TYROSINE REQUIREMENTS AND RESTING ENERGY EXPENDITURE IN PATIENTS WITH PHENYLKETONURIA

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Nutritional Sciences
University of Toronto

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Doctor of Philosophy, 1997
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ABSTRACT

Tyrosine requirements and resting energy expenditure (REE) were determined in subjects with Phenylketonuria (PKU). In order to study amino acid kinetics in children, a noninvasive method was developed in 16 adults which involved oral infusion of stable isotope-labelled amino acids (phenylalanine and lysine) and measurement of isotope enrichment in urine (verified in plasma) and breath. This noninvasive method was incorporated into the Indicator Amino Acid Oxidation protocol in which the oxidation of an infused indicator amino acid (lysine) decreases as the dietary intake of a test amino acid (tyrosine) is increased by increments, until the dietary requirement is reached. In this study, the hypothesis that tyrosine requirements in PKU account for 45% of the aromatic amino acid requirement was tested. The mean daily tyrosine requirement determined by lysine oxidation and F$^{13}C_2O$ were 16.3 and 19.2 mg·kg$^{-1}$, respectively. These values represent ~40 to 45% of the aromatic amino acid intake and are ~5 times lower than current recommendations. This study is the first application of an amino acid oxidation technique in determining amino acid requirements in a pediatric population and in subjects with an inborn error of metabolism. With respect to REE, it was hypothesized that excessive weight gain in PKU may in part be attributable to decreased REE. Twenty-three subjects with PKU, 7 subjects with MSUD and 28 control subjects had REE and body composition assessed by indirect calorimetry and bioelectrical impedance analysis, respectively. After adjusting for lean tissue differences, REE was determined to be reduced in
the PKU group (279 kJ·d⁻¹). Reduced REE in PKU may contribute to their tendency to be overweight.
FOR NATHAN MITCHELL
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# Abbreviations

<table>
<thead>
<tr>
<th>Term</th>
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<tbody>
<tr>
<td>α-keto acid dehydrogenase</td>
<td>BCKD</td>
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<td>3-methoxy-4-hydroxyl phenylethleneglycol</td>
<td>MHPG</td>
</tr>
<tr>
<td>5-hydroxyindoleacetic acid</td>
<td>5-HIAA</td>
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<td>Analysis of variance</td>
<td>ANOVA</td>
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<tr>
<td>Area</td>
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<td>Bioelectrical impedance analysis</td>
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<td>Blood brain barrier</td>
<td>BBB</td>
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<td>Body cell mass</td>
<td>BCM</td>
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<td>Body Mass Index</td>
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<td>Branched chain amino acid</td>
<td>BCAA</td>
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<td>Branch chain ketoacid</td>
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<td>Brown adipose tissue</td>
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<td>Calorie</td>
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<td>Carbon dioxide</td>
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<td>Central nervous system</td>
<td>CNS</td>
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<td>Cerebrospinal fluid</td>
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<td>Diet induced thermogenesis</td>
<td>DIT</td>
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<td>Dihydropteridine reductase</td>
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<td>Dihydroxyphenylalanine</td>
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<td>Extracellular fluid</td>
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<td>Fat free mass</td>
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<td>Flux</td>
<td>Q</td>
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<td>Height</td>
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<td>Homovanillic acid</td>
<td>HVA</td>
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<td>Term</td>
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<tr>
<td>Indicator amino acid oxidation</td>
<td>IAAO</td>
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<tr>
<td>Indispensable amino acid</td>
<td>IAA</td>
</tr>
<tr>
<td>Intelligence quotient</td>
<td>IQ</td>
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<tr>
<td>Joule</td>
<td>J</td>
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<td>Large neutral amino acids</td>
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<td>Lean body mass</td>
<td>LBM</td>
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<td>Length</td>
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<td>Magnetic resonance imaging</td>
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<td>Maple syrup urine disease</td>
<td>MSUD</td>
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<td>National center for health statistics</td>
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<td>Nitrogen</td>
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<td>qBH₂</td>
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<td>Rate of oxygen production</td>
<td>VO₂</td>
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<td>Recommended dietary allowance</td>
<td>RDA</td>
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<td>Resistance</td>
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<td>Respiratory quotient</td>
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<td>Resting energy expenditure</td>
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xvii
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<td>Tetrahydropteridine</td>
<td>BH₄</td>
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<td>TEF</td>
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<tr>
<td>Thermic effect of activity</td>
<td>TEA</td>
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<tr>
<td>Total body water</td>
<td>TBW</td>
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<tr>
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<td>TBN</td>
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<tr>
<td>Total Energy Expenditure</td>
<td>TEE</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>T₃</td>
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<tr>
<td>Vanillylmandelic acid</td>
<td>VMA</td>
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<tr>
<td>Volume</td>
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1. INTRODUCTION

Phenylketonuria (PKU) is a disorder of aromatic amino acid metabolism in which phenylalanine cannot be converted to tyrosine due to a total or near total deficiency in the hepatic enzyme phenylalanine hydroxylase (Scriver et al. 1989; Nyhan 1984). As a result of this enzyme defect, tyrosine is an indispensable amino acid in PKU. The major associated clinical manifestation of hyperphenylalaninemia in untreated PKU is severe mental retardation (Thompson et al. 1990; Waisbren et al. 1987). Treatment, which consists of a phenylalanine restricted, tyrosine supplemented diet, aims to lower plasma phenylalanine concentrations and keep plasma concentrations of tyrosine and other amino acids within normal ranges, thereby allowing for normal growth and brain development (Elsas and Acosta 1994; Scriver et al. 1989; van Spronsen et al. 1996). However, in much the same way that exogenous insulin cannot duplicate the body’s ability to control blood glucose, dietary manipulations in PKU do not achieve the same degree of phenylalanine homeostasis as does the normal hydroxylation reaction.

Early treatment in PKU benefits cognitive development (Nyhan 1984), however there is ample evidence that the outcome is not quite normal (Scriver et al. 1989). The causes of these cognitive deficits are not fully understood, but quality of treatment or poor compliance to treatment, reflected in high plasma phenylalanine levels, is one determinant (Pennington et al. 1985). Elevations in plasma phenylalanine levels also disrupt the homeostatic mechanisms that ensure the stability of precursor amino acid concentrations in the brain and produce defective synthesis of serotonin, dopamine and norepinephrine (Butler et al. 1981; Lou 1985; Krause et al. 1985). Neurotransmitter deficiency has been proposed as a factor in the changes in neuropsychological function associated with hyperphenylalaninemia (Krause et al. 1985).

The efficacy of dietary treatment in PKU is also judged by the maintenance of normal physical growth and weight gain. Here again, the outcome is not quite normal. Children with
PKU are reported to have significantly lower height standard deviation scores (Allen et al. 1996; Verkerk et al. 1994; Dhondt et al. 1995), lower body nitrogen (Allen et al. 1996) and excess body weight (McBurnie et al. 1991; White et al. 1982; Holm et al. 1979). The mechanisms by which these alterations occur have not been fully described, however dietary imbalances and reduced energy expenditure are two factors implicated in the process.

At present, the dietary management of patients with PKU is empirical, primarily because there have been no comprehensive studies of tyrosine or phenylalanine requirements and only one study on energy metabolism in PKU. The less than normal cognitive and physical development of some individuals with PKU clearly indicates that the current treatment regimen requires modification. Therefore, the objective of this thesis was to begin to reevaluate the nutritional requirements in PKU by: (i) measuring the tyrosine requirements by Indicator Amino Acid Oxidation in children with classical PKU and (ii) measuring the resting energy expenditure of individuals with PKU and determining the relationship between excess body weight, alterations in serotonin and catecholamine synthesis and energy expenditure, using healthy controls and patients with Maple Syrup Urine Disease as comparison groups.

The literature review which follows provides an overview of the three areas of study covered by this thesis. The first part reviews the basic concepts of energy balance and the determinants of energy expenditure. This is followed by an overview of protein and amino acid metabolism and methods to determine indispensable amino acid requirements. The final section reviews the genotype and phenotype, body composition and dietary treatment of PKU. A rationale for and description of the research plan of the present thesis is then presented.
2. LITERATURE REVIEW

2.1. ENERGY BALANCE AND THE DETERMINANTS OF ENERGY EXPENDITURE

Obesity is excessive fat storage and fat storage results from energy imbalance. Therefore, the study of obesity or excess body weight is a study of energy balance and the major terms of energy balance deals with intake and expenditure (Webb 1992).

Some of the fundamental questions posed in the study of excess body weight in children with PKU, concern whether there are differences between people who gain weight easily and those who do not. Do they differ in the components of daily energy expenditure - resting energy expenditure, thermic effect of eating and activities of daily living? Are those who gain weight more easily more efficient metabolically (Webb 1992)?

Although all the components of energy expenditure are of interest in the study of obesity, the resting component is the primary concern in PKU, since a low REE, adjusted for fat free mass, is a risk factor for body weight gain (Ravussin et al. 1988; Saltzman and Roberts 1995). The text which follows reviews the basic concepts of energy balance, (with emphasis placed on the resting component and those factors which affect resting energy expenditure), body composition and finally the techniques for measuring energy expenditure and body composition.

2.1.1. Definitions and Basic Concepts

2.1.1.1. Calorie (c) the amount of energy required to raise the temperature of
1 g of water from 3.5°C to 4.5°C

2.1.1.2. Joule (J) the unit of work and energy equal to the work done by a
force of one newton acting through a distance of one
metre

= 0.239 calories
2.1.1.3. **Thermodynamics**

The First Law of Thermodynamics is that energy can neither be created nor destroyed (Brown and Brengelmann 1965; Park et al. 1992). The human body loses energy continuously to the environment in the form of heat; to sustain life, this energy must be restored. The source of this energy is the chemical free energy in the highly structured carbon bonds in food, namely carbohydrate, fat and protein, which is extracted through oxidation. The energy actually available from food is the gross energy from complete oxidation less that which is not absorbed and that which is not oxidized (Webb 1992). In summary, the human body cannot destroy or produce energy; it takes in energy from food, converts it into usable forms to perform life functions, and then passes it on to the environment (Brown and Brengelmann 1965).

The Second Law of Thermodynamics is that the amount of entropy in a system and its surroundings always increases in a spontaneous reaction, where entropy is the energy of the system not available for work (Brown and Brengelmann 1965; Park et al. 1992). Enthalpy is the total heat content of a system and is made up of entropy and the free energy of a system, which is energy capable of doing work (Brown and Brengelmann 1965; Park et al. 1992). When a nutrient is oxidized, part of its energy is thermodynamically obligated for conversion to heat since the heat energy content of the metabolic end products is greater than the chemical free energy content of the initial nutrient. Man increases the entropy of his surroundings by discharging end products (increased energy) of metabolism of nutrient molecules (low entropy) ingested. The amount of heat lost is not large, with approximately 95% of ingested energy available as free energy. However, the conversion of food energy into usable high energy compounds (such as adenosine triphosphate, ATP) is not very efficient, with more than half of potentially available free energy lost to the environment as heat. This heat loss is essential since it is the basis for unidirectionality of metabolic pathways and their regulators. When hydrolysis of ATP is coupled with other reactions, energy retained in ATP can be transferred to
other chemical compounds, or transformed into mechanical or electrical energy. ATP is the source of energy for: (a) muscle contraction; (b) maintenance of ion gradients; (c) electrical conductance; and (d) biosynthesis of new molecules (Bursztein et al. 1989).

2.1.1.4. **Energy Balance**

The concept of energy balance is based on the first law of thermodynamics: energy is conserved (Webb 1992).

\[
\text{Energy Balance} = \text{Energy Intake} - \text{Energy Output}
\]

where energy intake consists of the metabolizable energy of the foods consumed and energy output represents the total energy expended (Schutz and Jequier 1994).

If energy intake is greater than energy output, a person is in positive balance and is storing energy as fat, carbohydrate and protein. If energy output is greater than energy intake, a person is in negative balance and must be oxidizing endogenous stores of energy (Bursztein et al. 1989).

When humans ingest carbohydrates, proteins, or fats, they either oxidize them immediately and use the energy released to do work, or transform them into forms that can be stored as potential energy (Bursztein et al. 1989). The body stores energy as fat, glycogen and protein. Differences in the cost of deposition of adipose tissue from dietary fat and carbohydrate mean that an increase in the quantity of fat in the diet is more critical than a comparable increase in carbohydrate in determining whether stores of triglycerides in adipose tissue will be increased (Sims and Danforth, Jr. 1987). When exogenous energy is not available, as occurs during an overnight fast, the body derives energy from its endogenous fuels (Bursztein et al. 1989). Most of the energy stored in the body is as fat. A normal 70 kg man has about 13 kg of fat, which can yield approximately 125,000 kcal (525,000 kJ) on oxidation. These stores can theoretically allow an individual to survive for 50 days, given a daily
energy requirement of 2500 kcal. Body protein is also lost during a total fast (Brown and Brengelmann 1965; Danforth, Jr. 1985). A normal man has about 11 kg of protein of which 7 kg are intracellular and 4 kg are extracellular. The extracellular or structural proteins in bone and connective tissue are very stable, and are considered to be unavailable for metabolic needs. The intracellular proteins, which make up the body's active cell mass, serve many functions but are chiefly enzymes and contractile proteins; there are no storage proteins. The estimated 11 kg of protein can potentially yield approximately 45000 kcal (188,000 kJ), or a maximum of 18 days' energy at 2500 kcal/day. However, due to the essential function of protein, loss of protein leads to adverse functional consequences which are conventionally considered to be the cause of death in long term starvation (Wu and Marliss 1992; Hoffer 1994). In addition to fat and protein, the body contains between 1000-3000 kcal of carbohydrates stored in the liver and muscles as glycogen. This is available for immediate energy needs, such as exercise, but is of little value during long term fasting by virtue of its limited supply.

The energy requirement of an individual is defined by the World Health Organization (WHO) as "the level of energy intake that will balance energy expenditure when the individual has a body size and composition, and a level of physical activity consistent with long-term good health. In children and pregnant or lactating women, the energy requirement includes the energy needs associated with the deposition of tissues or the secretion of milk at rates consistent with good health" (Food and Agriculture Organization of the United Nations-Rome 1985).

The variability of measured energy intake between individuals is greater than that of energy expenditure and additionally, it is difficult to measure energy intake without influencing intake behaviours (Schutz and Jequier 1994). This suggests that energy requirements can be
estimated more accurately from energy expenditure than from energy intake measurements (Schutz and Jequier 1994).

"Energy expenditure" refers ultimately to the hydrolysis of ATP, or of other high-energy bonds. Each substrate/fuel has a different heat of combustion and generates a different amount of heat, per litre of oxygen used to burn it. For example, the oxidation of 1 mol (180 g) glucose involves the uptake of 6 mol of oxygen ($O_2$), and the production of 6 mol of carbon dioxide ($CO_2$), 6 mol water and the liberation of 673 kcal (Ferrannini 1988). Each fuel also has a characteristic respiratory quotient (RQ), the volume of $CO_2$ divided by the volume of $O_2$ consumed for oxidation. The RQ for carbohydrate is 1.0, for fat is 0.7 and for protein is 0.8. Despite the large differences in the heat of combustion and RQ for the different fuels, they all produce similar amounts of energy per litre of $O_2$ used, 5.05 kcal for carbohydrate, 4.46 for protein and 4.74 for fat (Park et al. 1992).

2.1.2. Determinants of Energy Expenditure

Total energy expenditure (TEE) can be divided conceptually into three major components. The largest component is the resting energy expenditure (REE) which accounts for approximately 65-75% of total daily energy expenditure. The second largest component is the thermic effect of activity (TEA) which includes work done on the environment. The TEA of an individual not engaged in heavy labour accounts for 15-20% of total daily energy expenditure, but it can increase two-fold with heavy exercise. The third component of energy expenditure is the thermic effect of food (TEF) or diet-induced thermogenesis (DIT). The TEF accounts for 10% of daily energy expenditure, but can vary depending on the amount of energy consumed and on the nutrient composition of the diet (Danforth, Jr. 1985; Schutz and Jequier 1994; Simonson and DeFronzo 1990; Owen et al. 1993). For the purposes of this thesis, only REE will be reviewed.
2.1.2.1 Resting Energy Expenditure

The REE is the amount of energy expended by a resting individual in a thermoneutral environment without the effects of meal consumption, physical activity, or other physiological or mental stress (Shetty et al. 1996; Owen et al. 1993; Danforth, Jr. 1985; Bursztein et al. 1989). The value may be slightly greater than the true basal metabolic rate which, in addition to the above conditions, is measured in the morning upon awakening after 12-18 hours of rest and includes the definition of conditions with respect to circadian rhythms (Danforth, Jr. 1985). The energy expended at rest includes the costs of maintaining the biochemical and structural integrity of the body, and the costs of performing internal work, ion pumps, synthesis and degradation of cell constituents, biochemical cycles, and leakage of protons across the mitochondrial membrane (Bursztein et al. 1989).

In a review of the literature pertaining to the issue of intra-individual variability in REE, Shetty et al. (1996) reported that variations in REE measured over periods of days, weeks, months or years are in the range of 1.8 to 3.5%. By contrast, in subjects matched for age, gender, height and weight, inter-individual variations are much larger, with between subject variability in the order of 7.5 to 17.9% (Shetty et al. 1996). Inter-individual variability in REE is an important issue within the context of assessing energy requirements. Since REE constitutes 65-75% of the total energy expenditure, it forms the basis of the factorial approach for the assessment of energy requirements in children and adults (Torun et al. 1996; Durnin 1996; Shetty et al. 1996). TEE is obtained by multiplying the estimate of REE by a factor that covers the energy cost of increased muscle tone, physical activity, the TEF and the energy requirements for growth and lactation (Food and Agriculture Organization of the United Nations-Rome 1985).

Since 1919, when Harris & Benedict (1919) published their predictive equations, numerous equations have been derived for predicting REE from height, weight, age and gender
(Shetty et al. 1996; Owen et al. 1993). Schofield (1985) published predictive equations which represented the largest and most comprehensive analysis of REE to date. These equations were used for the 1985 FAO/WHO/UNU report and have become the present basis for estimating energy requirements. Recently, the reliability of the Schofield equations has been questioned. Thompson et al. (1995), as well as consensus reports of researchers who participated in a forum on energy and protein requirements, concluded that the Schofield equations may be overestimating the REE in many pediatric and adult populations (Shetty et al. 1996; Torun et al. 1996). It was noted, however, that there were differences between developed and developing countries with respect to the reliability of the equations. In the 1 to 18 year old population, the equations tended to coincide with measured REE in populations from developed countries. In contrast, several investigators have concluded that the Schofield equations reliably predict REE in healthy and in pediatric patient populations (Finan et al. 1997; Firouzbakhsh et al. 1993; Bandini et al. 1995; Kaplan et al. 1995). Clearly, when accurate estimates of energy requirements are needed, especially in disease states, REE should be measured. In lieu of that, the Schofield equations remain the best available predictive equations, based on the sample size and extensiveness of the analysis on which they were based.

The REE is influenced by a number of basic factors, including fat free mass, temperature and hormones/neurotransmitters (Simonson and DeFronzo 1990; Brown and Brengelmann 1965; Owen et al. 1993; Bursztein et al. 1989; Woo et al. 1985). In fact, most of the effect age and gender have on the REE relates to differences in fat free mass.

2.1.2.1.1. Fat-Free Mass (FFM)

Since 1915, when Benedict suggested that the "active body mass" determines the REE, it has been recognized that the amount of fat free mass in the body (body mass minus total fat
mass) is highly predictive of the REE (Shetty 1990; Goran et al. 1994; Brown and Brengelmann 1965; Holliday 1971; Weinsier et al. 1992; Ravussin and Bogardus 1989; Food and Agriculture Organization of the United Nations-Rome 1985). Fat free mass is thought to most closely reflect the active body mass, but controversy over the proper reference for the active body mass continues up to the present day. Even though REE and FFM are strongly correlated, the correlation is not linear in normal weight individuals, probably because of the varying compositions of the FFM at different total fat-free masses (Weinsier et al. 1992).

According to Holliday (1971), the average REE of skeletal muscles is 73.7 kJ.kg\(^{-1}\).d\(^{-1}\) (17.6 kcal) and that of visceral organs (brain, heart, kidney and liver) is 1497.2 kJ.kg\(^{-1}\).d\(^{-1}\) (357.7 kcal).

During growth the FFM increases, with muscle mass increasing more rapidly than organ mass (Holliday 1971; Weinsier et al. 1992). On average, the FFM of infants and preschoolers comprises 30% muscle and 20% other organs with the remaining 50% being bone mass, extracellular fluid etc. (Weinsier et al. 1992). Muscle contributes only 7% to REE (Holliday 1971). By adulthood, the less metabolically active muscle mass represents about 85% of the combined weight of muscle and organ tissue but accounts for only 25% of the REE (Holliday 1971). Across the full range of FFM, from infants (0.2 years old) to adults (40 years old), the relationship of REE plotted against FFM is nonlinear (Garby and Lammert 1992; Holliday 1971). REE per unit FFM falls disproportionately to the rise in FFM because of the change in composition of the metabolically active portions of the FFM (Weinsier et al. 1992). Furthermore, with aging in adulthood, the composition of the FFM changes (Forbes 1994), which further explains why the relationship between REE and FFM is not linear. Ravussin and Bogardus (1989) demonstrated that differences in FFM account for 82% of the variance in REE. However, because the relationship between REE and FFM does not regress through the zero intercept, use of the REE-FFM ratio will result in significant error when values of FFM are different from the mean. Thus, for comparisons of REE between groups with different FFMs, it...
is recommended to compare slopes and intercepts of the regression lines between metabolic rate and FFM (Ravussin and Bogardus 1989). Failure to account for differences in FFM between different groups when comparing REE can lead to incorrect conclusions. For example, without accounting for differences in FFM, one would conclude that women have a lower REE than men or that obese individuals have a higher REE than nonobese individuals.

In obesity, where FFM is increased, REE is proportional to FFM. However, the scatter about the regression line suggests that at any given body size, individuals can have high, average or low metabolic rate relative to the predicted REE defined by the regression line (Ravussin and Swinburn 1992). Several studies have reported that a low relative REE, after adjustment for differences in FFM, age and gender, predicts subsequent weight gain (Saltzman and Roberts 1995; Ravussin et al. 1988).

2.1.2.1.2. Temperature and REE

Humans, being homeothermic, regulate their core temperature independently of environmental temperature. Early in this century, DuBois (1954) concluded that metabolic rate increases by 13% per degree Celsius (7.2% per degree Fahrenheit) of body temperature when the increase in temperature is not due to an increased insulation. The effect of increasing body temperature on metabolic rate is related to acceleration of the rate of biochemical reactions (Bursztein et al. 1989). In the cold, energy expenditure increases to maintain a normal core temperature, termed cold-induced thermogenesis (Brown and Brenzelmann 1965). This occurs in two forms, shivering and nonshivering thermogenesis. Nonshivering thermogenesis involves increased rates of metabolic reactions involving hydrolysis of ATP (Bursztein et al. 1989). In the brown adipose tissue (BAT) of rodents, norepinephrine regulates the mechanisms by which oxidation and phosphorylation are uncoupled (Simonson and DeFronzo 1990). While BAT does buffer the entry of the newborn into a cold world, its role in the adult is less clear and the
degree to which humans retain the ability to adapt to cold by this mechanism remains controversial (Sims and Danforth, Jr. 1987; Brown and Brengelmann 1965; Simonson and DeFronzo 1990). However, the presence of histologically detectable BAT in infants and adults is well established (Lean 1992). Furthermore, the mitochondrial uncoupling protein, which is needed for nonshivering thermogenesis can be demonstrated in adults. The content of this protein is generally low, but high levels of circulating norepinephrine from pheochromocytoma, stimulate thermogenesis in adipose tissue at sites of BAT (Lean 1992). When ambient temperature rises to 27°C, energy expenditure is at a minimum in normal subjects. Above this, energy is expended to bring about cooling by sweating or panting, and total energy expenditure increases (Bursztein et al. 1989; Brown and Brengelmann 1965). Other deviations from thermoneutrality alter the REE. For example, the slight increase in body temperature during the post-ovulatory phase (days 17-26) of the menstrual cycle has been reported to result in about a 5% increase in REE (Webb 1992).

2.1.2.1.3. Hormones, Neurotransmitters and REE

There is evidence that norepinephrine (NE) released from sympathetic neurons and epinephrine and NE released from the adrenal medulla can influence metabolic processes (Leiter et al. 1984). In humans about 15% of the total energy expenditure has been estimated to be sympathetically mediated (Landsberg and Young 1983). Catecholamines increase energy expenditure in two ways: (a) by increasing the rate of cellular metabolism (Himms-Hagen 1976) and (b) by stimulating the conversion of stored fuels into usable energy (Young and Landsberg 1977). The increase in cellular metabolism is shown through an elevation in heat production, fuel utilization and O₂ consumption, while the stimulatory effects on the breakdown of complex fuels supplies the energy required for the increased cellular metabolism (Shetty 1990). When the sympathetic nervous system (SNS) is stimulated, all types of adrenoreceptors are activated,
but thermogenesis is primarily mediated through β-adrenoreceptor stimulation (DeFronzo et al. 1984). Infusion of epinephrine, norepinephrine and dopamine results in increases in REE, the effects being greatest for epinephrine (Jequier et al. 1992). Catecholamines are also important components of the metabolic adaptations associated with energy restriction and overfeeding (Shetty 1990).

When considering catecholamines and the regulation of energy metabolism, the SNS must be distinguished from the adrenal medulla. Increased levels of epinephrine in urine or plasma is good evidence of adrenal medullary stimulation, however, when the adrenal medulla is stimulated, changes in plasma or urinary norepinephrine cannot be assumed to originate from sympathetic nerves, since the adrenal medulla may contribute significantly to the circulating pool of norepinephrine (Landsberg and Young 1983). SNS activity can be accurately assessed by measurement of NE turnover in specific isolated sympathetically innervated tissue and is a technique not biased by simultaneous changes in adrenal medullary activity (Landsberg and Young 1983; Young and Landsberg 1981). This technique is commonly used in animal studies, while studies involving humans assess SNS activity by plasma and urine levels and to a lesser extent by tracer techniques. Measuring SNS activity through plasma or urine concentrations remains controversial. The majority of circulating NE arises from stimulation of the SNS. The fraction of neurotransmitter measurable in the blood (the "spillover concentration") however, is quite small and depends on many complex processes including synthesis, release, re-uptake, metabolism and clearance (Fernandez et al. 1988). An alternative to measuring NE in plasma is to determine its rate of excretion in urine. Animal studies (Kopp et al. 1983) suggest that there is a reasonably good relationship between arterial NE concentration and the rate of urinary NE excretion, however, it is not clear to what extent this applies in man. Furthermore, it is not clear how changes in catecholamine metabolism may change the proportions of free NE, conjugated NE and the methylated or deaminated
metabolites excreted, thus weakening the relationship between SNS activity and urinary NE excretion (MacDonald 1992). There are limited data reporting on the validity of using urinary catecholamines as a measure of SNS activity. One study reported intraindividual correlations between plasma and urine measurements of 0.7 (p<0.001) for epinephrine and NE in experimentally stress-stimulated catecholamine levels in young men (Akerstedt et al. 1983). Many researchers, however, rely on urinary catecholamines to demonstrate the effects nutritional factors have on SNS activity (McCargar et al. 1988; Kopp et al. 1983; Johnston et al. 1983; Schoni et al. 1985; Hoeldtke and Wurtman 1973).

Thyroid hormones play an important role in the regulation of REE. Triiodothyronine (T$_3$) administered to obese patients increases $O_2$ consumption (Hollingsworth et al. 1970) even when combined with an energy restricted diet (Shetty 1990). The thermogenic effects of thyroid hormones are mediated primarily through nuclear T$_3$ receptors in most tissues and is the result of increased membrane bound Na$^+$.K$^+$ ATPase activity (Guemsey and Edelman 1983). Thyroid hormones are also important components of the metabolic adaptations associated with energy restriction and overfeeding (Shetty 1990). Thyroid hormones and catecholamines interact at the periphery (Shetty 1990). For example, epinephrine is known to enhance the peripheral conversion of T$_4$ to T$_3$; thyroid hormone deficiency is associated with an enhanced SNS activity (NE turnover and plasma appearance rates increase); and T$_3$ increases the number of tissue NE receptors (Shetty 1990). These interactions may influence their mutual roles in regulating thermogenesis.

Insulin is another pre-eminent hormone that regulates energy balance. In the fed state, insulin regulates the disposition of absorbed nutrients and in the postabsorptive state, with decreases in blood glucose levels, reductions in circulating insulin levels allow for the mobilization of endogenous fuels (Hoffer 1994). The thermogenic roles of insulin are demonstrated through its stimulatory effect on Na$^+$.K$^+$ pumping across the cell membrane.
Serotonin, like the catecholamines and histamine, is a monoamine neurotransmitter. It is produced in axon terminals from the indispensable amino acid, tryptophan (Figure 2.1.). Changes in blood concentrations of tryptophan, resulting from changes in macronutrient intake, or changes in the rate of tryptophan transport from the blood into the extracellular space of the nervous system and into the synaptic terminals, can affect the rate at which serotonin is produced (Anderson 1994). Serotonin pathways exist in both the central nervous system (CNS) and the peripheral nervous system. "In the periphery, serotonin acts in collaboration with other neuromodulators in a complex network that links sensory receptors generating moment to moment information on the state of the gastrointestinal tract with effectors in musculature, secretory, and absorptive epithelium, blood vasculature; and entero-endocrine cells" (Blundell 1992). Serotonin releasing neurons occur in virtually all regions of the brain. When considering the diffuse serotonergic innervation of the brain, it is easy to understand that the serotonergic system is involved in many cerebral functions, such as control of emotional behaviour, sleep and wakefulness, endocrine function, appetite, body temperature, blood pressure and pain perception (Gothert 1992).

Among the varied cerebral functions involving the serotonergic systems, thermoregulation is a function that may be implicated in the control of energy expenditure. Direct injection of serotonin into the anterior hypothalamus causes a dose-dependent rise in body temperature (Myers and Waller 1978). Fenfluramine, a serotonin agonist used clinically to treat obesity has also been shown to produce temporary hyperthermia after a single administration in rats and chickens (Tagliaferro et al. 1982). Fluoxetine, a serotonin reuptake
Figure 2.1. Serotonin Synthesis
inhibitor, has been shown to produce chronic basal temperature elevations in obese women (Bross and Hoffer 1995).

The involvement of serotoninergic neurons in the expression of appetite has been recognized for more than 20 years (Blundell 1992). Diet-induced changes in the concentration of nutrient precursors within the brain can alter the rate of neurotransmitter synthesis. Serotonin is one of the best known neurotransmitters under precursor control. Increased concentration of serotonin metabolites are seen in cerebrospinal fluid and/or plasma after tryptophan ingestion (Wurtman and Fernstrom 1975; Fernstrom and Wurtman 1972; Anderson 1994). Research in animals has shown that the relative proportion of protein and carbohydrate in a meal can influence brain amino acid levels and therefore serotonin synthesis. Ingestion of a meal high in carbohydrate preferentially enhances brain tryptophan uptake and serotonin synthesis. In contrast, ingestion of a meal high in protein, results in a decrease in tryptophan uptake into the brain (Lehnert and Wurtman 1993; Anderson 1994; Fernstrom et al. 1979).

Serotonin does not cross the blood brain barrier, but serotonergic effects can be modulated by drugs that alter its pharmacology in the brain (Wurtman and Wurtman 1977). Available experimental evidence clearly demonstrates that serotonin releasing agents (fenfluramine), serotonin reuptake inhibitors (fluoxetine) and autoreceptor stimulatory agents, that enhance synthesis or release of serotonin, possess appetite suppressive effects (Cawthorne 1992; Munro et al. 1992; Lehnert and Wurtman 1993; Wurtman and Wurtman 1977).

Another interesting characteristic of serotonin and serotonergic drugs are their thermogenic properties. Numerous studies of dl-fenfluramine and dexfenfluramine indicate that these drugs have a thermogenic effect in animals and humans (Cawthorne 1992). Resting 

\[O_2 \text{ consumption was increased by 21\% and 40\% in rats singly injected with } 5\text{-hydroxytryptamine and } \text{dl-fenfluramine, respectively (Rothwell and Stock 1987).}\] In humans, both REE and the
TEF were increased after fenfluramine administration (Troiano et al. 1990; Scalfi et al. 1993). Recently, Bross and Hoffer (1995) reported that within three days of fluoxetine administration, REE increased 4.4% in obese women consuming a 1.76-MJ/day diet. The authors proposed that the increase in energy expenditure was secondary to increases in basal body temperature.

2.1.3. Techniques for Measuring Body Composition and Energy Expenditure

2.1.3.1. Body Composition

Body weight and height and values derived from these measurements (ex. body mass index, BMI = weight in kg ÷ height squared in metres) are used to assess growth and nutritional status (Forbes 1994). Body weight is a crude measure of the body's mass, but its relationship to the size of the body's energy stores is not simple, nor are changes in energy stores necessarily or fully reflected in weight changes. Because of the limitations associated with body weight, more complex methods are used to describe body composition, and changes in the body's energy stores as a result of growth and energy imbalances (Forbes 1994; Heymsfield and Lichtman 1992).

The methods currently in use to describe body composition are based on either a two or four compartment model for body composition. The two-compartment model assumes that the total body mass is composed of two chemically distinct compartments, fat and fat-free mass, whereas the four-compartment model divides the body into four chemical groups, water, protein, ash or bone mineral, and fat (Lukaski 1987). Total body water (TBW), protein and minerals (calcium, phosphorus, potassium, sodium and chloride) combined account for 98% of the nonlipid body mass (Heymsfield and Lichtman 1992).

Considering the living body as comprising fat mass and fat-free body mass, two terms, the lean body mass (LBM) and the fat-free mass (FFM), are often used synonymously, but are conceptually different (Roubenoff and Kehayias 1991). The LBM was initially defined as the
weight of the body totally devoid of fat except for a small amount of essential lipid. FFM is the mass of the body minus the total fat mass.

The FFM is a hetrogenous compartment containing both metabolically active and inactive components. An important aim of body composition research is to determine the size of the metabolically active tissues of the body. The metabolically active component of the FFM is the oxygen-exchanging, potassium-rich, glucose- and/or free fatty acid-oxidizing, work-performing tissue known as the body cell mass (BCM). This includes all the cellular components of the body including muscle, viscera, central nervous system and hematopoietic system, as well as cells in cartilage, tendon, bone and adipose tissue (Roubenoff and Kehayias 1991; Moore 1980). The BCM is approximately 75% water and comprises 50% of the total FFM of a normal adult (Foster et al. 1990; Forbes 1987; Barrows and Snook 1987). The metabolically inactive component of the FFM includes the extracellular fluid (ECF), extracellular solids (bone and connective tissue), and glycogen (and the water associated with it) (Foster et al. 1990; Heymsfield and Lichtman 1992; Barrows and Snook 1987). The ECF is distributed both within (1/4) and without (3/4) the circulation, and in total makes up about 20% of adult body weight. The solid structural component of bone and connective tissue makes up about 15% of body weight.

The other compartment of the human body is the adipose tissue. It is 80% lipid, 15% water, (3% intracellular fluid and 12% ECF) and about 5% protein (Heymsfield and Lichtman 1992). Since FFM is defined by subtracting body fat from total mass, the water of adipose tissue is included in the FFM.

Body composition also changes with growth and age. TBW decreases with age. The hydration of FFM falls during early life from 81% at birth to 73% in the adult (Forbes 1994). FFM increases progressively from birth to adulthood, followed thereafter by a slow fall in FFM (Forbes 1994; Forbes 1987). With respect to minerals, FFM potassium (K) and nitrogen (N)
increase from birth to adulthood, as does FFM density. However, with aging total body K, N and calcium progressively decline (Forbes 1994). Finally, body fat reaches a peak of 25% of body weight at 6 months then falls to a nadir of 13% and 16% in boys and girls in late childhood. Body fat then increases with aging (Forbes 1987; Forbes 1994).

The two and four compartment models serve as the basis upon which all body composition methods have developed. Methods based on the two compartment model include total body water, total body potassium, densitometry, Bioelectrical Impedance Analysis (BIA) and Total Body Electrical Conductivity (Heymsfield and Lichtman 1992; Lukaski 1987). Neutron activation analysis for protein and minerals and isotope dilution for total body water are techniques which utilize the four compartment model (Heymsfield and Lichtman 1992; Forbes 1994; Lukaski 1987). This review will focus on anthropometry and BIA, two simple, indirect bedside methods used to assess body composition in children and adults.

2.1.3.1.1. Anthropometry

Anthropometry is the surface measurement of the human body, including height, weight, skeletal build, circumferences and skinfolds (Forbes 1994). Age- and sex-adjusted anthropometric measurements allow comparisons of nutritional status across age groups and populations (Dibley et al. 1987). Skinfold measurements are used to determine and evaluate fat stores and FFM (Forbes 1994).

Standard deviation (SD) scores are used to evaluate anthropometric indices of individuals with respect to the distribution of the population. The method measures the deviation of the anthropometric measurement (height or weight) from the reference median.
The SD score is calculated as follows:

\[ \text{SD Score} = \frac{\text{Individual's value} - \text{Median value of reference population}}{\text{Standard deviation value of reference population}} \]

(Frisancho 1993; Dibley et al. 1987).

The measurement of skinfold thickness includes two layers of skin and subcutaneous adipose tissue (Lukaski 1987). It is based on two assumptions: "the thickness of subcutaneous adipose tissue reflects a constant proportion of the total body fat and the sites selected for measurement represent the average thickness of the subcutaneous adipose tissue" (Lukaski 1987). Neither of these assumptions is fully proven (Lukaski 1987). Regression equations to predict body density using the logarithmic transformation of the sum of four skinfolds (tricep, bicep, subscapula and ileac crest), plus age and gender are needed because body density is not linearly related to subcutaneous fat mass and furthermore, the relationship changes with age and gender (Reilly et al. 1995; Durnin and Womersley 1974; Durnin and Rahaman 1967; Brook 1971; Weststrate and Deurenberg 1989). With densitometrically determined body composition as the reference method, the error in determining body fat content from skinfolds ranges from 3 - 9% body fat (Lukaski 1987).

2.1.3.1.2. Bioelectrical Impedance Analysis (BIA)

BIA is a noninvasive method to estimate FFM in children and adults (Azcue et al. 1993; Roche 1993; Lukaski et al. 1986; Chumlea and Guo 1994; Reilly et al. 1996). Electrodes are attached to the wrist and ankle; a constant, alternating current (800 μA, 50 kHz) is applied and produces an impedance to the spread of the current which is frequency dependent (Lukaski 1987; Forbes 1994). Body fluids and electrolytes are responsible for electrical conductance (1/resistance) and cell membranes are involved in capacitance (brief storage of voltage) (Roche 1993; Lukaski 1987; National Institutes of Health 1994). Because FFM contains
almost all the water and conducting electrolytes in the body, conductivity is greater in the FFM than in the fat mass (Lukaski 1987).

The hypothesis that BIA measurements can be used to determine FFM is that the resistance (R) to the electrical current is directly proportional to the length (L) of the conductor and inversely proportional to its cross-sectional area (A), so

\[ R = \rho \frac{L}{A} \]

where \( \rho \) is volume resistivity.

Multiplying both sides of the equation by \( L/L \) gives

\[ R = \rho L^2/AL \]

where \( AL \) is volume (V) (National Institutes of Health 1994; Forbes 1994; Lukaski 1987).

The major assumptions on which this equation is based include uniform cross-sectional area and homogenous conductivity, both of which are not fulfilled in humans (National Institutes of Health 1994). As a result, the equation has been altered, height (Ht) replaces length, and the total conductive volume of the body, V, is related to \( Ht^2/R \) (Chumlea and Guo 1994). The parameter \( Ht^2/R \) is then used as one of the independent variables (others include weight, gender, age) in a statistical regression procedure that defines a relationship with the dependent variable, TBW (Lukaski 1987; National Institutes of Health 1994; Forbes 1994).

Validation studies have been conducted in children and adults in which TBW by isotope dilution (Suprasongsin et al. 1995; Lukaski et al. 1986) and densitometry (Reilly et al. 1996; Houtkooper et al. 1992) have been used as the standard method. There are many published equations for estimating body composition by BIA, most are population specific (National Institutes of Health 1994). Cross-validity (general applicability) of published equations has been studied (Reilly et al. 1996) and is recommended before equations are used. Validation and cross-validation studies predicting FFM derived by body density or total body water equations from BIA measures have resulted in prediction errors for children of 11% (Roche 1993) and for adults of 5% (coefficient of variation) (National Institutes of Health 1994). These errors reflect
the sum of the error of measurement of the criterion method, the error in the BIA measurement, 
errors in measurement of height and weight, and errors attributable to the predictive equation 
(National Institutes of Health 1994).

2.1.3.2. Energy Expenditure

The energy expended by an individual can be assessed by two different methods: the 
calorimetric techniques which include direct and indirect calorimetry and noncalorimetric 
techniques which include isotope dilution method (ex. doubly labelled water), kinematics 
recordings (ex. mechanical activity metres), human observations and records (ex. time and 
motion studies) and physiologic measurements (ex. heart rate, energy intake,
electromyography) (Schutz and Jequier 1994). This discussion will focus on the calorimetric 
methods used to determine energy expenditure.

2.1.3.2.1. Direct Calorimetry

The direct method for measuring metabolic rate involves the measurement of heat 
losses. Direct calorimetry measures the sum of radiant heat exchange and of convective-, 
conductive- and evaporative heat transfer. The total heat loss is equal to the rate of energy 
utilization when body temperature is constant (Webb 1992; Simonson and DeFronzo 1990; 
Ferrannini 1988). Direct calorimetry has the advantage of being based solely on conservation 
of energy and not on any assumptions about the physiology of energy metabolism, but it 
requires expensive instrumentation. The subject is placed in a thermically isolated chamber 
and the heat he/she dissipates is collected and measured (Ferrannini 1988). Furthermore, 
direct calorimetry cannot be used to measure short-term effects of thermogenic stimuli, such as 
food, on heat exchange due to the large heat storage capacity of the body (Brown and 
Brengelmann 1965; Ferrannini 1988). As a result, direct calorimetry is infrequently used.
2.1.3.2.2. Indirect Calorimetry

Indirect calorimetry is the method by which metabolic rate is estimated from measurements of oxygen consumption and carbon dioxide production and through a series of assumptions and equations (McClave and Snider 1992; Simonson and DeFronzo 1990; Ferrannini 1988). The measurement is based on the assumption that all the O₂ consumed (VO₂) is used to oxidize degradable fuels and all the CO₂ produced (VCO₂) is recovered (Bursztein et al. 1989). Calculating energy production in this situation is equivalent to converting the chemical free energy of nutrients into the chemical energy of ATP plus loss of some energy (as heat) during the oxidation process, plus the ultimate conversion of the chemical energy to heat lost to the environment, (plus any external work performed on the environment). Eventually all energy is converted into heat so direct and indirect calorimetry should and do, provide identical rates of energy expenditure under steady state conditions (Simonson and DeFronzo 1990).

