein catheter using a pressure-sensitive infusion pump. Piglets received 15g amino acids/kg*d and 1.1 MJ metabolizable energy/kg*d with glucose and lipid (Intralipid 20%, Pharmacia Upjohn, Stockholm, Sweden) each supplying 50% of non-protein energy intake. Vitamins, oil and water-soluble, were supplied by a prefabricated commercial solution (Multi-12/$K_1$ Pediatric; Sabex Inc., Boucherville PQ, Canada) and provided 115% of the estimated NRC (1998) requirement for piglets 3-5 kg. The vitamin solution was added to the elemental diet with iron sulfate (Ferroforte; Bimeda-MTC, Cambridge ON, Canada) and a mineral solution. The mineral solution contained zinc sulfate, copper sulfate, manganese sulfate, chromium sulfate, selenium sulfate and sodium iodide and provided 200% of the NRC (1998) recommendation for piglets 3-5 kg. Immediately following surgery all piglets received diet parenterally at 50% of the total infusion rate (6.75 mL/kg*d). 12 hours following surgery the infusion rate was increased to 75% of the total infusion rate (10.125 mL/kg*d). On day 1 all piglets received the diets at 13.5 mL/kg*d and which was maintained for the duration of the study. Tyrosine was provided in all diets as glycyl-tyrosine (House et al. 1997a). Lysine-HCl and ornithine-HCl were also used due to solubility issues. At noon of day 5 piglets were randomly allocated to continue on the same dietary treatment (commercial diet) or the commercial diet plus supplemental proline for a period of 24 hours. At noon of day 6 piglets were randomly assigned to receive the opposite dietary treatment for the remainder of the trial (approximately 24 hours). The composition of both diets is provided in **Table 3.3.1**.

**Table 3.3.1:** Amino acid concentrations of the commercial profile, and the commercial profile plus the addition of individual proline administered to parenterally-fed neonatal piglets

| | Concentration g $L^{-1*}$ | |
|---|---|---|
| **Amino Acids** | Commercial Profile | Commercial Profile plus Proline |
| L-Alanine[1] | 4.1489 | 1.7630 |
| L-Arginine | 4.4114 | 4.4114 |
| L-Aspartate | 3.1511 | 3.1511 |
| L-Glutamate | 5.1993 | 5.1993 |
| Glycine | 1.6544 | 1.6544 |
| L-Histidine | 1.9957 | 1.9957 |
| L-Isoleucine | 3.5187 | 3.5187 |
| L-Leucine | 5.1993 | 5.1993 |
| L-Lysine | 7.1522 | 7.1522 |
| L-Methionine | 1.2604 | 1.2604 |
| L-Phenylalanine | 2.2058 | 2.2058 |
| L-Proline | 1.5756 | **4.5956** |
| L-Serine | 2.1007 | 2.1007 |
| Taurine | 0.3151 | 0.3151 |
| L-Threonine | 1.9432 | 1.9432 |
| L-Tryptophan | 1.0504 | 1.0504 |
| L-Tyrosine | 0.4412 | 0.4412 |
| L-Valine | 3.9914 | 3.9914 |
| Glycyl-Tyrosine[2] | 1.5780 | 1.5780 |
| L-Cysteine | 0.9978 | 0.9978 |
| Ornithine | 1.4739 | 1.4739 |

* This solution was provided to the piglets at a rate of 272 mL/kg/d
[1] Alanine concentrations vary to allow for all treatments to be isonitrogenous
[2] Glycyl tyrosine supplying 0.32 g $L^{-1}$ of glycine and 1.19 g $L^{-1}$ tyrosine
Lysine and Ornithine were supplied as Lysine-HCl and Ornithine-HCL to provide the concentration shown.

*3.3.2.6 $^{14}C$ Phenylalanine Infusion, $^{14}CO_2$ and blood collection, and analytical procedures*

On days 6 and 7 piglets were given a primed (7 μCi/kg), constant (3.5 μCi/kg) intravenous infusion of L-[1-$^{14}$C]phenylalanine via the jugular vein catheter to determine phenylalanine flux and oxidation. To reach plateau in both breath $CO_2$ and blood phenylalanine labelling, the duration of constant infusion was 4h and blood samples (1 mL) were taken at time 0, 120, 150, 180, 210 and 240 minutes. On day 7, additional blood samples were taken one hour (–60 minutes) and half an hour (-30 minutes) prior to the start of isotope infusion to correct for the background specific radioactivity of phenylalanine in the blood, and breath samples were collected at -30 and 0 minutes to correct for any residual labelling of the breath $CO_2$. Details of infusion protocol, $^{14}CO_2$ collection and blood collection procedures have been described previously (House et al, 1997b). Following the infusion on d 7, piglets were killed by injection of 1000 mg of sodium pentobarbital into a venous catheter.

Plasma amino acids were determined using reverse-phase high performance liquid chromatography with the use of phenylisothiocyanate derivatives as previously described (Bidlingmeyer et al., 1984 and House et al., 1994). The internal standards norleucine and L-[4,5-$^{3}$H]leucine (1920 GBq/mmol; Amersham Pharmacia Biotech, St. Louis MO, USA) were added to each 300 μL plasma sample. Post-column radioactive derivatives of phenylalanine and tyrosine were collected in 2 mL fractions corresponding to the respective peaks, 14 mL of scintillant (Biodegradable Counting Scintillant; Amersham Canada, Ltd., Oakville ON, Canada) was added, and samples were counted on a scintillation counter.

*3.3.2.7 Calculations*

The formulas and procedures used to calculate intake, oxidation, flux, non-oxidative disposal, release from protein breakdown, balance and percent dose oxidized were previously described by House et al. (1997b). Protein breakdown and non-oxidative loss were calculated using the Stochastic model of amino acid metabolism [Q (Flux) = S (Non-oxidative loss) + E (Oxidation) = B (Protein breakdown) + I (Intake)] (Waterlow et al. 1978).

*3.3.2.8 Statistical Analysis*

All data were analyzed using SAS Version 9.1 (2003 SAS Institute, Cary NC, USA), and data were considered statistically significant if $p < 0.05$.

The dependent variables plasma amino acid concentration, phenylalanine flux, oxidation, non-oxidative disposal and release from protein, were analyzed using the mixed procedure where the fixed effect was diet (commercial profile and commercial profile plus proline) and the random variable was piglet. The variances were compared using equality of variance. When the effects were significant ($p < 0.05$), least squares means were separated using the pdiff option.

### 3.3.3 Results

*3.3.3.1 Piglet Performance*

All piglets remained healthy and active during the course of the study. Piglet weight upon arrival (2 d, 1.63 kg, pooled SD= 0.15) and weight at the end of the study (7d, 2.29 kg, pooled SD= 0.28) did not differ among dietary treatments.

*3.3.3.2 Phenylalanine Intake, Oxidation, Flux, Non-oxidative disposal, Release from Protein Breakdown, and Percent Dose Oxidized*

Diet had no significant effect on phenylalanine flux, oxidation, non-oxidative disposal or release from protein ($p>0.05$, **Table 3.3.2**). Although proline supplementation had no significant effect on phenylalanine flux, oxidation, synthesis or breakdown it was found that the standard error and variances appear to decrease with proline supplementation (**Table 3.3.2 and Table 3.3.3**). It was found that proline supplementation of the commercial profile significantly decreased ($p<0.05$) the variances in phenylalanine flux and release from protein breakdown (**Table 3.3.3**).

*3.3.3.3 Plasma Amino Acid Concentrations*

No significant differences were found when comparing dispensable and indispensable amino acids. For the conditionally indispensable amino acids proline supplementation only had a significant effect on plasma proline concentrations. When the diet was supplemented with proline, plasma concentrations increased significantly from 152 μmol/L to 498 μmol/L (**Figure 3.3.1**). This finding suggests that the diet supplemented with proline is adequate in meeting the proline requirement of the neonatal piglet whereas the original commercial profile was not.