The abbreviated Weir equation is frequently used to determine energy expenditure from VO₂ and VCO₂ [energy expenditure = (3.94 x VO₂) + (1.11 x VCO₂)] (McClave and Snider 1992). The accuracy of respiratory gas exchange has been reported to be accurate to within 2% (Webb 1992). Indirect calorimetry is therefore ideal as a method for assessing acute effects of thermogenic stimuli on metabolic rate and for the clinical measurement of REE.
2.2. PROTEIN AND AMINO ACID HOMEOSTASIS

The current estimates of amino acid requirements for healthy humans are based almost entirely on nitrogen balance studies (Food and Agriculture Organization of the United Nations-Rome 1985). In PKU, plasma amino acid concentrations are used in addition to nitrogen balance estimates, as the basis for defining indispensable amino acid requirements (Elsas and Acosta 1994). Both methods have a number of significant shortcomings which limit their accuracy and which question their validity (Dewey et al. 1996; Zello et al. 1995; Young and Bier 1987; Young 1987; Young and Scrimshaw 1978).

Advances in the use of stable isotopes has increased the availability of methods for the study of amino acid and protein metabolism. Since the early 1980's, several isotope studies have been applied to evaluate healthy adult indispensable amino acid requirements either by direct or indicator amino acid oxidation (Zello et al. 1995). In disease states, such as in PKU, the ability to directly measure whole body amino acid kinetics represents a powerful tool for the study of indispensable amino acid requirements.

There are currently no amino acid requirement estimates, derived by oxidation techniques in infants or children, in part because of the invasive and impractical interventions employed in the oxidation studies. However, there is substantial evidence in the literature that these interventions can be replaced by less invasive protocols (Picou and Taylor-Roberts 1969; Waterlow et al. 1978; de Benoist et al. 1984; Wykes et al. 1992; Wykes et al. 1990; Basile-Filho et al. 1997). Thus, amino acid oxidation techniques can potentially be modified for use in vulnerable groups, such as PKU.

The section which follows reviews the basic concepts of amino acid metabolism (with emphasis placed on phenylalanine, tyrosine and lysine), amino acid transport and past and contemporary methods for assessing indispensable amino acid requirements.
2.2.1. **Basic Concepts**

2.2.1.1. **Dispensable and Indispensable Amino Acids**

Protein is required in the diet as a source of amino acids. Some amino acids are essential (indispensable) because their carbon skeletons cannot be synthesized in the body, whereas others are nonessential (dispensable) because sufficient quantities can be synthesized in the body from carbon and nitrogen precursors (Crim and Munro 1994). In humans, the indispensable amino acids (IAA) are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and arginine. Cysteine and tyrosine are synthesized from methionine and phenylalanine, respectively, and an additional eight amino acids (alanine, aspartic acid, asparagine, glutamic acid, glutamine, glycine, proline and serine) are considered dispensable (Food and Agriculture Organization of the United Nations-Rome 1985). These 20 L-α-amino acids are the units from which the body makes proteins.

Each amino acid contains both an amino (-NH₂) and a carboxylic acid (-COOH) functional group, as well as a unique radical (R) group. In an α-amino acid, both functional groups are attached to the same carbon atom. With the exception of glycine, all amino acids have a carbon atom bearing four different substituents (chiral carbon). The orientation of the four different groups about the chiral carbon confers optical activity (L-α-amino acids rotate the plane of polarized light to the left) (Crim and Munro 1994).

2.2.1.2. **Amino Acid Transport**

Most dietary proteins are enzymatically hydrolysed to oligopeptides and free amino acids. Amino acids are absorbed through the mucosal cells of the small intestine (Crim and Munro 1994). Movement of free amino acids into the mucosa and across cell membranes involves energy dependent carriers with some specificity for neutral, basic and acidic classes of
amino acids (Crim and Munro 1994; Souba and Pacitti 1992). Within each class, competition for the carrier exists between any two amino acids of that class (Crim and Munro 1994). The neutral class transport mediator has three transport systems: two sodium dependent transport mechanisms, one system, 'A', has a high affinity for alanine, and the other, designated 'ASC', has a high affinity for alanine, serine and cysteine; and one sodium independent transport system, designated 'L' for leucine, which has a high affinity for the branched chain and aromatic amino acids (Crim and Munro 1994; Souba and Pacitti 1992). The activity of a specific cell membrane transport system refers to its capacity to transport the substrate into or out of the cell and to its intrinsic ability to bind the amino acid. The former relates to the number of functional carriers in the cell membrane, whereas the latter reflects the affinity of the transporter ($K_m$) (Souba and Pacitti 1992). A low $K_m$ is suggestive of a high affinity of a transporter for a substrate, whereas a high $K_m$ suggests a low affinity. In most tissues, transport systems for amino acids have high $K_m$ values (1-10 mM), so entry rates of amino acids are considered unlikely to limit their metabolism (Harper 1994). Conversely, in the brain, $K_m$ values for uptake of amino acids across the blood-brain barrier are low (25-100 µM) and rates of entry and sizes of brain pools are strongly influenced by competition among amino acids transported by the same carrier (Harper 1994).

From the mucosal cells, free amino acids pass into the portal vein and go to the liver. The liver is the main site for catabolism for seven of the indispensable amino acids; the branched chain amino acids are degraded mainly in the muscle and kidney (Crim and Munro 1994). The liver functions to maintain amino acid concentration in the plasma and body tissues in dynamic equilibrium by degrading indispensable amino acids and synthesizing dispensable amino acids, and also by synthesizing amino acids into proteins for transport through the blood to cells and tissues (Crim and Munro 1994; Ball 1984).
A significant amount of amino acid metabolism occurs in splanchnic tissues (intestinal mucosa and liver in particular) (Krempf et al. 1990). Splanchnic uptake of amino acids refers to the difference in the amount of amino acid entering the splanchnic tissue from the intestine and portal vein and the amount which leaves the splanchnic tissue and enters circulation. Within the splanchnic tissues uptake of amino acids results in their oxidation or use for protein synthesis. First pass metabolism of amino acids in the splanchnic bed has been studied in humans by adding labelled amino acid tracers to a feeding solution during simultaneous intravenous infusion of different isotopes of the same amino acid (Krempf et al. 1990; Hoerr et al. 1993; Matthews et al. 1993; Biolo et al. 1992; Krempf et al. 1993; Biolo and Tessari 1997). First pass splanchnic uptake of dietary leucine ranges from 20 to 40% (Biolo and Tessari 1997; Matthews et al. 1993; Hoerr et al. 1993; Krempf et al. 1993), ~30% for lysine (Hoerr et al. 1993) and ~30 to 58% for phenylalanine (Biolo et al. 1992; Matthews et al. 1993). Of the amount sequestered on the first pass, only small amounts of leucine are converted to α-ketoisocaproate (Biolo and Tessari 1997) or are oxidized (Matthews et al. 1993), whereas a significant proportion (80%) of phenylalanine is converted to tyrosine (Matthews et al. 1993). These studies and others demonstrate that the fates of different dietary amino acids within the splanchnic region vary and the effects of changes in nutritional status on splanchnic amino acid uptake is also variable (Matthews et al. 1993; Hoerr et al. 1993; Krempf et al. 1990; Krempf et al. 1993; Biolo et al. 1992).

2.2.1.3. Model for Whole Body Amino Acid and Protein Metabolism

Hellerstein and Munro (1988) composed a scheme in which the exchange of amino acids in various body compartments of a 70 kg adult male were estimated. In this scheme approximately 100 g of protein is consumed and 250 to 300 g of protein is synthesized daily. The difference between intake and synthesis indicates extensive reutilization of amino acids in
protein metabolism. The turnover of body proteins is associated with significant reutilization of amino acids for the synthesis of new proteins and the salvaging of urea nitrogen by the colon (el-Khoury et al. 1994b; Danielsen and Jackson 1992b). Urea nitrogen circulating in the blood can enter the intestinal tract and be hydrolysed by resident flora and become fixed in bacterial protein. Danielsen and Jackson (1992) have demonstrated increased urea nitrogen cycling under conditions of restricted protein intake or enhanced demand. They argue that the degree to which urea cycles between body and gut is a measure of the degree to which dietary supply of all amino acids meets the metabolic demand for amino acids. Despite a constant turnover or flux (Q) through the body, the quantity of protein (or amino acids) remains relatively fixed.

Waterlow et al. (1978a) proposed a single pool amino acid model for the study of whole body protein metabolism (Figure 2.2.). This single free pool of amino acid N has two inflows: amino acids from dietary protein and amino acids from endogenous protein breakdown, and two outflows: amino acid oxidation to end products (CO₂, urea and ammonia) and amino acid uptake for protein synthesis (Waterlow et al. 1978a). This model can also be used to study the kinetics of a single indispensable amino acid (IAA). Briefly stated, "an IAA enters the free pool from dietary intake (Iₐₐ) and protein breakdown (Bₐₐ); it disappears from the free pool by oxidation (Oₐₐ) and uptake for protein synthesis (Sₐₐ)" (Matthews 1992). Under steady state conditions (energy and N balance), the basic flux equation may be written as follows:

\[ Q_{aa} = I_{aa} + B_{aa} = S_{aa} + O_{aa} \]  

(Waterlow et al. 1978a).

Measurement of amino acid or protein kinetics in vivo has relied on the use of isotopically labelled amino acids. Details of the methodology will be discussed in section 2.2.5.3.

The main pathways responsible for the maintenance of body protein and amino acid homeostasis are protein synthesis, protein degradation, amino acid oxidation and amino acid synthesis (nutritionally dispensable amino acids) (Young and Marchini 1990). The rates of
Figure 2.2. Model of Whole Body Amino Acid Turnover
these pathways change in response to various nutritional or disease states which permits adjustments in nitrogen (N) balance and the attainment of new steady states (Young 1981). The processes of protein synthesis and degradation are critical to the body's physiology. In fact, at least 20% of the basal energy expenditure is used in maintaining whole body protein homeostasis (Welle and Nair 1990).

2.2.2. Phenylalanine

The molecular structure of phenylalanine is shown in Figure 2.3. Phenylalanine is classified as a neutral aromatic amino acid because the radical group is aromatic and contains no acidic or basic groups (Crim and Munro 1994). L-Phenylalanine is an indispensable amino acid. Its essentiality was first demonstrated by Rose (1957) who found that dietary phenylalanine was required to maintain nitrogen balance in adult men. Phenylalanine also provides the body with an endogenous supply of the dispensable amino acid tyrosine via hydroxylation (Scrimer et al. 1989). The current international (Food and Agriculture Organization of the United Nations-Rome 1985) values for aromatic amino acid (phenylalanine + tyrosine) requirements in infants, preschoolers, children and adults are 125, 69, 22-27, and 14 mg·kg⁻¹·d⁻¹, respectively. The validity of the adult requirement has been questioned and new requirement estimates have been proposed. Data generated from ¹³C-labelled amino acid tracer studies suggest the phenylalanine and tyrosine requirement in adult males ranges from 30 (Zello et al. 1990b) to 39 mg·kg⁻¹·d⁻¹ (Marchini et al. 1994).

"The phenylalanine hydroxylation reaction is an obligatory and rate limiting step in the catabolic pathway that leads to the complete oxidation of phenylalanine to CO₂ and water" (Milstien and Kaufman 1975) (Figure 2.4.). The first step in phenylalanine catabolism is the irreversible oxidation of phenylalanine to tyrosine, catalysed by the phenylalanine hydroxylase (PAH) complex (L-phenylalanine-4-monooxygenase, EC 1.14.16.1) (Scrimer et al. 1989).
Figure 2.3. Structure of Phenylalanine, Tyrosine and Lysine
Figure 2.4. Metabolism of Phenylalanine and Tyrosine
Phenylalanine hydroxylase is present in mammalian liver (Udenfriend and Cooper Jr. 1952), absent in the brain (Abita et al. 1974), and its status in the kidney remains unclear (Murthy and Berry 1975; Ayling et al. 1975). The hydroxylation reaction was first demonstrated by Moss and Schoenheimer (1940) in rat liver and direct evidence of the reaction has been demonstrated in humans using stable isotope tracer methods (Thompson et al. 1989; Kilani et al. 1995; Clarke and Bier 1982; Curtius et al. 1972).

The hepatic phenylalanine hydroxylating system contains three components, PAH, dihydropteridine reductase (DHPR) and tetrahydropteridine (BH₄) (Scriver et al. 1989). PAH catalyses a coupled reaction in which phenylalanine is oxidized to tyrosine and BH₄ is oxidized to quinoid dihydrobiopterin (qBH₂). During the reaction one atom in the oxygen molecule is incorporated into the para position of phenylalanine, while the other is reduced (by BH₄) producing water. BH₄ is regenerated by DHPR at the expense of reduced NADH (nicotinamide adenine dinucleotide). It is interesting to note that BH₄ and DHPR play the same role in the hydroxylating systems for tyrosine and tryptophan (Friedman et al. 1972; Brenneman and Kaufman 1964).

Phenylalanine can also be metabolized by minor pathways which become functionally significant when the major catabolic pathway is insufficient and phenylalanine concentrations increase, as is the case in PKU (Langenbeck et al. 1992; Michals and Matalon 1985; Scriver et al. 1989). Transamination of phenylalanine to phenylpyruvate is the best known initial step which leads to the ultimate formation of phenylacetate and excretion in the urine as phenylacetylglutamine (Scriver et al. 1989). A second minor pathway involves the decarboxylation of phenylalanine to phenylethylamine (Scriver et al. 1989). The catecholamines are 3,4-dihydroxy derivatives of phenylethylamine (Molinoff and Axelrod 1971).
2.2.3. **Tyrosine**

The molecular structure of tyrosine is shown in Figure 2.3. Like phenylalanine, tyrosine is a neutral aromatic amino acid (Crim and Munro 1994). L-Tyrosine is a dispensable amino acid. In most situations its requirements can be fully met by the hydroxylation of phenylalanine. However, when the phenylalanine hydroxylation system is immature, there is evidence that phenylalanine cannot be relied upon as the primary source of tyrosine (McIntosh and Mitchell 1990). Since part of the requirement for phenylalanine is as a source of tyrosine, dietary tyrosine can reduce the requirement for exogenous phenylalanine. The extent to which tyrosine is able to spare the phenylalanine requirement has not been fully resolved. Rose and Wixom (1955b) reported phenylalanine requirements of young men to be 1.10 g·d⁻¹ and found that tyrosine had a sparing effect of 70 - 75% upon the phenylalanine requirement. These results were based on two subjects with energy intakes of approximately 56 kcal·kg⁻¹. The high energy intakes in this study would clearly lead to an underestimate of the total aromatic amino acid requirement, however it is not clear what effect it would have on the ratio of phenylalanine to tyrosine. Leverton et al. (1956) found that when 900 mg·d⁻¹ of tyrosine was provided to ten young women, 220 mg of phenylalanine was the lowest intake that kept all subjects in nitrogen balance. Out of a total aromatic amino acid intake of 1120 mg·d⁻¹, tyrosine represented 80.35% which is similar to the estimate described by Rose and Wixom (1955b). However in the same study, four subjects on an intake of 450 mg tyrosine with 220 mg or 420 mg of phenylalanine also achieved nitrogen balance. This suggests that the nitrogen balance technique is not sensitive enough to distinguish phenylalanine to tyrosine ratios of 1:4.1, 1:2.1 and 1:1.1. Tolbert and Watts (1963) studied the sparing effects of tyrosine on the phenylalanine requirement in 3 female subjects and found that tyrosine exerted a sparing effect of 70%. Nitrogen balance was the technique used and energy intakes were high (50-55...
kcal·kg\(^{-1}\)). Burrill and Schuck (1964) also studied the quantitative relationship between phenylalanine and tyrosine in 22 young men and women. Zero nitrogen balance was the criterion of adequacy and energy intakes were adjusted to maintain a consistent body weight. The results showed that with no tyrosine in the diet, the female and male subjects required 600-700 mg and 900-1000 mg of phenylalanine per day respectively. Two hundred milligrams of tyrosine had a replacement value of 35-40% for the females and 50% for the men. The replacement value of 400 mg tyrosine was approximately 50% for both female and male subjects. When expressed as a ratio of phenylalanine to tyrosine, the Burrill and Schuck (1964) ratio of 1:0.67 to 1:1 is significantly higher than the Rose and Wixom (1955b) ratio of 1:2.3. The difference in the ratios between these studies is in part due to the use of excessive energy intakes used by Rose and Wixom (1955b) versus the maintenance energy intakes used by Burrill and Schuck (1964). The precise effect this would have on the relative proportion of phenylalanine and tyrosine to the total aromatic amino acid requirement is unclear, but certainly overall aromatic amino acid requirements would be reduced by a positive energy balance. Another possible reason for the difference is that Rose and Wixom (1955b) studied only two subjects, while 22 subjects were included in the other study. The reliability and applicability of the Rose and Wixom results are highly questionable given their small sample.

The higher Burrill and Schuck (1964) ratios are consistent with the animal literature in which tyrosine was shown to spare 40%, 45%, 46% and 46% of phenylalanine requirements (House et al. 1997a; Milner et al. 1984a; Stockland et al. 1971a; Williams et al. 1987a). They are also consistent with plasma phenylalanine to tyrosine ratios of 1:0.81 to 1:1.23 (Mitchell et al. 1995; Scriver et al. 1989) (based on adult plasma phenylalanine and tyrosine levels of 43-73 uM and 35-90 uM respectively) and with the mixed piglet body protein phenylalanine to tyrosine ratio of 1:0.76 (Aumaitre and Duee 1974). This literature seems to favour a more
equivalent contribution of phenylalanine and tyrosine to the total aromatic amino acid requirement than was originally reported (Rose and Wixom 1955b).

The major fates of tyrosine are incorporation into protein or degradation via the series of reactions shown in Figure 2.4. The catabolic reactions are cytoplasmic and occur predominantly in hepatocytes (Mitchell et al. 1995). Physiological degradation of tyrosine is controlled at the level of tyrosine aminotransferase (TAT, EC 2.6.1.5) (Mitchell et al. 1995). Tyrosine is both gluconeogenic (fumarate can be converted to oxaloacetate and enter the gluconeogenic pathway) and ketogenic (acetoacetate can be used as an energy source in nonhepatic tissue or enter lipogenic pathways) (Mitchell et al. 1995).

Tyrosine is also the starting point of synthetic pathways leading to catecholamines (Figure 2.4.), thyroid hormone and the melanin pigments (Mitchell et al. 1995). Dopamine, norepinephrine and epinephrine are synthesized in the chromaffin cells of the adrenal medulla, postganglionic sympathetic nervous system and central nervous system (Molinoff and Axelrod 1971). Tyrosine hydroxylase (TH, EC 1.14.3) is the rate limiting step in catecholamine biosynthesis. TH is dependent on both \( \text{O}_2 \) and \( \text{BH}_4 \) for the conversion of tyrosine to dihydroxyphenylalanine (DOPA). This intermediate is present in very small quantities in catecholaminergic neurons, owing to its immediate decarboxylation to dopamine by excess L-aromatic amino decarboxylase. Further conversion to norepinephrine or epinephrine is dependent on the presence of dopamine-\( \beta \)-hydroxylase and phenylethanolamine-N-methyltransferase in the neuron (Schmidt and Lovenberg 1992). Catecholamines are rapidly metabolized by catechol-O-methyltransferase and monoamine oxidase to form the inactive O-methylated metabolites, metanephrine and normetanephrine, and the deaminated metabolites, homovanillic acid, dihydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid (also known as vanillylmandelic acid, VMA) (Molinoff and Axelrod 1971; Schmidt and Lovenberg 1992).
Unlike tryptophan and brain serotonin synthesis, it is not clear whether tyrosine supplementation increases the synthesis of catecholamines under basal conditions (Milner and Wurtman 1986). However, Johnston et al. (1983) demonstrated that catecholamine metabolite production is related to dietary tyrosine availability in normal weight women. It has been clearly demonstrated that enhanced activity of TH containing neurons will increase synthesis and release of dopamine and norepinephrine following tyrosine supplementation (Milner and Wurtman 1986). Tyrosine hydroxylase is regulated primarily by feedback inhibition by the catecholamines, as well as by tyrosine derivatives (ex. α-methyltyrosine) (Molinoff and Axelrod 1971). Another noteworthy effect of TH is that it catalyses the conversion of phenylalanine to tyrosine in the brain, at a rate comparable to its ability to hydroxylate tyrosine (Ikeda et al. 1967). This suggests that even in PKU, where the phenylalanine hydroxylation pathway via PAH is blocked, there can never be a complete absence of the metabolic conversion of phenylalanine to tyrosine. In fact, the conversion of phenylalanine to tyrosine catalysed by the TH enzyme is thought to represent part of the conversion that is observed in classical PKU (Scriver et al. 1989).

2.2.4. Lysine

Lysine is an indispensable six-carbon dibasic amino acid (Figure 2.3.). Current international requirement estimates for lysine are 103, 64, 44-60, and 12 mg·kg⁻¹·d⁻¹ in infants, preschoolers, children and adults (Food and Agriculture Organization of the United Nations-Rome 1985). These estimates are based on growth and nitrogen balance data. Stable isotope studies have been used to reassess lysine requirements in adult males. Mean lysine requirements were determined to be 35 mg·kg⁻¹·d⁻¹ (Meredith et al. 1986), 36.9 mg·kg⁻¹·d⁻¹ at a protein intake of 1.0 gr·kg⁻¹ (Zello et al. 1993) and 41.2 mg·kg⁻¹·d⁻¹ at a protein intake of
0.8 g·kg⁻¹ (Duncan et al. 1996). As with the phenylalanine requirements, adult lysine requirements determined by tracer methodology are 2-3 times greater than the current estimate based on nitrogen balance data (McLarney et al. 1996). Lysine is a strongly conserved indispensable amino acid. The factors involved in this conservation include the following: the activity of lysine-ketoglutarate reductase is responsive to low dietary lysine levels; lysine does not undergo classic transamination; and there is little catabolism outside the liver (Flodin 1997; Young and Pellett 1994). Lysine also has a capacity for temporary storage in the muscle pool as demonstrated by Bergstrom et al. (1990) who showed that after a protein containing meal, 60% of the adult daily lysine requirement may be deposited in the intracellular skeletal muscle pool within three hours (Flodin 1997; Young and Pellett 1994). Flodin (1997) suggested that these characteristics of lysine may explain, in part, the low values obtained for the adult lysine requirement when measured by nitrogen balance (Flodin 1997). However, the degree of underestimation of the nitrogen balance derived adult lysine requirement, and the similar degree of underestimation of the adult phenylalanine requirement, indicates that limitations in the nitrogen balance technique are likely the most critical factors in explaining the low values obtained.

The metabolism of lysine follows two main paths, protein synthesis and oxidative catabolism (Figure 2.5). Catabolism of lysine occurs almost entirely in the liver (Flodin 1997). The main catabolic pathway for lysine, via saccharopine, is a mitochondrial pathway leading to acetyl-CoA (Vianey-Liaud et al. 1991). The major degradative pathway involves the transfer of the ε-amino group to α-ketoglutarate through the intermediate, saccharopine (Flodin 1997; Cox and Dancis 1995). The end result of transamination is achieved by a different mechanism for lysine (Cox and Dancis 1995). Two enzymatic steps, carried out by a bifunctional enzyme, amino adipic semialdehyde synthase, are involved in this mechanism. "In the first step,
Figure 2.5. Metabolism of Lysine
saccharopine is formed as a ligand between lysine and α-ketoglutarate. In the second step, saccharopine is cleaved to α-amino adipic acid semialdehyde and glutamic acid, which completes the transfer of the ε-amino group. The aldehyde is oxidized to α-amino adipic acid" (Cox and Dancis 1995). Transamination of the α-amino group to form α-keto adipic acid and successive decarboxylation and oxidation reactions leads to CO₂ and acetyl CoA as final products (Flodin 1997; Cox and Dancis 1995). There is evidence for an overflow pathway for L-lysine catabolism which involves pipecolic acid as an intermediate product (Flodin 1997; Cox and Dancis 1995). The peroxisomal pathway, via pipecolic acid, is of less physiological importance and is mainly active in the brain (Vianey-Liaud et al. 1991). L-pipecolic acid is known as a nonprotein amino acid that is widely distributed in plants and is derived via lysine metabolism from both plants and animals. It has also been shown to increase in plasma in humans with liver damage (Kawasaki et al. 1988) and hyperpipecolicemia has been observed in rats with peroxisomal defects (Kramar et al. 1989). Pipecolic acid is also a product of D-lysine catabolism in rats (Chang 1976). Both L- and D-lysine (¹⁴C labelled) injected intraventricularly into the brain, effectively labelled pipecolic acid (Chang 1976). It is unlikely that D-lysine could be equivalently metabolized if it were infused orally or by vein, since it would not be transported across the blood brain barrier or into peroxisomes elsewhere. L-pipecolic acid is oxidized to α-amino adipic acid semialdehyde, thereby joining the saccharopine pathway (Figure 2.5.). With respect to the peroxisomal pathway and the use of lysine as an indicator amino acid, the minimal activity of the pipecolic acid pathway under normal conditions suggests that the rate of lysine oxidation would not likely be affected. There does not appear to be data on the percent of lysine that is metabolized via the pipecolic acid pathway under normal physiological conditions. However, since the pipecolic acid and
saccharopine pathways ultimately join prior to complete oxidation, any labelled carboxyl carbon in lysine would still be recovered in breath CO₂.

2.2.5. Methods For Measuring Amino Acid Requirements

2.2.5.1. Nitrogen Balance and Growth

A major feature of whole body protein or amino acid turnover is the quantitatively significant reutilization of amino acids for the continuing synthesis and maintenance of cellular body protein content (Young 1991). This recycling process is not completely efficient and some amino acids are lost by oxidative catabolism (Young and Pellett 1987). In order to maintain adequate protein nutritional status, a dietary supply of protein and specific indispensable amino acids is needed to replace these losses in addition to the intake needed for growth (Young 1991). The overall losses of nitrogen (urinary urea, ammonia, uric acid, creatinine, as well as N in feces, sweat, hair and losses via other miscellaneous routes) can be measured. With a simultaneous measure of nitrogen intake, an estimate of the status of body nitrogen balance can be obtained (Young 1991).

Growth rates are also used to reflect nutritional adequacy (Denne 1992). Various measurements of growth (weight, length, head circumference) have been combined with nitrogen balance studies to estimate amino acid requirements in growing individuals. The requirement of an indispensable amino acid is determined by measuring nitrogen balance at varying dietary levels of the amino acid, which range from deficient to excessive, while an otherwise adequate diet is consumed (Fuller and Garlick 1994). The requirement estimate is the intake which results in zero balance in adults and a defined level of positive balance in children. Through this method, valine, methionine, threonine, isoleucine, leucine, phenylalanine, lysine and tryptophan were found to be required to maintain nitrogen equilibrium.
(Rose 1957). However, it should be noted that histidine was not identified as an indispensable amino acid and was thus left out of the diets during subsequent nitrogen balance studies (Fuller and Garlick 1994; Leverton et al. 1956; Rose 1957). In fact the essentiality of histidine was not established until much later (Kopple and Swenseid 1975). This error highlights the limitation of the nitrogen balance method with respect to its apparent lack of sensitivity. It also raises the question of whether the results of the studies carried out with inadequate histidine are at all valid. The implication is that nutritional supplements made with amino acid contents patterned after the nitrogen balance derived requirements may not be meeting the needs of the people using them. Perhaps some of the complications associated with PKU relate to the fact that the medical foods do not contain an appropriate balance of all the amino acids.

The FAO/WHO/UNU (1985) relied on growth and nitrogen balance studies in infants, children and adults to define protein and indispensable amino acid requirements. In the 1985 report, the requirement pattern for adults included a lower proportion of indispensable amino acids out of the total amino acid requirement than in the other age groups. This suggests that in addition to total protein, indispensable amino acid requirements (per kg) also decrease with age (Dewey et al. 1996). However, except for the first month of life, more than half the need for amino acids is for maintenance of body protein and not for growth, thus the plausibility of this trend is questionable (Dewey et al. 1996). In fact, a recent FAO/WHO review (Food and Agriculture Organization of the United Nations-Rome 1990) concluded that it was unlikely that there is an age related fall in indispensable amino acid requirements (per kg body weight) given the slow rate of growth of the human. There is clearly a need for a new independent method to define amino acid requirements.

During the last decade there has been a renewed research interest in the question of amino acid requirements. Specifically, the current estimates for adults have been challenged
on the basis of methodological problems in the nitrogen balance experiments (Young and Bier 1987; Young 1987; Young et al. 1989; Young 1994). As reviewed previously (Zello et al. 1995; Young 1994; Young 1987), there are several reasons to question the requirement estimates based on nitrogen balance. First, there is a tendency to overestimate nitrogen intake and underestimate nitrogen excretion. This is due in part to nitrogen balance determinations that did not include an estimate of miscellaneous losses, which amount to approximately 0.5 mg·kg⁻¹·day⁻¹ nitrogen in adults (Food and Agriculture Organization of the United Nations-Rome 1985). If these losses had been included in the calculation of nitrogen balance, a higher amino acid intake would have been needed to achieve zero nitrogen balance. Second, body nitrogen equilibrium does not reveal possible changes in the intensity, quality, and/or distribution of tissue and organ protein metabolism. Third, even if all routes of nitrogen losses were considered, many of the early balance studies (Tolbert and Watts 1963; Rose and Wixom 1955; Rose et al. 1954) used excess energy intakes to ensure weight maintenance. Finally, it is difficult to interpret the metabolic and nutritional significance of a specific nitrogen balance value. In many studies with increasing nitrogen intakes above requirement levels, nitrogen balance in adults became progressively and unrealistically more positive (Hegsted 1976). Similar interpretational problems were described in the Leverton et al. (1956) paper where three different combinations of phenylalanine and tyrosine intakes produced similar nitrogen balance results. Clearly, the nitrogen balance approach should not be the only means by which indispensable amino acid requirements are established (Young 1987).
2.2.5.2. **Plasma Amino Acids**

After a protein-containing meal is consumed, the liver serves to regulate the concentration of most indispensable amino acids in the plasma. However, amino acids can still be presented to peripheral tissues in amounts that surpass their use for protein synthesis (Crim and Munro 1994). As a result, plasma levels of some indispensable amino acids increase when dietary supply exceeds requirements. Plasma amino acid concentrations could be used to predict dietary amino acid needs (McLaughlan and Illman 1967). In one study, as the concentration of tryptophan in the diet was increased from deficient to more than sufficient, the tryptophan content in plasma rose after the requirement was reached (Young et al. 1969). The point of inflection in the plasma tryptophan curve agreed with the intake needed for nitrogen balance. Meal ingestion is associated with an increase in plasma concentration of a limiting amino acid when the meal contains adequate amounts of the amino acid. However, below requirement level, meal ingestion decreases the plasma concentration of the limiting amino acid (Young and Scrimshaw 1978). Studies conducted in adults with lysine (Meredith et al. 1986), leucine (Meguid et al. 1986a) and valine (Meguid et al. 1986b) showed that plasma concentration of each amino acid decreased with decreased dietary intake. Conversely, postprandial plasma lysine increased when lysine intake was greater than required (Zello et al. 1993). Ozalp et al. (1972) reported that free phenylalanine and tyrosine concentration in plasma from postabsorptive subjects showed little change when they had consumed a completely deficient aromatic amino acid diet for 12 days. Postprandial aromatic amino acid concentrations were lower compared to a control diet period but it was not clear that this fall was proportional to the inadequacy of the aromatic amino acid intake.

A clear inflection point in the amino acid concentration curve is not observable with all indispensable amino acids and the use of this method as a measure of requirement has been disputed (Young et al. 1972). Fasting plasma amino acid concentrations clearly have no
relationship to the nutritional adequacy of recent past intake. The pattern of change in plasma amino acids varies depending on the age of the subject, the timing of plasma sampling, the length of adaptation to the amino acid level and the specific amino acid under study (Young et al. 1972). Young and Scrimshaw (1978) concluded that the plasma amino acid technique was not sensitive enough and required further refinement. Plasma levels of amino acids may not provide a sensitive measure of requirement, since they are the sum of many homeostatic processes controlled by the liver (Ball 1984). Furthermore, many factors unrelated to nutritional intake can influence plasma amino acid concentrations, including disease, enzyme defects, and sampling and analytical procedures (Denne 1992). Finally, plasma amino acid concentrations represent a static measurement that provides no information about the movement of amino acids in or out of protein. For example, lower concentrations of plasma amino acids may result from a decreased protein intake, reduced absorption, decreased rate of protein breakdown, increased protein synthesis or increased amino acid oxidation (Denne 1992). Despite these limitations, plasma amino acid concentrations continue to be used as indicators of nutritional adequacy in individuals with inborn errors of amino acid metabolism.

2.2.5.3. **Stable Isotope Tracer Techniques**

The use of isotopically labeled amino acids as biological tracers enables the dynamic aspect of protein and amino acid metabolism to be estimated and the IAA requirements defined. Protein or amino acid kinetics can be monitored following the administration of an isotopically labeled amino acid with subsequent measurement of isotope appearance in the plasma (tracer amino acid or metabolite), urine (tracer amino acid, urea or ammonia) or in expired air (CO₂) (Waterlow 1995; Waterlow et al. 1978).

Several approaches have been used to measure protein or amino acid turnover. Labeled tracer may be administered orally, enterally or intravenously, as a bolus dose or
continuous infusion, and with or without priming of the relevant pool(s). In the primed continuous infusion protocol (for example Zello et al. 1990b; Zello et al. 1993b), metabolic steady state is achieved by feeding each subject hourly, beginning three hours prior to the isotope infusion and continuing hourly throughout the four hour infusion. Baseline enrichment levels are determined in blood and breath samples collected prior to the isotope infusion. The infusion of isotope begins by administering priming bolus doses of NaH$^{13}$CO$_3$ and the $^{13}$C labeled IAA. Immediately following the priming doses, the constant infusion solution of labeled amino acid is started. Isotopic steady state is generally achieved within 120 minutes in both breath and blood using this protocol, thus enabling the study to be complete in seven hours.

The oral infusion is another method that has been used to measure protein and amino acid turnover. Picou and Taylor-Roberts (1969) developed the original method whereby $^{15}$N-glycine is administered orally at short intervals (every 3 hours). The $^{15}$N glycine becomes diluted in the free amino acid pool by unlabeled glycine entering from protein breakdown, endogenous synthesis, and dietary intake. The dilution of $^{15}$N glycine is measured indirectly via urea or ammonia end products in urine. Urine samples are collected after 60 hours, when isotopic steady state is achieved. Waterlow et al. (1978b) measured whole body protein synthesis with $^{15}$N glycine in infants and adults by administering the tracer with food every hour for 31 - 34 hours. Equilibrium in urea was achieved at 26 hours. The long time needed to achieve isotopic equilibrium is that the urea pool is a large pool and that in both of these studies, neither the glycine nor the urea pool was primed.

De Benoist et al. (1984) measured whole body protein turnover in the preterm infant with a primed intragastric infusion of $^{13}$C leucine and with sampling of the urinary leucine pool. The infant feed, mixed with tracer, was administered by constant intragastric infusion for 24 hours. Isotopic equilibrium was achieved in 8 hours in urine and within 9 hours in breath CO$_2$. Wykes et al. (1990) tested the validity of measuring isotopic enrichment of amino acids in urine.
Infants received a primed nasogastric infusion of $^{15}$N glycine, $^{13}$C phenylalanine or $^{13}$C leucine for 12 - 16 hours. Isotopic equilibrium in urine was achieved in 8 hours. Wykes et al. (1992) subsequently studied the kinetics of these amino acids by infusing the labeled amino acids enterally and parenterally along with the feeds and measuring isotopic enrichment in urine. This primed infusion took 12 hours and equilibrium was achieved after 8 hours. The length of these studies is much less than those described above because the sizes of the amino acid pools are significantly smaller than the urea pool. Amino acid turnover is also faster than urea turnover, thereby enabling isotopic steady state to be achieved faster. Furthermore, both De Benoist et al. (1984) and Wykes et al. (Wykes et al. 1992; Wykes et al. 1990) primed the primary pools and were therefore able to reach equilibrium sooner than the earlier studies.

Another important outcome of these studies is that serial urine collection was found to be a physiologically valid alternative to blood sampling. Isotope enrichment of amino acids in plasma and urine were similar during the enterally and parenterally administered infusions.

Depending on the experimental design, data are assessed by stochastic or compartmental analysis. Stochastic analysis requires the attainment of isotope plateau labeling in either precursor or end-product, or measurement of the area under an enrichment versus time curve which is derived from integral analysis of the decline in enrichment following a bolus injection of isotope. Whereas compartmental analysis is used to quantitate aspects of protein metabolism from the rate of change of labeling of appropriate plasma or urinary amino acid or end-product (Waterlow et al. 1978a).

In humans, the most frequent technique used is the primed constant infusion, described in detail by Waterlow and colleagues (1978a). The basic model is a form of stochastic analysis. When a labeled amino acid enters its free amino acid pool by the continuous infusion method, its enrichment will reach a constant plateau. This means that the amount of tracer entering the pool in unit time is equal to the net amount leaving (Matthews 1992). The time it takes to
achieve plateau is reduced by including an initial priming (or bolus) dose of the tracer which in a very short time (less than two hours) labels the substrate pool to the level that would eventually be reached as a consequence of a continuous infusion alone (Waterlow et al. 1978a). It should be noted that the plateau achieved with the constant infusion is really a "pseudo" plateau. This is because the amino acids which are incorporated into protein are not irreversibly lost. There is a slow return of tracer, so that the enrichment in the amino acid pool will slowly rise. Since this rise in enrichment takes a long time, an infusion lasting several hours can produce a plateau with a negligible slope (Waterlow et al. 1978a).

The continuous infusion of an amino acid tracer, either orally or intravenously, may be coupled with measurement of end product of nitrogen (¹⁵N label) or carbon (¹³C-label) metabolism or determination of enrichment of isotope in plasma or urine (¹⁵N or ¹³C) (Matthews 1992). The turnover rate (or flux, Q) of an amino acid can be measured if the tracer enrichment, infusion rate and tracer dilution in the free amino acid pool at plateau are known (Matthews 1992). For a ¹³C-labelled tracer, the amino acid oxidation rate can also be measured from the rate of ¹³CO₂ excretion (Waterlow et al. 1978a). The rate of amino acid release from protein breakdown (Bₐ) and uptake for protein synthesis (Sₐ) is determined by subtracting dietary intake and oxidation from the flux (Figure 2.2.) (Matthews 1992; Waterlow et al. 1978).

The basic assumptions upon which the estimates of flux, synthesis and breakdown are made, include the following: (1) synthesis of protein and excretion of labelled end product occur from a single precursor pool of a constant size; (2) recycling of tracer that has been incorporated into protein is negligible within the time period of measurement; (3) routes of disposal of amino acids into other metabolic pathways are insignificant; and (4) plateau represents steady state within the context of the time course of the study (Waterlow et al. 1978a).
The first assumption raises the question as to what extent is the true precursor of protein synthesis (i.e. amino-acyl-tRNA) labelled with tracer and what relationship does this bear to the labelling of amino acids delivered by the blood (Rennie et al. 1991). Intracellular amino acid pools are compartmentalized within cells and the isotope enrichment of tRNA-bound amino acids is not the same as that of the extracellular amino acid pools (Reeds et al. 1992). For example, intracellular leucine enrichment, as measured by plasma α-ketoisocaproic acid, which is formed from intracellular leucine and released into plasma, is ~20% lower than the plasma leucine enrichment (Matthews 1992). Different approaches have been used to indirectly measure the true precursor pool. The flooding dose technique (Hunter et al. 1995) involves the infusion of a large dose of the amino acid along with the isotopic tracer. Equilibrium labelling of apolipoprotein B-100 has also been used to measure isotopic enrichment of hepatic amino acids (Reeds et al. 1992). Finally, labelling of α-ketoisocaproic acid has been used to infer the isotopic enrichment of the intracellular leucine pool (el-Khoury et al. 1995; Biolo et al. 1992; Goulet et al. 1993; Bennet et al. 1993; Nair et al. 1992; Pacy et al. 1991). Unfortunately, not all amino acids have comparable intracellular markers, therefore partitioning of amino acids between intracellular and extracellular spaces must be considered.

With respect to assumptions 2 and 4, reentry of isotope from protein breakdown is negligible in short studies due to the fact that the half lives of most proteins are longer than the duration of the studies which thereby has no measurable effect on plateau enrichment (Waterlow et al. 1978a).

The third assumption does not hold true for amino acids with metabolic fates other than protein synthesis and oxidation. For example, niacin is formed from tryptophan and melanin from tyrosine and phenylalanine (Waterlow et al. 1978a). In the case of tryptophan, adequate niacin must be provided in the diet. Similarly, tyrosine must be provided in the diet in order to minimize the conversion of phenylalanine to tyrosine. Thus any conversion of tyrosine to
melanin will derive from tyrosine and not from phenylalanine. Assumption three is of little consequence if the appropriate label is chosen. The continuous end product method requires that the position of the isotope be located at a point of irreversibility in which the amino acid is committed to metabolic degradation. Thus, the use of amino acids with uniformly labelled carbon skeletons would lead to distribution of the label among many metabolites. Similarly, carboxyl labelled amino acids in which the CO₂ is not exchanged with the body’s bicarbonate pool and released in expired breath directly upon catabolism are not desirable (Waterlow et al. 1978a).

One final limitation of the continuous end product method occurs when the tracer is administered intravenously and the subject is simultaneously being fed orally (or intragastrically). As was discussed earlier, the splanchnic bed is the site of a significant proportion of whole body amino acid metabolism. The flux and amino acid released from protein breakdown will be underestimated by an amount equal to that amount sequestered on the first pass through the splanchnic bed (Matthews 1992). This problem is avoided if the tracer is administered by the same route as the food.

With respect to indispensable amino acid requirements, the use of stable isotope tracer techniques has enabled the relationship between amino acid intake, amino acid oxidation and amino acid requirements to be described. As indicated in Figure 2.2., the status of amino acid oxidation plays an important role in determining body amino acid homeostasis. A series of studies in rats (Kang-Lee and Harper 1977; Harper and Benjamin 1984; Brookes et al. 1972) and humans (Young 1991; Motil et al. 1981) reveals that low intakes of specific indispensable amino acids brings about an efficient utilization of amino acids associated with a reduced rate of amino acid oxidation. This increased efficiency is due in part to a decrease in tissue amino acid concentration, which in turn affects the rate of amino acid oxidation because the Kₘ for the various amino acid degrading enzymes are relatively high (ie. low affinity). In contrast, the Kₘ of
the amino acyl-tRNA synthetases are low (high affinity), so the net effect of these differences in the kinetics of these enzymes is an efficient channelling of amino acids into pathways of protein synthesis. Thus, the efficiency of dietary and endogenous amino acid retention would increase when the intake is reduced (Young and Marchini 1990). Furthermore, a significant increase in the oxidation of the indispensable amino acids occurs when its dietary intake exceeds the requirement levels since the amounts in excess of those needed for protein synthesis are oxidized (Brookes et al. 1972).

2.2.5.3.1. Direct Amino Acid Oxidation

In the early 1980's, Young and collaborators initiated a series of studies designed to reassess the minimum physiological requirements for specific indispensable amino acids in healthy adult males. The premise of these experiments was that a direct measure of the oxidation of a specific indispensable amino acid, coupled with a determination of the minimum intake required to balance this oxidation, would yield a better estimate of requirement than is possible from the classical nitrogen balance measurements (Young 1991). In the initial four studies (Zhao et al. 1986; Meredith et al. 1986; Meguid et al. 1986; Meguid et al. 1986), estimates of 24 hour amino acid oxidation were made and compared with the levels of test amino acid intake required to just balance this rate of irreversible oxidation. The test subjects had been adapted for 5-6 days to an L-amino acid diet which provided graded levels of the test amino acid. In subsequent studies (Cortiella et al. 1988), amino acid oxidation was measured during three hour fasted and five hour fed periods, and were then extrapolated to 24 hours (12h fasted, 12h fed). More recently, a series of 24 hour studies have been conducted to measure daily rates of whole body leucine (el-Khoury et al. 1994b; el-Khoury et al. 1994b) and phenylalanine (Basile-Filho et al. 1997; Sanchez et al. 1995) oxidation and to reevaluate their requirements. The requirement estimates derived by direct amino acid oxidation are 2-3 times
greater than those derived from the nitrogen balance studies (Food and Agriculture Organization of the United Nations-Rome 1985). These findings have been challenged on theoretical and experimental grounds (Millward 1994; Millward 1993; Fuller and Garlick 1994). Some of the most critical limitations identified with direct amino acid oxidation are: (1) that direct oxidation studies are restricted to amino acids whose degradation results in direct release of the carboxyl carbon as CO₂; (2) variable dilution of the labelled amino acid with unlabelled amino acid occurs as the dietary intake of the test amino acid changes, thereby changing the size of the free amino acid pool; (3) studies at lower amino acid intakes are restricted by the amount of isotope infused which is included as part of dietary intake; and (4) not all amino acid losses are accounted for if amino acids are sequestered elsewhere in the body (for example, the carbon skeletons of amino acids that enter the colon may be sequestered in the microbial biomass (Fuller and Garlick 1994; Zello et al. 1995; Waterlow 1996; Fuller and Garlick 1994). Nevertheless, others have confirmed the validity of the direct oxidation estimates of lysine (Duncan et al. 1996; Zello et al. 1993).

2.2.5.3.2. Indicator Amino Acid Oxidation (IAAO)

Using the piglet model, the corollary concept of indirect or indicator amino acid oxidation was developed (Ball et al. 1986; Ball and Bayley 1986; Ball and Bayley 1984; Kim et al. 1983; Kim et al. 1983). This concept is based on the hypothesis that the partition of any IAA between oxidation and protein synthesis is sensitive to the dietary level of the most limiting amino acid. Therefore, when the dietary intake of an IAA is below requirement level, protein synthesis is limited by the intake of that amino acid. Therefore, all other IAA present in relative excess to the limiting IAA are oxidized. As the intake of the limiting amino acid increases, uptake of the other IAA present for protein synthesis increases, which in turn reduces their oxidation. Once the requirement level of the limiting IAA is reached, further increases in its intake have
no further effect on the oxidation or protein synthesis rates of the other IAA. The point at which the oxidation of the indicator amino acid no longer decreases, despite further increases in test amino acid is referred to as the breakpoint. The relationship of the oxidation of an indicator amino acid in response to the dietary intake of an indispensable amino acid is illustrated in Figure 2.6.

The IAAO concept is supported by the results of Ball and Bayley (1986), who demonstrated that indicator (14C phenylalanine) oxidation (determined by recovery of radioactivity in expired CO₂) was inversely proportional to its uptake for hepatic protein synthesis (determined by recovery of radioactivity in liver tissue). Phenylalanine oxidation was used to indicate the requirements for proline (Ball et al. 1986), arginine (Ball et al. 1986), histidine (Kim et al. 1983b), lysine (Kim et al. 1983a), threonine (Kim et al. 1983a) and tryptophan (Ball and Bayley 1984). Phenylalanine was selected and was considered preferable as the indicator amino acid for several reasons: it has a small relative pool size in liver and muscle; the phenylalanine pool is closely regulated; phenylalanine uptake for protein synthesis is most responsive to changes in dietary protein level; the liver is the site of phenylalanine oxidation, implying that its level of oxidation is affected by the pattern of amino acids entering the liver after a meal; and the carboxyl carbon of phenylalanine is lost in CO₂ during oxidation (Ball and Bayley 1984). Lysine has also been used as an indicator because it too is primarily oxidized in the liver and its carboxyl carbon is lost in CO₂ (Ball and Bayley 1984). Both phenylalanine and lysine were used as indicator amino acids in a study which sought to define tryptophan requirements in piglets (Ball and Bayley 1984). The oxidation of phenylalanine and lysine suggested that the tryptophan requirement was 1.8 to 2 g·kg⁻¹. Despite similar requirement estimates, phenylalanine was considered a more sensitive indicator of dietary adequacy. However, it should be noted that while phenylalanine was studied at seven tryptophan levels, lysine oxidation was only studied at four tryptophan levels, which makes
Figure 2.6. Indicator Amino Acid Oxidation
definition of a breakpoint mathematically tenuous. Furthermore, DL-[1-14C] lysine was used as the labelled amino acid, given that D-lysine is not metabolized by mammals, it is not clear what effect this would have on the pattern of lysine oxidation observed. In the piglet studies, the specific activity of the liver free phenylalanine (direct measure of precursor pool) was not influenced by the changes in test amino acid intake so that the release of 14CO2 in response to changes in test amino acid intake was equivalent to the rate of hepatic phenylalanine oxidation (Kim et al. 1983a).

Zello et al. (1993) carried out the first IAAO study in human adult males to determine the lysine requirement at a protein intake of 1.0 g·kg⁻¹·d⁻¹. L-[1-13C] phenylalanine was used as the indicator amino acid. Duncan et al. (1996) repeated this study at a protein intake of 0.8 g·kg⁻¹·d⁻¹ to test whether a lower protein intake would result in a lower estimate of lysine requirement. Lysine requirements were not different in these two studies and furthermore, supported the results of the direct lysine oxidation study (Meredith et al. 1986). IAAO has also been used to define tryptophan requirement in adult females (Lazaris-Brunner 1994) and threonine requirements in adult males (Wilson et al. 1997). Both of these studies resulted in higher requirement estimates than are currently proposed (Food and Agriculture Organization of the United Nations-Rome 1985). As in the piglet studies, the effect of changes in test amino acid intake on the rate of 13CO2 release were similar to the effects on the rate of phenylalanine oxidation (Lazaris-Brunner 1994; Zello et al. 1993). This suggests that expired 13CO2 alone may be used to determine indispensable amino acid requirements in situations where plasma or urine collections are not possible.

The IAAO method avoids some of the experimental pitfalls described for the direct oxidation studies. In IAAO, neither the level of the indicator amino acid in the diet, nor the ratio of labelled amino acid to unlabelled amino acid changes. Therefore, the dilution issue is
avoided since the pool size of the amino acid that is oxidized remains the same throughout the study (Zello et al. 1993). This is clearly demonstrated in the adult IAAO studies in which phenylalanine flux is not affected by changes in test amino acid intake. Second, in IAAO all dietary levels of amino acid intake can be studied, including an intake of zero. Thirdly, the requirement of any indispensable amino acid can be studied, this includes those amino acids whose carboxyl carbon is not released to CO₂ directly upon oxidation or those with complicated degradative pathways. The indicator method uses phenylalanine or lysine to measure the requirements of those amino acids which cannot be measured by direct oxidation. This suggests further applications of IAAO in conditions with blocked metabolic pathways due to inborn errors of metabolism. Finally, the IAAO studies employs a two-phase linear regression model that defines a mean breakpoint and a mean plus 95% confidence interval which is used as an estimate of the population requirement (Zello et al. 1995).

The IAAO studies have all been carried out without prior adaptation to the intake level of the test amino acid (Zello et al. 1995). Zello et al. (1995) suggest that because IAAO is carried out in the fed state, no, or minimal prior adaptation is required to measure the amino acid oxidation response to a change in intake level. This contention is not widely accepted (Waterlow 1996). It may be that differences in experimental design with respect to adaptation, may reflect differences in how indispensable amino acid requirements are defined. In keeping with the FAO/WHO/UNU report (Food and Agriculture Organization of the United Nations-Rome 1985), Young and colleagues appear to define indispensable amino acid requirements as the lowest intake that will maintain functional needs of the individual (Dewey et al. 1996). As such, their subjects are adapted to each test level for 5-6 days before amino acid oxidation is measured. This adaptation period is thought to be long enough (based on nitrogen balance studies) but not so long that accommodation would occur (Dewey et al. 1996). Zello et al. (1993), Duncan et al. (1996) and Lazaris-Brunner (1994) appear to define requirements as the
intake that will maintain *optimum* needs of the individual. Adaptation is clearly undesirable under this definition. It is interesting to note that the requirement estimates derived by direct and indicator oxidation for lysine are very similar despite the differences in adaptation periods. Perhaps this suggests that eventual adaptation can be predicted from acute studies without prior adaptation.

Currently there are no amino acid requirement estimates derived by oxidation techniques in either children or infants. Practical and ethical reasons related to the methodologies of the two oxidation protocols preclude their use in these and other vulnerable groups. Incorporation of noninvasive methods such as, oral infusions and measurement of urinary amino acid enrichment could expand the scope of application of stable isotope studies.
2.3. PHENYLKETONURIA (PKU) AND MAPLE SYRUP URINE DISEASE (MSUD)

Inborn errors of aromatic amino acid metabolism were reported to be the first to respond to nutrition support. PKU was discovered in 1933 and prevention of its resultant mental retardation by nutritional intervention is classic (Elsas and Acosta 1994). However, even with dietary treatment, cognitive development, growth and body composition are not quite normal in this population (Holm et al. 1979; Allen et al. 1996; Dhondt et al. 1995; Scriver et al. 1989). The precise pathogenesis of mental retardation and the mechanisms leading to growth and body composition alterations are not known. From a nutritional point of view, these sequelae of PKU highlight the limitations of current dietary management and represent the motivation for this body of work. The text which follows describes the genotype and phenotype of PKU, it then reviews the various hypotheses on the mechanisms which lead to mental and growth abnormalities and finally, an overview of the dietary management of PKU and its associated limitations are provided. The final portion of this section is a brief description of MSUD. This serves to explain why MSUD was selected as a comparison group for PKU in the area of energy metabolism.

2.3.1. Genotype in PKU

PKU is a disorder of aromatic amino acid metabolism in which phenylalanine cannot be converted to tyrosine (Okano et al. 1991; Anonymous 1993). It is one of several disorders of phenylalanine hydroxylation (known as hyperphenylalaninemas) characterized by a recessively inherited deficiency in the hepatic enzyme phenylalanine hydroxylase (PAH) (Anonymous 1993; Scriver et al. 1989). The PAH deficiency exhibits a wide range of clinical and biochemical severity due to residual enzyme activity of 0 to 35% (Lehmann et al. 1986; Thompson and Halliday 1990; Scriver et al. 1989). This variation can be explained by the multitude of mutations identified with the PAH gene. The PAH gene is on chromosome 12, contains 13
exons, has over 70 associated RFLP (restriction-fragment-length polymorphism) haplotypes, and harbors over 200 mutations (missense, nonsense, insertions, deletions, recurrent) associated with PKU and non-PKU hyperphenylalaninemas (Scriver et al. 1989; Eisensmith et al. 1996; Lehmann et al. 1984). In fact, the majority of affected individuals have combination mutations (compound heterozygotes), rather than one single mutation (homozygotes) (Scriver et al. 1989). PKU is present in many human populations and the majority of PAH mutations are not randomly distributed, but cluster in geographic locations (Scriver et al. 1989). The incidence among Caucasians is approximately 1 in 10,000, whereas the incidence among Chinese is 1 in 16,500 (Scriver et al. 1989).

2.3.2. Phenotype in PKU

Hyperphenylalaninemia is a generic term for a phenotype characterized by a phenylalanine concentration above 120 μM (Scriver et al. 1989). The clinical distinction between PKU and other hyperphenylalaninemas is higher plasma phenylalanine in PKU (>1000 μM) and lower tolerance for dietary phenylalanine (< 500 mg·day⁻¹) (Scriver et al. 1989). The PAH deficient hyperphenylalaninemas encompass a broad range of clinical phenotypes (Eisensmith et al. 1996). The major associated clinical manifestations in untreated PKU includes mental retardation (Holtzman et al. 1986; Waisbren et al. 1987; Thompson et al. 1990; Seashore et al. 1985), eczema (Drummond et al. 1966; Scriver et al. 1989), musty body odour (Scriver et al. 1989), irritability / hyperactivity (Michals and Matalon 1985; Yannicelli and Ryan 1995) and abnormal magnetic resonance images of the brain (Pietz et al. 1995a; Cleary et al. 1994a; Walter et al. 1993a). In contrast, individuals with mildly deficient PAH activity display only slight increases in plasma phenylalanine and have normal or near normal intelligence even in the absence of treatment (Advisory Committee on Inborn Errors of Metabolism 1976; Scriver 1991).
There are different variants of PKU which depend on genetic differences (Hanley et al. 1997; Advisory Committee on Inborn Errors of Metabolism 1976). Individuals with classical PKU are thought to have little to no residual PAH activity and present with plasma phenylalanine concentrations of more than 1200 µM before treatment and their phenylalanine tolerance at 5 years is below 20 mg·kg⁻¹ body weight. "Atypical", "mild" or "type II" PKU describes the condition of subjects with enzyme activities of 0.1 to 5% of normal and plasma phenylalanine levels before treatment of 600 - 1200 µM, and a phenylalanine tolerance at 5 years of 21 - 50 mg·kg⁻¹ (Burgard et al. 1997; Advisory Committee on Inborn Errors of Metabolism 1976).

The metabolic phenotype (degree of hyperphenylalaninemia) is the link between genotype and the associated clinical phenotype (disease manifestation) (Scriver et al. 1989). Several studies have reported strong correlations between the level of PAH activity predicted from the genotype (based on previous in vitro expression studies of the mutant proteins) and pretreatment serum phenylalanine levels, dietary phenylalanine tolerance, and clinical classification (i.e. disease variant) (Okano et al. 1991; Svensson et al. 1994; Eisensmith et al. 1996). It should be noted that widespread newborn screening for PKU has contributed to the identification of the different PKU variants and to the reduction in the overt clinical manifestations of the disorder (Scriver et al. 1989; Advisory Committee on Inborn Errors of Metabolism 1976).

2.3.3. Phenylalanine Homeostasis and Metabolic Phenotype

The phenylalanine concentration in plasma is thought to be in equilibrium with other fluid compartments at a steady state. The normal plasma phenylalanine values for children, adolescents and adults are 60 ± 13, 62 ± 18 and 58 ± 15 µM, respectively (Scriver et al. 1989). Regulation of the flux of phenylalanine through the body in a healthy individual is not
fully understood but is thought to involve two inflows: dietary phenylalanine and endogenous phenylalanine from free pools and polypeptides; and three outflows: incorporation into protein, hydroxylation to tyrosine and conversion to minor metabolites (Scriver et al. 1989).

Dietary phenylalanine and endogenous protein breakdown make up the inflow components. If net protein catabolism occurs because of a dietary deficiency, plasma phenylalanine levels increase because tissue protein contains 5.5% phenylalanine (Elsas and Acosta 1994). Input of phenylalanine will expand the plasma phenylalanine compartment when there is no compensating outflow. Incorporation of phenylalanine into protein is one source of outflow in PKU, however, this outflow is limited to the anabolic needs of the individual. The other outflow sources in PKU includes transamination of phenylalanine to phenylpyruvate and decarboxylation to phenylethylamine (Figure 2.4.).

2.3.4. PKU, hyperphenylalaninemia and Neurotoxicity

Although the location of the PAH deficiency is in the liver, the major clinical effect of hyperphenylalaninemia is on brain function and development. It follows that the main cause of neurotoxicity in PKU is high plasma phenylalanine levels (Scriver et al. 1989). Derivatives of phenylalanine are not found at sufficiently high concentrations to be toxic in the human disease (Kaufman 1989). Without dietary treatment, or when treatment is delayed, severe irreversible intellectual and neurologic impairments occur, whereas early initiation of a phenylalanine reduced diet results in development that is close to normal (Smith 1994). Nevertheless, a high proportion of early treated subjects exhibit some degree of intellectual impairment and have mean intelligence quotients (IQ) half a standard deviation lower than those of unaffected siblings (Koch et al. 1984) and population norms (Smith et al. 1990).

It is not clear whether a threshold value of plasma phenylalanine exists for neurotoxicity. The view that such a threshold exists is based on the apparently normal IQ of subjects with the
mildest forms of the disorder, whose plasma phenylalanine concentrations are <1000 μM (Anonymous 1993). Krause et al. (1985) induced short term high plasma phenylalanine levels in 10 subjects with PKU (aged 6-24 years) and reported associated impairments of higher integrative brain function. Performance on the same neuropsychological tests improved with the initiation of a low phenylalanine diet and lower plasma phenylalanine levels. In a subsequent study, Krause et al. (1986) identified abnormal electroencephalographic tracings in subjects with PKU (aged 7-10 years) treated with a high phenylalanine diet. The degree of brain dysfunction was correlated with plasma phenylalanine. These findings were reversed and correlated in the reverse direction when plasma phenylalanine was reduced by a low phenylalanine diet. The measures of brain dysfunction were positively associated with plasma phenylalanine values, which in turn were inversely associated with decreased urine dopamine and plasma L-dopa levels. There is further evidence that high plasma phenylalanine levels alter brain chemistry (Lykkelund et al. 1988; Antoshechkin et al. 1991), but these acute effects are observed when plasma phenylalanine exceeds 1300 μM (Scriver et al. 1989).

The threshold plasma phenylalanine value in the acute effect may be different from the threshold value in the long term with respect to the causation of neurotoxicity. The fact that individuals with milder variants of PKU still exhibit intellectual impairments, suggests that lower plasma phenylalanine concentrations may be toxic. The other side of this argument deals with an individual's susceptibility to the toxic effects of phenylalanine. The greatest susceptibility is thought to be in the first years of life, during the phase of most rapid brain growth (Thompson et al. 1990; Holtzman et al. 1986). Variable susceptibility to damage because of age differences may complicate the definition of a specific threshold plasma phenylalanine value at which neurotoxicity occurs.

In early treated preschool children, the degree to which intellect is impaired is associated with the degree of hyperphenylalaninemia to which the subjects have been exposed.
Relaxation or withdrawal of treatment before mid-childhood has also been associated with declines in intellectual ability (Holtzman et al. 1986; Seashore et al. 1985). The age at which plasma phenylalanine exceeded 900 μM (Holtzman et al. 1986) and the duration of unrestricted diet (Seashore et al. 1985) were correlated with the loss of intellectual function. Diet discontinuation and blood phenylalanine levels (off diet) were determined to be the best predictors of IQ loss in 91 subjects with PKU followed for up to 22 years. Improvements in behaviour and the physical manifestations of PKU have also been demonstrated in previously untreated adults started on a phenylalanine restricted diet (Yannicelli and Ryan 1995). Smith (1994) later made recommendations on PKU management that suggest that the plasma phenylalanine threshold level of 360 μM in preschool children and <700 μM in children 5-12 years of age, can be regarded as safe. These recommendations were recently supported in a study in which only children with a mean phenylalanine level below 360 μM performed as well in neuropsychological functions as control children (Burgard et al. 1997). In that same study, mean performance of patients with mild PKU off diet was the same as patients with classical PKU or mild PKU on diet, which lead the authors to question whether treatment is warranted in mild PKU. However, in all groups, mean plasma phenylalanine exceeded 360 μM. Current recommendations are to maintain strict phenylalanine control as long as possible. How strict and for how long continues to be debated. Finally, it should be noted that no randomized trials have been conducted in older children or adults, so there are little data to compare the long term effects of different treatment approaches on IQ (Smith 1994).

Hyperphenylalaninemia can interfere with the development and function of the central nervous system by different mechanisms. The two theories that will be reviewed here include: (a) high plasma phenylalanine levels in the brain disturb myelination and (b) high phenylalanine levels result in inhibition of neurotransmitter synthesis.
2.3.4.1. Defective Brain Myelination in PKU

Myelin turnover is increased in the brains of rats with experimentally induced hyperphenylalaninemia with myelin protein breakdown exceeding synthesis (Hommes et al. 1982). Recently, evidence based on magnetic resonance images (MRI) of demyelination in the brains of PKU patients who were inadequately treated or in whom treatment was terminated was reported (Walter et al. 1993; Cleary et al. 1994; Thompson et al. 1990). Thompson et al. (1991) also reported a high incidence of MRI abnormalities in 26 children and young adults with PKU, most of whom were still on dietary treatment and had been well controlled during the first 8 years of life. Some authors have been able to establish a relationship between the degree of MRI abnormality and biochemical severity (Cleary et al. 1994; Thompson et al. 1993). Reversal of MRI changes has been demonstrated by improvements in plasma phenylalanine control (Walter et al. 1993), which suggests that white matter changes on MRI represent a reversible structural defect of myelin rather than a permanent demyelination (Cleary et al. 1994). The available data suggest that MRI changes in PKU are due to current, dynamic changes in white matter water content directly related to the degree of hyperphenylalaninemia at the time of investigation (Smith 1994; Cleary et al. 1994; Walter et al. 1993; Thompson et al. 1993). To date, no one has been able to show a relationship between the severity of neurophysiological abnormalities and severity of MRI changes (Smith 1994; Cleary et al. 1994). The pathogenesis and significance of abnormal MRI readings remain to be elucidated.

2.3.4.2. Defective Neurotransmitter Synthesis in PKU

Tryptophan (as a precursor for serotonin) and tyrosine (catecholamines) are critical for brain neurotransmitter synthesis and dependent physiological functions. These CNS functions depend on homeostatic mechanisms that guarantee the stability of precursor amino acid
concentration. In PKU, elevations in plasma phenylalanine levels disrupt the homeostatic mechanisms and produce defective synthesis of serotonin, dopamine and norepinephrine (NE) (Krause et al. 1985; Lou et al. 1985; Butler et al. 1981; McKean 1972). It has been argued that the changes in neuropsychological function associated with hyperphenylalaninemia may be due to neurotransmitter deficiency (Krause et al. 1985).

Research in animals has shown that experimental PKU influences the synthesis of dopamine and serotonin by: (a) competitively inhibiting tyrosine 3-hydroxylase and tryptophan 5-hydroxylase (Ikeda et al. 1967; Udenfriend 1967; Yuwiler et al. 1965); and (b) limiting the transport of tyrosine and tryptophan across the blood brain barrier (BBB) at either the endothelial level and/or at the neuronal membrane (Pardridge and Oldendorf 1975; Oldendorf 1973). There is also evidence that neutral amino acids have low plasma concentrations in PKU (Christensen 1986) which further limits the availability of neurotransmitter precursors.

Outside the brain, tryptophan is also converted to 5-hydroxytryptophan in the liver, which in turn crosses the blood brain barrier and is available for serotonin synthesis (Yuwiler et al. 1965). To test if inhibition of 5-hydroxylation of tryptophan is involved in the decrease in brain serotonin in PKU, Yuwiler et al. (1965) administered high phenylalanine diets to rats in order to create experimental hyperphenylalaninemia and measured hepatic tryptophan hydroxylase activity. Tryptophan hydroxylase activity was dramatically inhibited in these animals. The authors reported that a 65% inhibition of enzyme activity had no effect on brain serotonin levels, while a 75% inhibition resulted in a 25% decrease in brain serotonin levels. These results suggest that since liver tryptophan hydroxylase activity is not limiting for serotonin synthesis until reduced to 1/4 of basal activity, this mechanism is likely not the primary explanation for reduced serotonin synthesis in PKU.
Ikeda et al. (1967) showed that tyrosine hydroxylase catalyzes the hydroxylation of both tyrosine and phenylalanine and that hydroxylation of phenylalanine to 3,4-dihydroxyphenylalanine occurs in two independent steps with free tyrosine as an intermediate. Also, that phenylalanine and tyrosine are mutually inhibitory. This means that high concentrations of phenylalanine inhibit dopamine formation from tyrosine without completely inhibiting the formation of tyrosine. In *in vitro* observations, Udenfriend (1967) found that with phenylalanine at $10^{-3}$ M and tyrosine at $5 \times 10^{-5}$ M (concentrations which are observed in PKU), as much as 80-90% of tyrosine hydroxylase activity is inhibited. Since phenylalanine is a substrate as well as an inhibitor, complete inhibition of the enzyme does not occur. Instead phenylalanine becomes limiting with respect to NE formation. Thus, when phenylalanine levels are sufficiently high, phenylalanine is converted to tyrosine in sympathetic tissue and brain. This conversion may represent the small amount of conversion observed in PKU (Udenfriend 1967).

With respect to amino acid transport, Christensen et al. (Christensen et al. 1986; Christensen and de Cespedes 1988) have postulated that hyperphenylalaninemia not only inhibits the passage of endogenous amino acids across the blood brain barrier, but also causes sequestration of amino acids into muscle and liver tissue, both of which deprives the brain of its supply of amino acids for neurotransmitter synthesis.

The system-L carrier achieves a net intracellular accumulation of phenylalanine that is enhanced in PKU (Thalhammer et al. 1980). Other neutral amino acids achieve uptake into parenchymal cells by other carrier systems, as such phenylalanine does not interfere with this uptake (Scriver et al. 1989). However, these same amino acids leave the cell on system L, and high intracellular levels of phenylalanine interfere with this process (Christensen 1986; Pardridge and Oldendorf 1975; Scriver et al. 1989). In the brain, the opposite occurs, an excess of phenylalanine blocks the transport of amino acids across the blood brain barrier via
the system-L carrier (Christensen 1986). Transport of phenylalanine and other large neutral amino acids through the blood brain barrier is mediated by a carrier that has a high affinity ($K_m \sim 22 \mu M$) for these amino acids (Pardridge and Oldendorf 1975). This means that the brain is acutely sensitive to the increases in concentration of single amino acids. Of the large neutral amino acids (LNAA), phenylalanine has the highest affinity for this transporter (Pardridge and Oldendorf 1975; Pardridge 1988). Knudsen et al. (1995) measured blood brain barrier permeability to phenylalanine and leucine in PKU and healthy controls. They reported that in subjects with PKU, brain permeability to phenylalanine and leucine was decreased by 55% and 46% respectively, compared to controls. The significance of this finding is that the plasma phenylalanine levels seen in PKU apparently saturate neutral amino acid transport sites at the blood brain barrier and may therefore cause derangements in amino acid availability to the brain (Pardridge 1988).

In animal studies, Oldendorf (1973) showed that the high free phenylalanine in PKU serum significantly reduced brain uptake of several LNAAAs when compared to control serum, but had no effect on arginine, ornithine or lysine. Tyrosine and tryptophan uptake were reduced by 40 and 50% respectively. Herrero et al. (1983) showed that phenylalanine at concentrations similar to those found in PKU, inhibits the uptake of tryptophan by membrane vesicles derived from rat synaptosomes.

A disturbance of the intestinal transport of L-tryptophan and L-tyrosine secondary to hyperphenylalaninemia has also been suggested. Tryptophan and tyrosine absorption were measured in six subjects with PKU before and after a low phenylalanine diet (Yarbro and Anderson 1966). Hyperphenylalaninemia was associated with low fasting plasma tryptophan values, limitations in the peak plasma tryptophan response to an oral tryptophan load, increased fecal tryptophan and tyrosine content and increased fecal and urinary indoles. These
abnormalities returned to normal (vs controls) with the low phenylalanine diet. Wapnir and Lifshitz (1974) also showed reduced rates of tryptophan absorption (-9.6% vs controls), but reported higher rates of tyrosine absorption in one adult with classical PKU when plasma phenylalanine was 1870 µM. Reduction of plasma phenylalanine to 840 µM normalized tryptophan absorption, however tyrosine absorption remained higher than controls. The difference in tyrosine absorption between these two studies may reflect small sample sizes.

In addition to transport inhibition, tyrosine deficiency is thought to be another cause for decreased availability to the brain. Several authors have reported low plasma tyrosine concentrations in treated PKU patients after an overnight fast (Koepp and Held 1977; Smith et al. 1990; Brouwer et al. 1977; Nord et al. 1988). These low values are considered problematic because "higher plasma levels in non-PKU individuals are, by definition, the optimal tyrosine concentrations" (Brouwer et al. 1977). The same authors (Nord et al. 1988) report no difference in plasma tyrosine levels between treated and nontreated PKU patients and no evidence of deficiency (such as growth retardation) despite low fasting plasma tyrosine levels. Clemens et al. (1993) showed that a high serum phenylalanine concentration is correlated with low concentrations of other LNAAs, including tyrosine. However, there is no consistent or significant reduction in plasma tyrosine in the untreated PKU patient (Koepp and Held 1977).

Van Spronsen et al. (1993) measured the plasma phenylalanine and tyrosine response to fasting and feeding in 9 patients (aged 1-20 years) with no evidence of tyrosine deficiency. They reported that plasma phenylalanine tended to remain stable, thus the patient’s nutritional state was not considered important in the evaluation of the adequacy of the phenylalanine intake. On the other hand, tyrosine decreased 27% after the overnight fast and increased up to 400% after one meal. The interindividual variability was very high and some patients experienced reductions in plasma tyrosine after one meal. This suggests that neither low overnight fasting nor low postprandial tyrosine concentrations necessarily represents an
insufficient intake of tyrosine and thus may lead to the false conclusion of tyrosine deficiency. Furthermore, several blood samples measured at different times appear to be needed to evaluate adequacy of intake. In a recent study, Van Spronsen et al. (1996) reported that both lower than normal and higher than normal postprandial plasma tyrosine levels were found in treated PKU patients, even when daily tyrosine intake was evenly distributed throughout the day. Overnight fasting resulted in a decrease in plasma tyrosine. Both of the previous studies reported decreases in tyrosine levels after an overnight fast, this finding is contrary to that of Ozalp et al. (1972), who reported no change in plasma phenylalanine or tyrosine in healthy adults despite previous insufficient intakes. Overall, these results suggest that plasma tyrosine concentrations in PKU are not reliable markers of dietary adequacy and furthermore, they seem to confirm the conclusion reached in section 2.2.5.2., that plasma amino acid levels may not represent valid markers for dietary adequacy.

The relative concentrations of the LNAA (phenylalanine, tyrosine, tryptophan, leucine, isoleucine, valine) affect their uptake into the brain because of competition between these amino acids on the brain transport system (Christensen 1986). Since brain serotonin synthesis and release depends on the availability of tryptophan and since catecholaminergic neurons are affected by increased availability of tyrosine (under conditions of neuronal activation) (Lehnert and Wurtman 1993), hyperphenylalaninemia could significantly reduce tryptophan and tyrosine availability for neurotransmitter synthesis in the brain (Krause et al. 1985; Lou et al. 1985; Butler et al. 1981; McKean 1972). Inhibition of brain tryptophan uptake in the presence of hyperphenylalaninemia can be predicted by computation of the ratio of tryptophan to other LNAA in plasma,

\[
\text{Tryptophan Ratio} = \frac{\text{Tryptophan}}{\Sigma\text{LNAA}} \quad \text{(Fernstrom and Wurtman 1972)}.
\]

Variations in the tryptophan ratio have been demonstrated to affect brain concentrations of tryptophan and serotonin (Lehnert and Wurtman 1993).
There are conflicting reports about the relative concentrations of phenylalanine, tyrosine, and tryptophan in cerebrospinal fluid (CSF). In untreated infants with PKU, both plasma and CSF phenylalanine levels are elevated as compared to controls, but no difference in CSF tyrosine are present (Berry et al. 1982; Snyderman et al. 1981). Plasma tyrosine was decreased (Snyderman et al. 1981) or within normal limits in these infants. Berry et al. (1982) also showed that CSF phenylalanine levels could be reduced by supplementing a low phenylalanine diet with branched chain amino acids. This reduction was not linked to increased neurotransmitter synthesis or to any other measure of neurological function.

Ratzmann and coworkers (1984) reported an increase in CSF phenylalanine and tyrosine together with an increased phenylalanine and decreased tyrosine concentration in plasma. The authors suggested that this disturbance in tyrosine levels indicates an inhibition of LNAA transport out of the brain. On the other hand, while Antoshechkin et al. (1991) found increased plasma and CSF phenylalanine levels (23 times control levels) and a small decrease in plasma tyrosine, equal CSF tyrosine concentrations were reported as compared to control values. The phenylalanine to tyrosine ratio in CSF (~26:1) and plasma (~31:1) were similar in PKU. Equal ratios in CSF and plasma were also reported in controls, indicating that inhibition of tyrosine transport across the blood brain barrier due to phenylalanine does not exist. Equal ratios also do not support the conclusions of Ratzman et al. (1984) regarding impaired transport out of the brain. They also found that the phenylalanine concentration in the CSF is about four times lower than in plasma both in PKU and controls. This suggests that the barrier function with respect to phenylalanine does not change in PKU.

In a recent study, cerebral concentrations of phenylalanine were measured by quantitative in vivo ¹H magnetic resonance spectroscopy in adult patients treated early for classical PKU (Pietz et al. 1995a). In agreement with the previous study, the plasma : brain ratio was 4.12:1.
The available data do not consistently suggest that phenylalanine and tyrosine compete for a carrier that serves their exit from brain and CSF back into blood. However, it may be that the composition of the CSF is not a good indicator of that of the extracellular fluid of the human brain (Pratt 1982). In PKU, it is suggested that increased levels of phenylalanine in the interstitial fluid of the brain may result in competitive inhibition of tyrosine and tryptophan transport across the cell membranes of neurons (Lykkelund et al. 1988). The findings of elevated CSF tyrosine and tryptophan support the postulation of impaired neuronal uptake, rather than inhibition of reuptake into circulation of these amino acids (Lykkelund et al. 1988).

The studies on the effects of hyperphenylalaninemia on serotonin and catecholamine metabolism in the brain are more consistent. McKean (1972) found that serotonin, dopamine and NE concentrations in autopsied brains of untreated patients with PKU were reduced to 30-40% of normal values and their amino acid precursors, tryptophan and tyrosine, in the same tissues, decreased to 40-50% of normal. McKean also found that the serotonin and catecholamine metabolites, 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) in CSF of PKU subjects increased significantly by lowering the blood phenylalanine concentration. Increases in CNS synthesis of serotonin and dopamine may be inferred from the increased rates of 5-HIAA and HVA accumulation. Butler et al. (1981) measured HVA, 5-HIAA and 3-methoxy-4-hydroxyphenylethylene (MHPG; NE metabolite) in lumbar CSF of three patients with classical PKU before and after treatment with a phenylalanine restricted diet. Mean values of all three metabolites were significantly decreased compared to age matched controls. Following treatment there were significant increases in HVA and MHPG levels, but the 129% increase in 5-HIAA was not statistically significant.

Lou et al. (1985) studied three subjects with PKU before and after discontinuation of dietary treatment. Cessation of phenylalanine restriction resulted in a two-fold increase in plasma and CSF phenylalanine, non-consistent changes in CSF tyrosine and tryptophan, a
decrease in plasma tyrosine and tryptophan, and a decrease in CSF HVA and 5-HIAA concentrations. In these patients, the decrease in neurotransmitter synthesis was associated with decreased vigilance. In a subsequent study, Lou et al. (1987) examined the effect of phenylalanine restriction and tyrosine supplementation with a free diet on vigilance and neurotransmitter synthesis (indicated by CSF levels of HVA and 5-HIAA) in 23 adults with classical PKU. HVA and 5-HIAA levels decreased with increases in plasma phenylalanine on an unrestricted diet. Addition of 160 mg·kg$^{-1}$ tyrosine to the free diet increased HVA and improved vigilance in patients with the poorest performance. The authors concluded that enhanced dopamine synthesis secondary to tyrosine supplementation may have contributed to improved vigilance and that large dose tyrosine supplementation may be a new therapeutic alternative when phenylalanine restriction is impractical. The authors did not address the fact that the supplemental intake of tyrosine resulted in a diet with a significantly lower phenylalanine to tyrosine ratio than exists in naturally occurring proteins and that this abnormal ratio may have produced amino acid imbalances which result in altered amino acid transport, nor did they consider the independent neurotoxicity of hyperphenylalaninemia.

Krause et al. (1985) tested 10 subjects with PKU (ages 6-24) at the end of each of three 1-week periods of high or low phenylalanine intakes. Tests included plasma amino acids, urine amino acids, dopamine and serotonin, and neuropsychological tests. In 9 of 10 subjects, there was an inverse relationship between plasma phenylalanine and urine dopamine excretion. Urinary serotonin fell with the high phenylalanine intake in 6 patients. Prolonged performance times on neuropsychological tests occurred when blood phenylalanine was elevated. Giovannini et al. (1988) measured serotonin and norepinephrine concentrations in platelets and the kinetic constants of platelet in vitro uptake of $^{14}$C-serotonin in untreated PKU, PKU and hyperphenylalanemic patients in good metabolic control, and control subjects. Platelet $^{14}$C-serotonin uptake (a peripheral marker for brain neuronal uptake) was normal in PKU and
hyperphenylalanemic patients whether or not they were being treated. Untreated PKU subjects had low platelet serotonin and norepinephrine levels and low plasma norepinephrine. Serotonin and norepinephrine were similarly reduced in platelets from PKU and hyperphenylalanemic patients in good metabolic control. The authors noted that since all subjects had normal neurological and psychomotor development, their data did not support a role for reduced neurotransmitter synthesis in the pathogenesis of neurotoxicity in PKU. Lykkelund et al. (1988) reported improvements in attention accompanied by increases in CSF levels of HVA and 5-HIAA in PKU patients in whom a phenylalanine restricted diet was reinstated. Similar finding were reported in PKU subjects on a free diet supplemented with tyrosine (106 - 194 mg·kg⁻¹). In both situations, significant change in attention or neurotransmitter synthesis only occurred in those subjects who performed poorly on an unrestricted/unsupplemented diet. Recently, Pietz et al. (1995b) concluded that 100 mg·kg⁻¹ tyrosine supplementation to adult subjects with PKU on an unrestricted diet was not beneficial with respect to brain function. In that study plasma tyrosine increased 200% and plasma phenylalanine did not vary. Finally, tyrosine supplementation in addition to a phenylalanine restricted diet did not produce any benefit to intellectual growth (Smith et al. 1997). It remains unclear whether the addition of certain free L-amino acids to the PKU diet is necessary or advisable (Scriver et al. 1989). It also remains unclear whether the current tyrosine supplemented formulas meet the nutritional needs of patients with PKU. At present, the limitations associated with the techniques used to assess the adequacy of a particular amino acid formulation do not allow for progress to be made in the nutritional management of these patients.
2.3.5. **Body Composition and PKU**

2.3.5.1. **Physical Growth in PKU**

The safety of dietary interventions in PKU have been judged by maintenance of normal physical growth and weight gain. Early treatment protocols were very restrictive and aimed at reducing plasma phenylalanine levels close to the normal range (60 - 180 µM). This resulted in growth failure and wasting in many patients (Hanley et al. 1970). After the treatment protocols were relaxed, and plasma phenylalanine was maintained at moderately elevated levels (120 - 600 µM), the United States Collaborative Study group reported normal rates of growth and skeletal maturation in the period from birth to 10 years of age (Holm et al. 1979; McBurnie et al. 1991). Since then, several longitudinal growth studies have reported on the physical growth of children with PKU (Allen et al. 1996; Dhondt et al. 1995; Verkerk et al. 1994; Schaefer et al. 1994).

Growth and skeletal maturation were evaluated in 82 children who participated in the German Collaborative Study of Children Treated for PKU (Schaefer et al. 1994). Height, weight, head circumferences and bone age were recorded every 6 months from birth to 6 years of age. A significant decrease in height standard deviation score (-0.65 SDS) during the second year of life was found in PKU compared to normal children. Thereafter, height SDS was not different from normal children. Boys were more affected than girls. Mean relative weight for height was close to the 50th percentile for both sexes, but a slight significant increase in weight for height SDS with time was observed. Skeletal maturation proceeded at a normal pace, while head circumference SDS was significantly decreased in boys during the first year of life. Growth tended to be compensated during later follow up. Plasma phenylalanine concentration in these children were on average 35% below the mean of the American children, however no correlation between phenylalanine intake or phenylalanine concentration and growth rate could be established.
Verberk et al. (1994) assessed growth in 137 early treated Dutch patients with PKU from birth to 10 years of age. They reported significantly reduced height for age SDS (-0.23) and head circumference SDS (-0.25) at the time treatment started. Thereafter to age 3, SDS for height decreased further (-0.74), while head circumference increased such that it was close to zero. Weight for height SDS was close to expected for all ages. After age 3 no further decreases in height SDS occurred. The authors suggested that differences in dietary treatment between American and other countries may explain the difference in results.

Ninety-four French children with PKU were studied from birth to 8 years of age during which time dietary treatment maintained plasma phenylalanine levels below 485 μM (Dhondt et al. 1995). At birth, length and head circumferences were normal but weight was slightly reduced compared to normal French children. From birth to 8 years, height for age SDS and weight for age SDS were reduced (-0.227 and -0.037). After 8 years of age, dietary treatment was relaxed, plasma phenylalanine levels were maintained between 910-1200 μM and there was a catch up growth such that height became normal. Seven children developed excessive weight gain after the relaxation of the diet.