**Table 3.3.2:** Phenylalanine kinetics (μmol $kg^{-1}$ $h^{-1}$) of piglets receiving total parenteral nutrition with the commercial profile, and commercial profile supplement with proline

| Diet ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) n | Profile Based on Primene 5 | SE | Profile Based on Primene Supplemented with Proline 5 | SE | ANOVA p value |
|---|---|---|---|---|---|
| Flux (Q) | 252.86 | 23.11 | 274.75 | 6.03 | NS |
| Intake (I) | 152.05 | 1.21 | 149.43 | 1.31 | NS |
| Oxidation (E) | 21.07 | 5.73 | 16.82 | 2.65 | NS |
| Non-Oxidative Disposal (S) | 231.79 | 18.57 | 257.93 | 8.29 | NS |
| Release from protein (B) | 101.52 | 23.91 | 123.41 | 6.02 | NS |

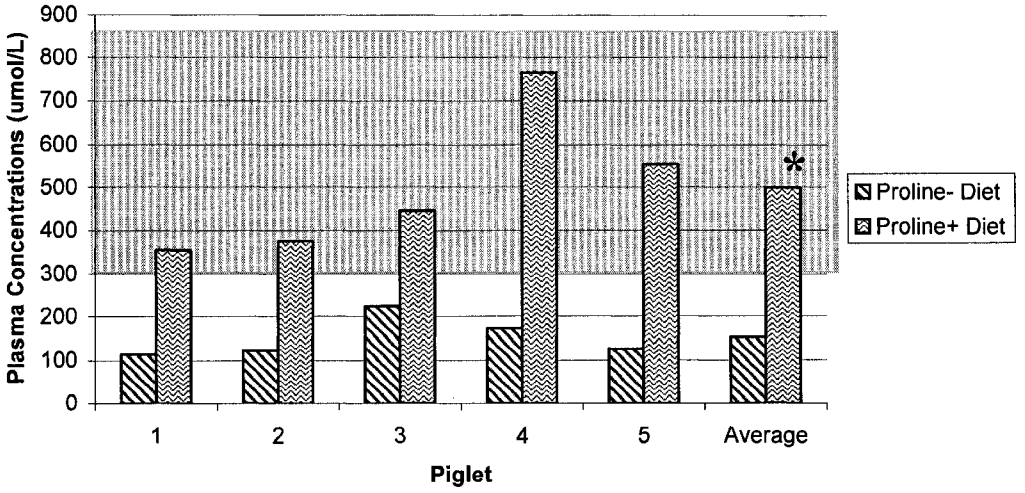
NS denotes no significance
SE denotes standard error

**Table 3.3.3:** Equality of variances between the commercial profile and the commercial profile supplemented with proline

| Diet ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) | Variance from the mean for the Profile Based on Primene | Variance from the mean for the Profile Based on Primene Supplemented with Proline | p Value |
|---|---|---|---|
| Flux (Q) | 18.91 | 4.39 | 0.0487 |
| Oxidation (E) | 54.41 | 31.50 | NS |
| Non-Oxidative Disposal (S) | 16.02 | 6.43 | NS |
| Release from protein (B) | 47.11 | 9.77 | 0.0487 |

NS denotes no significance

**Figure 3.3.1:** Comparison of plasma proline concentrations (μmol/L) for the commercial diet vs. the commercial diet supplemented with proline




* indicates a significant difference ($p<0.05$)
*Grey shaded area denotes the plasma amino concentration range taken from Wykes et al. 1994*

### 3.3.4 **Discussion**

There is no general consensus in the scientific and pharmaceutical industries concerning the optimal amino acid profile for TPN solutions for neonates (Brunton et al., 2000). Most parenteral profiles are based on enterally fed biological reference proteins such as breast milk. This commonly results in an overestimation of specific amino acid requirements since first pass splanchnic metabolism can account for up to 60% of protein metabolism and is bypassed when parenterally fed (Stoll et al., 1998). Although most amino acid profiles provide individual amino acids in excess there are some cases in which certain amino acids may be deficient. One of the conditionally indispensable amino acids that is thought to be under provided in neonatal TPN solutions is proline. Compared to other amino acid profiles currently available, the commercial profile used in previous experiments (Chapter 3.1 and 3.2) contained the lowest concentration (g/100g) of proline.

Proline is critical in the urea cycle and is primarily synthesized in the small intestine. Because parenteral feeding results in gut atrophy proline synthesis is directly affected. Previous experiments done by our research group have shown that the conversion of orthinine to proline is localized to the gut with the fractional net conversion being approximately 75% (Bertolo et al., 2003). Therefore in the absence of healthy intestinal metabolism, it seems reasonable to assume that gut proline synthesis would be severely affected, making proline an indispensable amino acid in the parenterally fed neonatal piglet (Bertolo et al., 2003). The original commercial profile contains a concentration of proline that is much lower than the concentrations available in sow's milk (Davis et al., 1994), providing additional evidence that the adequacy of proline in

the commercial profile requires evaluation. The present study used phenylalanine kinetics to assess how whole-body protein metabolism was affected by the supplementation of proline to a commercial profile.

Although not significantly different, the oxidation rate of the commercial profile supplemented with proline was lower than the original commercial profile (**Table 3.3.2**). The commercial profile resulted in the protein synthesis as a percent of phenylalanine flux of approximately 92%; whereas the profile supplemented with proline resulted in an increase to 94%. Both of these values are higher than the results found in previous study (**Chapter 3.2**), suggesting that the commercial amino acid profile, with or without supplemented proline, better meets the metabolic needs of the parenterally-fed neonatal piglet when compared to the B/P profile. These data suggest that proline may not be limiting in the commercial profile and is adequate in meeting the metabolic needs of the neonatal piglet.

When tested it was found that the variances were not equal between treatments when comparing phenylalanine flux and protein breakdown. Although not significant oxidation was lower thus protein synthesis higher when the profile was supplemented with proline. The high variation phenylalanine flux and protein breakdown in animals fed the commercial profile makes it nearly impossible to detect a significant difference between treatments with this sample size. One of the possible explanations of this discrepancy could be due to a high variability in metabolic response to an amino acid deficiency. The piglets could be increasing proline de-novo synthesis in varying degrees in response. Proline needs to be supplemented in the commercial profile and the original

B/P profile to decrease the variation which will enable us to detect a significant difference with a smaller small size in subsequent trials.

When the diet was supplemented with proline, plasma concentrations increased significantly to levels described as physiological normal (**Figure 3.3.1**), suggesting that the current commercial profile is inadequate in meeting the metabolic demands of the parenterally-fed piglet (Wykes et al., 1994). The oxidation data and plasma data seem to contradict one another which indicates that arginine may have played a role and has a sparing effect on proline.

The arginine requirement of the parenterally fed piglet was determined to be metabolically adequate at approximately 1.20 $g \cdot kg^{-1} \cdot day^{-1}$ and the commercial profile selected was adequate at 1.99 $g \cdot kg^{-1} \cdot day^{-1}$ (Wilkinson et al., 2004). It has been stated that arginine may have a sparing effect, of approximately 40%, on proline requirement (Murch et al., 1996). Therefore some arginine in excess of the requirement may have been used to increase de-novo proline synthesis, resulting in no significant effect on phenylalanine oxidation in piglets receiving the low proline diet (**Table 3.3.2**). In future research the conversions between arginine and proline need to be assessed in parenterally-fed piglets receiving a variety of arginine intakes. It is not possible to state that proline is not an indispensable amino acid because the commercial profile did contain some proline and was not at absolute zero. To irrefutably determine the indispensability of proline, an amino acid profile with no proline and low arginine needs to be tested; but when attempted piglets experienced hyperammonemia which resulted in death (Brunton et al., 1999). An IAAO requirement study may help to prove that proline is required in the TPN fed neonatal piglet.

Proline supplementation did not have a significant effect on protein synthesis therefore it was determined that the commercial profile may contain sufficient proline concentrations. However, proline may still be an indispensable amino acid in the parenterally fed piglet due to gut atrophy, and the interrelationship with arginine or other amino acids.

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## 3.4 RE-DETERMINATION OF THE FIRST LIMITING OR CO-LIMITNG AMINO ACID IN THE BALL/PENCHARZ AMINO ACID PROFILE

### 3.4.1 Introduction

Failure to accurately assess the parenteral requirement of the neonate can result in an amino acid deficiency or excess. The amino acid patterns of current TPN diets contain under and over-supply of amino acids compared to experimentally determined parenteral requirements of the human neonate, which thus can result in an amino acid imbalance (Brunton et al, 2000). As previously described, many TPN patterns are based on enteral protein sources which have been found to result in a significant excess of certain amino acids when used for parenteral administration (Bertolo et al., 1998, Elango et al., 2002, and Shoveller et al., 2003). In contrast, some of our findings have suggested that some indispensable (Chapter 3.1) and conditionally indispensable amino acids (Chapter 3.3) in the parenterally fed neonatal piglet may be underestimated (Bertolo et al., 2003, Wilkinson et al., 2004). The initial trial in this research (Chapter 3.1) indicated that at least one amino acid was limiting in the B/P profile. When testing individual potential candidates for the limiting amino acid (Chapter 3.2), using the IAAO technique, it was found that oxidation values of the supplemented diets did not differ significantly from the original B/P pattern. In combination, these findings suggested that the B/P profile may contain co-limiting amino acids. Due to the inadequacy of the B/P profile in meeting metabolic demands of the parenterally fed piglet, a sensitive method of determining the first or co-limiting amino acid is required.