Recently, Allen et al. (1996) reported that prepubertal children with classical PKU were shorter (height SDS -0.42) and had a lower total body nitrogen (TBN) than healthy controls. PKU children younger than 7.5 years had a significantly lower height SDS (-0.82) than those aged over 7.5 years (0.17). As in the previous study (Schaefer et al. 1994), it was the males in the younger age group who had the lower height SDS. A subset of children with PKU and controls were studied again after approximately one year in order to assess change in nitrogen with age. Similar rates of nitrogen accretion were found between the PKU and control children. Nitrogen accretion rates in PKU were greater than values from published reference data leading the authors to conclude that the PKU children exhibited a catchup in protein deposition. This conclusion is questionable given that the control children experienced the same protein gain.
The decrease in height for the children with PKU is consistent with the European studies, however unlike the European studies, there was no evidence of a difference in dietary practice between this Australian group and the Americans. Furthermore, plasma phenylalanine levels (652 μM) were higher than previously reported (Schaefer et al. 1994). Because this was a cross-sectional study, it was not possible to relate dietary intake to observations of reduced height and TBN. The recently observed reductions in height in children with PKU suggests that current dietary regimens aimed at strict plasma phenylalanine control may require modification. More specifically, they imply that phenylalanine intakes and/or energy intakes may have been inadequate. However, without the knowledge of phenylalanine requirements in PKU, no definite conclusion can be drawn. In addition, more sensitive or functional markers of dietary adequacy need to be established in order to assess the nutritional adequacy of changes made to dietary regimens.

2.3.5.2. Excess Body Weight in PKU

The Collaborative Study of Children Treated for Phenylketonuria reported that the children in the study were becoming overweight on average compared to the national standards for children their age (Holm et al. 1979). The Study collected information on nutrient and energy intake, socioeconomic factors, parents' heights and weights, and periodic measures of blood phenylalanine levels. The results indicated that by age 4, both the girls and boys averaged above the 70th percentile of weight for height. Seventy-two percent of the girls and 65% of the boys were above the median weight for their height based on National Centre for Health Statistics (NCHS) standards, when 50% would be expected.

White et al. (1982) conducted a subsequent analysis of the data collected in the Study with the aim of identifying the factors most highly associated with a child's overweight status. For the analyses, the PKU children who were above the 93rd percentile of weight for height and
sex at age 4 were classified as overweight (n=24) and were compared to the normal weight PKU children (n=127). Normal weight children had weight for heights between the 0 and 92nd percentiles. All the children in the study had previously been randomly assigned to either a low treatment group (in which subjects were to maintain a serum phenylalanine level of 60 - 328 µM) or to a moderate treatment group (serum phenylalanine levels of 333 - 605 µM). Therefore, children within the overweight and normal weight groups were in addition, heterogenous with respect to their dietary management and serum phenylalanine levels and children within the normal weight group were heterogenous with respect to their weight for height. The factors most highly associated with an increased risk of being overweight were subjects who were overweight at 2 years, had a lower socioeconomic status, and were female. The extent of dietary restriction and serum phenylalanine levels were not found to relate to overweight status for either boys or girls. Energy intake in the overweight group was insignificantly higher than in the normal weight group, with overweight girls and boys averaging 64 kcal/d and 75 kcal/d more than normal weight girls and boys. The authors implied that sample size may have been the reason for failing to detect significant differences in energy intake. Furthermore, they concluded "that energy intake (as opposed to energy output) could completely explain the weight differences between the two groups." The authors failed to account for several important factors in making their conclusions. First, the proportion of children assigned to the low or moderate treatment groups within the two weight groups is not described. It is unclear how an imbalance in this proportion may have affected the power of the results. Second, the rationale for classifying children as normal weight is questionable. The large range of weight for height percentiles among this group may have hidden or exaggerated intake differences between the two groups. For example, the mean energy intake in the normal weight group may have been artificially reduced if there were more children classified in the lower percentiles (0-10) than in the higher percentiles (82-92). This would result in an artificially large difference in energy
intakes between the two groups. Finally, the authors rule out energy output as a factor that may explain the excess weight without measuring energy expenditure or monitoring activity levels of the subjects.

The White et al. (1982) study focused on differences between overweight and normal weight PKU children. However the true cause for excess weight in these children may be related to factors that distinguish them as a group from non-PKU children (White et al. 1982). McBurnie et al. (1991), in a follow up to the White et al. study, compared the growth of 133 children, participating in the PKU Collaborative Study, with national standards for ages 2-10 years. The authors wanted to see if the PKU group at older ages still demonstrated a tendency toward higher than expected weight, and whether diet status as measured by plasma phenylalanine levels, explained the excess weight. To compare the growth of the PKU children with NCHS standards, median height and weight versus age, median weight versus median height, and median head circumference versus age were plotted against the 50th percentile of the NCHS children. These growth curves indicated that height and head circumference for both groups were very close. Similar results were found for median weight for age and median weight for height up to the age of 3 years. Thereafter, measurements for weight for age fell close to the NCHS 75th percentile and measurements for weight for height were between the 50th and 75th percentile. With respect to diet status, the authors reported a positive relationship between plasma phenylalanine levels and weight (after controlling for height) (p<0.001 for female and p = 0.08 for males). Another significant finding in this study is that the heights and weights of parents of the PKU children were not different from the national standards. Thus parental weight does not explain the observed weight differences. McBurnie et al. (1991) concluded that the relationship between weight and plasma phenylalanine is explained by differences in dietary intake, where high phenylalanine levels reflect a greater intake of phenylalanine and energy. It should be noted that the phenylalanine and energy intakes were
not described or analysed in this study and the conclusions regarding intakes are therefore speculative. Furthermore, the authors clearly fail to recognize the significant limitations on the use of plasma amino acids to reflect dietary intakes (Denne 1992).

The significant relationship between plasma phenylalanine and weight may in fact indicate a unique factor that impacts upon energy balance and that distinguishes PKU children from non-PKU children. Schulz and Bremer (1995) reported a high proportion of slightly overweight PKU patients (12 - 29 years old). BMI correlated positively with phenylalanine levels. The authors related these findings to an inability of PKU patients to control intakes, once their dietary treatment is stopped. However, despite the availability of food intake records, they do not comment on the presence or absence of a relationship between energy intake and BMI. Both White et al. (1982) and McBurnie et al. (1991) attribute the excess weight among children with PKU to excess energy intake. However neither study contained a thorough examination of both sides of the energy balance equation, namely, intake and expenditure. Also, a recent study which compared the nutrient intakes of adolescents with PKU (n=30) to those of age and gender matched controls (n=30), showed that energy intakes tended to be lower, and protein and fat intakes were consistently lower (p<0.01) among the PKU group (Gropper et al. 1993). The factors which contribute to the overweight status of children with PKU remain to be fully elucidated. A complete exploration of energy balance in PKU is clearly indicated by these observations and would help to explain the overweight status of some children. Also, the relationship between serotonin, catecholamines and energy expenditure and the relationship between these neurotransmitters and PKU, together are further justification for the study of energy expenditure in PKU.
2.3.6. **Dietary Treatment of PKU**

Dietary treatment of PKU requires the use of synthetic or semi-synthetic elemental or modified protein hydrolysate formulas. Medical foods omit phenylalanine and provide the majority of an individual's protein needs. Only a small amount of protein containing natural foods are permitted to provide the remaining indispensable amino acid and nitrogen needs. Medical foods also supply the majority of vitamins and minerals in the diet because natural foods are severely restricted (Gropper et al. 1993). "The objective of nutritional support in PKU is to maintain blood phenylalanine concentrations that will allow optimum growth and brain development by supplying adequate energy, protein and other nutrients, while restricting phenylalanine and supplementing tyrosine intakes" (Elsas and Acosta 1994). At present the dietary management of patients with PKU is empirical, primarily because of a lack of direct measure of phenylalanine and tyrosine requirements. Plasma phenylalanine and tyrosine, blood urea nitrogen, weight and height are some of the measures used to assess the adequacy of the PKU diet.

PKU and normal subjects have been reported to have similar energy and nitrogen requirements (Kindt et al. 1984; Kindt et al. 1983; Elsas and Acosta 1994; Acosta et al. 1977). However, a recent report (Acosta and Yannicelli 1994) in infants suggests that mean protein intakes 24% greater that the Recommended Dietary Allowance (RDA) is associated with better phenylalanine tolerance and growth than is found when protein intake is 9% greater than the RDA. With respect to phenylalanine, the tolerance for dietary phenylalanine to maintain plasma phenylalanine at nontoxic levels in 1 to 6 years old PKU children is 25% of normal or less (Scrimer et al. 1989; Acosta et al. 1983). Ruch and Kerr (1982) reported that the average dietary tolerance for phenylalanine in 6 infants (0 - 24 months of age) with PKU is approximately 50 - 68% of estimated normal phenylalanine requirement. Requirements were based on plasma phenylalanine levels. The recommended phenylalanine and tyrosine intakes
in PKU are shown in Table 2.1. With respect to tyrosine, since it is not supplied endogenously from phenylalanine, it in turn becomes an indispensable amino acid in PKU and thus specific intake recommendations are made. The main differences between the general recommendations and those for individuals with PKU include the wider range of phenylalanine requirements within each PKU age group and the supplemental tyrosine requirements, which when added to the phenylalanine requirements, far exceed the general aromatic amino acid requirements. In fact, the median tyrosine requirement across the different age groups, represents 5 to 7 times the corresponding phenylalanine requirement. This suggests that out of the total of aromatic amino acid requirement, phenylalanine contributes ~20% and tyrosine ~80%. These values are significantly different from those ratios described earlier in animals (House et al. 1997a; Milner et al. 1984a; Williams et al. 1987a; Stockland et al. 1971a) and humans (Burrill and Schuck 1964).

Dietary treatment aims to not only lower plasma phenylalanine concentrations, but to keep plasma tyrosine levels within the normal range. Current recommendations for tyrosine intake are based on plasma levels which as described earlier, may not be a sensitive marker of nutritional adequacy. Recently, it was reported that plasma tyrosine levels vary from below normal to higher than normal, depending on the distribution of the medical food throughout the day (van Spronsen et al. 1996). The authors suggested that an alternative distribution of the daily individual tyrosine intake may be more effective in preventing inappropriate tyrosine concentrations than would additional tyrosine supplementation (van Spronsen et al. 1996).

Early dietary treatment in PKU benefits cognitive development, however there is ample evidence that the outcome is not quite normal (Scriver et al. 1989). The causes of these cognitive deficits are not fully understood, but quality of treatment or poor compliance to treatment, reflected in blood phenylalanine levels is likely one determinant. There are several limitations in the nutritional care of individuals with PKU. First, there is no conclusive evidence
Table 2.1. Aromatic Amino Acid Requirements

<table>
<thead>
<tr>
<th>Source</th>
<th>Age Group</th>
<th>Phenylalanine (mg·kg⁻¹)</th>
<th>Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mg·kg⁻¹</td>
</tr>
<tr>
<td>FAO/WHO/UNU, 1985</td>
<td>Infants</td>
<td>25-90</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Preschool Children</td>
<td>69</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Older Children</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Young Adults</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>Elsas &amp; Acosta, 1994</td>
<td>Infants</td>
<td>15-70</td>
<td>250-350</td>
</tr>
<tr>
<td>PKU recommendation</td>
<td>Preschool Children</td>
<td>15-40</td>
<td>115-200²</td>
</tr>
<tr>
<td></td>
<td>Older Children</td>
<td>15-35</td>
<td>90-160²</td>
</tr>
<tr>
<td></td>
<td>Young Adults</td>
<td>10-30</td>
<td>63-126²</td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td>58-101²</td>
<td>3.75-6.5</td>
</tr>
</tbody>
</table>

¹ Recommendation is for phenylalanine plus tyrosine.

² Actual requirements (g/day) ÷ average weight (Nutrition Recommendations, 1990)

where mean weight of male & female:
- preschool children aged 1-6 is 15 kg
- older children aged 7-9 is 25 kg
- young adults aged 13-18 is 54 kg
- adults aged >18 is 65 kg.
to support the recommendation for similar energy requirements in healthy and phenylketonuric
individuals. Rose and coworkers (1957) reported a greater than normal need for energy in
adult men consuming an L-amino acid mix (similar to that consumed in PKU). On the other
hand, Zello et al. (1990a) found no change in energy requirements in adult men consuming a
diet which consisted of a free amino acid mixture and protein free formula and cookies. Pratt
(1955) found that infant's energy needs were 25% above normal when a complete amino acid
mix was the source of nitrogen (N) in the diet. Allen et al. (1995) tested the hypothesis that a
lower body protein content per unit of fat free mass (FFM) in children with classical PKU would
result in a lower resting energy expenditure (REE). Thirty children aged 4.6-17.0 years with
classical PKU were compared with 76 control children aged 4.3-18.4 years. FFM was indirectly
estimated from multiple skinfold thickness measurement. FFM was not different between the
male PKU and control subjects, but was significantly lower in female PKU subjects compared
with controls (23.1±8.1 kg vs 30.9±8.2 kg, P=0.007). However, female PKU subjects were also
significantly younger than controls (8.9±3.2 y vs 11.8±3.3 y, P=0.006). The technique employed
by Allen et al. to estimate FFM has significant limitations with respect to precision (Lukaski
1987) and furthermore, it is not clear how skinfold thickness measurements would measure the
protein content in the FFM. REE was similar in the PKU and control subjects, however when
measured REE was compared with predicted REE (Schofield 1985), female PKU subjects had
a significantly higher rate (105±6% of predicted, P=0.009). FFM is reported to be the best
predictor of REE in normal children and is highly predictive of REE in adults (Owen et al. 1986;
Owen et al. 1987). This accepted relationship between FFM and REE is inconsistent with the
findings of a significantly lower FFM and a significantly higher REE (vs. predicted) in female
PKU subjects. This contradiction may indicate that the protocol used to acclimatize the children
to the testing procedure was ineffective and/or that the FFM data are erroneous.
Although N requirements may be similar for PKU and normal subjects (Kindt et al. 1983), protein requirements are increased when either an L-amino acid mix or casein hydrolysate is the primary protein source rather than natural protein (Holt 1960). Nevertheless, recommended protein intakes in PKU are considerably less than actual intakes of normal infants and children (Albertson et al. 1992). Because amino acid mixtures are often consumed by PKU patients in only one or two portions and sometimes without any other nutrients, requirements may in fact increase since oxidation of amino acids increases under those consumption patterns (Herrmann et al. 1994). Herrman et al. (1994) demonstrated that $^{13}$CO$_2$ production (expressed as a percent of $[^{13}$C]leucine dose) doubled when one patient with PKU consumed her total amino acid mixture as compared to one third of the daily amount. The corresponding total nitrogen excretion in urine also increased from 4.3 to 6.9 g·24·1 hours. This suggests that requirements for amino acids in PKU may have to go beyond the definition of quantity, to how the diet is consumed.

The third limitation concerns the application of phenylalanine requirements assessed in healthy infants, children and adults (Food and Agriculture Organization of the United Nations-Rome 1985) to individuals with PKU. On one hand, studies have shown that loss of phenylalanine and its catabolites may result in a higher intake than is actually needed for protein synthesis (Kindt and Halvorsen 1980). On the other hand, Ruch and Kerr (1982) stated that, "..obligatory catabolism contributes a significant fraction of the normal infants requirement for essential amino acids and that loss of specific catabolic pathways due to enzymatic deficiency results in decreased requirements." In adults, Zello et al. (1990b) measured phenylalanine oxidation rates at deficient to excessive phenylalanine intakes. The oxidation rates at intakes below requirement represent obligatory phenylalanine oxidation. The oxidation rates below requirement were stable and they averaged 1.4 mg·kg$^{-1}$·d$^{-1}$. Given the mean phenylalanine requirement of 9.1 mg·kg$^{-1}$·d$^{-1}$, the obligatory catabolism of phenylalanine
represented ~15%. If there is no oxidation of phenylalanine, as is the case in classical PKU, these results provide some evidence that dietary phenylalanine requirements in PKU may be 15% lower than requirements in the general population.

The final limitation relates to the combined tyrosine and phenylalanine intakes in PKU. Scientific evidence could not be found to support the implied contention that tyrosine requirements in PKU are significantly greater than in healthy individuals.

2.3.7. Maple Syrup Urine Disease (MSUD)

Leucine, isoleucine and valine, the three branched chain amino acids (BCAA), are indispensable amino acids. The metabolic fates of the BCAA are either incorporation into protein or catabolism for energy. The anabolic pathway is regulated by the availability of tRNA’s for the BCAAs (Danner and Elsas II. 1989). Figure 2.7. illustrates the catabolism of the BCAA (Elsas and Acosta 1994). The first step is reversible transamination of the BCAA to its keto acid through transfer of the α-amino group to a keto acceptor (principally α-ketoglutarate). This reversible step does not commit the amino acid to catabolism. A single cytosolic amino transferase converts all three BCAAs to their respective branched chain ketoacid (BCKA) (Danner and Elsas II. 1989). The amino transferase enzyme is widely distributed among different tissues in humans but skeletal muscle is considered the main site for BCAA transamination (Danner and Elsas II. 1989). The second step is irreversible oxidative decarboxylation, which uses the branched chain α-ketoacid dehydrogenase complex (BCKD; EC 1.2.4.4) (Elsas and Acosta 1994). This four-protein, three-enzyme complex is located in the inner mitochondrial membrane and requires the coenzymes thiamin pyrophosphate, lipoic acid, CoA and NAD⁺ (Elsas et al. 1993; Danner and Elsas II. 1989; Elsas and Acosta 1994). BCKD activity is minimal in muscle and maximal in liver (Wagenmakers and Veerkamp 1982). So, the
Figure 2.7. Metabolism of Branched Chain Amino Acids
catabolic flow for BCAAs is thought to involve deamination by the muscle and oxidation of BCKAs by the liver (Danner and Elsas II. 1989).

MSUD is a group of disorders of BCAA metabolism with an autosomal recessive inheritance pattern (Elsas and Acosta 1994; Peinemann and Danner 1994). These disorders result from several different mutations that impair BCKD (Peinemann and Danner 1994; Danner and Elsas II. 1989). This impaired reaction results in increased concentrations of BCAAs and BCKAs in plasma, urine and CSF (Danner and Elsas II. 1989). MSUD is treated by dietary restriction of the BCAAs to reduce blood concentrations of the BCAAs and BCKAs into the normal range (Westwall 1963). Since this treatment lessens the clinical manifestations of MSUD, the BCAAs and BCKAs are believed to be toxic to the CNS. MSUD appears in all ethnic groups and has a general incidence of 1:290 000 live births (Danner and Elsas II. 1989).

The MSUD phenotype is influenced by the age at which the disease is identified, the degree of enzyme impairment and the time at which dietary therapy is started. Direct correlation of severity of the phenotype with BCKD activity is therefore difficult to show (Danner and Elsas II. 1989). Several forms of MSUD are defined depending on age of onset, plasma amino acid levels and amount of residual enzyme activity. Table 2.2. describes the three main forms of MSUD (Danner and Elsas II. 1989; Peinemann and Danner 1994). There is also a thiamin responsive form of MSUD in which daily pharmacological doses of vitamin B1 enable an individual to tolerate a higher protein diet while maintaining normal plasma leucine (Scrimer et al. 1971). Most recently, immunological classifications based on polyclonal antibodies specific for BCKD proteins have been used to define mutations resulting in MSUD with immunologically altered proteins (Danner and Elsas II. 1989). Untreated patients with classic MSUD who survive past early infancy have retarded physical and mental development (Danner and Elsas II. 1989).
Table 2.2. Clinical Classification of MSUD

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Age of Onset</th>
<th>Symptoms</th>
<th>Plasma Leucine&lt;sup&gt;1&lt;/sup&gt; (μM)</th>
<th>BCKD Activity&lt;sup&gt;2&lt;/sup&gt; (% normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic</td>
<td>Infant</td>
<td>Poor feeding, apnoea, ketoacidosis, seizures</td>
<td>1000 to 5000</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Infant to young adult</td>
<td>Ataxia, failure to thrive, mild ketoacidosis</td>
<td>400 to 2000</td>
<td>2 to 20</td>
</tr>
<tr>
<td>Mild</td>
<td>Childhood to young adult</td>
<td>Intermittent ataxia and ketoacidosis during infection or high protein intake</td>
<td>50 to 1000</td>
<td>2 to 40</td>
</tr>
</tbody>
</table>

<sup>1</sup> Normal plasma leucine: 56 - 179 μM (children)  
71 - 175 μM (adults)

<sup>2</sup> Leucine decarboxylation by intact cells

<sup>3</sup> Danner and Elsas (1989) and Elsas et al. (1993)
Mental retardation is a prominent clinical feature of both MSUD and PKU. Several attempts have been made to establish corresponding biochemical similarities between these two diseases. Tashian (1961) demonstrated that both BCAA and aromatic amino acids, inhibit serotonin and 5-hydroxytryptophan uptake by rat brain slices. Yuwiler and Geller (1965) showed that brain serotonin levels were reduced by dietary leucine in a manner analogous to that observed using dietary phenylalanine. More recently, leucine supplementation caused significant reductions in rat brain serotonin and 5-HIAA, tyrosine and NE (Yokogoshi et al. 1987).

In vivo studies of brain serotonin and catecholamine concentrations in subjects with MSUD do not appear to have been performed, nor does this area seem to be a focus of MSUD researchers. It remains to be determined whether subjects with MSUD have reduced serotonin, dopamine and NE synthesis.
3. RATIONALE, HYPOTHESIS AND OBJECTIVES

3.1. Rationale

There is evidence which suggests that the objectives of nutritional care in PKU are not fully met. Cognitive deficits associated with elevated plasma phenylalanine concentrations continue to be present in many patients (Scrver et al. 1989). Although the precise pathogenesis is not known, the accumulation of phenylalanine or its catabolites, a deficiency of tyrosine or its products, disruption of the homeostatic mechanisms that guarantee the stability of precursor amino acid concentration in the brain for neurotransmitter synthesis or all of these circumstances will affect central nervous system function (Elsas and Acosta 1994; Scrver et al. 1989).

Children with PKU tend to be overweight on average compared to national standards (McBurnie et al. 1991; White et al. 1982; Holm et al. 1979). The cause of excess weight in children with PKU is not clear. Excess energy intake may partially explain this tendency, however the available data on energy intakes are not definitive. There is one report which studied energy expenditure in classical PKU (Allen et al. 1995), however, it has methodological limitations that justify further exploration of energy balance in PKU. A significant relationship between plasma phenylalanine levels and weight has been described (Holm et al. 1979). Similarly, a relationship between plasma phenylalanine levels and altered synthesis of serotonin, dopamine, and norepinephrine has been observed (Krause et al. 1985). Since serotonin and the catecholamines influence energy expenditure, the presence of a previously unrecognized mechanism which relates energy expenditure, hyperphenylalaninemia and defective neurotransmitter synthesis to weight gain may be indicated.

The sequelae of PKU stem from the abnormalities in aromatic amino acid metabolism. Normal phenylalanine homeostasis in individuals with PKU is difficult to achieve and our lack of
knowledge of their tyrosine and phenylalanine requirements, in part, accounts for this difficulty. The current intake recommendations for IAAs in PKU are in part based on plasma concentrations and have been derived almost exclusively from requirements in healthy populations (Elsas and Acosta 1994). The application of requirements in the healthy population to the PKU population is questionable (Ruch and Kerr 1982; Kindt and Halvorsen 1980). Furthermore, the methods previously used to determine IAA requirements in healthy populations have conceptual and technical limitations (Zello et al. 1995; Young 1994). The current recommendations for the intake of aromatic amino acids in PKU have tyrosine contributing 5 times the corresponding phenylalanine intake (median values), or 80% of the total aromatic amino acid recommended intake. This proportion is not supported by the literature which suggests that tyrosine and phenylalanine contribute almost equally to the total aromatic amino acid requirement (Tolbert and Watts 1963; House et al. 1997a; House et al. 1997b). Direct and Indicator amino acid oxidation are new techniques used to determine IAA requirements. The Indicator technique has been validated in healthy adults and can be used in conditions where direct oxidation of an amino acid is blocked due to an enzyme defect. The Indicator amino acid oxidation method is a potentially powerful tool which can be used to assess IAA requirements in PKU.

3.2. **Hypothesis**

The purpose of the research presented in this thesis was to test the following hypotheses:

(i) The tyrosine requirement in PKU accounts for approximately 45% of the total aromatic amino acid requirement.
(ii) The REE of individuals with hyperphenylalaninemia due to PKU is lower than controls.

3.3. Objectives

(i) To develop a primed continuous oral infusion protocol using L-[1-13C]-lysine that can accurately and reproducibly measure amino acid flux and oxidation within an eight hour period. The prime to constant infusion ratio for L-[1-13C]-phenylalanine will be determined for an oral infusion protocol and will then be used as a model on which to base the prime to constant infusion ratio for L-[1-13C]-lysine. Further, the duration of a baseline period that is adequate to obtain a constant enrichment of 13CO2 (coefficient of variability < 5.0%) without prior adaptation to the study diet must be determined.

(ii) To define the tyrosine requirements in children with classical PKU by examining the effect of incremental increases in dietary tyrosine on lysine oxidation and flux.

(iii) To measure the REE of patients with PKU and MSUD and of healthy controls and compare the results of the three groups with each other and with predicted values.

(iv) To compare the tryptophan:LNAA ratio (an indicator of serotonin production), urinary catecholamine levels and catecholamine metabolite levels (indicators of SNS activity) and determine their relation to REE (expressed as a percent of predicted REE) in patients with PKU and MSUD.
4. DEVELOPMENT OF A NONINVASIVE METHOD TO MEASURE INDICATOR AMINO ACID OXIDATION

4.1. Introduction

Advances in the use of stable isotopes have increased the availability of suitable methods for the study of human amino acid and protein metabolism. Direct and Indicator Amino Acid Oxidation are currently the only two tracer techniques that have been used to reassess the human adult requirements for several indispensable amino acids (Lazaris-Brunner 1994; Basile-Filho et al. 1997; Sanchez et al. 1995; Zello et al. 1990; Zhao et al. 1986; Zello et al. 1993; Meredith et al. 1986; Meguid et al. 1986; Meguid et al. 1986; el-Khoury et al. 1994a; el-Khoury et al. 1994b).

No reports were found in the literature of amino acid requirement estimates derived by oxidation techniques for infants or children. The experimental designs used in both oxidation protocols involve invasive and impractical interventions such as: intravenous infusion of the stable isotope labelled amino acid; measurement of precursor isotopic abundance in blood; and adaptation to an experimental, metabolic diet for 2 to 7 days. All of which preclude their use in these and other vulnerable groups.

Noninvasive methods have been incorporated into the stochastic model for the study of different aspects of protein and amino acid metabolism. Oral or intragastric infusion of isotope has been used to study amino acid and protein turnover in both infants and adults (Basile-Filho et al. 1997; Cortiella et al. 1992; Sanchez et al. 1995; Krempf et al. 1990; Wykes et al. 1992; Wykes et al. 1990; de Benoist et al. 1984; Waterlow et al. 1978; Picou and Taylor-Roberts 1969). Furthermore, urine has been used to sample plasma amino acid enrichment. De Benoist et al. (1984), Wykes et al. (1992) and Zello et al. (1994) have shown good correlation between plasma and urinary amino acid enrichments using the isotopes L-[1-13C] leucine and
[\textsuperscript{15}N] glycine, L-[\textsuperscript{1-13}C] phenylalanine and L-[\textsuperscript{1-13}C] leucine, and L-[\textsuperscript{1-13}C] phenylalanine, respectively, which eliminates the need for blood sampling for these labelled amino acids. With respect to adaptation to experimental diets, consumption of a diet deficient in or containing excessive amounts of an amino acid would be contraindicated in healthy infants and in specific clinical pediatric or adult populations. For example, lengthy exposure to an imbalanced amino acid intake could compromise metabolic control in individuals with an inborn error of amino acid metabolism.

Despite the development and application of noninvasive methods, no one study has combined these methods into a one day protocol that could be followed to study amino acid kinetics. The objective of the current study was therefore, to develop a simpler model that would allow the study of indispensable amino acid requirements by indicator amino acid oxidation in vulnerable groups. The goals of the model were to: (i) limit the need for the experimental diet to only the day of study; (ii) orally administer the isotope; and (iii) use urine to sample plasma amino acid enrichment. Successful development of a one day protocol will allow for the application of the indicator amino acid oxidation technique to be expanded to include groups previously excluded from study because of age or clinical condition.

4.2. Materials and Methods

4.2.1. Experimental Design

Four experiments were conducted. The objective of experiment 1 was to determine whether the experimental diet could be limited to only the day of the study. This was evaluated by determining the pattern and stability of background \textsuperscript{13}C enrichment in expired CO\textsubscript{2}, while subjects consumed the experimental diet without any prior adaptation. Six subjects participated in experiment 1. The objectives of experiments 2 and 3 were to determine
whether L-[1-\textsuperscript{13}C] phenylalanine and L-[1-\textsuperscript{13}C] lysine respectively, administered orally, could produce an isotopic steady state in plasma, urine and expired CO\textsubscript{2} within four hours. Five subjects each, participated in experiments 2 and 3. Experiment 4 was conducted to evaluate the impact of D-lysine in the tracer in Experiment 3. Two subjects participated in Experiment 4.

All subjects were screened by questionnaire for chronic diseases and atypical exercise or dietary habits. Subjects were nonsmokers who had maintained a stable body weight for several months and had taken no medication in recent weeks. Subject characteristics are shown in Table 4.1. Written informed consent was obtained for the study, which was approved by the University of Toronto Human Experimentation Committee and the Human Subject Review Committee of the Hospital for Sick Children (HSC), Toronto (Appendix 9.1.).

4.2.2. Diet and Study Protocols

The experimental diet was developed for amino acid kinetic studies and its composition has previously been reported in detail (Zello et al. 1990a). A flavoured liquid formula (protein-free powder, Product 80056, Mead Johnson, Evansville, IN; Tang, Don Mills, Ont.; Koolaid, Don Mills, Ont.) and protein-free cookies (HSC Research Kitchen) supplied the main source of energy in the diet. A crystalline amino acid mixture, based on the amino acid composition of egg protein, was consumed at 1.0 g·kg\textsuperscript{-1}·day\textsuperscript{-1} and provided the only source of amino nitrogen in the diet. The macronutrient composition of the experimental diet, expressed as a percent of dietary energy, was 53% carbohydrate, 38% fat and 9% protein. Vitamins and minerals were provided by Product 80056, Tang and Koolaid. The diets were prepared and weighed (Mettler Scale, model PE 2000, Switzerland) in the research kitchen of the HSC and were portioned into isoenergetic, isonitrogenous meals. The diet was consumed as hourly meals and each meal represented 1/12\textsuperscript{th} of the subjects total daily requirement. Total energy intakes were
Table 4.1. Subject Characteristics of Adult Volunteers

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (y)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>BMI$^1$ (kg·m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>27</td>
<td>60.9</td>
<td>162.6</td>
<td>23.03</td>
</tr>
<tr>
<td>MC</td>
<td>23</td>
<td>46.9</td>
<td>150.4</td>
<td>20.73</td>
</tr>
<tr>
<td>GZ$^2$</td>
<td>27</td>
<td>68.2</td>
<td>175.3</td>
<td>22.2</td>
</tr>
<tr>
<td>SR</td>
<td>28</td>
<td>55.8</td>
<td>164.5</td>
<td>20.62</td>
</tr>
<tr>
<td>JV</td>
<td>32</td>
<td>92.3</td>
<td>171.8</td>
<td>31.27</td>
</tr>
<tr>
<td>AD</td>
<td>25</td>
<td>67.5</td>
<td>164.1</td>
<td>25.07</td>
</tr>
<tr>
<td>JT</td>
<td>26</td>
<td>57.9</td>
<td>170.3</td>
<td>19.96</td>
</tr>
<tr>
<td>MR</td>
<td>32</td>
<td>40.3</td>
<td>145.6</td>
<td>19.01</td>
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<td>CM</td>
<td>32</td>
<td>63.0</td>
<td>165.4</td>
<td>23.03</td>
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<tr>
<td>WS</td>
<td>37</td>
<td>73.9</td>
<td>172.8</td>
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<td>50.8</td>
<td>165.5</td>
<td>18.56</td>
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<td>54.3</td>
<td>158.8</td>
<td>21.61</td>
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<tr>
<td>SRI</td>
<td>26</td>
<td>55.1</td>
<td>163.3</td>
<td>20.67</td>
</tr>
<tr>
<td>KL</td>
<td>33</td>
<td>66.3</td>
<td>164.0</td>
<td>24.72</td>
</tr>
</tbody>
</table>

Mean ±SEM: 28.4 ± 1.1  60.9 ± 3.4  163.9 ± 2.2  22.73 ± 0.94

$^1$ Body Mass Index

$^2$ GZ, male subject who participated in Experiment 1.
based on each subjects’ calculated resting metabolic rate (Food and Agriculture Organization of the United Nations-Rome 1985), multiplied by a factor of 1.7 (Bell et al. 1985). Subjects were instructed to maintain their usual level of physical activity and to fast for 10 - 12 hours overnight, prior to the study. Standing height was measured without shoes, to the nearest 0.1 cm, with a wall mounted stadiometer and subjects were weighed (Toledo Scale, model 2020, Winsor, Ontario) within one week of the study day and again on the morning of the study.

Figures 4.1. and 4.2. illustrate the protocols followed in the three experiments. On each study day subjects were maintained in a temperature controlled metabolic facility at the HSC. In experiment 1, $^{13}$CO$_2$ enrichment in expired breath was measured in six subjects while they consumed hourly meals of the experimental diet for a period of six hours. Simultaneous breath collections and VCO$_2$ measurements occurred at minutes 30, 75, 105, 135, 165, 195, 225, 255, 285, 315, and 345 while the subjects lay in a semirecumbant position on a hospital bed. The subjects breathed in a normal fashion while wearing a ventilated face mask (Scott 80216730, Sensormedics, Anaheim, CA). Once the subject’s air flow had stabilized (CO$_2$ concentration = 0.5-0.8%), the expired breath was collected using a vacuum extraction system (Pump VB0025, Vortex Blower, Spencer Turbine Company, Winsor, CT) combined with a gas flow meter. In order to trap the respiratory CO$_2$, the expired breath was bubbled at a rate of 500 mL/min through 10 mL of a 1 M NaOH solution in a modified reflux condenser for 7 minutes. The resulting NaH$^{13}$CO$_3$ solution was then injected (Monojet, Sherwood Medical, St. Louis, MO) into vacutainer glass tubes (Vacutainer Brand 6441, 100 x 16 mm, Becton Dickinson Inc., Mississauga, Ontario). The vacutainers were evacuated with a syringe of any air introduced during the injection and frozen at -20°C until analysis. In order to determine CO$_2$ volume, the CO$_2$ analyzer (Beckman Medical Gas Analyzer LB-2, Fullerton, CA) was calibrated using
Figure 4.1. Experimental Design
Figure 4.2. Experimental Design for Experiment 2 ([\(^{13}\)C] Phenylalanine) and Experiment 3 ([\(^{13}\)C] Lysine)
standardized gases (Nitrogen, Linde Medical Gas, Union Carbide, Toronto, Ontario and CO₂ and O₂, Linde Medical Gas) and barometric pressure and temperature were accounted for to ensure that CO₂ volumes were obtained at standard temperature and pressure.

In experiments 2 and 3 respectively, the following stable isotopes were used: L-[1-\(^{13}\)C]phenylalanine with an enrichment of 99% (Tracer Technologies, Somerville MA) and L-[1-\(^{13}\)C]lysine-HCl·H₂O with an enrichment of 99% (Cambridge Isotopes Laboratories, Woburn, MA). The chemical and isotopic purity of the labelled amino acids were confirmed by gas chromatography-mass spectrometry. Isometric purity (< 2% D-isomer) was assessed by gas chromatography using a chiral column for \([^{13}\text{C}]\) phenylalanine and by chiral high performance liquid chromatography for \([^{13}\text{C}]\) lysine. The stock solutions of \([^{13}\text{C}]\) phenylalanine (20 mg·mL⁻¹) and \([^{13}\text{C}]\) lysine (10 mg·mL⁻¹) were prepared with sterile water by passage through a 0.22 μm Millipore filter (Millipore, Bedford, MA) under a laminar flow hood, and then dispensed into multiple dose vials. Each batch was demonstrated to be sterile and free from bacterial growth over seven days in culture. Phenylalanine and lysine were selected for study because both amino acids have been shown to be suitable indicator amino acids in animal studies (Ball and Bayley 1984; Kim et al. 1983; Kim et al. 1983; Ball et al. 1986; Ball and Bayley 1986) and phenylalanine has been used successfully as an indicator amino acid in human studies (Lazaris-Brunner 1994; Duncan et al. 1996; Zello et al. 1993) of indispensable amino acid requirements.

As shown in Figure 4.2., hourly meals were consumed beginning at time minus 240 minutes. The level of dietary phenylalanine or lysine in the meals was reduced by an amount that corresponded to the amount of \([^{13}\text{C}]\) phenylalanine or \([^{13}\text{C}]\) lysine administered during the oral tracer infusion period. One hundred and fifty millilitres of water was consumed with each meal to ensure hourly production of urine. Subjects voided hourly but urine was not collected.
before time minus 60 minutes. Three baseline samples of breath and two urine samples were taken during the hour before the administration of the isotopes. The priming doses of \([^{13}\text{C}]\) phenylalanine (19.37 \(\mu\text{mol}\cdot\text{kg}^{-1}\)) or \([^{13}\text{C}]\) lysine (21.89 \(\mu\text{mol}\cdot\text{kg}^{-1}\)) were administered at time 0. Equal infusion doses of \([^{13}\text{C}]\) phenylalanine (4.24 \(\mu\text{mol}\cdot\text{kg}^{-1}\)) or \([^{13}\text{C}]\) lysine (4.79 \(\mu\text{mol}\cdot\text{kg}^{-1}\)) were administered every 30 minutes beginning 15 minutes after the prime. Isotope administration involved the subjects swallowing the prime or equal infusion dose, followed by water, which was used to rinse the tube which contained the isotope. Once the isotope infusion was started, hourly urine collections continued, breath collections occurred at minutes 45, 75, 105, 135, 165, 195, 210, and 225 and blood samples were taken at minutes 160, 200 and 240.

Hourly urine samples were mixed with 1 mL of 3.4 M HCl as preservative and stored at -20\(^\circ\)C until analysis. Breath collections and VCO\(_2\) measurements were performed as described above. Blood was collected via a 21 gauge needle inserted into a superficial dorsal vein in the left hand. The line was kept patent by administering heparin (10 USP Units/mL) between blood sampling. Arterialized venous blood was obtained by heating the hand inside a thermostatic chamber maintained at 60\(^\circ\)C for 15 minutes before the blood was sampled (Zello et al. 1990c). Three mL of the arterialized venous blood was drawn into heparinized syringes (Aspirator\textsuperscript{TM}, Marquest Medical Products, Englewood, CO). The blood samples were kept on ice until centrifugation at 4\(^\circ\)C. The plasma was then frozen at -20\(^\circ\)C until analysis.

Due to the presence of 1.6% D-[1-\(^{13}\text{C}\)] lysine in the lysine, an additional experiment was conducted in two parts. Part 1: 30 urine samples and 42 blood samples taken from two adult subjects participating in another amino acid kinetics study carried out in this lab were analysed to determine background \([^{13}\text{C}]\)lysine enrichment. Samples were taken during the baseline period of the protocol, after the subjects had consumed four hourly meals of the same experimental diet as described above, but prior to isotope administration. Part 2: two infusion
studies were conducted with L-[1-\textsuperscript{13}C]lysine (≤ 0.2% D-[1-\textsuperscript{13}C]lysine, Mass Trace Inc., Woburn, MA) to determine whether urine and plasma enrichments of [\textsuperscript{13}C]lysine were equal when D-lysine was not present in the infusion. The first of the two infusion studies was identical to the protocol described in Fig 4.2. The second study was conducted in the fasted state and involved a four hour oral primed equal dose L-[1-\textsuperscript{13}C] lysine infusion. Three paired urine and blood samples were collected in 30 minute intervals in the hour preceding the start of the isotope infusion (minutes -60, -30 and 0). Water was consumed with each dose of isotope to ensure adequate production of urine. The subjects voided but urine was not collected during the first 120 minutes of the infusion. Four paired urine and blood samples were collected during the final 120 minutes of the infusion protocol, at minutes 140, 180, 220 and 240, which corresponded to the time when isotopic plateau was achieved in the fed state lysine infusion protocol. For both parts of this experiment, expired breath was not collected. The procedures followed for collecting and storing urine and blood samples and for the administration of isotope were identical to the fed state lysine infusion study described above.

4.2.3. Analytical procedures

Plasma (200 \textmu L) and urine samples (500 \textmu L) were deproteinized and acidified with an equal volume of 20\% trichloroacetic acid (wt:vol) and centrifuged. Amino acids were separated from the supernatant by a cation exchange resin (Dowex 50W-X8, 100-200 mesh H\textsuperscript{+} form, Bio Rad Laboratories, Richmond, CA) and were derivatized by the method described by Patterson et al. (1991) to their N-heptafluorobutyryl n-propyl esters. Amino acid enrichment was measured on a gas chromatograph (Hewlett-Packard model 5890 Series II, Mississauga, Ont) attached to a quadrupole mass spectrometer (VG Trio-2). Separation of the amino acid derivatives was performed with helium as the carrier gas on a 30m X 0.32mm (inside diameter)
X 1.0 μm (film thickness) fused silica capillary column (HP5, Hewlett-Packard) coupled directly to the ion source, which was operated under conditions of negative chemical ionization with ammonia as the reactant gas. Each amino acid was analyzed by splitless injection, on an automatic sampler (HP 7673 injector). Selected ion chromatographs were obtained by monitoring mass-to-charge ratio of 383 and 384 for [13C]phenylalanine, and 560 and 561 for [13C]lysine, corresponding to the unenriched (m) and enriched (m+1), respectively. The areas under the peaks were integrated by a Digital DECp 450D,LP computer, using a Lab-Base program (VG Biotech, Altrincham, U.K.).

The percentage enrichment of the expired 13CO2 was measured on a dual inlet magnetic sector isotope ratio mass spectrometer (VG Micromass 602D, Cheshire, England) using techniques described in earlier work (Jones et al. 1986). Breath enrichments from baseline samples and from those taken during the isotope infusion were expressed as atoms percent excess (APE) 13CO2 over a reference standard of compressed CO2 gas.

4.2.4. Data Analysis

A stochastic model was used to evaluate phenylalanine and lysine kinetics (Waterlow et al. 1978a). Isotopic steady state in the metabolic pool was represented by plateaux in 13CO2 enrichment in breath, for [13C] phenylalanine and [13C] lysine, in [13C]phenylalanine and [13C] lysine enrichment in plasma and urine. This state was achieved in breath, urine and plasma by 120 minutes from the start of both the [13C]phenylalanine and [13C]lysine isotope infusions and was maintained to the end of the study at 240 minutes. The mean breath isotope enrichment values of the 3 baseline samples and the 5 plateau samples were used to determine atoms percent excess (APE) above baseline at isotopic steady state. The mean ratio of the enriched peak (m+1) to the unenriched peak (m) in urine for both baseline and plateau samples was.
used to calculate molecules percent excess (MPE) for $[^{13}\text{C}]$ phenylalanine and $[^{13}\text{C}]$ lysine (< 0.2% D-isomer). The mean ratio of the enriched peak (m+1) to the unenriched peak (m) in urine for baseline samples and the mean ratio of the enriched peak (m+1) to the unenriched peak (m) in plasma for plateau samples was used to calculate molecules percent excess (MPE) for $[^{13}\text{C}]$ lysine (1.6% D-isomer). Plasma enrichments at plateau were used because urinary enrichment of $[^{13}\text{C}]$lysine was affected by the presence of 1.6% D-$[^{13}\text{C}]$lysine in the infused $[^{13}\text{C}]$lysine. The background $[^{13}\text{C}]$lysine enrichment in urine was used with the plateau plasma enrichment to calculate MPE for $[^{13}\text{C}]$lysine due to interference from the D-isomer.

The isotopic enrichment was calculated using the following equation:

$$\text{APE} = \frac{R_s - R_b}{1 + R_s - R_b} \times 100$$

where $R_s$ and $R_b$ were the ratios of (m+1) to (m) for the enriched sample and sample at natural abundance respectively (Rosenblatt et al. 1992; Matthews et al. 1980).

Flux (Q) was measured from the dilution of the infused tracer L-$[^{13}\text{C}]$phenylalanine and L-$[^{13}\text{C}]$lysine in the plasma and urine metabolic pools at isotopic steady state:

$$Q = i \left[ \frac{E_i}{E_p} - 1 \right]$$

where $i$ was the rate of $[^{13}\text{C}]$phenylalanine or $[^{13}\text{C}]$lysine isotope infused ($\mu$mol.kg$^{-1}$.h$^{-1}$), $E_i$ and $E_p$ were the enrichments of infused and urine or plasma $[^{13}\text{C}]$phenylalanine or $[^{13}\text{C}]$lysine, molecules percent excess (MPE), at isotopic steady state (Matthews et al. 1980).

The rate of phenylalanine or lysine oxidation (O, $\mu$mol.kg$^{-1}$.h$^{-1}$) was calculated using the equation:

$$O = F_{[^{13}\text{C}]\text{CO}_2} \left( \frac{1}{E_p} - 1 \right) \times 100$$

where $F_{[^{13}\text{C}]\text{CO}_2}$ was the rate of release of $^{13}\text{CO}_2$ by the oxidation of the tracer $[^{13}\text{C}]$phenylalanine or $[^{13}\text{C}]$lysine ($\mu$mol.$^{13}\text{CO}_2$.kg$^{-1}$.h$^{-1}$) (Matthews et al. 1980). $F_{[^{13}\text{C}]\text{CO}_2}$ was calculated as follows:
\[ F^{13}\text{CO}_2 = (FCO_2) (ECO_2) (44.6) (60) / (W) (0.82) (100) \]

where FCO₂ was the production rate of CO₂ (ml.min⁻¹); ECO₂ was the \(^{13}\text{CO}_2\) enrichment in expired breath at isotopic steady state (APE); and W was the weight (kg) of the subject (Matthews et al. 1980). The constants 44.6 \(\mu\text{mol.ml}^{-1}\) and 60 min.h⁻¹, converted FCO₂ to micromoles per hour and the factor 100 changed APE to a fraction. The correction factor of 0.82 represented the \(^{13}\text{CO}_2\) that was released by \([^{13}\text{C}]\text{phenylalanine}\) or \([^{13}\text{C}]\text{lysine}\) oxidation, but not liberated from the body bicarbonate pool into expired air. This correction factor was based on the fraction of infused Na\([^{13}\text{C}]\text{bicarbonate}\) recovered in the breath of adult males under fed state conditions (Hoerr et al. 1989).

4.2.5. **Statistical Analysis**

Results are expressed as mean ± standard error of the mean (SEM). In experiment 1, the significance of the change in background APE of \(^{13}\text{C}\) over the six hour study period was tested by regression analysis and by one way analysis of variance (ANOVA) on the data from individual subjects. In experiments 2 and 3, isotopic steady state in the metabolic pool was represented by plateaux in urine and plasma for \([^{13}\text{C}]\text{phenylalanine}\), in plasma for \([^{13}\text{C}]\text{lysine}\), and in breath \(^{13}\text{CO}_2\) for both labelled amino acids. In experiment 4, for \([^{13}\text{C}]\text{lysine}\) (< 0.2% D-isomer), isotopic steady state was represented by plateaux in urine and plasma. Attainment of isotopic steady state (plateau) was evaluated in each subject in breath, plasma and urine data. Plateau was evaluated by visual inspection followed by repeated linear regression analysis in which data points, beginning at time 0 minutes, were removed until a regression line with a slope not different from zero was achieved. Similar data analysis could not be performed for urinary amino acid enrichment in the fed-state \([^{13}\text{C}]\text{phenylalanine}\) and \([^{13}\text{C}]\text{lysine}\) (1.6% D-isomer) studies, since only two urine collections were obtained during the last 2 hours of the
isotopic infusions. In experiments 2, 3, and 4, the difference in amino acid enrichment of plasma and urine was evaluated by paired t-test. For each subject, the mean ratio of amino acid enrichment in plasma to the mean ratio of amino acid enrichment in urine was calculated for [13C]phenylalanine and [13C]lysine. The difference of the mean ratio from 1 was evaluated by t-test. The relationship between enrichment in plasma and urine of each amino acid was also assessed by linear regression analysis. For the [13C] lysine (< 0.2% D-isomer) infusion studies carried out in experiment 4, the enrichments in each pair of urine and plasma samples were compared by paired t-test and the differences in the ratio of plasma to urine enrichment from 1 was compared by t-test. Agreement between plasma and urine [13C] phenylalanine and [13C] lysine enrichment was also assessed by the method of Bland and Altman (1986) in which the difference between plasma and urine enrichment is plotted against the mean of the two measurements. The limits of agreement and the presence of a systematic error in one measurement (urine) against the other standard measurement (plasma) are determined. Results were considered statistically significant at p values less than or equal to 0.05.

4.3. Results

Complete data sets of 11 breath samples were obtained in four subjects. The final CO₂ sample in subject RB was unsuccessfully trapped in NaOH and subject GZ became ill for reasons unrelated to the experiment and did not provide the final two samples. The effect of diet on background ¹³CO₂ enrichment in each subject is shown in Table 4.2. The effect of small hourly meals on background ¹³C enrichment in breath CO₂ in six subjects is shown in Figure 4.3. The mean change in background ¹³CO₂ enrichment for the six subjects after four meals was 0.003471 ± 0.00041 APE and after six meals for five subjects was 0.003505 ± 0.00033 APE. This corresponds to a ~20% increase in ¹³CO₂ enrichment due solely to the
Table 4.2. Experiment 1: Effect of Experimental Diet on $^{13}$CO$_2$ Enrichment in Expired Breath, Change in Enrichment and Slope of Enrichment Curve During Isotopic Equilibrium

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>$^{13}$CO$_2$ Enrichment in Expired Breath (A.P.E.) Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RB</td>
</tr>
<tr>
<td>30</td>
<td>0.0172</td>
</tr>
<tr>
<td>75</td>
<td>0.0173</td>
</tr>
<tr>
<td>105</td>
<td>0.0173</td>
</tr>
<tr>
<td>135</td>
<td>0.0189</td>
</tr>
<tr>
<td>165</td>
<td>0.0195</td>
</tr>
<tr>
<td>195</td>
<td>0.0199</td>
</tr>
<tr>
<td>225</td>
<td>0.0208</td>
</tr>
<tr>
<td>255</td>
<td>0.0208</td>
</tr>
<tr>
<td>285</td>
<td>0.0204</td>
</tr>
<tr>
<td>315</td>
<td>0.0208</td>
</tr>
<tr>
<td>345</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Change in $^{13}$CO$_2$ (Final - Initial)</th>
<th>0.0036</th>
<th>0.0036</th>
<th>0.0042</th>
<th>0.0037</th>
<th>0.0055</th>
<th>0.0018</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to reach plateau$^1$ (minutes)</td>
<td>225</td>
<td>255</td>
<td>225</td>
<td>195</td>
<td>255</td>
<td>225</td>
</tr>
<tr>
<td>Slope$^2$ (225 - 360 minutes)</td>
<td>$5 \times 10^{-6}$</td>
<td>$6 \times 10^{-6}$</td>
<td>$0.14 \times 10^{-6}$</td>
<td>$3 \times 10^{-6}$</td>
<td>$0.12 \times 10^{-6}$</td>
<td>$6 \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>$p = 0.32$</td>
<td>$p = 0.13$</td>
<td>$p = 0.22$</td>
<td>$p = 0.22$</td>
<td>$p = 0.19$</td>
<td>$p = 0.13$</td>
</tr>
</tbody>
</table>

$^1$ Plateau was determined by visual inspection.

$^2$ Slope was determined by linear regression analysis. $P > 0.05$, slope of enrichment curve not significantly different from zero between 225 and 360 minutes.
Figure 4.3. Experiment 1: Effect of Experimental Diet on $^{13}$CO$_2$ Enrichment (open squares) and on Rate of CO$_2$ Production (VCO$_2$) (filled squares). Plateau in $^{13}$CO$_2$ enrichment and VCO$_2$ was achieved for all subjects beginning at 225 minutes. Slopes of the $^{13}$CO$_2$ enrichment or VCO$_2$ versus time regression lines were not significantly different from zero, $p>0.05$. 
experimental diet. One way ANOVA on the change in $^{13}$CO$_2$ enrichment from baseline showed that there was a significant effect of meal consumption on background $^{13}$C enrichment in expired CO$_2$ ($p<0.0001$). The change in background enrichment between 225 and 360 minutes was almost significant ($p=0.0525$), suggesting that isotopic steady state had not been reached by 225 minutes. Between 225 and 255 minutes, background enrichment did not change significantly ($p=0.1$), suggesting that isotopic steady state began at 255 minutes. The mean difference in enrichment between 225 and 255 minutes was negligible (0.0003608 APE), and accounted for only 1.69% of the mean enrichment at time 255 minutes. For each subject, the slope of the line for breath $^{13}$CO$_2$ enrichment data collected after 225 minutes, was not significantly different from zero (Table 4.2.). For data collected between 225 and 360 minutes, the coefficient of variation between points within a slope was 1.52 - 3.31%.

Meal consumption had a significant effect on VCO$_2$ such that VCO$_2$ increased from 230 ± 15 mL/min to 282 ± 24 mL/min ($p=0.002$) between the first and last breath samples (Figure 4.3.). VCO$_2$ increased with each meal until 225 minutes, after which VCO$_2$ did not change significantly until the end of the study.

Mean isotopic enrichment of breath $^{13}$CO$_2$ measured during the $[^{13}$C] phenylalanine and $[^{13}$C] lysine infusion studies is shown in Figure 4.4. Isotopic steady state was achieved in breath $^{13}$CO$_2$ by 120 minutes from the start of the $[^{13}$C] phenylalanine and by 150 minutes from the start of the $[^{13}$C] lysine infusions and was maintained to the end of the study at 240 minutes. The oral infusion protocol for $[^{13}$C] phenylalanine and $[^{13}$C] lysine produced mean enrichment curves for data collected between 120 and 240 minutes, with slopes not significantly different from zero ($p=0.73$ for $[^{13}$C]phenylalanine and $p=0.16$ for $[^{13}$C]lysine). The coefficients of variation for breath $^{13}$CO$_2$ enrichment determinations for baseline and plateau
Figure 4.4. Experiment 2 & 3: $^{13}\text{CO}_2$ Enrichment in Expired Breath Following Oral, Primed, Equal Dose Infusions of L-[1-$^{13}\text{C}$] Phenylalanine (filled squares) or L-[1-$^{13}\text{C}$] Lysine (1.6% D-isomer) (open squares).
enrichments were, respectively, 2.90 ± 0.53% and 2.61 ± 0.23% for the \([^{13}C]\)phenylalanine infusion and 2.67 ± 0.76% and 2.40 ± 0.30% for the \([^{13}C]\)lysine infusion.

From the 30 urine and 42 plasma samples taken from subjects participating in another study in the same laboratory (experiment 4), the mean ratio of plasma to urine \([^{13}C]\)lysine enrichment was 0.994 ± 0.008, which was not significantly different from 1 (p = 0.49). Furthermore, the mean baseline urinary enrichment from these 30 samples was not significantly different from the mean baseline urinary enrichment of \([^{13}C]\) lysine in the 5 subjects who participated in experiment 3 (0.229097 ± 0.00212 APE vs 0.231681 ± 0.00176 APE, p = 0.36), both of which reflected the natural enrichment in the dietary source of lysine (0.228291 ± 0.00093 APE).

The mean isotopic enrichment of \([^{13}C]\) phenylalanine and \([^{13}C]\) lysine in urine before isotope infusion and at isotopic steady state in plasma and urine is shown in Table 4.3. Plasma samples were not available for one subject (AD) in experiment 2 during the expected plateau period. At isotopic steady state, plasma \([^{13}C]\)phenylalanine was similar to that of urine (p=0.31) and the P:U ratio was not different from 1 (p=0.36). From the Bland and Altman (1986) analysis, the mean bias in \([^{13}C]\) phenylalanine enrichment between urine and plasma was 0.015 MPE, with the limits of agreement (mean bias ± 2 standard deviations) ranging from 0.054 to 0.085. This mean bias represents 2.5 to 4.8% of the range of average enrichments observed at plateau in plasma. Conversely, the mean plasma \([^{13}C]\)lysine enrichment in subjects who received the \([^{13}C]\)lysine which contained 1.6% D-[\(^{13}C\)]lysine was approximately 1/3 that of urine (p=0.002) and the resulting P:U ratio was significantly different from 1 (p<0.0001). Independent qualitative analysis of the \([^{13}C]\)lysine isotope, a plateau urine sample

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Table 4.3. Mean Isotopic Enrichment (moles percent) of L-[1-13C] Phenylalanine and L-[1-13C] Lysine in Urine (U) and Plasma (P) Before Isotope Infusion and at Isotopic Steady State

<table>
<thead>
<tr>
<th>Amino Acid Tracer</th>
<th>[13C] Phenylalanine</th>
<th>[13C] Lysine (D-lysine 1.6%)</th>
<th>[13C] Lysine (D-lysine &lt;0.2%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Infusion</td>
<td>n=5</td>
<td>n=5</td>
<td>n=2</td>
</tr>
<tr>
<td>Isotopic Enrichment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>22.21 ± 0.09</td>
<td>23.11 ± 0.11</td>
<td>21.49 ± 0.38</td>
</tr>
<tr>
<td>Plasma</td>
<td>ND</td>
<td>ND</td>
<td>21.91 ± 0.55</td>
</tr>
<tr>
<td>Enrichment Ratio (P:U)</td>
<td>ND</td>
<td>ND</td>
<td>0.98 ± 0.04 (NS)³</td>
</tr>
<tr>
<td>Isotopic Steady State</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isotopic Enrichment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>40.71 ± 5.41</td>
<td>92.14 ± 9.04</td>
<td>30.35 ± 0.53</td>
</tr>
<tr>
<td>Plasma</td>
<td>43.22 ± 7.45</td>
<td>35.55 ± 2.22</td>
<td>31.39 ± 0.55</td>
</tr>
<tr>
<td>Enrichment Ratio (P:U)</td>
<td>1.05 ± 0.04²</td>
<td>0.39 ± 0.03 (P&lt;0.0001)⁴</td>
<td>0.97 ± 0.02 (P=0.27)³</td>
</tr>
</tbody>
</table>

¹ mean ± SEM. Each value represents the mean of the subjects studied in experiments 2, 3, or 4. ND, not determined. NS, not significant, p>0.05.

² P:U ratio is based on the mean of 4 subjects (JT, MR, WS, CM).

³ NS, p=0.36, p=0.27, P:U ratios not different from 1.

⁴ p<0.0001, P:U ratio significantly different from 1.
and a plateau plasma sample, by electron impact GCMS using a chiral column (Mass Trace Inc., Woburn, MA), revealed a significant m+1 peak of D-[1-13C]lysine in the urine sample. Although the results were not quantitative, the plasma sample contained only negligible amounts of the D-isomer. At isotopic steady state, mean plasma [13C]lysine enrichment in the 2 subjects who received the [13C]lysine with less than 0.2% D-isomer, was similar to that of urine (p=0.24) and the P:U ratio was not different from 1 (p=0.27). Furthermore, the Bland and Altman (1986) analysis confirmed this agreement, with a mean bias in [13C] lysine enrichment in urine and plasma of 0.0007 (limits of agreement ranged from 0.044 to 0.045). The mean bias represents 0.23 to 0.35% of the range of average enrichments observed at plateau in plasma.

Figures 4.5a and 4.5b illustrate that the plateau isotopic enrichment of amino acids in plasma was significantly correlated with the enrichment in urine for [13C]phenylalanine (r²=0.9981, p=0.0019), however, no correlation was observed for [13C]lysine with 1.6% D-isomer (r²=0.2562, p=0.384).

Flux and oxidation rates for [13C]phenylalanine and [13C]lysine are shown in Table 4.4 and 4.5. Results from the current study are compared to those reported in the literature, where the ratio of phenylalanine or lysine intake to total protein intake was kept constant.

4.4. Discussion

This investigation was prompted by the lack of indispensable amino acid requirement estimates derived by oxidation techniques in infants and children. This lack is due in part to invasive and impractical experimental designs that preclude their use in these and other vulnerable groups. The main objective of the present study was to develop a one-dimensional noninvasive isotope infusion method that would produce the necessary isotopic steady state.
Figure 4.5. Relationship Between Isotopic Enrichment (mole percent excess, M.P.E.) of (A) L-[13-C] Phenylalanine (filled squares) and (B) [1-13C] Lysine (1.6 % D-[1-13C] lysine) (open squares) in Plasma and Urine (symbols represent sample pairs).
Table 4.4. Phenylalanine Oxidation and Flux From the Present Study Compared to Data Reported in the Literature

<table>
<thead>
<tr>
<th>Reference</th>
<th>Protein Intake (g·kg⁻¹·d⁻¹)</th>
<th>Phenylalanine Intake (mg·kg⁻¹·d⁻¹)</th>
<th>Isotope</th>
<th>Route of Administration</th>
<th>Oxidation (μmol·kg⁻¹·h⁻¹)</th>
<th>Flux (μmol·kg⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present study</td>
<td>1</td>
<td>14</td>
<td>[1-¹³C]Phe</td>
<td>O</td>
<td>2.27 ± 0.47</td>
<td>49.24 ± 13.69</td>
</tr>
<tr>
<td>Zello et al (1993)</td>
<td>1</td>
<td>14</td>
<td>[1-¹³C]Phe</td>
<td>IV</td>
<td>1.58 ± 0.25</td>
<td>40.6 ± 2.2</td>
</tr>
<tr>
<td>Duncan et al (1996)</td>
<td>0.8</td>
<td>14</td>
<td>[1-¹³C]Phe</td>
<td>IV</td>
<td>2.13 ± 0.27</td>
<td>35.0 ± 2.17</td>
</tr>
<tr>
<td>Lazaris-Brunner (1994)</td>
<td>1</td>
<td>14</td>
<td>[1-¹³C]Phe</td>
<td>IV</td>
<td>2.34 ± 0.29</td>
<td>37.78 ± 2.02</td>
</tr>
<tr>
<td>Sanchez et al (1995)</td>
<td>1</td>
<td>21.9</td>
<td>[1-¹³C]Phe</td>
<td>O</td>
<td>0.93 ± 0.31</td>
<td>46.8 ± 3.58</td>
</tr>
</tbody>
</table>

¹ mean ± SEM. Phe, phenylalanine. O, oral infusion of isotope. IV, intravenous infusion of isotope.
Table 4.5. Lysine Oxidation and Flux From the Present Study Compared to Data Reported in the Literature

<table>
<thead>
<tr>
<th>Reference</th>
<th>Protein Intake (g·kg⁻¹·d⁻¹)</th>
<th>Lysine Intake (mg·kg⁻¹·d⁻¹)</th>
<th>Isotope</th>
<th>Route of Administration</th>
<th>Oxidation (μmol·kg⁻¹·h⁻¹)</th>
<th>Flux (μmol·kg⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present study</td>
<td>1.0</td>
<td>54.06</td>
<td>[1-¹³C]Lys</td>
<td>O</td>
<td>9.33 ± 1.27</td>
<td>76.38 ± 11.38</td>
</tr>
<tr>
<td>Zello et al (1992)</td>
<td>1.0</td>
<td>78.8</td>
<td>[1-¹⁵N]Lys</td>
<td>IV</td>
<td>-</td>
<td>98.80 ± 4.70</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>61.8</td>
<td></td>
<td></td>
<td></td>
<td>88.50 ± 6.70</td>
</tr>
<tr>
<td>Conway et al (1980)</td>
<td>0.8</td>
<td>72.07</td>
<td>[1-¹⁵N]Lys</td>
<td>IV</td>
<td>-</td>
<td>93.00 ± 9.0</td>
</tr>
<tr>
<td>Motil et al (1981)</td>
<td>0.6</td>
<td>83.9</td>
<td>[1-¹⁵N]Lys</td>
<td>IV</td>
<td>-</td>
<td>87.80 ± 4.80</td>
</tr>
<tr>
<td>Motil et al (1981a)</td>
<td>0.6</td>
<td>83.9</td>
<td>[1-¹⁵N]Lys</td>
<td>IV</td>
<td>-</td>
<td>90.50 ± 2.00</td>
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<tr>
<td>Motil et al (1994)</td>
<td>1.0</td>
<td>108.7</td>
<td>[1-¹⁵N]Lys</td>
<td>IV</td>
<td>-</td>
<td>118.50</td>
</tr>
</tbody>
</table>

¹ mean ± SEM. Lys, lysine. O, oral infusion of isotope. IV, intravenous infusion of isotope.
conditions required to estimate amino acid kinetic parameters. This method could in turn be used to estimate indispensable amino acid requirements in vulnerable populations by Indicator Amino Acid Oxidation.

The goal of the first experiment was to determine the duration of a fed state baseline period that would produce a stable VCO₂ and a constant enrichment of ¹³CO₂ in breath without prior adaptation to the experimental diet. In experiments 2 and 3, the goals were to develop an oral primed, equal dose infusion protocol using either L-[1-¹³C] phenylalanine or L-[1-¹³C] lysine that could produce isotopic steady state in breath and urine and thus allow for the estimation of amino acid flux and oxidation. These infusion studies were conducted under steady fed state conditions and followed the baseline protocol determined in experiment 1. The stable isotope L-[1-¹³C] phenylalanine has been shown to be an acceptable tracer in studies of amino acid flux and oxidation in infants and adults using urine (Wykes et al. 1992; Zello et al. 1994) and in adults using plasma (Sanchez et al. 1995; Zello et al. 1993; Zello et al. 1990; Lazaris-Brunner 1994; Duncan et al. 1996). L-[1-¹³C] lysine has also been used as a tracer in amino acid kinetic studies (Thomas et al. 1991; Irving et al. 1986; Robert et al. 1982; Nair et al. 1992), but the use of urine to sample the arterialized plasma enrichment has not been verified for lysine. This series of experiments represents the first attempt to combine several noninvasive methods and incorporate them into an amino acid oxidation technique applicable to vulnerable populations.

Measurement of ¹³CO₂ following administration of a ¹³C-labelled substrate occurs in the presence of a large background of naturally occurring isotope of approximately 1.1% ¹³C (Schoeller et al. 1980). The abundance of ¹³C in breath varies depending on substrate oxidation and on the enrichment of ¹³C in the diet (Bray 1989). Because there is a natural background of ¹³C in expired breath, the labelled CO₂, derived from oxidation of the ¹³C-
labelled substrate, is actually the amount of \(^{13}\text{C}_2\) in excess of the natural background \(^{13}\text{C}_2\) (Schoeller et al. 1980). The background \(^{13}\text{C}\) enrichment in breath must therefore be measured prior to isotope administration. The stability of background \(^{13}\text{C}_2\) during the study period can be achieved by consuming a diet with a fixed percentage of \(^{13}\text{C}\) and by consuming the diet as equally spaced, identical meals, thereby ensuring that the rate of substrate oxidation is kept constant throughout the measurement period.

Results from experiment 1 show that consumption of the experimental diet increased \(^{13}\text{C}_2\) and \(\text{CO}_2\) production between 0 and 225 minutes. A constant background \(^{13}\text{C}\) enrichment in breath and a stable rate of \(\text{CO}_2\) production was achieved between 225 and 255 minutes and was maintained through to the end of the study. The experimental diet increased both \(^{13}\text{C}_2\) enrichment and \(\text{VCO}_2\) from the first to the last measurement by approximately 20%. One way ANOVA of the change in \(^{13}\text{C}_2\) enrichment showed a borderline significance from data collected between 225 and 360 minutes, but showed no significance for data collected between 255 and 360 minutes. This might suggest that five hourly meals are required to achieve a constant \(^{13}\text{C}_2\) enrichment, however, the change in enrichment between 225 and 255 minutes represented less than 2% of the average \(^{13}\text{C}_2\) enrichment at 255 minutes. The difference between the enrichment at 225 and 255 minutes is less than the measurement error associated with the isotope ratio mass spectrometer. Furthermore, the slope of the \(^{13}\text{C}_2\) enrichment curve measured between 225 and 360 minutes in each individual was not significant.

Using the same experimental diet, Zello et al. (1990a) measured breath \(^{12}\text{C}_2\) content in four adult males while consuming hourly meals of the diet, after having received the same diet for two days. From 30 minutes after the first meal, to 30 minutes after the sixth meal, no effect of time on background \(^{13}\text{C}_2\) enrichment was detected. Both endogenous and
exogenous substrates are oxidized prior to the establishment of a steady fed state. Therefore, the \(^{13}\text{C}\) content of the endogenous substrates in the Zello et al. study (Zello et al. 1990a) must be the result of prior consumption of the experimental diet, in order for the \(^{13}\text{CO}_2\) enrichment to be constant for the duration of the study. With the same experimental diet, but without prior consumption of the diet, \(~3.7\) hours and \(~4\) meals are needed to establish the same constancy in expired \(^{13}\text{CO}_2\) content. Presumably, this was the time required to shift from oxidizing endogenous substrates, to oxidizing a mixture of endogenous and exogenous substrates and finally, to oxidizing only exogenous substrates. This suggests that the experimental diet consumed as four meals over four hours and measurement of \(^{13}\text{CO}_2\) enrichment and \(\text{VCO}_2\) during the last 20 minutes of the fourth hour could be used as a baseline protocol to precede an isotope infusion study. This protocol could be used in place of a longer adaptation period in populations where adaptation to an imbalanced metabolic diet is prohibited. The effect of the macronutrient composition of an individual's usual intake on amino acid kinetic parameters is not addressed by this experiment. Therefore, at the present time the application of this baseline protocol is restricted to those populations whose habitual diets are relatively constant and similar, and where the macronutrient composition of the experimental diet can be matched to the population's usual intake. Inborn errors of amino acid metabolism and insulin dependent diabetes mellitus are examples of such populations and represent groups whose indispensable amino acid requirements have not been assessed.

Oral or intragastric infusion of amino acid tracers such as \(^{15}\text{N}\) glycine, \(^{13}\text{C}\) leucine and \(^{13}\text{C}\) phenylalanine, have produced isotopic equilibrium in urine, plasma and breath \(\text{CO}_2\) within 2 to 60 hours (Basile-Filho et al. 1997; Cortiella et al. 1992; Sanchez et al. 1995; Krempf et al. 1990; Wykes et al. 1992; Wykes et al. 1990; de Benoist et al. 1984; Waterlow et al. 1978; Picou and Taylor-Roberts 1969). A significant amount of amino acid metabolism occurs in
splanchnic tissues (Krempf et al. 1990). Matthews et al. (1993) reported that there is a significant first pass uptake by the splanchnic bed of an enterally derived phenylalanine tracer in healthy adults, with a significant oxidation of phenylalanine during its initial passage through the liver. Sanchez et al. (1995) also reported significantly higher rates of phenylalanine oxidation when L-[1-13C] phenylalanine was given orally as compared to when it was administered intravenously. Conversely, Hoerr et al. (1991) found similar rates of leucine oxidation when L-[1-13C] leucine was administered by vein or via the intestine. Matthews et al. (1993) suggest that the fraction of amino acid taken up during first pass that is irreversibly oxidized is apparently greater for phenylalanine than for leucine. These findings relate in part, to the fact that while leucine is catabolized to a great extent in the peripheral tissues (Harper et al. 1997), phenylalanine oxidation occurs primarily in the liver (Udenfriend and Cooper Jr. 1952). Sanchez et al. (1995) concluded that the oral tracer technique allows for a truer estimate of phenylalanine oxidation than the intravenous protocol. This conclusion may also apply to other amino acids predominantly oxidized in the liver, for example, lysine.

Development of an oral infusion protocol for phenylalanine and lysine appears to be doubly justified, in terms of the ability to study infants and children and in terms of the accurate estimation of oxidation rates.

Oral, primed, equal dose infusion of L-[1-13C] phenylalanine produced isotopic steady states in urine, plasma and breath CO₂, similar to steady states produced by intravenous tracer protocols (Duncan et al. 1996; Zello et al. 1990; Zello et al. 1993; Zello et al. 1994). The similarity between the enrichment of 13C phenylalanine in plasma and urine is consistent with the results of previous studies (Wykes et al. 1990; Zello et al. 1994). As in the Zello et al. (1994) study, the urinary 13C phenylalanine enrichment was approximately 5% lower than in plasma (P:U enrichment ratio = 1.05) at isotopic equilibrium. The P:U ratio was not
significantly different from 1, the correlation \( R^2 = 0.99 \) was highly significant and the mean bias in enrichment suggested good agreement between the two measurements. The nonsignificant 5% difference in enrichment observed in this study may be the result of the urine measurements being an average value obtained from the small amount of amino acids excreted in the urine over hourly periods, whereas the plasma represented the enrichment of the plasma metabolic pool at a specific time and sampling site. More frequent urine samples would likely bring the P:U ratio closer to 1 by providing a better average value for the plasma enrichment. Zello et al. (1994) suggested that the lower enrichment in urine versus plasma was because isotopic steady state had not been reached in urine during the four hour infusion period. However, in both the Zello et al. (1994) study and the current study, isotopic steady state in breath \(^{13}\text{CO}_2\) was achieved in the last two hours of the four hour infusion. Isotopic steady state in expired \(^{13}\text{CO}_2\) reflects a steady state in the \(^{13}\text{C}\) bicarbonate pool, which in turn reflects a steady state in the precursor \(^{13}\text{C}\) phenylalanine pool. Since isotopic steady state was achieved in the secondary body pool (bicarbonate), it is unlikely that steady state was not achieved in the primary pool.

Our estimates of phenylalanine flux and oxidation are similar to those reported in the literature, where the phenylalanine intake was constant in relation to the total protein intake (Table 4.4.) (Sanchez et al. 1995; Lazaris-Brunner 1994; Duncan et al. 1996; Zello et al. 1993). As in a previous study, in which all subjects were female (Lazaris-Brunner 1994), flux rates of the five female subjects in this study were not different from data obtained in male subjects. Comparison of the studies from this laboratory (Lazaris-Brunner 1994; Duncan et al. 1996; Zello et al. 1993), where phenylalanine and tyrosine intakes were the same as in this study (phenylalanine, 14.0 mg·kg\(^{-1}\)·day\(^{-1}\) and tyrosine, 40.0 mg·kg\(^{-1}\)·day\(^{-1}\)), revealed similar rates of phenylalanine oxidation. Phenylalanine oxidation in this study was approximately two times
greater than that of Sanchez et al. (1995) despite a lower phenylalanine intake (14 mg·kg⁻¹·d⁻¹ vs. 21.9 mg·kg⁻¹·d⁻¹, respectively). However, tyrosine intake in this study was significantly higher than in the Sanchez et al. (1995) study (40.0 mg·kg⁻¹·d⁻¹ vs. 5.3 mg·kg⁻¹·d⁻¹, respectively). Tyrosine intake of 40 mg·kg⁻¹·day⁻¹ has been demonstrated to minimize the conversion of phenylalanine to tyrosine (Zello et al. 1990b). Furthermore, in animals, when tyrosine was present in excess, tyrosine derived from the hydroxylation of phenylalanine in the liver was oxidized without first equilibrating with the tyrosine in the plasma pool (Moldawer et al. 1983). These studies suggest that the lower rate of phenylalanine oxidation observed by Sanchez et al. (1995) was due to conversion of phenylalanine to tyrosine without subsequent oxidation. Phenylalanine therefore, provided a source of tyrosine for protein synthesis in the presence of an inadequate dietary tyrosine intake. In the present study, with excess dietary tyrosine, phenylalanine was not needed as a source for tyrosine and any excess phenylalanine consumed above that which was needed for protein synthesis, was oxidized. Hence the rate of phenylalanine oxidation was higher. Flux rates are similar, perhaps because an equivalent proportion of dietary phenylalanine was removed from the phenylalanine pool either via complete oxidation (in the present study) or hydroxylation to tyrosine (Sanchez study). The similarity between the phenylalanine kinetic data in the present study and data from the literature (in which phenylalanine intakes are similar), suggests that the oral, primed, equal dose infusion protocol with measurement of isotopic enrichment in urine and breath is a viable alternative for the measurement of phenylalanine kinetics in populations where more invasive methods are contraindicated.

Oral equal dose infusion of [1-¹³C] lysine (1.6% D-isomer) produced isotopic steady states in breath ¹³CO₂ and plasma ¹³C lysine. Urinary and plasma isotope enrichments were significantly different, with urinary enrichments being 61% higher. These data could be
interpreted to indicate that $^{13}$C lysine is not a good tracer to use in studies of amino acid kinetics when the free amino acid pool to be sampled is in urine. However, baseline data from Experiment 4 clearly showed comparable enrichment of $^{13}$C lysine in urine and plasma before isotope administration. Urinary $^{13}$C lysine enrichment was less than 1% higher than in plasma, which indicates that the process of renal filtration and reabsorption was not affected by the naturally occurring isotope. If $^{13}$C isotopes were biologically discriminated against by transport mechanisms in the kidney, they would be preferentially retained or eliminated over the long term. This would result in the baseline urine samples having a higher or lower enrichment compared with plasma samples. If plasma and urinary enrichments are similar before an isotopic infusion, then the enrichment in urine should be reflective of the enrichment in the plasma pool during an infusion of amino acid tracers, provided that there is no isotopic effect due to the tracer. Therefore, the difference in plasma and urine enrichment during infusion of $[1^{-13}C]$ lysine could be attributed to the presence of 1.6% D-$[1^{-13}C]$ lysine in the $^{13}$C lysine tracer that was administered and not due to an isotopic effect of the $^{13}$C lysine tracer itself.

Amino acids in arterial blood are freely filtered through the glomerulus, and active transport carriers in the proximal tubules reabsorb nearly 100% of the amino acids from tubular fluids (Crim and Munro 1994; Souba and Pacitti 1992). However, since these transporters are specific to the L-isomers of the amino acids, D-isomers are not reabsorbed and are lost in the urine. Discrimination in the process of reabsorption would account for the higher urinary enrichment of $^{13}$C lysine as compared to plasma. Even if discrimination of the D-$[1^{-13}C]$ lysine at the lumen of the proximal tubule was quantitatively small, the effect on the enrichment ratio would be large because of the low concentration of free amino acids in urine and because 99% of the D-lysine was $[1^{-13}C]$ lysine. This conclusion was further supported by the statistically similar plasma and urinary enrichment when the $^{13}$C lysine tracer with less than
0.2% D-isomer was used. In those two studies, baseline urinary $^{13}$C lysine enrichment was <2% higher than in plasma. At isotopic equilibrium, urinary enrichment was 3% higher than in plasma. The agreement between urine and plasma enrichment was further demonstrated by a negligible bias of less than 0.5% (Bland and Altman 1986).

The isotope effect of an amino acid tracer which contains small amounts of the D-isomer of the amino acid has been reported before. Waterlow (1967) originally investigated the use of urine as an alternative to blood sampling in humans using U-$[^{14}$C] lysine. In both a human (Waterlow 1967) and a rat study (Waterlow and Stephen 1967), lysine specific activity was measured in blood and urine during a constant infusion of $^{14}$C lysine. They reported significantly higher levels of specific activity of free lysine in urine as compared to plasma. The authors related these findings to the presence of D-lysine in their tracer. The Waterlow studies (Waterlow and Stephen 1967; Waterlow 1967) and the current study point to the hazards of using tracers contaminated with D-isomers. This problem would not have been identified, had urine not been studied as an alternative to plasma. Plasma enrichments do not appear to be significantly affected by the D-isomers for the reciprocal of the reasons that urine is affected. Namely, the plasma amino acid pool is large and the relative amount of D-isomer to the total free L-amino acid pool is insignificant, since the majority of the D-isomer is removed from circulation. Experimental evidence for this position is that lysine flux based on plasma $^{13}$C lysine enrichment, was consistent with flux rates reported in the literature (Table 4.5.). This study does not provide information about a threshold value of D-lysine, above which an isotope effect will be seen, however, a tracer with > 0.2% D-isomer cannot be recommended for any study in which urine will be used to sample the arterial plasma pool.

In the [1-$^{13}$C] lysine infusion study, the $^{13}$CO$_2$ enrichment (Figure 4.4.) was slightly higher between 50 and 100 minutes of the infusion as compared to the final 120 minutes, when
isotopic steady state was achieved. This was evidence of over priming of the lysine pool. We would therefore recommend reducing the prime of 21.89 μmol·kg⁻¹ to 17.1 μmol·kg⁻¹.

Lysine flux and oxidation rates are shown with other results reported in the literature (Motil et al. 1994; Motil et al. 1981; Motil et al. 1981a; Conway et al. 1980; Zello et al. 1992) in Table 4.5. Direct comparisons between lysine flux rates could not be made because of significant differences in lysine intake. Figure 4.6. depicts the relationship between lysine intake and lysine flux based on published data. At an intake of 54.06 mg·kg⁻¹·day⁻¹, a lysine flux of 79.57 μmol·kg⁻¹·h⁻¹ would be predicted. The measured lysine flux at that intake was 76.38 μmol·kg⁻¹·h⁻¹. The similarity between measured and predicted flux values suggests that the oral, primed, equal dose infusion protocol did not alter the relationship between lysine intake and lysine flux. The results also suggest that the gender of the subjects in this study (female) did not significantly affect the relationship between lysine intake and flux. This, in addition to equivalent enrichment in urine and plasma, suggests that the noninvasive protocol can be used as an alternative method for the measurement of lysine kinetics in populations where an intravenous infusion is prohibited.

In conclusion, a simpler model to measure amino acid kinetics was developed for L-[1-¹³C] phenylalanine and L-[l-¹³C] lysine. With this model, a four hour baseline period can be combined with a four hour oral, primed, equal dose infusion protocol and measurement of isotopic enrichment in breath and urine, to estimate phenylalanine and lysine kinetics. This noninvasive protocol was developed in part, to assess indispensable amino acid requirements in vulnerable populations by the Indicator Amino Acid Oxidation technique. However, the use of the model is not limited to this application alone.
Figure 4.6. Linear relationship between lysine intake and lysine flux ( ). Published (filled circle), Predicted (open circle) and Measured (star) Lysine Flux. Regression line for published lysine flux data: flux = 46.86 + 0.6(lysine intake) \( (r^2 = 0.67, p=0.04) \). Regression line ( - - - ) indicates predicted lysine flux at a lower lysine intake. Lysine flux = 79.57 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) predicted for an intake of 54.06 \( \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \). Measured mean lysine flux = 76.38 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) for an intake of 54.06 \( \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \).
5. TYROSINE REQUIREMENTS IN CHILDREN WITH CLASSICAL PKU DETERMINED BY INDICATOR AMINO ACID OXIDATION

5.1. Introduction

Phenylketonuria (PKU) is a disorder of aromatic amino acid metabolism in which phenylalanine cannot be converted to tyrosine (Okano et al. 1991; Anonymous 1993). Thus, tyrosine is an indispensable amino acid in PKU because it is supplied endogenously to a very limited extent from phenylalanine hydroxylation (Anonymous 1993; Scrival et al. 1989). Without a dietary restriction of phenylalanine, these patients show extremely high plasma phenylalanine levels which are associated with the development of severe mental retardation (Thompson et al. 1990; Seashore et al. 1985).

Natural foods are sources of phenylalanine, thus their use in PKU is limited by the need to limit phenylalanine intake. As a result, dietary treatment of PKU requires the use of synthetic or semi-synthetic elemental or modified protein hydrolysate formulas. The treatment aims to lower blood phenylalanine levels and to keep plasma concentrations of tyrosine and other amino acids within the normal range, thereby allowing for optimum growth and brain development. Treatment consists of supplying adequate energy, other amino acids and nutrients, while phenylalanine intake is restricted and tyrosine intake is supplemented (Elsas and Acosta 1994). At present, the dietary management of patients with PKU is empirical because it is based on plasma amino acid concentrations, blood urea nitrogen and growth indices and not on direct measures of tyrosine and phenylalanine requirements.

The recommended phenylalanine and tyrosine intakes in PKU and in the general population are shown in Table 2.1. The main differences between the general recommendations and those for individuals with PKU include the wider range of phenylalanine requirements within each PKU age group and the supplemental tyrosine requirements, which when added to the phenylalanine requirements, far exceed the recommendations for aromatic
amino acid requirements in the general, healthy population. In fact, the median tyrosine requirement across the different age groups, represents 5 to 7 times the corresponding phenylalanine requirement. This suggests that out of the total of aromatic amino acid requirement, phenylalanine contributes ~20% and tyrosine ~80%. These values are significantly different from those ratios described earlier in animals (House et al. 1997a; House et al. 1997b; Milner et al. 1984b; Williams et al. 1987b; Stockland et al. 1971b) and humans (Burrill and Schuck 1964) in which dietary tyrosine was shown to spare 40 to 50% of the phenylalanine requirement. This almost equivalent contribution of phenylalanine and tyrosine to total aromatic amino acid intake is consistent with the plasma (Mitchell et al. 1995; Scriver et al. 1989) and mixed piglet body protein (Aumaitre and Duee 1974) ratio of phenylalanine to tyrosine.