The IAAO technique has been successfully applied to the determination of limiting amino acids in parenterally fed piglets (Brunton et al., 2007). A previous experiment done by our research group used the IAAO technique to determine whether the aromatic and sulphur amino acids were limiting in an amino acid profile. By supplementing the parenteral diet with either the aromatic or sulphur amino acids or both they were able to distinguish which amino acids were limiting by a decrease in lysine oxidation (Brunton et al., 2007). The IAAO technique was applied in the previous study (Chapter 3.2) to determine which amino acid was first limiting in the B/P profile. When piglets were fed the B/P profile, the plasma concentrations of leucine, threonine and methionine were lower than the plasma concentrations found by Wykes *et al.* (1994) when examining piglets fed intravenously and orally (Chapter 3.1). Supplementation of threonine and methionine did not result in an increase in plasma amino acid concentrations, whereas leucine supplementation did (**Table 3.2.3**, Chapter 3.2). However in all treatments, phenylalanine flux and protein synthesis remained unchanged from the B/P profile (**Table 3.2.3**, Chapter 3.2). Individual supplementation of B/P pattern with leucine, methionine, or threonine did not significantly improve protein synthesis, suggesting that a limiting amino acid was still present or that there were co-limiting amino acids.

Upon further examination, it was found that plasma proline concentrations were low across all treatments, suggesting that proline may be limiting in the B/P profile. The B/P profile was then supplemented with proline (Chapter 3.3) and tested to determine whether proline was the first limiting amino acid. Protein synthesis did not increase significantly, however plasma proline concentrations did, and fell within the reference

range (Wykes et al., 1994). This finding warranted the supplementation of additional proline in the B/P pattern to eliminate the chance of it being either limiting or co-limiting with either leucine, threonine or methionine in a subsequent experiment.

By using the IAAO method, we hypothesized that by feeding the B/P profile supplemented with a combination of two of the three amino acids (methionine, leucine or threonine), the combination containing the co-limiting amino acids would decrease phenylalanine oxidation, indicating an increase in protein synthesis in the TPN-fed neonatal piglet.

### 3.4.2 **Materials and Methods**

#### *3.4.2.1Animals and study protocol*

The Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta approved all procedures performed in this study. A total of 12 Duroc-Landrace/Large White cross piglets were used within the study. Piglets were obtained from the Swine Research and Technology Centre at the University of Alberta Research Farm (Edmonton, Alberta, Canada) at the ages of 1-2 days and weights of 1.1-1.8 kg. No changes were made to the animal and surgical protocols previously described in section 3.1.2.1.

#### *3.4.2.2 Animal housing*

The animal housing in this experiment was as described in section 3.1.2.2.

*3.4.2.3 Daily care*

Piglets were monitored constantly throughout the day and night. General health was observed and recorded. Daily care consisted of weighing the piglet, temperature reading, treatment of incision sites and ensuring symptoms associated with diet-induced hyperammonia were not present.

Diet bags were also weighed at this time to calculate pump efficiency to allow for an accurate calculation of the amount of diet received on a mL/kg*day basis.

*3.4.2.4 Blood Sampling*

Blood samples were taken on day 5, 7 and 9 as described under $^{14}C$ Phenylalanine Infusion, $^{14}CO_2$ and blood collection, and analytical procedures section.

*3.2.2.5 Diet Regimen*

Following surgery, piglets were fed a complete diet with the amino acid profile similar to the TPN solution, Primene®. All diets were infused into the jugular vein catheter using a pressure-sensitive infusion pump. Piglets received 15g amino acids/kg*d and 1.1 MJ metabolizable energy/kg*d with glucose and lipid (Intralipid 20%, PharmaciaUpjohn, Stockholm, Sweden) each supplying 50% of non-protein energy intake. Vitamins, oil and water-soluble, were supplied by a prefabricated commercial solution (Multi-12/$K_1$ Pediatric; Sabex Inc., Boucherville PQ, Canada) and provided 115% of the estimated NRC (1998) requirement for piglets 3-5 kg. The vitamin solution, iron sulfate (Ferroforte; Bimeda-MTC, Cambridge ON, Canada) and a micromineral solution were added to the elemental diet immediately prior to feeding.

The micromineral solution contained zinc sulfate, copper sulfate, manganese sulfate, chromium sulfate, selenium sulfate and sodium iodide and provided 200% of the NRC (1998) recommendation for piglets 3-5 kg. Immediately following surgery, all piglets received diet parenterally at 50% of the targeted infusion rate (6.75 mL/kg*d). Twelve hours following surgery the infusion rate was increased to 75% of the targeted infusion rate (10.125 mL/kg*d). On the morning of day 1, all piglets received the diets at 13.5 mL/kg*d and continued at that rate for the remainder of the study.

At noon of day 4, 6 and 8, diets were randomly assigned and piglets were fed one of the five treatment groups. All piglets received the B/P profile at one point during the oxidation schedule and no piglet received the same diet twice. In total each piglet was fed the B/P profile and two test diets. The five test diets were the B/P amino acid profile (n=10), the B/P profile supplemented with a combination of either methionine/threonine (n=5), methionine/leucine (n=5), threonine/leucine (n=5) or all three, methionine, leucine, and threonine (n=5), at 150% of the previously determined requirement (Brunton et al., 2007). The composition of the test diets are presented in **Table 3.4.1**.

**Table 3.4.1:** Amino acid concentrations of B/P Profile, and the B/P profile plus additional methionine/threonine (MT), methionine/leucine (ML), threonine/leucine (TL), or methionine/leucine/threonine (MLT) administered to parenterally-fed neonatal piglets

| | Concentration g $L^{-1}$* | | | | |
|---|---|---|---|---|---|
| **Amino acid** | B/P Profile | B/P + MT | B/P + ML | B/P + TL | B/P + MLT |
| L-Alanine[1] | 11.7540 | 11.1690 | 10.6300 | 10.6375 | 10.3410 |
| L-Arginine | 4.4118 | 4.4118 | 4.4118 | 4.4118 | 4.4118 |
| L-Aspartate | 3.1511 | 3.1511 | 3.1511 | 3.1511 | 3.1511 |
| L-Cysteine | 0.5515 | 0.5515 | 0.5515 | 0.5515 | 0.5515 |
| L-Glutamate | 5.1993 | 5.1993 | 5.1993 | 5.1993 | 5.1993 |
| Glycine | 1.6544 | 1.6544 | 1.6544 | 1.6544 | 1.6544 |
| L-Histidine | 1.9956 | 1.9956 | 1.9956 | 1.9956 | 1.9956 |
| L-Isoleucine | 2.4386 | 2.4386 | 2.4386 | 2.4386 | 2.4386 |
| L-Leucine | 2.4386 | 2.4386 | **3.6579** | **3.6579** | **3.6579** |
| L-Lysine | 3.8585 | 3.8585 | 3.8585 | 3.8585 | 3.8585 |
| L-Methionine | 0.9926 | **1.4889** | **1.4889** | 0.9926 | **1.4889** |
| L-Phenylalanine | 2.2058 | 2.2058 | 2.2058 | 2.2058 | 2.2058 |
| L-Proline | 4.5956 | 4.5956 | 4.5956 | 4.5956 | 4.5956 |
| L-Serine | 2.1007 | 2.1007 | 2.1007 | 2.1007 | 2.1007 |
| Taurine | 0.3151 | 0.3151 | 0.3151 | 0.3151 | 0.3151 |
| L-Threonine | 0.7721 | **1.1581** | 0.7721 | **1.1581** | **1.1581** |
| L-Tryptophan | 0.6618 | 0.6618 | 0.6618 | 0.6618 | 0.6618 |
| L-Tyrosine | 0.4412 | 0.4412 | 0.4412 | 0.4412 | 0.4412 |
| L-Valine | 2.4386 | 2.4386 | 2.4386 | 2.4386 | 2.4386 |
| Glycyl-tyrosine[2] | 1.5074 | 1.5074 | 1.5074 | 1.5074 | 1.5074 |
| Ornithine | 1.4739 | 1.4739 | 1.4739 | 1.4739 | 1.4739 |

**Bold value** indicates supplementation

* This solution was provided to the piglets at a rate of 272 mL/kg/d

[1] Alanine concentrations vary to allow for all treatments to be isonitrogenous

[2] Glycyl tyrosine supplying 0.32 g $L^{-1}$ of glycine and 1.19 g $L^{-1}$ tyrosine

Lysine and Ornithine were supplied as Lysine-HCl and Ornithine-HCL to provide the concentration shown.

*3.4.2.6 $^{14}C$ Phenylalanine Infusion, $^{14}CO_2$ and blood collection, and analytical procedures*

On days 5, 7 and 9, piglets were given a primed (7 μCi/kg), constant (3.5 μCi/kg) intravenous infusion of L-[1-$^{14}$C] phenylalanine via the jugular vein catheter to determine phenylalanine flux and oxidation. To reach plateau in both breath and blood labelling, the duration of constant infusion was 4h, and blood samples (1 mL) were taken at time 0, 120, 150, 180, 210 and 240 minutes. On day 7 and 9, additional blood samples were taken one hour (–60 minutes) and half an hour (-30 minutes) prior to the start of isotope infusion to correct for the background specific radioactivity of phenylalanine in the blood, and breath samples were collected at -30 and 0 minutes to correct for any residual labelling of the breath $CO_2$. Details of infusion protocol, $^{14}CO_2$ collection and blood collection procedures have been described previously (House et al, 1997). Following the infusion on d 9, piglets were killed by injection of 1000 mg of sodium pentobarbital into a venous catheter.

Plasma amino acids were determined using reverse-phase high performance liquid chromatography with the use of phenylisothiocyanate derivatives as previously described (Bidlingmeyer et al., 1984 and House et al., 1994). The internal standards norleucine and L-[4,5-$^{3}$H]leucine (1920 GBq/mmol; Amersham Pharmacia Biotech, St. Louis MO, USA) were added to each 300 μL plasma sample. Post-column radioactive derivatives of phenylalanine and tyrosine were collected in 2 mL fractions corresponding to the respective peaks, 14 mL of scintillant (Biodegradable Counting Scintillant; Amersham Canada, Ltd., Oakville ON, Canada) was added, and samples were counted on a scintillation counter.

*3.4.2.7 Calculations*

The calculations within this experiment are as described in the previous chapter in section 3.2.2.7.

*3.4.2.8 Statistical Analysis*

All data were analyzed using SAS Version 9.1 (2003 SAS Institute, Cary NC, USA), and data were considered statistically significant if $p < 0.05$.

The dependent variables plasma amino acid concentration, phenylalanine flux, oxidation, non-oxidative disposal and release from protein, were analyzed using proc mixed where the fixed effect was diet (B/P profile, B/P profile plus MT, B/P profile plus ML, B/P profile plus TL and B/P profile plus MLT) and the random variable was piglet. When the effects were significant ($p < 0.05$), least squares means were separated using the pdiff option.

### 3.4.3 **Results**

*3.4.3.1 Piglet Performance*

Most piglets remained healthy and active during the course of the study, in contrast to the piglets on the original B/P diet (Chapter 3.1). Two piglets were removed from the trial due to health concerns unrelated to treatment. Piglet weight upon arrival was 1.24 kg (2 d, SD= 0.15) and 2.47 kg at the end of the study (9d, SD= 0.33). These values did not differ among all dietary treatments.

*3.4.3.2 Phenylalanine Intake, Oxidation, Flux, Non-oxidative disposal, Release from Protein Breakdown, and Percent Dose Oxidized*

Diet had no effect on phenylalanine flux, oxidation, non-oxidative disposal or release from protein ($p>0.05$, **Table 3.4.2**).

*3.4.3.3 Plasma Amino Acid Concentrations*

Diet had a significant effect on the plasma concentrations of histidine, isoleucine, leucine, phenylalanine, threonine and valine ($p<0.05$, **Table 3.4.3**). When compared to the B/P diet, plasma histidine concentrations decreased when diets were supplemented with ML, MT and TL. When the diet was supplemented with leucine, plasma isoleucine and valine concentrations significantly decreased when compared to the B/P profile. Leucine supplementation resulted in a significant increase in leucine concentrations in the ML and MLT treatment groups. Threonine concentrations increased significantly for the ML and MLT treatment groups, when compared to the B/P profile, however when the diet was supplemented with MT and TL, no significant increase was observed. Plasma concentrations of phenylalanine decreased significantly when the diet supplemented with MT was fed. Supplementation of methionine had no effect on plasma methionine levels ($p>0.05$, **Table 3.4.3**). Threonine and methionine plasma concentrations were substantially lower than the sow-fed reference range across all treatment groups.

Of the conditionally indispensable amino acids, arginine, glutamine and tyrosine, were significantly affected by diet ($p<0.05$, **Table 3.4.3**). When compared to the B/P profile, arginine concentrations were significantly lower when the diets were

supplemented with MT and TL. Glutamine and tyrosine concentrations also decreased significantly when the MT, and TL and MLT treatments were fed. Diet had no effect on cysteine, glycine or proline plasma concentrations ($p>0.05$, **Table 3.4.3**). Diet had a significant effect on the plasma concentrations of aspartate, asparagine, taurine, citrulline and serine ($p<0.05$, **Table 3.4.3**). Diet had no effect on concentrations of hydroxy-proline, ornithine glutamate or alanine ($p>0.05$, **Table 3.4.3**). Compared to the B/P profile, the plasma concentration of asparagine was significantly lower in all supplemented treatment groups; whereas aspartate only decreased significantly if the diet was supplemented with TL. The plasma concentration of serine differed significantly between MT and TL treatment groups. Taurine concentrations were significantly higher when piglets were fed a diet supplemented with ML relative to the other diets. Plasma citrulline concentrations were lowest in the piglets fed a diet supplemented with TL. Plasma amino acid concentrations of serine and glutamate were higher than the sow-fed reference range when piglets were fed the B/P amino acid pattern. However, citrulline concentrations were lower than the sow-fed reference range for all supplemented diets.

**Table 3.4.2:** Phenylalanine kinetics (mmol $kg^{-1}$ $h^{-1}$) of piglets receiving total parenteral nutrition with the B/P profile, or the B/P profile supplemented either methionine and leucine (ML), methionine and threonine (MT), threonine and leucine (TL) or methionine, leucine and threonine (MLT)

| Diet ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) | B/P Profile | SE | B/P Profile + MT | SE | B/P Profile + ML | SE | B/P Profile + TL | SE | B/P Profile + MLT | SE | P value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| n | 10 | | 5 | | 5 | | 5 | | 5 | | |
| Flux (Q) | 249.53 | 8.91 | 253.36 | 12.60 | 235.36 | 13.80 | 243.81 | 12.60 | 248.49 | 12.60 | NS |
| Intake (I) | 149.66 | 1.60 | 149.92 | 1.42 | 149.09 | 1.94 | 151.63 | 2.48 | 148.23 | 1.83 | NS |
| Oxidation (E) | 28.37 | 5.15 | 35.21 | 7.08 | 26.09 | 7.72 | 19.70 | 7.08 | 25.53 | 7.08 | NS |
| Non-Oxidative Disposal (S) | 221.16 | 7.82 | 201.48 | 11.06 | 204.06 | 12.11 | 220.75 | 11.06 | 222.96 | 11.06 | NS |
| Release from protein (B) | 99.87 | 8.91 | 103.44 | 12.60 | 86.27 | 13.80 | 92.18 | 12.60 | 100.26 | 12.60 | NS |