Therefore, the aim of this study was to test the hypothesis that the tyrosine requirement in PKU would account for approximately 45% of the total aromatic amino acid requirement. The Indicator Amino Acid Oxidation technique was used to define the tyrosine requirements in children with classical PKU. In this technique, the effect of incremental increases in dietary tyrosine on lysine oxidation and flux were examined. Both phenylalanine and lysine have been shown to be suitable indicator amino acids in animals (Ball and Bayley 1984) and phenylalanine has been used as an indicator in several human studies (Wilson et al. 1997; Lazaris-Brunner 1994; Duncan et al. 1996; Zello et al. 1993). Due to the enzyme defect in PKU, lysine was used as the indicator amino acid.
5.2. **Methods**

5.2.1. **Study Population**

Five children with classical PKU treated by the PKU clinic of the Hospital For Sick Children (HSC), Toronto, participated on an outpatient basis in the Clinical Investigation Unit at the HSC. Subjects were screened for suitability according to the following criteria:

**Inclusion Criteria:**
- Plasma phenylalanine concentration $\geq 1200$ $\mu$M at diagnosis
- Prepubertal males or females, aged 4-10, in good health
- Treated by dietary phenylalanine restriction from early infancy

**Exclusion Criteria:**
- Concurrent use of any medication that may alter protein or energy metabolism
- Recent illness
- IQ < 80
- History of endocrine disease or any other medical condition that may alter protein or energy metabolism
- Significant weight change during the preceding three months (> 10% of body weight).

Subject characteristics at the start of the study are summarized in Table 5.1. The purpose of the study, the benefits and the potential risks involved were explained to the subjects and their parents. Written consent was obtained from the subject’s parents and detailed information sheets about the study day protocol were provided. The consent forms, information sheets and newsletter are found in Appendix 9.2. All procedures used in the study were approved by the University of Toronto Human Experimentation Committee and the Human Subjects Review Committee of the HSC.
Table 5.1. Subject Characteristics of the Children with Phenylketonuria (PKU) who Participated in the Tyrosine Requirement Study

<table>
<thead>
<tr>
<th>Subjects</th>
<th>TN</th>
<th>LF</th>
<th>AK</th>
<th>MJ</th>
<th>TJ</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
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<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>F</td>
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<td>6</td>
<td>9</td>
<td>7</td>
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<tr>
<td>Weight (kg)</td>
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<td>29.3</td>
<td>40.6</td>
<td>38.0</td>
<td>35.9 ± 2.3</td>
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<td>Height (cm)</td>
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<td>137.0</td>
<td>115.3</td>
<td>147.6</td>
<td>135.9</td>
<td>133.1 ± 5.3</td>
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<tr>
<td>% IBW²</td>
<td>142.9</td>
<td>90.9</td>
<td>139.5</td>
<td>101.5</td>
<td>111.8</td>
<td>117.3 ± 10.3</td>
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<tr>
<td>FFM - BIA (kg)³</td>
<td>29.77</td>
<td>26.69</td>
<td>21.78</td>
<td>33.76</td>
<td>28.2</td>
<td>28.0 ± 1.9</td>
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<tr>
<td>FFM - SF (kg)⁴</td>
<td>27.91</td>
<td>27.59</td>
<td>19.52</td>
<td>32.1</td>
<td>26.34</td>
<td>26.7 ± 2.0</td>
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</tbody>
</table>

¹ M = male, F = female

² IBW = Ideal body weight; determined by matching weight percentile to actual height percentile and dividing actual weight by matched weight x 100%

³FFM - BIA = Fat free mass determined by bioelectrical impedance analysis

⁴FFM - SF = Fat free mass determined by multiple skinfold thickness
5.2.2. **Experimental Design**

Each subject was studied on an outpatient basis for six nonconsecutive days during a two month period between March and September, 1996. On the study days, the subject received one of the test intakes of dietary tyrosine, 0, 8, 12, 16, 32, 64, or 130 mg·kg⁻¹·day⁻¹, assigned in random order. The resulting assignment of tyrosine intakes and the average number of days between study days is shown in Table 5.2. Each subject was studied at six intake levels, which enabled the estimation of individual tyrosine requirements. Subject MJ was only studied at five test levels because a minor illness prevented him from completing one study.

The range of dietary tyrosine levels tested was based on the consideration of two values: (i) the average phenylalanine intake of the subjects (used to calculate the predicted tyrosine requirement); and (ii) the published recommended tyrosine intakes for the subjects (used to determine the maximum tyrosine level). With respect to the predicted tyrosine requirement level, the average of the phenylalanine intakes of all five subjects was taken to equal 55% of the total aromatic amino acid requirement. For this group the average phenylalanine intake was 24 mg·kg⁻¹, the total aromatic amino acid requirement was therefore, \( 24 + 0.55 \times 43.6 \) mg·kg⁻¹. The predicted tyrosine requirement was the difference between the total aromatic amino acid requirement and the phenylalanine intake, \( 43.6 - 24 = 19.6 \) mg·kg⁻¹. This tyrosine level was set as the middle value of the range of tyrosine test levels and reflected the predicted requirement based on the hypothesis. The maximum tyrosine level tested, 130 mg·kg⁻¹, reflected the median value of the recommended range of tyrosine intakes (Table 2.1.). The lowest tyrosine test level of 0 mg·kg⁻¹ was designed to balance the test levels on either side of the predicted breakpoint such that three tyrosine levels would bracket the breakpoint. The preliminary breakpoint for the first three subjects was close to 16 mg·kg⁻¹, therefore the tyrosine test level of 12 mg·kg⁻¹ was studied in the last two subjects. This test level was added
Table 5.2. Test Tyrosine Intake Assignment

<table>
<thead>
<tr>
<th>Subject</th>
<th>0</th>
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<th>12</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>130</th>
<th>Days Between Studies^2</th>
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</thead>
<tbody>
<tr>
<td>TN</td>
<td>2</td>
<td>5</td>
<td>-</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>11.8 ± 2.5</td>
</tr>
<tr>
<td>LF</td>
<td>3</td>
<td>5</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>9.4 ± 2.7</td>
</tr>
<tr>
<td>AK</td>
<td>6</td>
<td>3</td>
<td>-</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>9.6 ± 1.1</td>
</tr>
<tr>
<td>MJ</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>-</td>
<td>6</td>
<td>1</td>
<td>-</td>
<td>16.3 ± 6.0</td>
</tr>
<tr>
<td>TJ</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>13.0 ± 4.3</td>
</tr>
</tbody>
</table>

^1 Numbers 1 to 6 refer to study days.

^2 Mean ± SEM.
because the descending regression line, defined by the first three intake levels (0, 8, 16) and the regression line with a zero slope, defined by the last three intake levels (32, 64, 130), included the point at 16 mg·kg⁻¹. Since it was unclear on which line the 16 mg·kg⁻¹ point should lie, the additional point of 12 mg·kg⁻¹ was added so that at least three test levels defined the descending regression line.

5.2.3. Experimental Diet

Table 5.3 describes the amino acid mixture used in this study. A flavoured liquid formula (Protein-Free Powder, Product 80056, Mead Johnson, Evansville, IN) and protein-free cookies (HSC Research Kitchen) supplied the main source of energy in the diet. A crystalline amino acid mixture, based on the amino acid composition of egg protein, was consumed at 1.5 g·kg⁻¹·day⁻¹ and provided the only source of amino nitrogen in the diet. The approximate macronutrient composition of the experimental diet, expressed as a percent of dietary energy, was 53% carbohydrate, 38% fat and 9% protein. The macronutrient composition of the subject’s usual diet was 55% carbohydrate, 35% fat and 10% protein. The diets were prepared and weighed (Mettler Scale, model PE 2000, Switzerland) in the research kitchen of the HSC and were portioned into eight isoenergetic, isonitrogenous meals. The diet was consumed as hourly meals and each meal represented 1/8th of the subjects total daily requirement. Study day intakes of tyrosine, phenylalanine and lysine were measured separately from the other amino acids within the mixture in order to ensure that intakes were accurate. These three amino acids were then added to the remaining study day amino acid mix, so that all amino acids were consumed together. Total energy intakes were based on each subject’s calculated resting metabolic rate (Food and Agriculture Organization of the United Nations-Rome 1985), multiplied by an activity factor of 1.5 (reflects sedentary activities).
Table 5.3. Composition of L-Amino Acid Mix

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>PKU Study(^1) (g·kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>variable</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>41.11</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>22.00</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>22.00</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>14.63</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>37.47</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>37.47</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>22.00</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>15.02</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>41.57</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>55.09</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>42.67</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>19.63</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>16.00</td>
</tr>
<tr>
<td>L-Proline</td>
<td>27.75</td>
</tr>
<tr>
<td>L-Serine</td>
<td>55.49</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>31.15</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>10.32</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>variable</td>
</tr>
<tr>
<td>L-Valine</td>
<td>46.48</td>
</tr>
</tbody>
</table>

\(^1\) Diet provided 1.5 g protein per kg body weight.
Subjects were instructed to maintain their usual level of physical activity and to fast for 10 - 12 hours overnight, prior to the study. Subjects were weighed (Toledo Scale, model 2020, Windsor, Ontario) within one week of the first study day and again on the morning of each subsequent study day. Body weight and height from one study day was used to calculate the total energy and protein (amino acids) content of the experimental diet for the next study day. Subjects were not adapted to the experimental diet because it was possible to match the protein and phenylalanine intakes of the experimental diet to the usual intakes of the subjects who follow a highly regulated diet. In addition, the protein source of these subjects with PKU was composed of crystalline amino acids, identical to the amino acid mix used in the experimental diet. It was therefore possible to apply the baseline protocol developed in experiment 1 of section 4. to this group.

With respect to protein, the usual intakes of the five subjects were variable (Table 5.4.). However a standard protein intake was required for the study since the ratio of the test amino acid (tyrosine) and the indicator amino acid (lysine) to total nitrogen intake must be constant across subjects because indispensable amino acid requirements are influenced by total protein intake (below requirement levels). An intake of 1.5 g·kg⁻¹·day⁻¹ was used for all subjects. The current safe level of protein intake for children, as proposed by the FAO/WHO/UNU (1985), is 1.02 g·kg⁻¹ (age 5-6), 1.01 g·kg⁻¹ (age 7-9), and 0.99 g·kg⁻¹ (age 9-10). The dietary recommendations for children with PKU (Elsas and Acosta 1994) are indicated in Table 5.4. An intake of 1.5 g·kg⁻¹·day⁻¹ clearly met and exceeded the recommended intakes of all five subjects, although it was below the actual intakes of two subjects. Subjects whose protein intakes were not covered during the study were instructed to consume the difference in protein from their phenylalanine free formula (this will supply the balance of amino nitrogen without
Table 5.4. Actual and Recommended Intakes of Phenylalanine and Protein in Children with Phenylketonuria (PKU)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Phenylalanine intake (mg·kg⁻¹)</th>
<th>Protein intake (g·kg⁻¹)</th>
<th>Recommended Protein intake (g·kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN</td>
<td>8</td>
<td>40.0</td>
<td>36.0</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>LF</td>
<td>8</td>
<td>31.8</td>
<td>22.0</td>
<td>1.6</td>
<td>1.3</td>
</tr>
<tr>
<td>AK</td>
<td>6</td>
<td>29.3</td>
<td>25.0</td>
<td>2.5</td>
<td>1.2</td>
</tr>
<tr>
<td>MJ</td>
<td>9</td>
<td>40.6</td>
<td>17.0</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>TJ</td>
<td>7</td>
<td>38.0</td>
<td>21.0</td>
<td>1.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Mean ± SEM 7.6 ± 0.51 35.9 ± 2.3 24.2 ± 3.2 1.9 ± 0.2 1.1 ± 0.05

1 Elsas & Acosta (1994).

2 Phenylalanine and protein intakes were derived from 2-3 day food intake records collected by the PKU Clinic dietitian within 6 months of the tyrosine requirement study.
adding phenylalanine). The phenylalanine intake provided by the experimental diet on the study day was 24 mg·kg⁻¹, which reflected the average phenylalanine intake of the five subjects. The lysine intake on the study day was 64 mg·kg⁻¹. This value reflects the upper limit of the recommended lysine intake for children aged 2 - 12 (Food and Agriculture Organization of the United Nations-Rome 1985).

5.2.4. Anthropometrics and Body Composition

Within one week of the first study day and on the morning of each subsequent study day, each subject had their height and weight measured. Standing height was measured without shoes, to the nearest 0.1 cm, using a wall-mounted stadiometer. Body weight was measured while wearing light clothing and without shoes, after an overnight fast and following voiding, on a balance scale (Toledo Scale, Model 2020, Windsor, Ont.) to the nearest 0.1 kg.

Body composition (fat and fat free mass) was determined by bioelectrical impedance analysis (BIA) and multiple skinfold thickness measurements. BIA and skinfolds were performed in the morning, prior to the first and second study days. Fat free mass (FFM) was estimated using a 4-terminal bioelectrical impedance analyser (RJL Systems, Model 101A, Detroit, MI). Subjects were instructed to lie in a supine position on a bed with arms placed away from the body trunk and with no contact between the legs. Two current-introducing electrodes were placed on the dorsal surfaces of the right hand and foot proximal to the metacarpal-phalangeal and metatarsal-phalangeal joints, respectively. Two detector electrodes were placed at the right pisiform prominence of the wrist, with the proximal edge dissecting the ulnar tubercle, and between the medial and lateral malleoli, with the proximal edge dissecting the medial malleolus. An excitation current of 800 μA at a fixed frequency of 50 Khz was introduced into the subject at the distal electrodes of the hand and foot, and the voltage drop was detected by the proximal electrodes. The lowest reading of three measurements was used
to calculate impedance, where:
\[ \text{Impedance} = (R^2 + X_C^2)^{1/2} \]  
(Nyboer 1970).

Age specific equations were then used to determine FFM (kg) as follows:

Age 1 - 15:  
FFM = \frac{0.61Ht^2}{R} + 0.25Wt + 1.31  
(Houtkooper et al. 1992).

Age 16*:  
FFM = \frac{0.756Ht^2}{R} + 0.110Wt + 0.107X_C - 5.463  
(Lukaski et al. 1986).

where, Ht = height (cm)  
R = resistance (ohms)  
Wt = weight (kg)  
X_C = reactance.

Skinfold measurements were taken at 4 sites, tricep, bicep, subscapular and suprailiac. Measurements were repeated three times by the same individual, and the average value was taken to the nearest 0.1 mm using a skinfold caliper (British Indicators Ltd.). Body density (d, kg·m⁻³) was derived from the sum of the 4 skinfolds (SSF) as follows:

Age 1 - 11 (Brook 1971).
Male  
d = 1.1690 - 0.0788\log(SSF)
Female  
d = 1.2063 - 0.0999\log(SSF)

Age 11 - 17 (Durnin and Rahaman 1967).
Male  
d = 1.1533 - 0.0643\log(SSF)
Female  
d = 1.1369 - 0.0598\log(SSF)

Age 18 - 30 (Durnin and Rahaman 1967).
Male  
d = 1.1610 - 0.0632\log(SSF)
Female  
d = 1.1581 - 0.072\log(SSF).
Body fat, expressed as a percentage of body weight was then calculated as follows:

\[
\text{Fat (\%) = } \frac{(4.95 - 4.5) \times 100}{d} 
\]

(Siri 1956).

5.2.5. **Oral Isotope Infusion Studies**

L-[\text{1-}{^13}\text{C}] lysine-HCl-H\text{H}_2\text{O} with an enrichment of 99\% (Cambridge Isotopes Laboratories, Woburn, MA) was used in this study. The chemical and isotopic purity of the labelled amino acid was confirmed by gas chromatography-mass spectrometry. Isometric purity (< 0.2\% D-isomer) was assessed by chiral high performance liquid chromatography. The stock solution of \text{[^13]C} lysine (10 mg mL\textsuperscript{-1}) was prepared with sterile water by passage through a 0.22 \mu m Millipore filter (Millipore, Bedford) under a laminar flow hood, and then dispensed into multiple dose vials. Each subject received a priming dose of 17.1 \mu M kg\textsuperscript{-1} and eight subsequent equal oral infusion doses of 4.79 \mu M kg\textsuperscript{-1}. The isotope was taken orally and was followed by water to rinse the tube which had contained the isotope. The prime and equal infusion dose were based on the results of experiment 3, as described in section 4.4.

Thirty isotope infusion studies were initiated (6 oxidation studies per subject), however only 29 studies were completed (see section 5.2.2. for details). Figure 5.1. illustrates the study day protocol followed for each subject. Hourly meals were consumed beginning at time -240 minutes. The level of lysine in each meal was the same. This was achieved when the dietary lysine content of the last four meals was reduced by an amount that corresponded to the amount of \text{[^13]C} lysine administered. The level of amino nitrogen in the diet was also kept constant despite the different tyrosine test levels. Molar equivalents of L-alanine were added to the diet to match the deficit in tyrosine intake created when test levels were below 130.
Figure 5.1. Tyrosine Requirement Study Day Protocol
mg·kg\(^{-1}\). See Appendix 9.3. for forms used in the diet and isotope calculations and for the collection of study day data.

At least 150 mL of water was consumed with each meal to ensure a steady production of urine. Subjects voided every hour but urine was not collected before time -60 minutes. Subjects were also acclimatized to the breath collection procedure during the second hour of the study, although no breath samples were actually collected. Three baseline samples of urine were collected every 20 minutes between -60 and 0 minutes and three baseline breath samples were collected between -21 and 0 minutes. The priming dose of \(^{[13]C}\) lysine was administered at time 0 minutes. Equal oral infusion doses were then administered every 30 minutes beginning 15 minutes after the prime. Subjects voided at 60 and 120 minutes, but urine was not collected. Five plateau urine samples were collected at 144, 168, 192, 216, and 240 minutes. Five plateau breath samples were collected at 135, 160, 195, 210, and 225 minutes. One 20 minute measure of the CO\(_2\) production rate (VCO\(_2\), mL·minute\(^{-1}\)) was determined between 0 and 120 minutes using the 2900 Metabolic Cart (Sensormedics, CA).

Urine samples were separated into 500 µL aliquots and stored at -20°C until analysis. Each breath sample was collected for 7 minutes while the subjects lay in a semirecumbant position on a hospital bed. The subjects breathed in a normal fashion while wearing a ventilated face mask (Scott 80216730, Sensormedics, Anaheim, CA). Once the subject's air flow had stabilized (CO\(_2\) concentration = 0.5-0.8%), the expired breath was collected using a vacuum extraction system (Pump VB0025, Vortex Blower, Spencer Turbine Company, Windsor, CT) combined with a mass flow meter. In order to trap the respiratory CO\(_2\), the expired breath was bubbled at a rate of 500 mL·min\(^{-1}\) through 10 mL of a 1 M NaOH solution in a modified reflux condenser for 7 minutes. The resulting NaH\(^{13}\)CO\(_3\) solution was then injected (Monojet, Sherwood Medical, St. Louis, MO) into vacutainer glass tubes (Vacutainer Brand
6441, 100 x 16 mm, Becton Dickinson Inc., Mississauga, Ontario). The vacutainers were evacuated of any air introduced during the injection with the syringe and frozen at -20°C until analysis.

\( \text{VCO}_2 \) was determined by continuous indirect calorimetry with the use of a ventilated hood. The hood was placed over the subjects' head while they lay supine on a hospital bed. A mass flow meter maintained the \( \text{CO}_2 \) concentration in the hood in the range of 0.5 to 0.8%. The 2900 Metabolic Cart is equipped with a paramagnetic \( \text{O}_2 \) analyser and an infrared \( \text{CO}_2 \) analyser. Prior to any \( \text{VCO}_2 \) measurement, gas analysers were calibrated with standard gases (Nitrogen, 20% Oxygen and 4% Carbon Dioxide, Linde Medical Gas, Union Carbide, Toronto, Ontario. \( \text{CO}_2 \) production was expressed under standard conditions (STPD): dry gas at 0°C and 760 mm Hg. Measured \( \text{VCO}_2 \) took place under ambient conditions, and the 2900 corrected the gas volumes to standard conditions.

5.2.6. **Analytical Procedures**

Urine samples (500 µL) were deproteinized and acidified with an equal volume of 20% trichloroacetic acid (wt:vol) and centrifuged. Amino acids were separated from the supernatant by a cation exchange resin (Dowex 50W-X8, 100-200 mesh H\(^+\) form, Bio Rad Laboratories, Richmond, CA) and were derivatized by the method described by Patterson et al. (1991) to their N-heptafluorobutyryl n-propyl esters. Amino acid enrichment was measured on a gas chromatograph (Hewlett-Packard model 5890 Series II, Mississauga, Ont) attached to a quadrupole mass spectrometer (VG Trio-2). Separation of the amino acid derivatives was performed with helium as the carrier gas on a 30m X 0.32mm (inside diameter) X 1.0 µm (film thickness) fused silica capillary column (HP5, Hewlett-Packard) coupled directly to the ion source, which was operated under conditions of negative chemical ionization with ammonia as
the reactant gas. Each amino acid was analysed by splitless injection, on an automatic sampler (HP 7673 injector). Selected ion chromatographs were obtained by monitoring mass-to-charge ratio of 560 and 561 for $[^{13}\text{C}]$ lysine, corresponding to the unenriched (m) and enriched (m+1), respectively. The areas under the peaks were integrated by a Digital DECp 450D2LP computer, using a Lab-Base program (VG Biotech, Altrincham, U.K.).

The percentage enrichment of the expired $^{13}$CO$_2$ was measured on a dual inlet magnetic sector isotope ratio mass spectrometer (VG Micromass 602D, Cheshire, England) using techniques described in earlier work (Jones et al. 1986). Briefly, approximately 0.25 mL of the sodium bicarbonate solution and an equal amount of 85% phosphoric acid were placed in either leg of a Rittenberg tube. Vacuum grease (Dow Corning Corp.) was applied to the Rittenberg stopper to ensure an air tight seal. The tube was then evacuated twice to less than 0.005 Torr using a vacuum extraction system (Vacuum Generators Ltd., Sussex, England). CO$_2$ was liberated when the sodium bicarbonate solution was mixed with the phosphoric acid. The liquid in the Rittenberg tube was frozen in liquid nitrogen. The tube was coupled to the inlet of the mass spectrometer and was kept frozen by being immersed in a methanol - dry ice bath. Breath enrichments from baseline samples and from those taken during the isotope infusion were expressed as atoms percent excess (APE) $^{13}$CO$_2$ over a reference standard of compressed CO$_2$ gas.

5.2.7. Data Analysis

Results are presented as mean ± standard error of the mean (SEM). A stochastic model was used to evaluate lysine kinetics (Waterlow et al. 1978a). Isotopic steady state in the metabolic pool was represented by plateaux in $^{13}$CO$_2$ enrichment in breath and in $[^{13}\text{C}]$ lysine enrichment in urine. This state was achieved in breath and urine by 120 minutes from
the start of the isotope infusion and was maintained to the end of the study at 240 minutes. The mean breath isotope enrichment values of the 3 baseline samples and the 5 plateau samples were used to determine atoms percent excess (APE) above baseline at isotopic steady state. The mean ratio of the enriched peak (m+1) to the unenriched peak (m) in urine for both baseline and plateau samples was used to calculate molecules percent excess (MPE) for [\textsuperscript{13}C] lysine. Figure 5.2. shows typical \textsuperscript{13}CO\textsubscript{2} and \textsuperscript{13}C lysine enrichments in breath and urine, respectively at baseline and plateau for an individual study.

The isotopic enrichment was calculated using the following equation:

\[
\text{APE} = \frac{(R_s - R_b)}{(1 + R_s - R_b)} \times 100
\]

where \(R_s\) and \(R_b\) are the ratios of (m+1) to (m) for the enriched sample and sample at natural abundance respectively (Rosenblatt et al. 1992; Matthews et al. 1980).

Flux (Q) was measured from the dilution of the infused tracer L-[\textsuperscript{1-13}C] lysine in the urine metabolic pool at isotopic steady state:

\[
Q = i \left[ \left( \frac{E_i}{E_p} \right) - 1 \right]
\]

where \(i\) is the rate of \([\textsuperscript{13}C]\) lysine isotope infused (\(\mu\text{mol.kg}^{-1}.\text{h}^{-1}\)), \(E_i\) and \(E_p\) are the enrichments of infused and urine \([\textsuperscript{13}C]\) lysine, molecules percent excess (MPE), at isotopic steady state (Matthews et al. 1980).

The rate of lysine oxidation (O, \(\mu\text{mol.kg}^{-1}.\text{h}^{-1}\)) was calculated using the equation:

\[
O = F^{13}\text{CO}_2 \left( \frac{1}{E_p} - \frac{1}{E_i} \right) \times 100
\]

where \(F^{13}\text{CO}_2\) is the rate of release of \(^{13}\text{CO}_2\) by the oxidation of the tracer \([\textsuperscript{13}C]\) lysine (\(\mu\text{mol.}^{13}\text{CO}_2.\text{kg}^{-1}.\text{h}^{-1}\)) (Matthews et al. 1980). \(F^{13}\text{CO}_2\) was calculated as follows:

\[
F^{13}\text{CO}_2 = (FCO_2)(ECO_2)(44.6)(60)/(W)(0.82)(100)
\]

where \(FCO_2\) is the production rate of \(CO_2\) (mL.min\(^{-1}\)); \(ECO_2\) is the \(^{13}\text{CO}_2\) enrichment in expired breath at isotopic steady state (APE); and \(W\) is the weight (kg) of the subject (Matthews et al. 1980).
Figure 5.2. Typical Study Day Data: $^{13}$CO$_2$ Enrichment in Breath (filled squares) and $^{13}$C-Lysine Enrichment in Urine (open squares).
1980). The constants 44.6 μmol.mL⁻¹ and 60 min.h⁻¹, convert FCO₂ to micromoles per hour and the factor 100 changes APE to a fraction. The correction factor of 0.82 represents the ¹³CO₂ that is released by [¹³C] lysine oxidation, but not liberated from the body bicarbonate pool into expired air. This correction factor is based on the fraction of infused Na[¹³C]bicarbonate recovered in the breath of adult males under fed state conditions (Hoerr et al. 1989).

Statistical analysis was performed on primary and derived values. For each subject, on each of the first two study days, the ratio of FFM, determined by BIA, to FFM determined by skinfolds, was calculated. The difference of the mean ratio from 1 was compared by t-test. Analysis of variance (SAS Institute 1985) was used to assess the relationship of lysine flux, lysine oxidation and lysine F¹³CO₂ to the experimental variables: (i) tyrosine test intake, (ii) subject, and (iii) sex of subject. Least Squares Difference multiple range test was used to test the significance of specific differences between variables grouped according to tyrosine test intake. Results were considered to be statistically significant at P ≤ 0.05).

The mean requirement for tyrosine was determined by breakpoint analysis using a two-phase linear regression crossover model (Seber 1977) similar to that described previously (Duncan et al. 1996; Zello et al. 1993; Zello et al. 1990). The 95% confidence limits for the level of tyrosine intake corresponding to the intersection of the two straight lines was determined using Fieller's theorem (Seber 1977). The estimates set by the upper confidence interval of the linear regression model have been suggested to closely represent the requirement to meet the needs of 95% of the population (Were 1989). This is based on the assumption that the sample of 5 subjects studied is representative of the PKU population. Details about this statistical method are provided in Appendix 9.4. Individual tyrosine requirements were estimated by visual inspection of the breakpoint from the F¹³CO₂ and oxidation curves.
5.3. Results

Tables 5.5. and 5.6. show observed weight and height changes for each subject and for the group, as well as the expected rate of weight and height changes over the two month study period (Roche and Himes 1980). Both mean weight and height changes were in the expected range compared to standard growth velocity curves for children of the same age and gender.

Subjects AK and TJ experienced weight gains that were above the 97th centile, subject TN experienced a greater gain and subject TJ experienced a lower gain in stature than was predicted. It should be noted that the extrapolation of observed growth velocities over a two month period to growth over a 12 month period is not strictly correct, since there is seasonal variation in growth rates (Marshall 1971).

Mean FFM determined by BIA and skinfolds on study day one was 28.0 ± 1.9 kg and 26.7 ± 2.0 kg, respectively, and on study day two was 28.2 ± 1.8 kg and 27.1 ± 1.9 kg, respectively. There was good reproducibility between study days, with mean coefficients of variation of 1.62 ± 0.61% and 2.88 ± 1.43% for FFM determined by BIA and skinfolds, respectively. There was also good agreement between the two methods such that the FFM determined by BIA was 4.2 ± 2.5% (P = 0.09) and 4.2 ± 2.6 (P = 0.18) greater than FFM determined by skinfolds, on study days one and two, respectively.

The possibility of isotope not clearing the body between infusion studies and thus interfering with determination of baseline isotope enrichment was examined by comparing baseline isotope enrichment before each study period. Mean baseline [13C] lysine enrichments were 0.212 ± 0.004, 0.215 ± 0.004, 0.215 ± 0.003, 0.219 ± 0.005, 0.220 ± 0.004 and 0.221 ± 0.004 MPE at study days one to six, respectively, and were not different (p=0.50).

Lysine flux of the individual subjects is shown at the seven test tyrosine intakes in Table 5.7. Lysine flux was not affected by tyrosine intake (P = 0.89). The individual subject had a
Table 5.5. Weight Change (kg) During Two Month Study Period in Children with Phenylketonuria (PKU)

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Weight Change (kg)</th>
<th>Expected Weight Velocity (kg/2 mo$^{-1}$) $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN</td>
<td>40.0</td>
<td>40.0</td>
<td>40.4</td>
<td>40.9</td>
<td>40.4</td>
<td>40.4</td>
<td>0.4</td>
<td>0.16 - 0.87</td>
</tr>
<tr>
<td>LF</td>
<td>31.8</td>
<td>32.0</td>
<td>32.0</td>
<td>31.9</td>
<td>32.5</td>
<td>32.5</td>
<td>0.7</td>
<td>0.13 - 0.87</td>
</tr>
<tr>
<td>AK</td>
<td>29.3</td>
<td>29.3</td>
<td>29.5</td>
<td>29.9</td>
<td>29.9</td>
<td>30.6</td>
<td>1.3</td>
<td>0.13 - 0.73</td>
</tr>
<tr>
<td>MJ</td>
<td>40.6</td>
<td>40.6</td>
<td>41.1</td>
<td>41.1</td>
<td>40.8</td>
<td>41.1</td>
<td>0.5</td>
<td>0.18 - 0.93</td>
</tr>
<tr>
<td>TJ</td>
<td>38.0</td>
<td>37.1</td>
<td>38.0</td>
<td>37.9</td>
<td>38.4</td>
<td>38.9</td>
<td>0.9</td>
<td>0.15 - 0.80</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>35.90 ± 2.3</td>
<td>35.8 ± 2.2</td>
<td>36.2 ± 2.3</td>
<td>36.3 ± 2.3</td>
<td>36.4 ± 2.7</td>
<td>36.7 ± 2.2</td>
<td>0.8 ± 0.2</td>
<td>0.15 ± 0.01 - 0.84 ± 0.3</td>
</tr>
</tbody>
</table>

$^1$ Weight velocity (kg per year + 6) for 3rd to 97th centile (Marshall, 1971).
Table 5.6. Height Change (cm) During Two Month Study Period in Children with Phenylketonuria (PKU)

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Height Change (cm)</th>
<th>Expected Height Velocity (cm:2 mo(^{-1}))(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN</td>
<td>129.7</td>
<td>130.1</td>
<td>130.5</td>
<td>131.0</td>
<td>131.0</td>
<td>131.1</td>
<td>1.4</td>
<td>0.67 - 1.16</td>
</tr>
<tr>
<td>LF</td>
<td>137.0</td>
<td>137.2</td>
<td>136.9</td>
<td>137.6</td>
<td>137.7</td>
<td>137.7</td>
<td>0.7</td>
<td>0.63 - 1.27</td>
</tr>
<tr>
<td>AK</td>
<td>115.3</td>
<td>115.3</td>
<td>115.3</td>
<td>115.3</td>
<td>115.3</td>
<td>116.4</td>
<td>1.1</td>
<td>0.7 - 1.3</td>
</tr>
<tr>
<td>MJ</td>
<td>147.6</td>
<td>147.6</td>
<td>147.6</td>
<td>147.6</td>
<td>148.4</td>
<td>148.4</td>
<td>0.8</td>
<td>0.65 - 1.13</td>
</tr>
<tr>
<td>TJ</td>
<td>135.9</td>
<td>135.9</td>
<td>135.9</td>
<td>136.2</td>
<td>136.2</td>
<td>136.2</td>
<td>0.3</td>
<td>0.68 - 1.23</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>133.1 ± 5.3</td>
<td>133.2 ± 5.3</td>
<td>133.2 ± 5.3</td>
<td>133.5 ± 5.3</td>
<td>133.7 ± 5.4</td>
<td>134.0 ± 5.2</td>
<td>0.9 ± 0.2</td>
<td>0.67 ± 0.01 - 1.22 ± 0.03</td>
</tr>
</tbody>
</table>

\(^1\) Height velocity (cm per year ± 6) for 3rd to 97th centile (Marshall, 1971).
Table 5.7. Effect of Tyrosine Intake on Lysine Flux in Children with Phenylketonuria (PKU) \(^1\)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Tyrosine Intake (mg·kg(^{-1})·day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>TN</td>
<td>158.7</td>
</tr>
<tr>
<td>LF</td>
<td>78.4</td>
</tr>
<tr>
<td>AK</td>
<td>122.2</td>
</tr>
<tr>
<td>MJ</td>
<td>100.7</td>
</tr>
<tr>
<td>TJ</td>
<td>89.4</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>109.9 ± 14.2</td>
</tr>
</tbody>
</table>

\(^1\) Lysine flux is expressed as μmol·kg\(^{-1}\)·hour\(^{-1}\). By use of analysis of variance, subject had a significant effect on flux (p<0.0001), however, tyrosine intake did not have a significant effect on lysine flux.
significant effect on lysine flux ($P = 0.0001$), whereas the sex of the subjects had no effect ($P = 0.14$).

The rate of $^{13}\text{CO}_2$ released by lysine tracer oxidation is shown in Table 5.8. There was a significant decrease in the mean rate of $^{13}\text{CO}_2$ released by lysine tracer oxidation between tyrosine intakes of 0 and 12 mg·kg$^{-1}$·day$^{-1}$ and no change in the mean rate of $^{13}\text{CO}_2$ released between 12 and 130 mg·kg$^{-1}$·day$^{-1}$. The individual subject ($P = 0.21$) and the sex of the subjects ($P = 0.67$) did not have significant effects on F $^{13}\text{CO}_2$. Figure 5.3. illustrates the mean breakpoint in the F $^{13}\text{CO}_2$ data, as analysed by two-phase linear regression crossover, occurring at a dietary intake of 19.2 mg·kg$^{-1}$·day$^{-1}$ of tyrosine (95% confidence limits of 13.3 - 25.2). The individual F $^{13}\text{CO}_2$ data are illustrated in Figure 5.4. For the F $^{13}\text{CO}_2$ data, the overall error for the two phase linear regression crossover model was lowest when tyrosine intakes of 0, 8, 12, and 16 mg·kg$^{-1}$·day$^{-1}$ were partitioned to the first regression line and tyrosine intakes of 32, 64 and 130 mg·kg$^{-1}$·day$^{-1}$ were partitioned to the second line.

Similar results were apparent from lysine oxidation rates (Table 5.9.). There was a significant decrease in the mean rate of lysine oxidation between tyrosine intakes of 0 and 12 mg·kg$^{-1}$·day$^{-1}$ and no change between 12 and 130 mg·kg$^{-1}$·day$^{-1}$. The individual subject had a significant effect on lysine oxidation ($P = 0.0001$), whereas the sex of the subjects did not ($P = 0.20$). Figure 5.5. shows the effect of tyrosine intake on the mean rates of lysine oxidation. A breakpoint in the lysine oxidation curve occurred at a dietary intake of 16.3 mg·kg$^{-1}$·day$^{-1}$ (95% confidence limits of 5.8 - 26.8). For the lysine oxidation data, the overall error for the two phase linear regression crossover model was lowest when tyrosine intakes of 0, 8, 12, and 16 mg·kg$^{-1}$·day$^{-1}$ were partitioned to the first regression line and tyrosine intakes of 32, 64 and 130 mg·kg$^{-1}$·day$^{-1}$ were partitioned to the second line.

Individual tyrosine requirements derived by visual inspection of the F $^{13}\text{CO}_2$ response curves are described in Table 5.10. Requirements varied from 16.0 to 25.0 mg·kg$^{-1}$·day$^{-1}$. 152
Table 5.8. Effect of Tyrosine Intake on the Rate of $^{13}$CO$_2$ Released From L-[1-$^{13}$C] Lysine oxidation (F$^{13}$CO$_2$) in Children with Phenylketonuria (PKU)$^1$

<table>
<thead>
<tr>
<th>Subject</th>
<th>Tyrosine Intake (mg·kg$^{-1}$·day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>TN</td>
<td>2.289</td>
</tr>
<tr>
<td>LF</td>
<td>2.170</td>
</tr>
<tr>
<td>AK</td>
<td>1.645</td>
</tr>
<tr>
<td>MJ</td>
<td>2.679</td>
</tr>
<tr>
<td>TJ</td>
<td>1.850</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>2.127 ± 0.179$^{a}$</td>
</tr>
</tbody>
</table>

$^1$ F$^{13}$CO$_2$ is expressed as μmol·kg$^{-1}$·hour$^{-1}$. By use of analysis of variance, tyrosine intake significantly affected F$^{13}$CO$_2$ (means with different superscripts are significantly different, P < 0.05), however, subject and sex of subject did not significantly affect the rate of $^{13}$CO$_2$ released.
Figure 5.3. Effect of Tyrosine Intake on Mean $^{13}$CO$_2$ in Children with Phenylketonuria (PKU). Mean (± SEM) rate of $^{13}$CO$_2$ release at 7 test tyrosine intakes. Pooled data of all observations ($n=29$) and all subjects ($n=5$). The breakpoint estimates the mean tyrosine requirement of the sample population.

Breakpoint = 19.2 mg·kg$^{-1}$
(95% confidence limits of 13.3 - 25.2)
Figure 5.4. Effect of Tyrosine Intake on Individual $^{13}$CO$_2$
Table 5.9. Effect of Tyrosine Intake on the Rate of Lysine Oxidation in Children with Phenylketonuria (PKU)\(^1\)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Tyrosine Intake (mg·kg(^{-1})·day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>TN</td>
<td>38.30</td>
</tr>
<tr>
<td>LF</td>
<td>17.94</td>
</tr>
<tr>
<td>AK</td>
<td>21.16</td>
</tr>
<tr>
<td>MJ</td>
<td>28.47</td>
</tr>
<tr>
<td>TJ</td>
<td>17.44</td>
</tr>
</tbody>
</table>

Mean ± SEM: 26.66 ± 3.94\(^a\), 19.72 ± 4.63\(^b\), 14.10 ± 2.15\(^c\), 16.49 ± 2.83\(^c\), 15.05 ± 3.94\(^b\), 13.95 ± 4.21\(^c\), 17.62 ± 6.25\(^b\)

\(^1\) Lysine Oxidation is expressed as μmol·kg\(^{-1}\)·hour\(^{-1}\). By use of analysis of variance, tyrosine intake and subject significantly effected lysine oxidation (means with different superscripts are significantly different, P< 0.05), however, sex of the subject did not significantly affect lysine oxidation.
Figure 5.5. Effect of Tyrosine Intake on Mean Lysine Oxidation in Children with Phenylketonuria (PKU). Mean (± SEM) rate of lysine oxidation at 7 test tyrosine intakes. Pooled data of all observations (n=29) and all subjects (n=5). The breakpoint estimates the mean tyrosine requirement of the sample population.
Table 5.10. Individual Tyrosine Requirements for 5 Children with Phenylketonuria (PKU) Based on $^{13}$CO$_2$ and Lysine Oxidation

<table>
<thead>
<tr>
<th>Subject</th>
<th>Tyrosine Requirement$^2$</th>
<th>F$^{13}$CO$_2$</th>
<th>Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg·kg$^{-1}$·day$^{-1}$</td>
<td>mg·kg FFM$^{-1}$·day$^{-1}$</td>
<td>mg·kg$^{-1}$·day$^{-1}$</td>
</tr>
<tr>
<td>TN</td>
<td>16.0</td>
<td>21.5</td>
<td>15.0</td>
</tr>
<tr>
<td>LF</td>
<td>17.0</td>
<td>20.2</td>
<td>19.0</td>
</tr>
<tr>
<td>AK</td>
<td>25.0</td>
<td>33.6</td>
<td>18.0</td>
</tr>
<tr>
<td>MJ</td>
<td>20.0</td>
<td>24.0</td>
<td>16.0</td>
</tr>
<tr>
<td>TJ</td>
<td>16.0</td>
<td>21.6</td>
<td>15.0</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>18.8 ± 1.5</td>
<td>24.2 ± 2.2</td>
<td>16.6 ± 0.7</td>
</tr>
</tbody>
</table>

$^1$ Individual tyrosine requirements were determined by visual inspection.

$^2$ Tyrosine requirements are expressed per kg body weight and per kg FFM (FFM = fat free mass determined by BIA on study day one).
Although the absolute rates of lysine oxidation varied between subjects, the overall effect of incremental increases in tyrosine intake on oxidation was similar (Figure 5.6.). Individual tyrosine requirements derived from visual inspection of the lysine oxidation response curves are shown in Table 5.10. Requirements varied from 15.0 to 19.0 mg·kg\(^{-1}\)·day\(^{-1}\).

5.4. Discussion

This investigation was prompted by the lack of direct measurement of aromatic amino acid requirements in individuals with PKU. It was further prompted by the observation that amino acid homeostasis, while disturbed in untreated PKU, is not fully normalized in treated PKU (Hjelm et al. 1994). If untreated, PKU patients show low to normal plasma tyrosine concentrations (Efron et al. 1969; Koepp and Held 1977). Other authors have reported low plasma tyrosine concentrations in treated PKU patients after an overnight fast (Smith et al. 1990; Brouwer et al. 1977; Nord et al. 1988). Treated patients with PKU have both lower than healthy control and higher than healthy control postprandial plasma tyrosine concentrations while consuming the current recommended tyrosine enriched amino acid mixtures (van Spronsen et al. 1996). The clinical implications of low fasting plasma tyrosine concentrations are not known, however, low plasma tyrosine levels have been the basis for tyrosine supplementation in PKU. The current recommendations for aromatic amino acid intake in PKU far exceed the recommended intake for the general population (Food and Agriculture Organization of the United Nations-Rome 1985). The source of this difference is the supplemental tyrosine intake provided to PKU patients. The objective of the present study was to test the hypothesis that the tyrosine requirement in PKU accounts for 45% of the total aromatic amino acid requirement, and not 80%, as is suggested by the current recommendations.
Figure 5.6. Effect of Tyrosine Intake on Individual Lysine Oxidation
Tyrosine was selected as the first of the aromatic amino acids to study because although phenylalanine homeostasis is directly dependent on tyrosine homeostasis, the partition of tyrosine between uptake for protein synthesis and oxidation is not directly dependent on phenylalanine homeostasis. Furthermore, the current phenylalanine intakes of this treated group are closely monitored with respect to plasma phenylalanine levels, growth rates and neuropsychological development. Therefore, the phenylalanine intakes represent empirical estimates of the true phenylalanine requirement in these subjects. The phenylalanine intakes of these subjects may be assumed to be in slight excess of the true requirement since plasma phenylalanine concentrations are routinely maintained above the normal range (> 500μM), while growth rates are within the normal range. Finally, since the phenylalanine requirement in PKU is primarily based on the requirement for protein synthesis, it can only be studied once tyrosine requirements are known.

In the current indicator amino acid oxidation study, the mean tyrosine requirement, determined by two-phase linear regression crossover analysis, was estimated at 19.2 mg·kg⁻¹·day⁻¹ and 16.3 mg·kg⁻¹·day⁻¹ by the F¹³CO₂ and oxidation response curves, respectively. From the F¹³CO₂ and oxidation data, the upper 95% confidence interval of the breakpoint estimate was calculated as 25.2 mg·kg⁻¹·day⁻¹ and 26.8 mg·kg⁻¹·day⁻¹, respectively. The mean requirement estimates determined by the rate of ¹³CO₂ released and by lysine oxidation represent 44.4% and 40.4%, respectively, of the total aromatic amino acid intake. The requirement estimates derived from these two data sets are similar and support the hypothesis that tyrosine contributes approximately 45% of the total aromatic amino acid requirement in PKU, assuming the phenylalanine intake of 24 mg·kg⁻¹·day⁻¹ is a reasonable estimate of the true phenylalanine requirement. The proportion of tyrosine and phenylalanine to the aromatic amino acid requirement in PKU is consistent with the animal literature in which tyrosine was shown to spare 40 to 46% of phenylalanine requirement by both nitrogen balance (Milner et al. 1984;
Williams et al. 1987; Stockland et al. 1971) and tracer oxidation methods (House et al. 1997a; House et al. 1997b). The proportion is consistent with the requirements estimated in humans by nitrogen balance (Tolbert and Watts 1963; Leverton et al. 1956; Burrill and Schuck 1964) and is also in keeping with human plasma phenylalanine to tyrosine ratios (Scrimer et al. 1989) and with the ratio in mixed piglet body protein (Aumaitre and Duee 1974).

The results from nitrogen balance studies in humans are highly variable. Some studies agree with the current recommendation for tyrosine requirements in PKU (Elsas and Acosta 1994) and report that tyrosine can spare 70% (Tolbert and Watts 1963; Rose and Wixom 1955) and 80% (Leverton et al. 1956) of the phenylalanine requirement. Whereas others (Burrill and Schuck 1964; Leverton et al. 1956; Tolbert and Watts 1963) are consistent with the results of this study and have shown that tyrosine can spare 40 to 50% of the phenylalanine requirement. The results from Rose and Wixom (1955b) are suspect because they used excess energy intake of 56 kcal·kg⁻¹ and studied just two subjects. In addition to the problems associated with drawing conclusions from a small sample, the high energy intake would have led to an underestimate of aromatic amino acid requirements and its not clear what effect this would have had on the relative proportions of tyrosine and phenylalanine. Both Leverton et al. (1956) and Tolbert and Watts (1963) reported similar nitrogen balance results with tyrosine intakes that ranged from 40 to 80% of the aromatic amino acid intake. This suggests that in addition to the previously reviewed limitations of the nitrogen balance technique (Zello et al. 1995; Young 1994), nitrogen balance is also not sensitive enough to distinguish between phenylalanine to tyrosine ratios as different as ~1:4 and ~1:1. The results of Burrill and Schuck (1964), which are consistent with the animal literature, are also the most credible because of the relatively large sample size (n=22) and their use of a weight maintenance diet. Finally, our results of the mean tyrosine requirement estimates and the safe level of tyrosine intake are consistent with the estimate of the tyrosine requirement of 25 mg·kg⁻¹·day⁻¹, in children with
hypertyrosinemia, aged 9 to 10 years (Acosta and Elsas II. 1970). The tyrosine requirements in hypertyrosinemia are analogous to the phenylalanine requirement estimates in PKU in that they reflect intakes associated with optimal clinical outcomes and are therefore reasonable estimates of the true requirement.

Each subject was studied at six test tyrosine levels in the present study, thus enabling an approximation of their individual requirement. Significant differences between individuals were observed with respect to the lysine oxidation response, but not with respect to the F$^{13}$CO$_2$ response to changes in tyrosine intake. The individual patterns of lysine oxidation were however, consistent with the group oxidation data and with the F$^{13}$CO$_2$ data. The oxidation pattern observed in these individual measurements suggests an inflection in oxidation occurring between 15.0 and 19.0 mg·kg$^{-1}$·day$^{-1}$ of tyrosine intake. Individual tyrosine requirements based on inflections in the F$^{13}$CO$_2$ curves ranged from 16.0 to 25.0 mg·kg$^{-1}$·day$^{-1}$. This range of requirement suggests that amino acid requirements are subject dependent. However, the range was smaller in this group than in previous studies of healthy adult males (Duncan et al. 1996; Zello et al. 1993), which may relate to the greater relative homogeneity of the PKU group and to the fact that two of the subjects (MJ and TJ) were siblings.

The significant subject effect on lysine oxidation rates at variable tyrosine intakes reflects the significant difference between subjects in lysine flux. Oxidation is the rate of $^{13}$CO$_2$ released by tracer amino acid oxidation (F$^{13}$CO$_2$) corrected for the enrichment of amino acid in the free metabolic pool at isotopic steady state (Ep), with flux inversely related to Ep. In healthy adults, lysine flux ranges from 76.38 to 98.8 µmol·kg$^{-1}$·hour$^{-1}$ (Zello et al. 1992; Conway et al. 1980; Motil et al. 1981; Motil et al. 1994; Bross et al. 1996). Although direct comparisons cannot be made between children and adults, the range of lysine flux observed in the current PKU group (81.7 to 128.2 µmol·kg$^{-1}$·hour$^{-1}$) was consistent with the adult studies.
Lysine flux was not affected by changes in tyrosine intakes. This provides evidence that the overall metabolism of the indicator amino acid pool was not altered by varying tyrosine intake.

The similar tyrosine requirements determined from $F^{13}CO_2$ and lysine oxidation suggest that the $F^{13}CO_2$ response can be used as an accurate and noninvasive means to determine requirements by indicator amino acid oxidation. For those amino acids which do not have an intracellular pool indicator, such as ketoisocaproic acid for leucine, determination of $F^{13}CO_2$ may be preferable to oxidation, given the potential error associated with using plasma enrichment to measure the intracellular enrichment. In the original indicator amino acid oxidation studies in animals, $F^{13}CO_2$ and amino acid oxidation (determined by recovery of radioactivity in liver tissue) were shown to estimate similar requirements (Ball and Bayley 1984; Ball et al. 1986; Kim et al. 1983). The specific activity of the liver free phenylalanine was not influenced by the changes in test amino acid intake so that the release of $^{14}CO_2$ in response to changes in test amino acid intake were equivalent to the rate of hepatic phenylalanine oxidation. This suggested that it should be possible to estimate amino acid requirements without sampling the free amino acid metabolic pool. Zello et al. (1993) also reported close agreement between breakpoints derived from $F^{13}CO_2$ data and phenylalanine oxidation (determined from plasma enrichment) in adult males. Thus, the results from this and previous studies suggest that estimation of the breakpoint in the $F^{13}CO_2$ curve is a valid alternative method to determine amino acid requirement by the indicator amino acid oxidation approach and may represent an avenue by which indispensable amino acid requirements can be assessed in vulnerable populations.

This study demonstrates the first use of lysine as an indicator for amino acid requirements in a human population. Like phenylalanine, lysine is an indispensable amino acid,
it is catabolized primarily in the liver and routes of disappearance of its carboxyl carbon are protein synthesis and oxidation to CO₂ (Flodin (1997). Thus it fulfills the criteria of an indicator amino acid. Both phenylalanine and lysine were used as indicators in a study of tryptophan requirements in piglets (Ball and Bayley 1984). The oxidation of phenylalanine and lysine suggested similar tryptophan requirements, although phenylalanine was identified as the preferred indicator amino acid. However, lysine oxidation was only studied at four tryptophan levels (whereas phenylalanine was studied at 7, which made definition of a breakpoint mathematically tenuous). In this study, the pattern of F \(^{13}\)CO₂ or lysine oxidation, was similar to those observed in previous human studies in which phenylalanine was used as the indicator (Lazaris-Brunner 1994; Wilson et al. 1997; Duncan et al. 1996; Zello et al. 1993). Both F \(^{13}\)CO₂ and lysine oxidation decreased and then reached a plateau as tyrosine intake increased from deficient levels to levels above requirement. The results from this study demonstrate that \([^{13}\text{C}]\text{lysine}\) can be used as an indicator amino acid in the estimation of amino acid requirement in humans.

In the present study, the mean tyrosine requirement was estimated to be 16.3 to 19.2 mg \(\cdot\text{kg}^{-1}\cdot\text{day}^{-1}\), which represents 40.4 to 44.4% of the total aromatic amino acid intake in children with PKU. This proportion is consistent with previous estimates determined by nitrogen balance, amino acid oxidation and plasma tyrosine levels (Acosta and Elsas II. 1970; Burrill and Schuck 1964; Tolbert and Watts 1963; Leverton et al. 1956; Milner et al. 1984; Williams et al. 1987; Stockland et al. 1971). However, direct measurement of the phenylalanine requirement in PKU is required before final conclusions can be drawn regarding the relative contribution of tyrosine and phenylalanine to the total aromatic amino acid requirement in PKU. Therefore, the current recommendations for tyrosine intake in PKU patients are overestimated by a factor of \(\sim 5\). The findings of this study have significant implications with respect to the dietary treatment of individuals with PKU. This study is the first use of the indicator amino acid
oxidation method with a noninvasive experimental design in a population with an inborn error of metabolism. The conclusive results produced by this study supports the application of the indicator method in the determination of indispensable amino acid requirements in other vulnerable groups.
6. RESTING ENERGY EXPENDITURE IN INDIVIDUALS WITH PHENYLKETONURIA AND MAPLE SYRUP URINE DISEASE

6.1. Introduction

The Collaborative Study of Children Treated for Phenylketonuria (McBurnie et al. 1991; White et al. 1982; Holm et al. 1979) reported that prepubertal children older than 3 years receiving treatment for PKU were becoming overweight on average compared to the national standards for children their age. The study further reported that energy intakes in 4 year old children above the 93rd percentile of weight for height were insignificantly higher than in children below the 93rd percentile. Despite this insignificant difference, the authors concluded that energy intake could completely explain the weight difference between the two groups (White et al. 1982). The study also reported that being overweight up to 10 years of age was positively associated with plasma phenylalanine levels (McBurnie et al. 1991). The authors suggested that the relationship between weight and plasma phenylalanine was explained by differences in dietary intake, however neither phenylalanine nor energy intakes were determined. In an older PKU population, Schulz and Bremer (1995) reported a high proportion of slightly overweight patients aged 12 to 29 years, no longer receiving dietary treatment. Body mass index (BMI) was positively correlated to plasma phenylalanine levels, suggesting a possible increase in energy intake associated with an increased phenylalanine intake (Schulz and Bremer 1995). However, despite the availability of food intake records, no relationship between intake and BMI was described. Dietary intake studies of children with PKU have not demonstrated an increase in energy intake when compared with control subjects (McMurray et al. 1992). Also, nutrient intake studies of adolescents with PKU showed that energy intakes tended to be lower, and fat and protein intakes were consistently lower when compared to control subjects (Gropper et al. 1993).
Weight gain occurs when energy intake is greater than energy expenditure. Data suggest that a low rate of energy expenditure is a major risk factor for weight gain in humans (Ravussin et al. 1986; Ravussin et al. 1988; Ravussin and Bogardus 1992). Children with PKU may be at risk to become overweight because of a reduced resting energy expenditure (REE). Since REE accounts for 60 - 75% of total energy expenditure (Ravussin et al. 1988; Goran et al. 1993; Danforth, Jr. 1985), any reduction in REE could have a significant effect on energy balance. Previous studies have reported defective synthesis of serotonin, dopamine, and norepinephrine in PKU (Butler et al. 1981; Lou 1985; Krause et al. 1985). Higher plasma phenylalanine concentrations have been associated with lower CSF serotonin and 5-HIAA (McKean 1972) and reduced urinary excretion of dopamine and HVA (Krause et al. 1985).

Serotonin and catecholamines are also involved in the regulation of energy expenditure (Troiano et al. 1990; Cawthorne 1992; Myers and Waller 1978; Shetty 1990; Landsberg and Young 1983), suggesting that alterations in neurotransmitter synthesis may result in reduced REE and hence lower total energy expenditure in PKU.

One previous study of REE in children with classical PKU found no evidence of a reduced REE (Allen et al. 1995). This study was limited by a weak method for assessment of body composition and a sample population that did not include treated patients with more mild variants of PKU, who represent 1/3 of all treated PKU patients in Ontario (Hanley et al. 1997). The aims of the present study were to: (i) measure REE in patients with PKU in comparison with a group of patients with maple syrup urine disease (MSUD) and a group of similarly aged, healthy control subjects and (ii) to compare the ratio of tryptophan to the sum of the large neutral amino acids (LNAA) (as a marker of central serotonin levels (Fernstrom and Wurtman 1972) and urinary catecholamine and catecholamine metabolite levels (as markers of sympathetic nervous system activity) in patients with PKU and MSUD and investigate their relationship to REE. MSUD patients were selected as a comparison group because like PKU,
MSUD is associated with disturbances in large neutral amino acid concentrations and mental deficits, however, unlike PKU, it is not associated with excess body weight (Elsas and Acosta 1994; Danner and Elsas II. 1989).

6.2. Methods

6.2.1. Study Population

Twenty-three patients with PKU treated by the PKU Clinic of the Hospital For Sick Children (HSC), Toronto, participated on an outpatient basis, in the Clinical Investigation Unit at the HSC. Subjects with classical PKU, defined as persistent elevation of fasting plasma phenylalanine levels $>1200$ µM on an unrestricted diet (Hanley et al. 1997) and subjects with mild PKU, defined as fasting plasma phenylalanine levels of 601 to 1200 µM on an unrestricted diet (Hanley et al. 1997), both treated from early infancy with dietary phenylalanine restriction, were recruited.

Seven patients with classical MSUD treated by the Genetic Metabolic Diseases Clinic at the HSC, Toronto, participated on an outpatient basis, in the Clinical Investigation Unit at the HSC. Each subject had presented in the neonatal period and had typical clinical features of the disorder.

Twenty-eight control subjects were also studied. Healthy subjects had no history of disease or illness and did not have any family history of PKU or MSUD in first degree relatives. They were matched for age with the subjects with PKU and MSUD. Some of the control subjects ($n=17$) were recruited from the Adolescent Medicine Clinic at the HSC and were part of a previous control group in a study conducted in this laboratory (Azcue 1992). The remaining 11 subjects were recruited from employees and children of employees at the HSC.

The purpose of the study, the benefits and the potential risks involved were explained to the subjects and their parents/guardians. Written consent was obtained from the subjects or
from the subject's parent/guardian and detailed information sheets about the study day protocol were provided. The consent forms, information sheet, and newsletter, are found in Appendix 9.5 and 9.1. All procedures used in the study were approved by the University of Toronto Human Experimentation Committee and the Human Subjects Review Committee of the HSC.

6.2.2. **Experimental Design**

Patients with PKU were studied in the Clinical Investigation Unit on two nonconsecutive mornings. On each study day, patients arrived at the unit in the morning after an overnight fast. Height, weight and body composition were measured, followed by a 20 minute rest period. REE was then measured for 20 minutes. The first study day was scheduled to coincide with a visit to the PKU clinic where fasting blood was drawn for determination of plasma amino acids. This REE measurement was treated as a training period in order to acclimatize the patients to the testing procedure. The second study followed the same protocol except no blood samples were taken. A 24 hour urine collection was performed one or two days before the second study day.

The REE of the comparison groups of MSUD patients and controls were only studied on one occasion. Therefore, subjects were given extra time to rest and familiarize themselves with the testing procedure prior to the actual measurement. For the MSUD patients, one blood sample was drawn immediately following the REE measurement. The 24 hour urine collection was performed within three days of the REE measurement. Blood and urine were not collected in the healthy control subjects.

6.2.3. **Anthropometrics and Body Composition**

Height, weight and body composition were measured in all subjects after an overnight fast and following voiding. The methods for these measurements have been described earlier.
The Ideal Body Weight (IBW) was calculated (Moore et al. 1985) by plotting the height and weight values on the Growth and Development Charts of Tanner and Whitehouse (1966) for subjects up to 18 years of age. IBW in subjects above 18 years was determined by calculating the weight that would produce a Body Mass Index (BMI) of 22.5 kg·m⁻² (midpoint of the normal range) for a subject's actual height (Health and Welfare Canada 1988). Height and weight measurements were normalized by being expressed as standard deviation scores (SDS) (Frisancho 1993).

6.2.4. Resting Energy Expenditure

REE was determined by continuous indirect calorimetry with the use of a ventilated hood. The hood was placed over the subject's head while they lay supine on a hospital bed. A mass flow metre maintained the CO₂ concentration in the hood in the range of 0.5 to 0.8%. The 2900 Metabolic Cart is equipped with a paramagnetic O₂ analyser and an infrared CO₂ analyser. Prior to any VCO₂ measurement, gas analysers were calibrated with standard gases (Nitrogen, ~20% Oxygen and ~ 4% Carbon Dioxide, Linde Medical Gas, Union Carbide, Toronto, Ontario. CO₂ production was expressed under standard conditions (STPD): dry gas at 0°C and 760 mm Hg. Measured VCO₂ took place under ambient conditions, and the 2900 corrected the gas volumes to standard conditions. External calibration was performed every month by combusting 3.18 g absolute ethanol. The accuracy of O₂ consumption and CO₂ production were verified by combusting and verifying the accuracy of the respiratory quotient (RQ = 0.667) and CO₂ production rate based on the weight of ethanol consumed. The 2900 has less than a 2% error in determining the concentration of O₂ and CO₂ over the full range of concentrations (i.e. 0 - 100%) and the stability of the CO₂ analyser over the full range of concentration has an error of less than 0.2%. REE was determined from the measured VO₂ and VCO₂ using the abbreviated Weir equation (McClave and Snider 1992). REE measurements were performed
by the same operator, at the same time of day (7-10 am). The measurements were performed on subjects after a 6 - 8 hour sleep and after a 10 - 12 hour overnight fast in a semi-darkened room with only the subject and tester present. One parent was permitted in the room for younger children. If they wished, subjects listened to music or watched television to help them relax and prevent them from falling asleep. The room temperature was maintained between 21 - 24°C. Prior to each test, subjects lay in a semi-recumbent position on a bed for at least 20 minutes. A minimum 20 minute REE determination followed. The first 5 minutes of data collected was not included in the analysis. A steady state was required for acceptance of the data. The steady state was defined as the time during which the coefficient of variability for VO₂ and VCO₂ was less than 5% of the respective means.

REE was also calculated for each subject using the predictive equations published by the World Health Organization (Food and Agriculture Organization of the United Nations-Rome 1985). Measured REE was expressed as a percentage of the predicted value by dividing measured REE by predicted REE and multiplying by 100.

6.2.5. Blood Collections and Analysis

Postabsorptive venous blood samples were drawn by the Phlebotomy Department at the HSC for patients with PKU and MSUD. Blood was collected into two heparinized syringes by venipuncture. One sample was used for the determination of phenylalanine, tyrosine, valine, leucine and isoleucine concentrations and the other sample was used for tryptophan analysis. The former sample was centrifuged immediately after it was drawn and aliquots were stored at -70°C. Plasma phenylalanine, tyrosine, valine, leucine and isoleucine concentrations were determined by ion exchange chromatography with post column ninhydrin reaction with visible colorimetric detection, using the Beckman System 7300 high-performance amino acid
analyser (Beckman Instruments Canada, Mississauga, On) in the Department of Clinical Biochemistry at the HSC. Blood drawn for tryptophan analysis was retrieved from the Phlebotomy Department immediately after collection, centrifuged at 13000 rpm for five minutes at 4°C, and the plasma was stored at -20°C until analysis. Plasma tryptophan concentration was determined by the fluorometric method of Denkla and Dewey (1967) with the Bloxam and Warren (1974) modification. The method is based on the formation of the fluorophore norharman from tryptophan, by condensation with formaldehyde followed by oxidation with FeCl₃. The ratio of tryptophan to LNAA (phenylalanine + tyrosine + valine + leucine + isoleucine) was calculated.

6.2.6. Urine Collections and Analysis

One complete 24 hour urine collection was made in all PKU and MSUD patients. Urine was collected in containers with 5 or 10 mL of 6N HCl (for ages 1-5 years and > 5 years, respectively) as preservative. Urine was refrigerated immediately upon collection and within 24 hours, urine volume was measured and multiple samples frozen at -70°C for analysis of creatinine, dopamine, epinephrine, norepinephrine, metanephrine, VMA and HVA. Creatinine, the catecholamines, VMA and HVA concentrations were determined in the Department of Clinical Biochemistry of the HSC. Urinary metanephrine was determined in the Special Chemistry Laboratory of St. Michael's Hospital. Urinary creatinine concentration was determined on the Kodak Ektachem Clinical Chemistry Analyser (Johnson & Johnson Diagnostics Inc., Rochester New York). Dopamine, epinephrine and norepinephrine concentrations were determined by high performance liquid chromatographic (HPLC) analysis (HPLC Series 2/2, Perkin-Elmer Corp., Norwalk, CT) (Soldin et al. 1980). Urinary VMA and HVA were determined by ion exchange purification and HPLC analysis with electrochemical detection, according to the method published by BIO-RAD (BIO-RAD, Richmond CA). Urinary
metanephrine was determined by HPLC following sample purification by cation exchange chromatography, according to the method published by BIO-RAD (BIO-RAD, Richmond, CA).

6.2.7. **Data Analysis**

Results are presented as mean ± standard error of the mean (SEM). A minimum sample size of 7.8 subjects per group was estimated, based on a power calculation for a one-tailed test and α and β levels of 0.05; given a 3% intra individual variation of REE on different days, an intra individual standard deviation of 3%, and a 5% difference between PKU and MSUD or control subjects (considered physiologically relevant). Statistical analyses were conducted using SAS software (SAS Institute, Cary, NC). One way analysis of variance (ANOVA) was used to compare differences between means in PKU patients with MSUD and control subjects. Comparisons of the REE in PKU, MSUD and control groups were made by analysis of covariance with subject classification (PKU, MSUD and Control) as the grouping variable and FFM as the covariate. In analysis of covariance, test of a significant group effect on REE is first assessed by comparing the variance in REE due to group, with variance in REE due to random error. When the variance in REE due to the group variable is greater than that due to random error, a significant group effect is concluded. Analysis of covariance then tests for differences in adjusted means (REE adjusted for FFM) based on the assumption that the slopes of the dependent variable (REE) on the covariate (FFM) were the same in each group. The method of least squares estimation was used in the comparison of REE between groups. A stepwise multiple linear regression analysis was used to determine the best predictor variables for REE, expressed as a percent of predicted REE for the PKU and MSUD groups. Differences between means of plasma amino acid concentration and urinary catecholamine and catecholamine metabolite levels in the PKU and MSUD groups were tested for significance using a Student’s unpaired t-test. The level of significance was defined as p less than 0.05.
6.3. Results

The physical characteristics of the patients with PKU and MSUD and the control subjects are shown in Table 6.1. There was no difference in age, height, weight, weight expressed as a percent of IBW (%IBW), FFM or weight for height SDS. However, patients with MSUD had a lower weight for age (p=0.03) and height for age (p=0.002) SDS than PKU or control subjects.

A significant correlation existed between REE and FFM in the PKU (r=0.93), MSUD (r=0.89), and control (r=0.91) groups and in the three groups combined (r=0.89). The relationship between FFM and REE is presented in Figure 6.1.

The results of indirect calorimetry are shown in Table 6.2. The PKU, MSUD and control groups showed a similar measured REE expressed in absolute terms (kJ per day, unadjusted for FFM). Patients with PKU had a lower measured REE expressed as a percent of predicted REE (PREE) than MSUD (p=0.006) or control groups (p=0.003). There were no differences in REE expressed as a percent of PREE between MSUD and control groups.

Using analysis of covariance, we compared the REE in PKU, MSUD and control groups, using FFM as a covariate. REE adjusted for FFM was significantly lower in PKU (adjusted mean REE = 1297 ± 22 kcal, 5427 ± 92 kJ) than in both MSUD (adjusted mean REE = 1420 ± 39 kcal, 5941 ± 164 kJ) and control groups (adjusted mean REE = 1364 ± 20 kcal, 5706 ± 84 kJ) (p=0.01) (Table 6.2.). There was no significant difference in REE adjusted for FFM between MSUD and control groups.

Plasma amino acid concentrations in patients with PKU and MSUD are shown in Table 6.3. The concentrations of the amino acids which characterize the two amino acid disorders (i.e. phenylalanine in PKU and leucine and isoleucine in MSUD) were significantly different between the two patient groups (p<0.0001). Plasma valine concentrations were not different between the two groups. Plasma tryptophan concentrations were not different between the two
Table 6.1. Characteristics of Patients with Phenylketonuria (PKU) Compared with Patients with Maple Syrup Urine Disease (MSUD) and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>PKU (n=23, 12M:11F)</th>
<th>MSUD (n=7, 4M:3F)</th>
<th>Control (n=28, 19M:9F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11 ± 1.2</td>
<td>15.9 ± 2.2</td>
<td>14.0 ± 1.5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>136.7 ± 4.9</td>
<td>150.4 ± 6.6</td>
<td>148.2 ± 4.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>37.6 ± 3.8</td>
<td>46.7 ± 5.6</td>
<td>45.7 ± 3.6</td>
</tr>
<tr>
<td>%IBW</td>
<td>105.3 ± 3.3</td>
<td>99.2 ± 4.3</td>
<td>101.7 ± 1.8</td>
</tr>
<tr>
<td>FFM-BIA (kg)</td>
<td>29.5 ± 2.7</td>
<td>33.7 ± 4.2</td>
<td>34.7 ± 2.3</td>
</tr>
<tr>
<td>Weight x Age (SDS)</td>
<td>0.53 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.79 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Weight x Height (SDS)</td>
<td>0.45 ± 0.33</td>
<td>-0.43 ± 0.34</td>
<td>0.08 ± 0.15</td>
</tr>
<tr>
<td>Height x Age (SDS)</td>
<td>0.12 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.34 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> mean ± SEM. IBW, ideal body weight. FFM-BIA, fat free mass determined by bioelectrical impedance. SDS, standard deviation score.

Means with different superscripts are significantly different, p<0.05.
Table 6.2. The Resting Energy Expenditure (REE) of Patients with Phenylketonuria (PKU) Compared with Patients with Maple Syrup Urine Disease (MSUD) and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>PKU (n=23)</th>
<th>MSUD (n=7)</th>
<th>Control (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REE (kJ·day⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>5722 ± 247</td>
<td>6088 ± 456</td>
<td>5971 ± 243</td>
</tr>
<tr>
<td>FFM Adjusted²</td>
<td>5427 ± 92a</td>
<td>5941 ± 164b</td>
<td>5706 ± 84b</td>
</tr>
<tr>
<td>PREE (kJ·day⁻¹)</td>
<td>5261 ± 241</td>
<td>5665 ± 375</td>
<td>5707 ± 226</td>
</tr>
<tr>
<td>REE/PREE (%)</td>
<td>98 ± 1a</td>
<td>107 ± 3b</td>
<td>105 ± 2b</td>
</tr>
</tbody>
</table>

¹ mean ± SEM. PREE, predicted resting energy expenditure based on the equations of the World Health Organization (FAO/WHO/UNU, 1985).

² By use of analysis of covariance, REE is adjusted for FFM.

Lower-case letters denote comparisons across the PKU, MSUD and control groups.

Means with different superscripts are significantly different, p<0.05.
Figure 6.1. Relationship Between Fat Free Mass (FFM) and Resting Energy Expenditure (REE) in Patients with Phenylketonuria (PKU; stars) Compared with Maple Syrup Urine Disease (MSUD; open triangle) and Control Subjects (open circles). Regression lines are as follows: PKU: REE = 2639.56 + 85.94FFM \( (r = 0.93) \); MSUD: REE = 2823.61 + 96.82FFM \( (r = 0.89) \); Controls: REE = 2700.86 + 94.39FFM \( (r = 0.91) \).
### Table 6.3. Plasma Amino Acid concentrations (μM) in Patients with Phenylketonuria (PKU) Compared with Patients with Maple Syrup Urine Disease (MSUD)

<table>
<thead>
<tr>
<th></th>
<th>PKU (n=23)</th>
<th>MSUD (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>746 ± 65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>44 ± 4</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>Leucine</td>
<td>103 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>416 ± 71&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Valine</td>
<td>190 ± 10</td>
<td>212 ± 36</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>50 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>107 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>45 ± 1</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>LNAA</td>
<td>1167 ± 68</td>
<td>821 ± 112</td>
</tr>
<tr>
<td>Trp:LNAA</td>
<td>0.039 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.053 ± 0.006&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> mean ± SEM. Trp:LNAA, ratio of tryptophan to the sum of the large neutral amino acids (phenylalanine, tyrosine, leucine, valine and isoleucine).

Means with different superscripts are significantly different, p<0.05.
patient groups, however, the PKU patients had a reduced tryptophan:LNAA (p=0.03) compared to the MSUD patients.

Urinary excretion of catecholamines and catecholamine metabolites in patients with PKU and MSUD are shown in Table 6.4. There were no significant differences in the urinary excretion of the catecholamines or their metabolites (normalized to creatinine excretion) between patients with PKU and patients with MSUD.

None of the amino acid or catecholamine variables measured were significant in predicting REE expressed as a percent of PREE in either the PKU or MSUD group.

6.4. Discussion

This study shows that REE, adjusted for FFM, is significantly reduced in patients with PKU compared to patients with MSUD and control subjects. Although the tryptophan:LNAA ratio was significantly lower in the PKU patients compared to MSUD patients, it did not predict differences in REE expressed as a percent of predicted REE. Furthermore, none of the amino acid or catecholamine variables measured were significant in predicting REE expressed as a percent of predicted REE. The patients with PKU had a body composition similar to that of the control subjects and there was no evidence of a higher frequency of excess body weight, indicated by %IBW, in PKU compared to MSUD and control subjects. The patients with MSUD had significantly lower weight for age and height for age SDS compared to patients with PKU and control subjects. The outcome of newborn screening, retrieval, diagnosis, and management of MSUD has improved significantly since 1981 (Danner and Elsas II. 1989). This may explain the below average SDSs in the MSUD group, since four out of the seven patients were born before 1981 and would therefore, not have been identified or treated as early or with the same level of management as those patients born after 1981. In contrast, mass newborn
Table 6.4. Urinary Excretion of Catecholamines and Catecholamine Metabolites in Patients with Phenylketonuria (PKU) Compared with Patients with Maple Syrup Urine Disease (MSUD)\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>PKU (n=23)</th>
<th>MSUD (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>(26.3 \pm 3.0)</td>
<td>(29.9 \pm 3.7)</td>
</tr>
<tr>
<td>((\mu)mol/mol creatinine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine</td>
<td>(7.9 \pm 1.2)</td>
<td>(6.1 \pm 0.9)</td>
</tr>
<tr>
<td>((\mu)mol/mol creatinine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>(223.6 \pm 32.9)</td>
<td>(184.3 \pm 37.5)</td>
</tr>
<tr>
<td>((\mu)mol/mol creatinine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metanephrine</td>
<td>(0.19 \pm 0.03)</td>
<td>(0.19 \pm 0.03)</td>
</tr>
<tr>
<td>(mmol/mol creatinine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VMA</td>
<td>(2282.2 \pm 159.0)</td>
<td>(2351.7 \pm 262.3)</td>
</tr>
<tr>
<td>((\mu)mol/mol creatinine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVA</td>
<td>(3.9 \pm 0.4)</td>
<td>(3.4 \pm 0.5)</td>
</tr>
<tr>
<td>(mmol/mol creatinine)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) mean \(\pm\) SEM.
screening for PKU began in 1965 (Advisory Committee on Inborn Errors of Metabolism 1976), therefore most of the patients studied here were diagnosed at birth and treated.

Fat free mass, representing the active metabolic tissue of the body, is highly correlated with REE (Ravussin et al. 1986; Weinsier et al. 1992) and is the best predictor of REE in children (Goran et al. 1994; Bandini et al. 1990). Therefore, the most appropriate comparison of REE between groups is as a function of FFM. As in previous studies (Goran et al. 1994; Bandini et al. 1990; Weinsier et al. 1992), a significant correlation existed between REE and FFM in the three study groups. Using analysis of covariance with subject classification (PKU, MSUD, control) as the grouping factor and FFM as the covariate, we found that REE was lower in the PKU group than in the MSUD or control groups. In contrast, Allen et al. (1995) found no evidence of a reduced REE in patients with classical PKU compared to control subjects. Several factors which distinguish the present study from that of Allen et al. (1995) may explain the different REE findings. Allen et al. (1995) tested the hypothesis that lower body protein content per unit of FFM in children with classical PKU would result in a lower REE. FFM was indirectly estimated from multiple skinfold measurements. This technique has significant limitations with respect to precision (Lukaski 1987) and furthermore, it is not clear how skinfold thickness measurements would measure the protein content of the FFM. Limitations of this technique may explain why body weight was used as the covariate for REE and not FFM, which is considered the best predictor of REE in children (Goran et al. 1994; Bandini et al. 1990). In the present study, FFM determined by BIA was significantly correlated to REE in PKU, MSUD and Control groups and was used as the covariate in the analysis of covariance. Another difference between the two studies which may relate to the different observations in REE is the composition of the PKU populations studied. All of the subjects studied by Allen et al. (1995) had pretreatment phenylalanine levels >1000 μM, consistent with a classification of classical PKU. In the present study 10 out of the 23 patients studied had atypical or mild PKU, the
remaining 13 patients had classical PKU. Finally, Allen et al. (1995) reported that female PKU subjects had a higher rate of REE compared with predicted REE (Schofield 1985). This may indicate that the protocol used to acclimatize the children to the testing procedures was ineffective and/or that the sample of patients studied was not representative of the population.

Predicted REE was based on the equations of the FAO/WHO/UNU (1985). The FAO/WHO/UNU (1985) devises total energy requirements for a group of individuals based on the predicted REE. The REE is predicted from a series of simple linear regression equations, partitioned into male versus female, and broken down into different age categories. In this study measured REE expressed as a percent of predicted REE was significantly lower in the PKU group than in the other two groups. The 7% mean difference between PKU and control groups is physiologically significant and supports the results of the analysis of covariance. However, the fact that all the groups had REE within ±10% of predicted REE and that the uncertainty of the prediction is about ±7 to 10% for a single individual (FAO/WHO/UNU 1985; Schofield 1985), suggests that no clear conclusions can be drawn about the significance of the energy expenditure data when it is expressed as a percent of predicted REE.

The synthesis and release of serotonin by brain neurons is influenced by the tryptophan concentration in the brain (Fernstrom and Fernstrom 1995). Brain tryptophan levels reflect its uptake from the circulation; uptake occurs via a transport carrier shared between tryptophan, tyrosine, leucine, isoleucine, valine and phenylalanine. Transport carrier competition is one postulated mechanism by which hyperphenylalaninemia is thought to limit the uptake of tryptophan and tyrosine from circulation and lead to defective serotonin and catecholamine synthesis in PKU (Butler et al. 1981; Lou 1985; Krause et al. 1985). Hyperphenylalaninemia may also competitively inhibit tryptophan- and tyrosine- 3-hydroxylase, thereby decreasing synthesis of serotonin and catecholamines (Udenfriend 1967; Ikeda et al. 1967). We measured the ratio of plasma tryptophan to the sum of the concentrations of the other LNAAs
which provides an index of tryptophan influx into the brain, and of serotonin levels in the brain (Wurtman and Fernstrom 1975). The ratio was significantly lower in the PKU group compared to the MSUD group, despite similar plasma tryptophan levels. This suggests that the degree to which plasma phenylalanine deviates from normal limits in PKU is greater than the degree to which the sum of plasma leucine, isoleucine and valine deviate from normal limits in MSUD, thus competition for tryptophan uptake into the brain is apparently greater in PKU than MSUD. The lower ratio may indicate reduced brain tryptophan availability and synthesis of serotonin in the PKU patients, however, the ratios in both groups were below the observed normal range reported in the literature (0.06 to 0.16) (Lehnert and Wurtman 1993).

Increases in dietary tryptophan cause increases in brain tryptophan concentration, which in turn produce proportionate increases in brain serotonin (Wurtman and Fernstrom 1975). But, this relationship ceases when tryptophan intakes produce brain tryptophan levels that are above the physiological range, such that no further increments in brain serotonin are observed (Wurtman and Fernstrom 1975). The corollary to this observation is that while reductions in the ratio of tryptophan to the sum of the LNAA s correlate to reductions in brain tryptophan and serotonin, this relationship must fail below a minimum ratio, such that no further decreases in brain serotonin are observed. Whether the tryptophan:LNAA ratio continues to be a significant index of brain tryptophan or of brain serotonin levels when it falls below the physiological range is unknown. It is also unclear whether the difference in the ratio observed between PKU and MSUD groups might predict a true difference in brain serotonin levels. The tryptophan:LNAA ratio was not a significant predictor of REE expressed as percent of predicted REE in PKU or MSUD groups. The lack of a relationship in the MSUD group may suggest that serotonin production is not significantly related to REE. However, due to the small sample size, a significant correlation between REE and the tryptophan:LNAA ratio would be difficult to describe. The fact that the ratio did not predict REE expressed as percent of predicted REE
may also suggest that serotonin production is not significantly related to REE in PKU and does not explain the observation of a reduced REE. However, the limitations of the ratio itself makes it impossible to draw any final conclusions in either group.

Urinary excretion of dopamine, epinephrine, norepinephrine, VMA (derived almost entirely from peripheral adrenal epinephrine and norepinephrine metabolism) (Molinoff and Axelrod 1971), HVA (derived in part from dopamine turnover) (Mass et al. 1980), metanephrine (a methoxy derivative of epinephrine) (Molinoff and Axelrod 1971) were measured as indicators of sympathetic nervous system activity. There were no significant differences between the two groups in any of the catecholamines or catecholamine metabolites. Furthermore, none of the catecholamine variables predicted REE expressed as percent of predicted REE in either the PKU or MSUD group.

Measuring SNS activity through plasma or urine concentrations remains controversial. The majority of circulating NE arises from stimulation of the SNS. The fraction of neurotransmitter measurable in the blood (the "spillover concentration") however, is quite small and depends on many complex processes including synthesis, release, re-uptake, metabolism and clearance (Fernandez et al. 1988). An alternative to measuring NE in plasma is to determine its rate of excretion in urine. Animal studies (Kopp et al. 1983) suggest that there is a reasonably good relationship between arterial NE concentration and the rate of urinary NE excretion, however, it is not clear to what extent this applies in man. Furthermore, it is not clear how changes in catecholamine metabolism may change the proportions of free NE, conjugated NE and the methylated or deaminated metabolites excreted, thus weakening the relationship between SNS activity and urinary NE excretion (MacDonald 1992). There are limited data reporting on the validity of using urinary catecholamines as a measure of a SNS activity. One study reported intraindividual correlations between plasma and urine measurements of 0.7 (p<0.001) for epinephrine and NE in experimentally stress-stimulated catecholamine levels in
young men (Akerstedt et al. 1983). SNS activity can be accurately assessed by measurement of NE turnover in specific isolated sympathetically innervated tissue (Landsberg and Young 1983; Young and Landsberg 1981). The lack of a relationship between urinary catecholamines or their metabolites and REE, expressed as a percent of predicted, may indicate that reduced SNS activity is not a factor in the reduced REE observed in the PKU group. However, the limitations of urinary catecholamines and their metabolites as indicators of SNS activity suggests that more accurate techniques of assessing SNS activity and catecholamine production should be evaluated before final conclusions can be drawn regarding the role of reduced catecholamine synthesis in PKU and reduced REE.

Excess body weight is caused by an imbalance between energy intake and expenditure. When energy intake exceeds energy expenditure the excess calories are stored as adipose tissue. In the presence of an abundant food supply, it is easy to understand how a low REE increases the risk for weight gain (Ravussin 1995; Ravussin et al. 1988; Ravussin and Bogardus 1992). In this study, the group with PKU were not overweight compared with MSUD or control subjects. This suggests that their energy intake did not exceed their expenditure. Energy intake was not measured in this study, however, in order for patients with PKU to be in energy balance (or gaining weight appropriate for gains in height), their intake would have had to be lower than in the other groups, or their activity level would have had to be higher by an amount equal to the difference in their REE. Previous studies found that energy intake in children with PKU is not different from control subjects (McMurray et al. 1992). If some of the PKU patients in the intake study (McMurray et al. 1992) had similar reductions in REE as seen in this study, and similar activity levels as control subjects, then a similar energy intake would produce a positive energy balance of ~293 kJ (70 kcal) per day, which over a one year period would result in a weight gain of 3.3 kg. This excess weight gain is consistent with the degree to which children with PKU are overweight. Without energy intake and expenditure data, the
cause for the increased weight in the PKU population in United States remains unclear, however, a reduced REE may in part explain this observation.

This study found a significantly lower REE, adjusted for FFM in patients with PKU compared to patients with MSUD and healthy control subjects. This provides some evidence to support the hypothesis that REE in patients with PKU is reduced and these results may in part explain the above average weight of children with PKU.
7. GENERAL DISCUSSION AND CONCLUSIONS

The main aim of this dissertation was to begin to reevaluate the nutritional requirements in patients with PKU. Specifically, the objectives were: (i) to estimate the tyrosine requirements by Indicator Amino Acid Oxidation in children with classical PKU and (ii) to measure the resting energy expenditure of individuals with PKU, using patients with MSUD and healthy control subjects as comparison groups.