NS denotes no significance, $p>0.05$

**Table 3.4.3:** Mean plasma amino acid concentrations of piglets receiving the B/P profile, and B/P profile supplemented with either, methionine and leucine (ML), methionine and threonine (MT), threonine and leucine (TL), or all three amino acids (MLT)

| | Plasma Amino Acid Concentrations umol/L Diet | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | B/P Profile | SE | B/P + ML | SE | B/P + MT | SE | B/P + TL | SE | B/P + MLT | SE | Orally-Fed Reference Range[1] |
| **Dispensable Amino Acids** | | | | | | | | | | | |
| Alanine | 991.98 | 171.33 | 574.02 | 164.18 | 982.43 | 164.18 | 499.57 | 164.18 | 799.88 | 164.18 | 413-1015 |
| Aspartate | 57.78[a] | 19.13 | 34.26[ab] | 16.50 | 23.87[ab] | 16.50 | 17.26[b] | 16.50 | 20.46[ab] | 16.50 | 22-63 |
| Asparagine | 332.45[a] | 179.11 | 49.41[b] | 85.48 | 30.40[b] | 85.48 | 36.92[b] | 85.48 | 37.89[b] | 85.48 | |
| Glutamate | 230.68 | 44.56 | 216.67 | 48.01 | 213.78 | 48.01 | 141.57 | 48.01 | 144.90 | 48.01 | 92-217 |
| Serine | 466.14[ab] | 65.51 | 354.97[ab] | 69.14 | 537.48[a] | 69.14 | 326.06[b] | 69.14 | 421.93[ab] | 69.14 | 137-442 |
| **Conditionally Indispensable Amino Acids** | | | | | | | | | | | |
| Arginine | 91.45[a] | 12.50 | 84.41[ab] | 12.96 | 53.96[b] | 12.96 | 55.75[b] | 12.96 | 78.86[ab] | 12.96 | 50-267 |
| Cysteine | 16.77 | 3.12 | 35.72 | 9.17 | 23.30 | 8.53 | 7.58 | 9.20 | 20.45 | 8.53 | |
| Glutamine | 444.51[a] | 63.14 | 354.68[ab] | 62.11 | 270.46[b] | 62.11 | 284.47[ab] | 62.11 | 303.37[ab] | 62.11 | |
| Glycine | 1010.10 | 133.27 | 886.66 | 149.28 | 1188.87 | 149.28 | 825.60 | 149.28 | 1069.08 | 149.28 | 450-976 |
| Proline | 720.77 | 95.56 | 501.79 | 94.94 | 666.12 | 94.94 | 493.64 | 94.94 | 571.52 | 94.94 | 304-890 |
| Tyrosine | 289.07[a] | 42.14 | 204.68[ab] | 43.03 | 169.10[ab] | 43.03 | 158.29[b] | 43.03 | 147.51[b] | 43.03 | 88-231 |
| **Indispensable Amino Acids** | | | | | | | | | | | |
| Histidine | 158.63[a] | 38.17 | 45.05[b] | 34.26 | 51.53[b] | 34.26 | 30.21[b] | 34.26 | 76.27[ab] | 34.26 | 47-121 |
| Isoleucine | 195.83[a] | 15.31 | 149.70[b] | 17.60 | 272.99[a] | 17.60 | 143.95[b] | 17.60 | 175.01[ab] | 17.60 | 81-143 |
| Leucine | 72.92[a] | 6.54 | 111.13[b] | 8.12 | 22.74[c] | 8.12 | 87.02[ab] | 8.12 | 104.42[a] | 8.12 | 102-175 |
| Lysine | 206.87 | 36.09 | 144.73 | 31.40 | 135.19 | 31.40 | 150.94 | 31.40 | 148.45 | 31.40 | 77-317 |
| Methionine | 6.90 | 1.34 | 10.09 | 3.72 | 13.44 | 2.63 | 2.10 | 5.26 | 9.62 | 2.63 | 33-74 |
| Phenylalanine | 139.98[a] | 21.79 | 92.46[a] | 19.88 | 95.34[b] | 19.88 | 98.25[ab] | 19.88 | 100.92[ab] | 19.88 | 34-86 |
| Threonine | 8.85[a] | 1.93 | 21.75[b] | 3.31 | 12.72[a] | 3.31 | 13.61[ab] | 3.31 | 22.75[b] | 3.31 | 214-500 |
| Tryptophan | 30.80 | 5.30 | 33.70 | 5.45 | 30.96 | 5.45 | 28.43 | 5.45 | 34.02 | 5.45 | |
| Valine | 320.76[a] | 27.77 | 209.31[b] | 26.22 | 291.54[a] | 26.22 | 170.69[b] | 26.22 | 195.29[b] | 26.22 | 175-318 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Other Amino Acids** | | | | | | | | | | | |
| Taurine | 149.90[a] | 17.70 | 178.02[b] | 18.84 | 168.41[ab] | 18.84 | 131.55[a] | 18.84 | 158.22[ab] | 18.84 | |
| Ornithine | 124.03 | 21.55 | 113.78 | 22.39 | 114.66 | 22.39 | 109.82 | 22.39 | 125.17 | 22.39 | 79-195 |
| Hydroxy-Proline | 42.68 | 5.94 | 40.74 | 6.54 | 55.92 | 6.54 | 43.19 | 6.54 | 50.34 | 6.54 | |
| Citrulline | 94.28[a] | 11.62 | 67.98[a] | 11.05 | 64.87[ab] | 11.05 | 59.17[b] | 11.05 | 62.79[ab] | 11.05 | 73-151 |

[1] Wykes et al., 1994

[abc]Values sharing a superscript are not significantly different ($p<0.05$).

### 3.4.4 **Discussion**

A limiting amino acid is generally an indispensable amino acid and is defined as the amino acid that is supplied in the lowest proportion relative to its requirement. Data from our first experiment suggested that an amino acid in the B/P profile was severely limiting, which resulted in hyperammonemia and death of some of the piglets (Chapter 3.1). In the subsequent trials, we have determined that threonine, leucine and methionine were most likely limiting in the B/P profile. The premise of the IAAO method is that when the intake of the limiting amino acid increases, oxidation of the indicator amino acid will decrease, therefore reflecting an increase whole body protein synthesis (Elango et al., 2008). This concept was applied and additional threonine, methionine and leucine in varying combinations were added to the B/P profile. However, no significant change in phenylalanine oxidation was observed suggesting that threonine, leucine and methionine were either not limiting or were co-limiting in the B/P profile.

In our initial trial (Chapter 3.1), low leucine plasma concentrations, as compared to an amino acid reference range (Wykes et al., 1994), were found when piglets were fed the B/P profile, suggesting that leucine may have been limiting. The family of branched chain amino acids, valine, leucine and isoleucine, are indispensable amino acids which are primarily incorporated into muscle protein and have a complex interrelationship. When fed in excess, the branched chain amino acids have been shown to have antagonistic effects on each other (Harper et al., 1984). Isoleucine and valine antagonisms are not as frequently reported and do not seem to have as adverse effects on whole body metabolism as does excess leucine supplementation (Elango et al., 2004). However, it is suspected that plasma leucine concentrations would only be depleted if

leucine was the first limiting of the branched-chain amino acids and isoleucine and valine were in excess (Harper et al., 1984). Previous research has defined the optimal enteral branched chain amino acid ratio to be 1:1.8:1.2 (isoleucine:leucine:valine) therefore if the ratio of 1:1:1 is incorrect, leucine would be the first limiting and isoleucine would be in excess, which is supported by our plasma amino acid values (**Table 3.4.3**). When leucine was supplemented to the diet, plasma concentrations increased significantly but there was no significant change in protein synthesis. Contrary to what has been previously described (Elango et al., 2004), this finding suggests that leucine may be the first limiting amino acid when the branched chain amino acids are fed at a ratio of 1:1:1 and therefore, the ratio of 1:1:1 may be incorrect. Further study into the optimal ratio for the branched chain amino acids in a parenterally fed piglet is warranted. However because no change was observed in protein synthesis, it may be concluded that leucine is unlikely to be the first limiting amino acid within the B/P pattern.

Methionine supplementation had no effect on protein synthesis; as well, plasma concentrations still remained lower than what is typically seen in a healthy parenterally fed piglet of the same age (Wykes et al., 1994)(**Table 3.4.2**). These data suggests that methionine concentrations in the diet may still be inadequate in meeting the metabolic demands of the growing piglet. It has demonstrated that parenteral feeding may have a higher requirement for cysteine than what was previously reported in the requirement study (Shoveller, 2004, Shoveller et al., 2003a). Since the amount fed to the piglets in the previous trials are based on a molar relationship between cysteine and methionine, and the sparing effect of cysteine, the possibility exists that methionine was not limiting in the B/P profile and that cysteine may be. These explanations may also account for the

increased plasma concentration values of serine present when piglets were fed the B/P pattern (**Table 3.4.3**), since serine is a precursor for synthesis of several amino acids, including cysteine. During parenteral feeding, cells are challenged by an increase in oxidants and in response to this oxidative stress the transsulfuration pathway is activated to enable the up-regulation of glutathione synthesis (Shahal et al., 1991). Therefore, glutathione synthesis results in an additional requirement for cysteine synthesis and/or dietary methionine. An inadequacy in dietary cysteine intake for glutathione synthesis would not result in a change in oxidation or an increase in protein synthesis if additional methionine and/or cysteine were added, therefore IAAO technique would be unable to determine if the cysteine requirement has been met (Shoveller, 2004). Futhermore, piglets fed the B/P profile clearly had a limiting amino acid that did not meet the metabolic needs of the parenterally fed piglet. This limiting amino acid would have resulted in an increase in oxidants, and may therefore result a higher requirement for cysteine synthesis and/or dietary methionine. Therefore, a decrease in sulphur amino acid concentrations in the blood may be also a secondary response to another amino acid being limiting, not directly because the sulphur amino acids were the limiting amino acids for protein synthesis.

The parenteral threonine requirement was found to be approximately 60% lower than the enteral requirement suggesting that an atrophied gut has a lower requirement (Bertolo et al., 1998). Even when the diet was supplemented with additional threonine, the parenteral level of feeding was still 40% lower than the safe level of intake required in an orally fed piglet (Bertolo et al., 1998). Threonine intake is also reflected in overall health status of the gut and the level of intestinal mucin production (Law et al., 2007).

The parenteral threonine requirement was determined at day 7 when piglets were experiencing complete gut atrophy and minimal mucin production. During the initial trial (Chapter 3.1), piglets were newly weaned, and fed at the safe level of intake for parenteral threonine beginning at two days of age. Due to being newly weaned, the piglets would not be experiencing any, or very minimal, gut atrophy at that time, and mucin production would have been normal. Threonine deficiency may have been more severe at the beginning of the experiment due to the demand for mucin production. The parenteral threonine requirement would have then slowly decreased as the gut atrophied and mucin production ceased to occur. This would explain why no significant change in oxidation or plasma amino acid concentration was observed when the diet was supplemented with threonine at day 7 (**Table 3.4.2 and Table 3.4.3**). This finding suggests that the determined parenteral threonine requirement may be reflective of gut health status and period of TPN feeding. Although the previous estimate (Bertolo et al., 1998) may be the correct value for a gut that is completely atrophied; this requirement value may be an underestimate of the threonine needed during initial parenteral feeding due to more normal gut function. Further study is warranted into the variability of the parenteral threonine requirement in relation to mucin production and gut atrophy.

The basis of the IAAO technique states that no amino acids provided above what is needed for protein synthesis are stored; all amino acids in excess will be oxidized and will reflect the severity of the limiting amino acid (Brunton et al., 2007). In our trials we supplemented the diets in hopes of decreasing the severity of the limiting amino acid present in the B/P TPN pattern. However, none of the supplemented diets resulted in a significant decrease in phenylalanine oxidized, thus indicating that either threonine,

methionine, or leucine were either not the first limiting amino acids, or that supplementation was still below requirement.

Parenteral amino acid metabolism is a complex series of events and interactions that are still not fully understood. In the quest to understand and determine the amino acid requirements of the parenterally fed neonatal piglet, it seems we have a better understanding, but not a complete knowledge of what the optimal amino acid profile would be. These series of experiments have shown that a limiting amino acid exists within the B/P pattern, and therefore, the total parenteral nutrition solution that optimizes protein synthesis remains undefined. Yet, in contrast to the piglets in the original study of the BP pattern (Chapter 3.1) the piglets in the current study were all healthy. Examination of the diets used show that the important difference was the additional proline from 1.58g/L to 4.6 g/L. Hence there is some evidence that proline was limiting in the original BP pattern. What is not clear is why the indicator oxidation was unable to demonstrate clearly this fact in chapter 3.3 and what interaction proline may have with methionine, leucine and/or threonine.

### 3.4.5 **Literature Cited**


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## 4.0 Summary, General Discussion and Future Directions

The main objective of the research described in this thesis was to combine the previously determined requirements for the indispensable amino acids into a single TPN solution, the B/P profile, and compare the performance of parenterally-fed neonatal piglets receiving this profile to piglets receiving another TPN profile. The first experiment demonstrated that the B/P profile did not meet the metabolic needs of the neonatal piglets and contained at least one severely limiting amino acid, based upon hyperammonaemia and death (Chapter 3.1). In the second experiment lysine, leucine, methionine or threonine were added to the B/P profile and oxidation was measured to determine which of these amino acids was the first limiting amino acid. Lysine was demonstrated to not be the first limiting amino acid in the B/P profile; however the data pertaining to leucine, methionine and threonine were inconclusive (Chapter 3.2). This finding suggested that another amino acid, that was not studied, was the limiting or co-limiting amino acid. In the third experiment, proline was determined to be an indispensable amino acid in parenterally-fed piglets and that the B/P profile used in the previous experiments provided an inadequate quality of proline (Chapter 3.3). Therefore, proline was supplemented in the subsequent experiment. In the fourth and final trial (Chapter 3.4), specific combinations of methionine, threonine and leucine were added to the B/P profile to determine if these were co-limiting amino acids. The addition of methionine, leucine or threonine to the B/P profile did not improve protein synthesis or oxidation. Therefore, we were unable conclude that any of these amino acids were limiting in the B/P profile. Possible explanations for the lack of conclusive results

include: a flaw in the original experimental design, use of incorrect formulations, age and breed of piglet or unknown amino acid interactions.

The indicator amino acid technique was used to determine the requirements, individually, for lysine, threonine and tryptophan (House et al., 1998, Bertolo et al., 1998, Cvitkovic et al., 2000). This technique was also used to evaluate the inter-relationships and requirements of the aromatic, sulphur, and branched chain amino acids (House et al., 1997a, Shoveller et al., 2003a, Elango et al., 2002). Although inter-relationships were tested between amino acids of the same structural family, they were not tested across families, which was the objective of this research. For the initial experiment (Chapter 3.1), all of the predetermined amino acid requirements were combined for the first time to form a complete parenteral amino acid solution. This solution resulted in high plasma urea values after approximately 48 hours, water retention and eventually death suggesting that one or more of the requirements were incorrect.

One of the primary concerns with the B/P profile was the low nitrogen content compared to other TPN solutions. Based on the NRC (1998) nutrient requirements for swine weighing between 1-5kg, piglets should receive 20 $g \cdot kg^{-1} \cdot d^{-1}$ of amino acids however the B/P TPN solution contained 11.8 $g \cdot kg^{-1} \cdot d^{-1}$. Previous experiments showed that piglets can sustain protein metabolism with a protein intake of 14.6 $g \cdot kg^{-1} \cdot d^{-1}$ and the NRC protein requirement may be over-estimated for the parenterally-fed piglet (Wykes et al., 1993). During the initial trial (Chapter 3.1) the B/P amino acid profile was tested against an amino acid profile based on Primene®, which contained 14.6$g \cdot kg^{-1} \cdot d^{-1}$ of amino acids. This difference in total protein intake was addressed by adding a third treatment group that was isonitrogenous to the amino acid profile based Primene® but had an

identical amino acid pattern, on g per 100g basis, to the B/P pattern. Both the B/P and isonitrogenous B/P profile resulted in poor nitrogen retention, and high blood urea concentrations, suggesting that a limiting amino acid was present in both profiles and the protein content had no effect.

The results from experiment 1 (Chapter 3.1) suggested that a protein intake of 14.6 $g \cdot kg^{-1} \cdot d^{-1}$ may be in excess of the requirement for the parenterally fed neonatal piglet. Previous studies showed that the parenteral protein requirement is substantially lower than the enteral requirement because splanchnic metabolism is bypassed when fed intravenously (Stoll et al., 1998, Stoll and Burrin, 2006). Indispensable amino acid requirements are approximately 40% less in parenterally fed piglets versus those enterally fed (Bertolo et al., 1998, Elango et al., 2002 Shoveller et al., 2003a); whereas Stoll et al. (1998) found that approximately one third of the dietary intake of indispensable amino acids were used extracted during first-pass metabolism.. This indicates that the intravenous protein requirement may only be 60-70% of the enteral protein requirement or approximately 9-10 g/kg/d. By this reasoning, even the low amino nitrogen intake provided by the B/P profile was well in excess of the parenteral requirement.

Another possible concern pertaining to the present experiments was the age of the piglets used to measure the amino acid requirements compared to the age of the piglets when they began receiving these dietary intakes of amino acids in the present experiments. The individual amino acid requirements were determined in piglets between 7 and 10 days of age (House et al, 1997a, Bertolo et al, 1998, Elango et al., 2002, Brunton et al., 2003, Shoveller et al, 2003a, Cvitkovic et al, 2004); whereas in the first experiment piglets were to be fed at this level starting at 1-2 days of age. Some

young animals lack some enzyme development to metabolically adapt to the adverse effects of an amino acid imbalance (Harper et al., 1970) and enzymes relating to amino acid metabolism undergo many changes during the first week of life (Wu et al., 1996). This lack of development would increase the sensitivity of the piglets to amino acid toxicity and imbalance, whereas a higher enzymatic activity can result in a deficiency. The high urea values observed in the first experiment (Chapter 3.1) suggests that an amino acid was limiting, and thus all other amino acids were in excess and therefore were catabolized. Therefore, lack of enzymatic development in the piglets is probably not the explanation for the findings of this experiment (Chapter 3.1).

However, age may still play an important role in determining amino acid requirements of neonatal piglet. One of the potential reasons for an effect of age on parenteral amino acid requirement, between 2 and 7 day old piglets, could be the extent of gut dysfunction and atrophy due to the length of parenteral feeding. When comparing enteral versus parenteral requirements, the parenteral requirements were found to be much lower due to the bypassing of splanchnic metabolism and gut atrophy (Stoll et al. 1998, Stoll and Burrin, 2006, and Bertolo et al., 1999, Elango et al., 2002, Shoveller et al., 2003a). The dietary threonine requirement seems to reflect the health status of the gastrointestinal tract and level of intestinal mucin production (Law et al., 2007). The parenteral threonine requirement was found to be substantially lower than the enteral requirement suggesting that a fully developed gut has a higher requirement (Bertolo et al., 1998). The parenteral threonine requirement was determined at day 7 when piglets were experiencing gut atrophy and minimal mucin production. Within our trial, piglets were fed at the determined parenteral threonine requirement from the time of surgery, before

the onset of intestinal atrophy (Chapter 3.1). Initially, these piglets would have had minimal atrophy, and mucin production and gut metabolism may not decline for the first few days of parenteral feeding. This could mean that the previously determined threonine requirement was inadequate for parenterally-fed piglets with a lesser degree of atrophy. Further research into the relationship between enzymatic activity and amino acid requirement during the first few days of life in the piglet is warranted.

The plasma amino acid pattern of the piglets in first experiment (Chapter 3.1) suggested that one of the sulfur amino acids, methionine, and/or one of the branched chain amino acids, leucine, was possibly limiting in the B/P amino acid pattern. To determine the inter-relationships of amino acids within structural families, and the requirements for each amino acid within that group, a minimum of three experiments are required (Ball et al., 2006). However, for both the sulfur and branched chain amino acids, only 2 experiments were conducted. For example, to successfully determine the sulfur amino acid requirements using the IAOO technique one must first determine the methionine requirement devoid of cysteine (experiment 1) (Shoveller et al., 2003a). This will value will represent the total sulfur amino acid requirement, assuming there is no limitation in the ability of methionine to form cysteine. The second experiment (experiment 2) would consist of feeding cysteine in excess of its requirement with varying levels of methionine (Shoveller et al., 2003b). The second experiment will demonstrate the minimum methionine requirement and the sparing effect of cysteine on the methionine requirement. Lastly (experiment 3), methionine should be fed at the minimum requirement, determined in experiment 2, and cysteine should be added at varying levels to determine the total sulfur amino acid requirement of the animal. The

aromatic, sulfur and branched-chain amino acid requirements were all determined by a set of two experiments (House et al., 1997a, House et al., 1997b, Shoveller et al., 2003a, Shoveller et al., 2003b, Elango et al., 2002, Elango et al, 2004). By not doing experiment 3, the estimate of total sulfur amino acid requirement may not be completely accurate. For example, when compared to enterally fed piglets, Shoveller (Shoveller, 2004) found higher levels of methionine oxidation in TPN fed piglets (8.07 vs. 5.16% of dose oxidized); this suggests that there may be an additional demand for cysteine synthesis in TPN fed piglets and that the total sulphur amino acid requirement may have been somewhat underestimated in the parenterally fed piglets (Shoveller, 2004). If maintaining the optimal sulfur amino acid ratio of 60:40 (methionine:cysteine) this finding suggests that the previously determined methionine requirement may only meet 56% of the actual requirement (Shoveller et al., 2003b), thus be limiting the B/P and methionine supplemented amino acid patterns.

Elango *et al.* (2002) determined the parenteral branched chain amino acid requirement to be 1.99 $g \cdot kg^{-1} \cdot d^{-1}$ (experiment 1) (Elango et al., 2002). The parenteral requirement was determined using a ratio of 1:1.8:1.2 (isoleucine/leucine/valine) but a subsequent experiment (Elango et al., 2004) suggested that the parenteral ratio may be closer to 1:1:1 (experiment 2). These trials suggest a total amount of branched chain amino acids to be fed parenterally as well as the ratio. However; the new parenteral ratio and individual amino acids requirements were not compared to the previous ratio (experiment 3). This series of experiments done by Elango *et al.* (2002, 2004) may have resulted in an underestimation of the requirement for one or more the branched chain amino acids. A series of experiments may be required to individually determine the

requirements for isoleucine, leucine and valine. This can be achieved by using the IAAO technique and titrating one branched chain amino acid into the diet with an excess of the other two and repeating for each amino acid (experiment 1a,b,c). However, caution is required because this may result in an over-estimation because excess intake of the branched chain amino acids tends to increase the oxidation of the others (Elango et al., 2002). To decrease the chance of an over-estimation the previous series of experiments should be repeated with titrating one branched chain amino acid into the diet with the intake of other two at the previous estimate of the requirement and repeating for each amino acid (experiment 2a,b,c). These series of experiment could be very labour intensive and expensive to conduct, however it may be the only way of most accurately determining the individual amino acid requirements and its interactions. Once the requirements have been determined individually a new parenteral ratio can be developed and compared to the previously determined ratio and total branched chain amino acid requirement. Finally, the potential difference between the requirements determined this way and the estimates of Elango *et al.* must be considered and a decision made as to whether the difference is likely to be large enough to warrant the additional time, effort and cost.

The use of the "ideal protein concept" has been suggested for determining amino acid requirements and it states that amino acids requirements should be expressed proportionally to the requirement of lysine (Ball et al., 2007). The ideal amino acid pattern in growing mammals, is similar to the amino acid patterns in milk protein and body tissue protein within that species (Ball et al., 2007). Lysine is commonly the first limiting amino acid in many grain or cereal-based livestock diets (Chung and Baker,

1992) and it was one of the first parenteral requirements determined in piglets using the IAAO technique (House et al., 1998). The subsequent amino acid requirements were then determined individually and independently of each other (with the exception of the aromatic, sulfur and branched-chain amino acids) resulting in values which may be dependent on inter-relationships among the amino acids that are not yet understood in the parenterally fed piglet. A comparison between the ideal protein profile for a enterally fed 10 kilogram pig to the B/P profile, expressed as a percentage of lysine, shows an interesting trend (**Table 4.1**). The three amino acids suggested to be limiting based upon the plasma amino acid pattern, methionine, threonine, and leucine, are all substantially lower in the B/P profile than what is suggested for the ideal protein profile. The ideal protein profile is based on enterally fed pigs, and first pass-metabolism is still intact therefore a suggested optimal parenteral amino acid pattern was calculated accounting for first-pass metabolism being bypassed (**Table 4.1**). This suggested ideal pattern for the partenterally fed pig still shows that methionine and threonine are lower in the B/P amino acid pattern whereas valine and isoleucine may be in excess. It has been also demonstrated that tissue free amino acids seem to be affected by parenteral vs. enteral feeding (Bertolo et al., 1999), however that the ideal protein concept is based on whole body protein composition (not free amino acids) and to our knowledge the effect of parenteral vs. enteral feeding on the free amino acid pool has not been investigated. The requirement values for the sulfur amino acids as well as threonine and leucine should be re-evaluated as a percentage of lysine to determine the ideal protein profile for parenterally-fed piglets.

**Table 4.1:** Comparison of Ideal Amino Acid profiles

| | % of Lysine Requirement | | | |
|---|---|---|---|---|
| | Ideal Amino Acid Profile (10kg pigs)[1] | Ideal Amino Acid Profile (5 kg pigs)[2] | Suggested Ideal Parenteral Amino Acid Profile[1] | B/P Profile |
| Isoleucine | 60 | 55 | 34[3] | 60[3] |
| Leucine | 100 | 100 | 56[3] | 60[3] |
| Valine | 68 | 68 | 38[3] | 60[3] |
| Phenylalanine +Tyrosine | 95 | 92 | 95 | 105[4] |
| Sulfur Amino Acids | 60 | 58 | 52[5] | 30[5] |
| Threonine | 65 | 65 | 29[6] | 25[6] |
| Tryptophan | 18 | 18 | 18[7] | 22[7] |
| Arginine | 42 | 39 | -- | 143[8] |
| Histidine | 32 | 32 | -- | 64* |

[1] Value obtained from Chung and Baker, 1992
[2] Values obtained and calculated from NRC 1998
[3] Values calculated from Elango et al, 2002.
[4] Values calculated from House et al, 1997a
[5] Values calculated from Shoveller et al, 2003a
[6] Values calculated from Bertolo et al, 1998
[7] Values calculated from Cvitkovic et al, 2004
[8] Value calculated from Brunton et al., 2003
* Histidine value from Primene®. Value not actually calculated for the B/P profile

One of the possible problems in the design of these experiments was the selection of the dispensable and conditionally indispensable amino acid profile to complete the B/P profile. The pattern selected for experiment 3.1 was based on the Primene® (Baxter Corporation, Mississauga, Ontario, Canada) amino acid profile, whereas the parenteral amino acid requirements were determined using the Vaminolact® (Fresenius Kabi AG, Bad Homburg, Germany) amino acid pattern. The Primene® pattern is based on cord blood, it is currently the only TPN solution based on an intravenous source of nutrition and is in use throughout many pediatric hospitals in North America. The initial goal of the first experiment (Chapter 3.1) was to test the B/P amino acid pattern against the current industry standard. The Primene® pattern met all the criteria and was selected as the "industry standard". However the Primene® pattern may contain inadequate amounts of proline, phenylalanine and tyrosine (Brunton et al., 2000); whereas the Vaminolact® pattern, based on milk protein, contains low amounts of arginine, lysine and aromatic amino acids as well (Ball et al., 1996). One of the initial discrepancies between profiles is that the Vaminolact® profile is based on an enterally fed reference; whereas Primene® is based on an intravenous source. Many studies have shown that when splanchnic metabolism is bypassed, as it is when being intravenously fed, the amino acid requirement can be substantially lower (Bertolo et al., 1998, Elango et al., 2002, Shoveller et al., 2003, Cvitkovic et al, 2004). These findings suggest that Vaminolact® may provide some of the indispensable amino acids in excess of their parenteral requirement. Although the indispensable amino acid concentrations were changed to reflect the previously determined amino acid requirements, the conditionally indispensable and dispensable amino acid remained the same as in Primene®. This

suggests that one or more the conditionally indispensable amino acids may be indispensable in the neonatal piglet. It has been shown previously that arginine and proline should both be considered indispensable in the parenterally fed neonatal piglet (Bertolo et al., 2003). To conclusively determine whether or not there is a significant difference between the conditionally indispensable and dispensable patterns of both diets we need to determine the rate of protein synthesis for both. A subsequent IAAO trial would consist of the B/P indispensable amino acid pattern being applied to both the Vaminolact® and Primene® dispensable pattern. The oxidation results would show which profile optimized protein synthesis while the plasma amino acid pattern may possibly provide some insight into which of the conditionally indispensable amino acid may be indispensable in the neonatal piglet based on the differences between the two B/P diet backbones.

The individual amino acid requirements were determined on three different breeds of pig, Yorkshire, Landrace/Large White cross and Duroc/Large White/Landrace cross and in two separate barn environments (House et al, 1997a (Yorkshire), Bertolo et al, 1998 (Yorkshire), Elango et al., 2002 (Yorkshire), Brunton et al., 2003 (Landrace/Large White), Shoveller et al, 2003a (Landrace/Large White), Cvitkovic et al, 2004(Yorkshire)). These requirements were combined to create the B/P TPN amino acid pattern and tested in a Duroc (Duroc/Large White/Landrace) cross-breed. Within 72 hours of receiving this profile the Duroc piglets experienced signs of distress and were immediately removed from the trial (Chapter 3.1). It was later thought that the B/P pattern may contain a limiting amino acid. Protein deposition can be significantly influenced by breed, gender, age and environment (Schinckel and de Lange, 1996);

suggesting that the protein requirements may be breed, gender and age specific. Zhu et al., (2005) examined a gene on chromosome nine that is thought to be responsible for loin muscle growth. Loin muscle area is a direct result of three genotypes, GG, TT or TG; where GG was found to have a significantly greater loin muscle area. It was also stated that Durocs had a higher frequency of the GG genotype than any of the other breed including Large White (Zhu et al., 2005). It has also been demonstrated that greater rates of protein deposition, synthesis, and degradation can be attributed to differences in body protein mass (Rivera-Ferre et al., 2006). This finding suggests that the Duroc breed may have a larger muscle area than other breeds thus resulting in a higher protein requirement. The branched chain amino acids are primarily metabolised in the extra-hepatic tissue (Harper et al., 1984). As a result of the larger muscle mass in Duroc pigs, a substantial increase in the branched chain amino acid requirements may occur. Because some of the requirements were determined with a Large White cross these estimates may not be sufficient in meeting the protein requirement of the Duroc breed. The influence of the rate of protein deposition on amino acid requirement needs to be tested and compared across modern swine breeds. The individual parenteral amino acid requirements also need to be re-evaluated in the Duroc cross piglets to determine if there is a significant difference amongst modern breeds of pigs.

The indicator amino acid technique was used to determine the requirements for all the indispensable amino acids that compromised the B/P amino acid pattern. The IAAO technique is based upon the appearance in breath of $^{14}C$ from phenylalanine. This measurement is believed to be inversely related to the rate of protein synthesis (Ball and Bayley, 1986). However, the recent paper by Rafii et al., (2008) shows that a direct

measurement of protein synthesis could be accomplished by measuring the incorporation of labelled carbon in Apolipoprotein B-100 (ApoB 100). ApoB-100 is a hepatic export protein synthesized from intrahepatocyte amino acids and has been shown to reflect the amino acid concentrations at the site of phenylalanine hydroxylation, the hepatic intracellular pool (Reeds et al., 1992). ApoB-100 has been shown to provide a more accurate assessment of hepatic intracellular amino acid enrichment and can be used to study amino acid metabolism in vivo (Rafii et al., 2008). Therefore, this may be a more correct way of measuring amino acid requirements than labelled $CO_2$ in breath. Perhaps this method could be used to re-evaluate the requirements of the amino acids, within the B/P profile, which are thought to be incorrect.

TPN feeding commonly results in liver dysfunction and gut atrophy (Wang et al., 2007). The primary site of oxidation for many amino acids is the hepatocytes in the liver (Stoll et al., 1997) therefore the level of oxidation of amino acids in the TPN fed piglet may be representative of the severity of liver disease. The primary assumption of the IAAO technique is that amino acids are not stored and that they are oxidized when there are in excess of what is required for protein synthesis (Brunton et al., 2007). By this logic, if one amino acid is limiting in the diet then all other amino acids are in excess, and therefore oxidized (Zello et al., 1995). Increasing the dietary intake of the limiting amino acid then in turn improves the uptake of all the other amino acids; thus increasing protein synthesis and inversely decreasing oxidation. Therefore if phenylalanine oxidation was affected not only by rates of protein synthesis, but also the extent of liver damage, then the IAAO technique may not be an accurate assessment of parenteral amino acid requirements. TPN liver tissue data was not collected in any experiment in which amino

acid requirement was measured by IAAO, therefore the effect liver dysfunction may have on oxidation is still unknown. However, the IAAO technique is a relative measure of liver function or dysfunction since the requirement studies were tested in piglets experiencing some form of liver failure. Liver function or lack there of is not an adequate explanation for the underestimation of an amino acid in the B/P profile.

Finally, the remaining portion of the diet must be considered. The vitamin and mineral requirements of piglets receiving TPN has never be determined. However, vitamin and mineral intakes were 115% and 200% respectively, of those recommended by NRC for 5 kg piglets (NRC 1998). All vitamins were supplied in a commercial solution, MVI Paediatric (Rhone-Poulenc Rorer Canada Inc, Montreal, PQ), which provided a combination of oil and water-soluble vitamins, uniquely formulated for incorporation into parenteral solutions. Piglets also received a mineral solution including zinc, copper, manganese, chromium, selenium and iodide. Because we provided equal intakes of these vitamins, lipids and minerals to all treatments, we assume that these did not affect any of our results.

In summary, this series of studies demonstrated that one or more of the neonatal piglet parenteral amino acid requirements, determined using the IAAO technique, is probably incorrect and should be re-evaluated. The results of these experiments also demonstrate the complexity and difficulty of determining parenteral amino acid requirements and indicate that amino acid interactions, piglet breed and age and conditionally indispensable amino acids may all have significant effects on protein metabolism. Additionally, when an animal is fed parenterally, differing levels of intestinal injury and organ dysfunction ensue, and the mechanisms involved in an

animal's metabolic and physiological response to this are still not fully understood (Wang et al., 2006). There is still much research that is required prior to composing and evaluating another TPN solution for neonatal piglets; consequently the optimal parenteral amino acid profile remains elusive.

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