The initial experiments conducted led to the development of a noninvasive model to measure Indicator Amino Acid Oxidation in children or other vulnerable groups. This model was subsequently used to test the hypothesis that tyrosine requirements in PKU account for ~45% of the aromatic amino acid requirement. In the final study, REE was measured in PKU, MSUD and control subjects to test the hypothesis that the REE of individuals with hyperphenylalaninemia due to PKU is lower than predicted because synthesis of serotonin and catecholamines are reduced secondary to imbalances in plasma large neutral amino acids.

A one day, noninvasive isotope infusion method was developed that produced the necessary steady state conditions needed to estimate amino acid kinetic parameters and could therefore, be used to estimate indispensable amino acid requirements by the Indicator Amino Acid Oxidation technique. This simplified infusion model was tested with [1-13C] phenylalanine and [1-13C] lysine, two amino acids that have been shown to be suitable indicator amino acids (section 2.2.5.3.2.). Although the noninvasive model was developed to study tyrosine requirements in children with classical PKU, it can be applied in other vulnerable populations to study amino acid metabolism as well as to estimate indispensable amino acid requirements. At the present time, the model is limited to those populations with relatively fixed diets, whose macronutrient compositions are similar, and where the macronutrient composition of the experimental diet can be matched to the population's usual intake. Studies to determine the effect of the macronutrient composition of an individual's usual diet on amino acid kinetics are
ongoing in this laboratory. The effect of adaptation to the experimental diet on amino acid kinetics and therefore, on estimates of requirements, needs further study before it can be determined whether this one day protocol can be applied in populations with variable nutrient intakes.

The one day, oral, primed, equal dose [1-13C] lysine infusion protocol was then used to study tyrosine requirements in children with classical PKU by examining the effect of incremental increases in dietary tyrosine on lysine oxidation (section 5.). At a fixed phenylalanine intake (24 mg·kg⁻¹·d⁻¹), the tyrosine requirement determined by the rate of ¹³CO₂ release and by lysine oxidation was 19.2 and 16.3 mg·kg⁻¹, respectively and accounted for 44.4 and 40.4%, respectively, of the total aromatic amino acid intake. This finding supported our hypothesis and indicates that the current tyrosine requirement recommendations are overestimated by a factor of ~5 (section 2.3.6.) and thus, need to be revised. The findings of this study and the recent reports of high plasma tyrosine concentrations in PKU patients consuming the current recommended tyrosine intakes (van Spronsen et al. 1996) may indicate a new mechanism contributing to abnormal cognitive development in PKU. Elevated plasma tyrosine levels in hypertyrosinemia (type II) is associated with a variable degree of mental retardation in some patients (Elsas and Acosta 1994), however it is not clear whether the high postprandial plasma tyrosine levels observed in PKU patients (van Spronsen et al. 1996) are in fact high enough to produce similar effects. Research efforts will be required to determine what effect excessive tyrosine intakes have on the transport of other LNAA, on the synthesis of serotonin and of protein in general and on indices of cognitive function.

The next step in reassessing nutritional requirements in PKU will be to estimate phenylalanine requirements by the Indicator Amino Acid Oxidation technique. A more accurate estimation of the proportion of tyrosine and phenylalanine to the aromatic amino acid requirement in PKU can then be made. This study was the first use of the Indicator Amino Acid
Oxidation method with a noninvasive protocol in a pediatric population with an inborn error of metabolism. The conclusive results from this study suggest that the Indicator Amino Acid Oxidation technique can be successfully applied in other vulnerable groups to estimate indispensable amino acid requirements. For example, this technique can be used to determine the requirements of the branched chain amino acids in MSUD.

In the final study (section 6.), REE was measured in patients with PKU in order to determine if the prevalence of above average weight in children with PKU was in part related to a low REE. When adjusted for FFM, REE was found to be significantly reduced in patients with PKU compared to MSUD and control subjects. Indirect measures of serotonin and catecholamine production did not significantly relate to REE, expressed as a percent of predicted in the PKU group. The mechanism leading to the reduced REE in PKU is unknown. Measurements of CSF concentrations of serotonin and the catecholamines together with measurement of REE would make it possible to determine if the reduced REE in PKU is associated with alterations in central neurotransmitter synthesis. The finding of a reduced REE in PKU suggests one factor which may contribute to the tendency of patients with PKU to gain weight. Detailed measures of energy intake and energy expenditure are needed to fully understand the cause for this tendency. Genomic information would also make it possible to determine whether a low REE is a phenotype associated with a specific genetic mutation.

This body of work makes significant and unique contributions to the areas of amino acid and energy metabolism. The noninvasive method to measure indispensable amino acid kinetics opens up new avenues by which previously excluded populations can now be studied. For patients with PKU, we now have a better understanding of their tyrosine requirements and future studies are planned to estimate phenylalanine requirements. The results reached in this study also suggest that the Indicator Amino Acid Oxidation technique can be used in patients with other inborn errors of metabolism. Finally, the data on REE contributes to the PKU
literature by identifying one possible cause for the tendency of PKU children to be overweight. The novel findings from this study also suggest new areas of research to pursue in the search to determine the cause for excess body weight in children with PKU.
8. BIBLIOGRAPHY


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9. APPENDIX

9.1. Consent and Information Forms

Title: The kinetics of orally administered $^{13}$C Phenylalanine or $^{13}$C Lysine.

Investigators: Dr P Pencharz, MD PhD
Rachelle Bross, RD MSc

I acknowledge that the research procedures have been explained to me and that all my questions have been answered satisfactorily. I understand the possible risks and discomforts associated with the study.

I understand that I am free to withdraw from the study at any time without consequence. Withdrawal from the study prior to completion will result in forfeit of compensation.

Subjects Name: ________________________

Signature: _________________________

Investigators signature: _________________________

Date: _________________________

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9.2. *Consent and Information Forms*

**RESEARCH CONSENT FORM**

*(For patients 16 years or older)*

**Title:** TYROSINE AND PHENYLALANINE REQUIREMENTS IN PATIENTS WITH PKU

**Investigators:**

Rachelle Bross M.Sc., R.D.  Division of Gastroenterology/Nutrition  Ph: 813-6175
Dr. P. Pencharz  Division of Gastroenterology/Nutrition  Ph: 813-6176
Ms. V. Austin  Division of Clinical Genetics  Ph: 813-5332
Dr. J. Clarke  Division of Clinical Genetics  Ph: 813-5753
Dr. A. Feigenbaum  Division of Clinical Genetics  Ph: 813-5333
Dr. W. Hanley  Division of Clinical Genetics  Ph: 813-6635
Ms. W. Schoonheyt  Division of Clinical Genetics  Ph: 813-6356

**Purpose of the Research:**

It is hard to achieve normal blood tyrosine and phenylalanine levels in patients with PKU, in part, because we don't have good estimates of their requirements for tyrosine and phenylalanine. We want to measure the tyrosine and phenylalanine requirements in individuals with PKU using a new technique. Increasing our knowledge about the requirements for these amino acids will improve the dietary treatment of individuals with PKU, which we believe will lead directly to better outcome.

**Description of the Research:**

If you decide to enter the study, we will be measuring the amount of tyrosine and/or phenylalanine that you require to be in metabolic control. This will involve six study days for each amino acid. You have the choice of entering the study for one set of six study days or for both. The six study days will not be consecutive, but you will be required to complete all six days within a 2 month period.

On each study day you will be required to spend 8 hours in the Clinical Nutrition Laboratory of the Hospital for Sick Children, where your breath and urine will be collected. You will arrive at the hospital after an overnight, 10 - 12 hour fast, during which time no food or drink (except water) is taken after the previous night's supper. During each study day you will lie comfortably on a bed or sit in a chair and watch TV, read, or listen to music. We will start by measuring your height and weight. Body fat will be determined by measuring skin thickness.
from the arm, waist and back using a caliper. After this we will measure the amount of muscle in your body by placing four small stickers on the right arm and leg. These stickers are connected to a machine by wires. The machine computes the amount of muscle in the body by passing a small current through the body. The current used in this test is extremely small and the body does not feel anything. The body fat and muscle measurements are completely safe and painless and will be done on the first and last study day.

During the study period you will eat 8 small meals spaced one hour apart. The meals will be made up of three parts: (1) a mixture of amino acids in which the amount of tyrosine or phenylalanine will either be less than adequate, adequate or more than adequate; (2) an amino acid free flavoured liquid formula; and (3) amino acid free cookies. Your individual nutritional requirements will be determined prior to beginning the study and the test diet will be designed to meet your energy and protein requirements. During the last 4 hours of each study day you will also consume a solution made up of water and an amino acid that contains a naturally occurring label. An amino acid which contains a label is different from a regular amino acid only in that it weighs more. This label (stable isotope), found naturally in small amounts in your body, is harmless and is used routinely in human nutrition research. It is the amount of this heavy isotope that we measure in your breath and urine that allows us to determine your requirements for tyrosine and phenylalanine.

**Potential Harms:**
All of the tests used in this protocol are harmless.

**Potential Benefits:**
You will benefit directly from participating in this study. The results of the study will enable us to determine your individual tyrosine and phenylalanine requirements which can lead to improved metabolic control. The results of the study will also be beneficial to other individuals with PKU since they will be used to estimate the requirements for tyrosine and phenylalanine of other similar individuals.

**Confidentiality:**
Confidentiality will be respected and no information that discloses your identity will be released or published without consent. For your information, the research consent form will be inserted in the patient's health record.

**Participation:**
Participation in research must be voluntary. If you choose not to participate, you and your family members will continue to have access to quality care at HSC. You can withdraw anytime during the study and again your family will continue to have access to quality care at HSC. You may ask any questions now or during the study.
Title: TYROSINE AND PHENYLALANINE REQUIREMENTS IN PATIENTS WITH PKU

Purpose of the Research:
It is hard to achieve normal blood tyrosine and phenylalanine levels in patients with PKU, in part, because we don't have good estimates of their requirements for tyrosine and phenylalanine. We want to measure the tyrosine and phenylalanine requirements in individuals with PKU using a new technique. Increasing our knowledge about the requirements for these amino acids will improve the dietary treatment of individuals with PKU, which we believe will lead directly to better outcome.

Description of the Research:
If you and your child decide that he/she will to enter the study, we will be measuring the amount of tyrosine and/or phenylalanine that your child requires to be in metabolic control. This will involve six study days for each amino acid. Your child has the choice of entering the study for one set of six study days or for both. The six study days will not be consecutive, but your child will be required to complete all six days within a 2 month period.

On each study day your child will be required to spend 8 hours in the Clinical Nutrition Laboratory of the Hospital for Sick Children, where your child's breath and urine will be collected. You may be present with your child during the entire study period. Your child will arrive at the hospital after an overnight, 10 - 12 hour fast, during which time no food or drink (except water) is taken after the previous night's supper. During each study day your child will lie comfortably on a bed or sit in a chair and watch TV, read, or listen to music. We will start by measuring your child's height and weight. Body fat will be determined by measuring skin thickness from the arm, waist and back using a caliper. After this we will measure the amount of muscle in your child's body by placing four small stickers on the right arm and leg. These stickers are connected to a machine by wires. The machine computes the amount of muscle in the body by passing a small current through the body. The current used in this test is extremely small and the body does not feel anything. The body fat and muscle measurements are completely safe and painless and will be done on the first and last study day.
During the study period your child will eat 8 small meals spaced one hour apart. The meals will be made up of three parts: (1) a mixture of amino acids in which the amount of tyrosine or phenylalanine will either be less than adequate, adequate or more than adequate; (2) an amino acid free flavoured liquid formula; and (3) amino acid free cookies. Your child's individual nutritional requirements will be determined prior to beginning the study and the test diet will be designed to meet your child's energy and protein requirements. During the last 4 hours of each study day your child will also consume a solution made up of water and an amino acid that contains a naturally occurring label. An amino acid which contains a label is different from a regular amino acid only in that it weighs more. This label (stable isotope), found naturally in small amounts in your child's body, is harmless and is used routinely in human nutrition research. It is the amount of this heavy isotope that we measure in your child's breath and urine that allows us to determine your child's requirements for tyrosine and phenylalanine.

**Potential Harms:**
All of the tests used in this protocol are harmless.

**Potential Benefits:**
Your child will benefit directly from participating in this study. The results of the study will enable us to determine your child's individual tyrosine and phenylalanine requirements which can lead to improved metabolic control. The results of the study will also be beneficial to other individuals with PKU since they will be used to estimate the requirements for tyrosine and phenylalanine of other similar individuals.

**Confidentiality:**
Confidentiality will be respected and no information that discloses the identity of your child will be released or published without consent. For your information, the research consent form will be inserted in the patient's health record.

**Participation:**
Participation in research must be voluntary. If you choose not to participate, your child and other family members will continue to have access to quality care at HSC. You can withdraw your child anytime during the study and again your family will continue to have access to quality care at HSC. You may ask any questions now or during the study.
TYROSINE AND PHENYLALANINE REQUIREMENTS IN PATIENTS WITH PKU

Consent

I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at The Hospital for Sick Children for my child and other members of my family. As well the potential harms and discomforts have been explained to me and I understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that the records relating to my child's care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I hereby consent for my child______________________________to participate.

Name of Parent

______________________________

Signature

The person who may be contacted about the research is______________________________

Who may be reached at telephone #______________________________

Name of person who obtained consent

______________________________

Signature

______________________________

Date

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RESEARCH INFORMATION FORM
(For patients 7 - 15 years)

Title: TYROSINE AND PHENYLALANINE REQUIREMENTS IN PATIENTS WITH PKU

Investigators:
Rachelle Bross M.Sc., R.D. Division of Gastroenterology/Nutrition Ph: 813-6175
Dr. P. Pencharz Division of Gastroenterology/Nutrition Ph: 813-6176
Ms. V. Austin Division of Clinical Genetics Ph: 813-5332
Dr. J. Clarke Division of Clinical Genetics Ph: 813-5753
Dr. A. Feigenbaum Division of Clinical Genetics Ph: 813-5333
Dr. W. Hanley Division of Clinical Genetics Ph: 813-6635
Ms. W. Schoonheyt Division of Clinical Genetics Ph: 813-6356

Why are we doing this study? It is hard for children with PKU to achieve normal blood tyrosine and phenylalanine levels partly because we don't know what the requirements for tyrosine and phenylalanine are. We want to measure the tyrosine and phenylalanine requirements in individuals with PKU using a new technique. This is important to know since it would make the dietary treatment of individuals with PKU better.

What will happen during the study? On 6 different days, you will spend 8 hours in the Clinical Nutrition Laboratory of the Hospital for Sick Children. We will start by measuring your height and weight. We will also measure the amount of fat and muscle in your body. This will only take a few minutes and is completely painless. Your breath and urine will be also be collected. To collect your breath you will have to put a mask on and sit or lie still. You will be able to breath normally and can watch TV, read, or listen to music. You will also need to pee into a bottle everytime you go to the bathroom during the study.

Every hour you will eat a small meal made up of a mixture of amino acids, a flavoured liquid formula and cookies. During the last 4 hours of each study day you will also drink a solution made up of water and an amino acid that contains a naturally occurring label. An amino acid which contains a label is different from a regular amino acid only in that it weighs more. This label (stable isotope), found naturally in small amounts in your body, is completely harmless. We will measure the amount of this heavy isotope in your breath and urine.

Are there good things and bad things about the study? All of the tests used in the study are harmless and when the study is over we will be able to tell you exactly how much tyrosine and phenylalanine you need to eat.
Who will know about what I did in the study? If you are part of this study your name and address will not be given to anyone.

Can I decide if I want to be in the study? If you do not want to be part of this study that is O.K. No one will be upset. If you say yes now and change your mind, you can say no to the doctor or nurse and that is O.K. Your mother or father are also given this information. They will talk to you about it. Please ask the doctor or nurse any questions that you may have.

Assent

I was present when_________________________read this form and gave his/her verbal assent.

_________________________ Name of person who obtained assent

_________________________ Signature  _____________ Date
Protein and Energy Metabolism in Patients with PKU: Tyrosine and Phenylalanine Requirements and Resting Metabolic Rates of Children, Adolescents and Adults

The Principal Investigators at The Hospital for Sick Children are Rachelle Bross, PhD Student in Nutritional Sciences; Dr. P.B. Pencharz, Division of Gastroenterology and Nutrition; and Dr. J.T.R. Clarke, Head of Clinical Genetics. Coinvestigators from the PKU programme also include, Dr. W. Hanley, Dr. A. Feigenbaum, Ms. W. Schoonheyt and Ms. V. Austin.

The following pages review the studies and describes which patients are eligible to be involved. We have only enough funding to enrol 12 patients for the Protein Metabolism study and no specific limit for the number of patients to enrol in the Energy Metabolism study.
OVERVIEW: PROTEIN METABOLISM STUDY

It is hard to achieve normal blood tyrosine and phenylalanine levels in patients with PKU, in part, because we don't have good estimates of their requirements for tyrosine and phenylalanine. High phenylalanine levels may contribute to restlessness, short attention span, school problems, and an I.Q. level lower than that of brothers or sisters who do not have PKU. Patients with PKU may also not be getting enough tyrosine since phenylalanine cannot be broken down to tyrosine.

Researchers at the Hospital for Sick Children have designed a research project to directly measure the tyrosine and phenylalanine requirements in individuals with PKU using a new technique. Knowing the requirements for these amino acids will improve the dietary treatment of individuals with PKU and will lead directly to improvements in care.

PATIENTS ELIGIBLE FOR THE STUDY

The study will be conducted on children, adolescents and adults with classical PKU treated by dietary phenylalanine restriction from early infancy.

The patient must be in good health and not taking any chronic medication that might alter protein metabolism.

The patient cannot be pregnant or lactating

HOW CAN THIS CHANGE THE TREATMENT OF PKU?

The significance of this research project will be to determine the requirements of tyrosine and phenylalanine in individuals with PKU. This will improve the treatment of Phenylketonuria, not only at the Hospital for Sick Children, but in PKU centres around the world.
The amount of tyrosine and/or phenylalanine required for metabolic control will be measured. This measurement will involve six study days for each amino acid. Each patient has the choice of entering the study for one set of six study days or for both. The six study days will not be consecutive, but each patient will be required to complete all six days within a 2 month period.

On each study day patients will spend 8 hours in the Clinical Nutrition Laboratory of the Hospital for Sick Children, where breath and urine will be collected. Patients will arrive at the hospital after an overnight, 10 - 12 hour fast, during which time no food or drink (except water) is taken after the previous night's supper. During each study day patients will lie comfortably on a bed or sit in a chair and watch TV, read, or listen to music. The study will with a measurement of height and weight. Body fat will be determined by measuring skin thickness from the arm, waist and back using a caliper. After this the amount of muscle in your body will be measured by placing four small stickers on the right arm and leg. These stickers are connected to a machine by wires. The machine computes the amount of muscle in the body by passing a small current through the body. The current used in this test is extremely small and the body does not feel anything. The body fat and muscle measurements are completely safe and painless and will be done on the first and last study day.

During the study period patients will eat 8 small meals spaced one hour apart. The meals will be made up of three parts: (1) a mixture of amino acids in which the amount of tyrosine or phenylalanine will either be less than adequate, adequate or more than adequate; (2) an amino acid free flavoured liquid formula; and (3) amino acid free cookies. Individual nutritional requirements will be determined prior to beginning the study and the test diet will be designed to meet each patient's energy and protein requirements. During the last 4 hours of each study day patients will also consume a solution made up of water and an amino acid that contains a naturally occurring label. An amino acid which contains a label is different from a regular amino acid only in that it weighs more. This label (stable isotope), found naturally in small amounts in the body, is harmless and is used routinely in human nutrition research. It is the amount of this heavy isotope that will be measured in breath and urine that allows for the determination of the requirements for tyrosine and phenylalanine.
OVERVIEW: ENERGY METABOLISM STUDY

A balance between the amount of food we eat and the energy (calories) we burn is essential for good health. Patients with PKU tend to be overweight on average compared to normal standards and we do not know why. We want to see if individuals with PKU burn energy slower and if changes in certain hormones and neurotransmitters (chemicals in the brain) that occur secondary to high concentrations of phenylalanine might play a role.

Researchers at the Hospital for Sick Children have designed a research project to directly measure energy requirements in individuals with PKU using indirect calorimetry. A better understanding of the way energy is burned will help us to develop better dietary treatment for PKU.

PATIENTS ELIGIBLE FOR THE STUDY

The study will be conducted on children, adolescents and adults with classical PKU treated by dietary phenylalanine restriction from early infancy.

The patient must be in good health and not taking any chronic medication that might alter energy metabolism.

The patient cannot be pregnant or lactating.

HOW CAN THIS CHANGE THE TREATMENT OF PKU?

The significance of this research project will be to determine the energy requirements in individuals with PKU. This will improve the treatment of Phenylketonuria, not only at the Hospital for Sick Children, but in PKU centres around the world.
THE PROCESS: ENERGY METABOLISM STUDY

The amount of energy required that each patient burns while resting will be measured. This will involve two 2 hour visits to the Clinical Nutrition Laboratory of the Hospital for Sick Children. On each study day, the patient will have to fast (no food or drink, except water) from 8 PM the evening before the test until the testing is complete. The study will start with a measurement of height and weight. Body fat will be determined by measuring skin thickness from the arm, waist and back using a caliper. After this the amount of muscle in the body by placing four small stickers on the right arm and leg. These stickers are connected to a machine by wires. The machine computes the amount of muscle in the body by passing a small current through the body. The current used in this test is extremely small and the body does not feel anything. The body fat and muscle measurements are completely safe and painless and will be done on both study days.

The patients will then lie down on a bed and rest for 30 minutes during which time they can watch TV, read or listen to music. A clear plastic hood will be placed over the patient’s head for another 30 minutes. The patient will be able to breathe air normally. From the oxygen breathed in and the carbon dioxide breathed out, it is possible to calculate how many calories the patient uses while resting. One of the study days will take place on the same day as a clinic visit. One teaspoon (5 ml) of blood will be needed to determine the amount of neurotransmitter present. This blood will be taken by the PKU nurse along with the blood usually taken during a clinic visit.

The patients will also be asked to collect all the urine that is passed over 24 hours prior to the second study day. A container will be provided for this purpose. The urine will be used to measure certain hormones.
How Can Our Family Get Involved?

Contact Wanda Schoonheyt Today.

(416) 813-6356

We have resources for a limited number of patients. Call Wanda to discuss your questions and concerns and, if interested, book your child into the study.

Remember, if you choose to become involved we need a commitment to the 2 month appointment schedule. However, if at any time you choose to withdraw your child from the study it will not affect the quality of care your child is receiving at the Hospital for Sick Children.
9.3. Diet and Study Day Forms

**Tyrosine Study: Diet**

<table>
<thead>
<tr>
<th>Study Date:______________</th>
<th>Subject:__________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height:________m</td>
<td>Weight:________kg</td>
</tr>
</tbody>
</table>

**Energy Requirements: kcal/day**

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 - 10 yrs: 22.5W + 499</td>
<td>3 - 10 yrs: 22.7W + 495</td>
</tr>
<tr>
<td>10 - 18 yrs: 7.4W + 482H + 217</td>
<td>10 - 18 yrs: 16.6W + 77H + 572</td>
</tr>
</tbody>
</table>

\[
\text{Energy} = \frac{\text{kcal/day} \times 1.5}{\text{[A] kcal/day}} = \frac{\text{kcal/day}}{\text{[A] kcal/day}}
\]

**Diet Constituents**

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

\[
\text{Amino Acids} = 1.5 \text{ g Prot/kg/day} \times \text{_______kg} = \text{_______g Prot/day}
\]

\[
\text{Formula} = 0.427 \text{ml/kcal} \times 65\% \times \text{[A] kcal/day} = \text{_______ml/day}
\]

\[
\text{BS Cookies} = 0.234 \text{g/kcal} \times 12.5\% \times \text{[A] kcal/day} = \text{_______g/day}
\]

\[
\text{CF Cookies} = 0.229 \text{g/kcal} \times 12.5\% \times \text{[A] kcal/day} = \text{_______g/day}
\]

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

Actual Energy Intake = ________ kcal/day

Energy Distribution = ________\% Fat, ________\% CHO, ________\% Prot
Amino Acid Mix (no alanine, tyrosine or lysine) (study day)

Study day includes 100% of the total intake.

\[
\text{Prot/day [B] x } \text{ % } = \text{ Prot/day }
\]

\[
\text{Prot/study day } \div 8 \text{ meals } = \text{ Amino acid mix /meal }
\]

[1-13C] Lysine Intake (study day)

Lys requirement/day = 64mg/kg x _______kg = _______mg [F]

Lys-HCl intake/day = [F] x 1.2494 = _______mg lys-hcl [G]

Lys-HCl intake/meal = [G] \div 8 = _______mg/meal [H]

Lys intake as tracer
Prime = 2.5mg/kg x _______kg = _______mg = _______mg Lys-HCl
CI = 1.4mg/kg/hr x _______kg = _______mg/hr = _______mg Lys-HCl

\[
\text{mg/1/2 hr } \times \text{mg/hr } \times \text{4 hr } = \text{ mg }
\]

Total (P + CI) = _______mg Lys x 1.2494 = _______mg Lys-HCl[I]

Vol of Lys tracer
Prime = _______mg \div _______mg/ml = _______ml

CI(per 1/2 hr) = _______mg/1/2 hr \div _______mg/ml = _______ml

Diet Lys intake/study day = [G]mg - [I]mg = _______mg Lys-HCl [J]

Diet Lys intake per meal (first 4 meals) = _______mg Lys-HClmg/meal

Diet Lys intake per meal 5= _______mg Lys-HClmg/meal

Diet Lys intake per meal 6-8= _______mg Lys-HClmg/meal

Phe Intake = 24mg/kg x _______kg = _______mg \div 8 meals = _______mg/meal

Tyrosine Test Intake and Extra Alanine on Study Day

Tyr test intake = _______mg/kg

Tyr test intake/study day= _______mg/kg x _______kg= _______mgTyr _______gTyr [K]

Tyr intake per meal = _______mg Tyr \div 8 meals = _______mg Tyr per meal

Tyrosine intake from reference diet is 130 g/1.5 kg of amino acid mix, therefore pts would need:

\[
130\text{mg } \times \text{ pt weight kg } = \text{ mg Tyr/d } = \text{ g Tyr/d [L]}
\]
Alanine will replace the difference between the test tyr intake and what would have been provided in a complete amino acid mix:

\[
([L] \div 181.19\text{g/mol}) - ([K] \div 181.19\text{g/mol}) = \text{mol Tyr}
\]

\[
\text{mol Ala} \times 89.09\text{g/mol} = \text{g Ala}
\]

Additional alanine intake per meal = \text{mg Ala} \div 8 \text{ meals} = \text{mg Ala per meal}

### Amino Acid Composition of Meals & Tracer Intake

<table>
<thead>
<tr>
<th></th>
<th>Meal Number</th>
<th></th>
<th>Daily Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Ala (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LysHCl (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA Mix (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LysHCl Tracer (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Formula Requisition (Study day)**

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

Formula required per meal  = [C] ml ÷ 8 = _______ ml/meal [M]

** Each meal is served in a 4oz bottle.

Formula requisition = 9 4oz bottles x _______ ml/meal [M] = _______ ml/day

**Formula Recipe**

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

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

**Butterscotch Cookies (study day)**

BS Cookies required per study day  = [D] = _______ g/day [N]

BS Cookies required per meal  = [N] ÷ 8 = _______ g/meal

**Cornflake Cookies (study day)**

CF Cookies required per study day  = [E] = _______ g/day [O]

CF Cookies required per meal  = [O] ÷ 8 = _______ g/meal

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

Subject's Name: 

**Formula Recipe:**

Study Day #: ___________________ Delivery Date: ___________________

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

Total volume of formula: _________ ml
________ 8oz Bottles _________ ml per bottle

**Cookie Requirements**

**Butterscotch Cookie:**

_______ g BS cookie per day

8 meals per day ___________g BS Cookie per meal

**Cornflake Cookie:**

_______ g CF cookie per day

8 meals per day ___________g CF Cookie per meal

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

-Subject name
-Day #1-6
-Meal Number
-Type of Cookie
-Weight (grams) of cookie per bag

Call Rachelle Bross at 813-6175 for more information (Pager # 235-8968)
Tyrosine Study Protocol
Equipment List: Calorimetry Room

Urine Collection:
- hat
- container
- 8 x 5 ml yellow top plastic tubes
- 16 x 1.5 ml microfuge tubes (labelled)
- 0.5 ml pipette

Breath Collection:
- 1 Normal NaOH (250 mL minimum)
- 1 NaOH glass tubes
- automatic dispenser bottle
- 50 mL syringe
- 18 gauge needle
- 9 brown top vacutainers (labelled)
- 200 mL beaker (NaOH refuse)
- permanent marker
- 2 test tube racks

Diet:
- ice bucket x 2
- food
- straw

Isotope:
- 9 x 50 ml pink plastic tubes
- pipette
**Study Day Schedule**

Pt Name: ______________________
Pt Weight: _____________________
Pt Height: _____________________

BIA/Skinfolds  Yes / No (if yes see body composition forms)

Lys intake as tracer
Prime = 2.5mg/kg x ________kg = ________mg
Cl = 1.4mg/kg/hr x ________kg = ________mg/hr
_______mg/½hr x 4hrs = ________mg
Total (P + Cl) = ________mg[1]

Vol of Lys tracer
Prime = ________mg + ________mg/ml = ________ml
Cl (per ½hr) = ________mg/½hr + ________mg/ml = ________ml

<table>
<thead>
<tr>
<th>Time (Hour:Min)</th>
<th>List of Events</th>
<th>Food/Isotope/Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Murphy</td>
<td></td>
</tr>
<tr>
<td>Pre-study</td>
<td>Calibrate Murphy</td>
<td>Prime = _______ml</td>
</tr>
<tr>
<td></td>
<td>Clean mask</td>
<td>C/I = _______ml/½hr</td>
</tr>
<tr>
<td></td>
<td>Check equipment</td>
<td>Collect diet + water</td>
</tr>
<tr>
<td></td>
<td>Patient set-up</td>
<td>Patient voids Yes/No</td>
</tr>
<tr>
<td></td>
<td>Collect ice</td>
<td></td>
</tr>
<tr>
<td>0:00</td>
<td></td>
<td>Meal #1 + 150ml water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine (no collection)</td>
</tr>
<tr>
<td>1:00</td>
<td>Sham Breath Collection</td>
<td>Meal #2 + 150ml water</td>
</tr>
<tr>
<td></td>
<td>Flow = __________</td>
<td>Urine (no collection)</td>
</tr>
<tr>
<td>2:00</td>
<td></td>
<td>Meal #3 + 150ml water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine (no collection)</td>
</tr>
<tr>
<td>3:00</td>
<td></td>
<td>Urine collection 1</td>
</tr>
<tr>
<td>3:05</td>
<td></td>
<td>Meal #4 + 250ml water</td>
</tr>
<tr>
<td>3:25</td>
<td></td>
<td>Urine collection 2</td>
</tr>
<tr>
<td>3:30</td>
<td>CO₂ trapping 1(wait 5 min)</td>
<td></td>
</tr>
</tbody>
</table>

240
<table>
<thead>
<tr>
<th>Time (Hour:Min)</th>
<th>List of Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:40</td>
<td>CO₂ trapping 2</td>
</tr>
<tr>
<td>3:50</td>
<td>CO₂ trapping 3</td>
</tr>
</tbody>
</table>
| 3:59           | Stop breath collection  
                  | Save data           |
| 4:00           |                  |
| 4:05           |                  |
| 4:15           |                  |
| 4:15           |                  |
| 4:45           |                  |
| 4:59           |                  |
| 5:00           |                  |
| 5:05           |                  |
| 5:15           |                  |
| 5:45           |                  |
| 5:59           |                  |
| 6:00           |                  |
| 6:05           |                  |
| 6:15           | Breath collection |
|                | CO₂ trapping 7   |
| 6:30           |                  |
| 6:40           |                  |
| 6:45           |                  |
| 6:45           |                  |
| 6:59           |                  |
| 7:00           |                  |
| 7:05           |                  |

**Food/Isotope/Urine**

<table>
<thead>
<tr>
<th>Time (Hour:Min)</th>
<th>Murphy</th>
<th>Food/Isotope/Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:00</td>
<td></td>
<td>Urine collection 3</td>
</tr>
<tr>
<td>4:05</td>
<td></td>
<td>Meal #5 + water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isotope prime</td>
</tr>
<tr>
<td>4:15</td>
<td></td>
<td>Isotope Cl 1</td>
</tr>
<tr>
<td>4:45</td>
<td></td>
<td>VCO₂</td>
</tr>
<tr>
<td>4:59</td>
<td></td>
<td>Isotope Cl 2</td>
</tr>
<tr>
<td>5:00</td>
<td></td>
<td>Urine 4</td>
</tr>
<tr>
<td>5:05</td>
<td></td>
<td>Meal #6 + water</td>
</tr>
<tr>
<td>5:15</td>
<td></td>
<td>Isotope Cl 3</td>
</tr>
<tr>
<td>5:45</td>
<td></td>
<td>Isotope Cl 4</td>
</tr>
<tr>
<td>5:59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:00</td>
<td></td>
<td>Urine 5</td>
</tr>
<tr>
<td>6:05</td>
<td></td>
<td>Meal #7 + water</td>
</tr>
<tr>
<td>6:15</td>
<td>Breath collection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ trapping 7</td>
<td></td>
</tr>
<tr>
<td>6:30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:45</td>
<td></td>
<td>CO₂ trapping 8</td>
</tr>
</tbody>
</table>
| 6:59            |        | Stop breath collection  
                  | Save data           |
| 7:00            |        |                    |
| 7:05            |        | Meal #8 + water    |

241
<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:15</td>
<td>Breath collection</td>
<td>CI 7</td>
</tr>
<tr>
<td></td>
<td>CO₂ trapping 9</td>
<td></td>
</tr>
<tr>
<td>7:20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:30</td>
<td>CO₂ trapping 10</td>
<td></td>
</tr>
<tr>
<td>7:45</td>
<td>CO₂ trapping 11</td>
<td>CI 8</td>
</tr>
<tr>
<td>7:59</td>
<td>Stop breath collection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Save data</td>
<td></td>
</tr>
<tr>
<td>8:00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>urine collection 10</td>
<td></td>
</tr>
</tbody>
</table>
9.4. Data Analysis

Two-Phase Linear Regression Crossover Model

A two phase linear regression crossover model (Seber 1977) was done allowing a partition of the data points between two separate regression lines that minimized the residual error. This resulted in a crossover value termed the breakpoint.

The data points used were all the individual observations for the rate of $^{13}$CO$_2$ released from L-[1-$^{13}$C]-lysine oxidation for all dietary tyrosine levels tested.

Before the program could be executed (SAS, 1985), visual inspection of the possible location of the breakpoint was made in order to separate the test tyrosine intakes (x axis) to the two regression lines. For each estimated separation of the x axis, the data was fitted into 4 different statistical models:

1. Both lines are sloping, unweighted regression.
2. Both lines are sloping, weighted regression.
3. Only one line is sloping, unweighted regression.
4. Only one line is sloping, weighted regression.

**SLOPING:** Given the concept behind IAAO, a slope in the second line should not be different from zero.

**WEIGHTING:** Variances along a sloping regression line are proportionally equal with respect to their distance from the lowest value. Therefore, the numerically higher values at the end of the regression lines have both a higher variance and more effect on the slope of the line than the points closer to the lowest value which have a proportionally smaller variance and less effect on the slope of the line.

The purpose of weighting the data is to reduce the numerical effect such that the points at the end of the line have a similar effect on the slope as the points along the line. This is done by multiplying each variance by its inverse. This causes all variances to become numerically equal, but not proportionally equal.

**REGRESSION EQUATIONS:**

The determining factors of the overall regression equations were whether one or both lines were sloping.

*Both lines sloping:* $y = A_2 + (A_1-A_2)C + B_2x + (B_1-B_2)Cx$

*One line sloping:* $y = A_2 + (A_1-A_2)C + (B_1-B_2)Cx$

where $A_1$ = intercept of first line  
$A_2$ = intercept of second line  
$B_1$ = slope of first line  
$B_2$ = slope of second line

The independent variable C (choose) was used to determine the data points to use for the individual regression lines. C=1 for observations before the breakpoint (first line) and C=0 for observations after the breakpoint (second line).
Both lines sloping:  First line (C=1)

\[ y = A_2 + (A_1-A_2) + B_2x + (B_1-B_2)x \]
\[ y = A_1 + B_1x \]

Second line (C=0)
\[ y = A_2 + B_2x \]

One line sloping:  First line (C=1)

(B2=0 since second line has no slope)
\[ y = A_2 + (A_1-A_2) + (B_1-B_2)x \]
\[ y = A_1 + (B_1-B_2)x \]

Second line (C=0)
\[ y = A_2 \]

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

Both lines sloping:  \[ A_1 + B_1x = A_2 + B_2x \]
\[ x = -(A_1-A_2) / (B_1-B_2) \]

One line sloping:  \[ A_1 + (B_1-B_2)x = A_2 \]
\[ x = -(A_1-A_2) / (B_1-B_2) \]

(A1-A2) and (B1-B2) are represented in the SAS output as the parameters choose and tyrosin*choose.

CHOOSING THE MODEL:

Once all 4 models were executed (on all possibilities of the separation of the x axis to the two regression lines), the model that best fit the data was chosen. The value that indicated this was the lowest standard error of the largest parameter (choose). The model with the lowest error was taken as the model which fit the data best. Other values which were looked at included the lowest root mean squared error. This is because the aim of linear regression is to minimize the sum of the squared distances of the points from the line. The highest \( r^2 \) was also considered as that indicated that the slopes of the lines most closely matched the means.

Calculation of 95% Confidence Limits

The upper 95% confidence limit was calculated to determine a safe tyrosine requirement estimate (Fieller's Theorem). The following is how the 95% confidence limits were determined from the two phase linear regression crossover model.

95% confidence levels = Breakpoint ± \( t \) \( (n) \) SE(Breakpoint)

where

Breakpoint = \( -\) choose / tyrosin*choose

\[ t = 1.699 \] (t distribution, 29 degrees of freedom; n-1)

SE(Breakpoint)= calculated from 3 sources of variation

(i) variation of choose
(ii) variation of tyrin*choose
(iii) covariance of (i) and (ii)

The variance (i) and (ii) are the standard error of the estimates squared.
The covariance (iii) is derived from the output of the two phase linear regression crossover model (proc reg outest = out123 covout).
9.5. Consent and Information Forms

RESEARCH CONSENT FORM
(For patients 16 years or older)

Title: ENERGY REQUIREMENTS IN PATIENTS WITH PKU

Investigators:
Rachelle Bross M.Sc., R.D. Division of Gastroenterology/Nutrition Ph: 813-6175
Dr. P. Pencharz Division of Gastroenterology/Nutrition Ph: 813-6176
Ms. V. Austin Division of Clinical Genetics Ph: 813-5332
Dr. J. Clarke Division of Clinical Genetics Ph: 813-5753
Dr. A. Feigenbaum Division of Clinical Genetics Ph: 813-5333
Dr. W. Hanley Division of Clinical Genetics Ph: 813-6635
Ms. W. Schoonheyt Division of Clinical Genetics Ph: 813-6356

Purpose of the Research:
A balance between the amount of food we eat and the energy (calories) we burn is essential for good health. Patients with PKU tend to be overweight on average compared to normal standards and we do not know why. We want to see if individuals with PKU burn energy slower and if changes in certain hormones and neurotransmitters (chemicals in the brain) might play a role. A better understanding of the way energy is burned will help us to improve the dietary treatment of PKU.

Description of the Research:
If you decide to enter the study, we will be measuring the amount of energy that you burn while resting. This will involve two 2 hour visits to the Clinical Nutrition Laboratory of the Hospital for Sick Children. On each study day, you will have to fast (no food or drink, except water) from 8 PM the evening before the test until the testing is complete. We will start by measuring your height and weight. Body fat will be determined by measuring skin thickness from the arm, waist and back using a caliper. After this we will measure the amount of muscle in your body by placing four small stickers on the right arm and leg. These stickers are connected to a machine by wires. The machine computes the amount of muscle in the body by passing a small current through the body. The current used in this test is extremely small and the body does not feel anything. The body fat and muscle measurements are completely safe and painless and will be done on both study days.

You will then lie down on a bed and rest for 30 minutes during which time you can watch TV, read or listen to music. A clear plastic hood will be placed over your head for another 30 minutes. You will be able to breathe air normally. From the oxygen you breathe in and the carbon dioxide you breathe out, we will calculate how many calories you use while resting.

One of the study days will take place on the same day as a clinic visit. We will need one teaspoon (5 ml) of blood to determine the amount of neurotransmitter present. This blood will be taken by the PKU nurse along with the blood usually taken during a clinic visit.
You will also be asked to collect all the urine that is passed over 24 hours prior to your second study day. A container will be provided for this purpose. The urine will be used to measure certain hormones.

**Potential Harms:**
All of the tests used in this protocol are harmless.

**Potential Benefits:**
You will benefit directly from participating in this study. The results of the study will enable us to determine your individual energy requirements which can lead to improved dietary treatment. The results of the study will also be beneficial to other individuals with PKU since they may help us to understand why some individuals with PKU are overweight.

**Confidentiality:**
Confidentiality will be respected and no information that discloses your identity will be released or published without consent. For your information, the research consent form will be inserted in the patient's health record.

**Participation:**
Participation in research must be voluntary. If you choose not to participate, you and your family members will continue to have access to quality care at HSC. You can withdraw anytime during the study and again your family will continue to have access to quality care at HSC. You may ask any questions now or during the study.
Consent

I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at The Hospital for Sick Children for me and other members of my family. As well the potential harms and discomforts have been explained to me and I understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that the records relating to me and my care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I ____________________________ hereby consent to participate.

Name of Patient & age

Signature

The person who may be contacted about the research is ________________________________

Who may be reached at telephone # ________________________________

Name of person who obtained consent

Signature

Date
Title: ENERGY REQUIREMENTS IN PATIENTS WITH PKU

Purpose of the Research:
A balance between the amount of food we eat and the energy (calories) we burn is essential for good health. Patients with PKU tend to be overweight on average compared to normal standards and we do not know why. We want to see if individuals with PKU burn energy slower and if changes in certain hormones and neurotransmitters (chemicals in the brain) might play a role. A better understanding of the way energy is burned will help us to develop better dietary treatment for PKU.

Description of the Research:
If you and your child decide that he/she will enter the study, we will be measuring the amount of energy that your child burns while resting. This will involve two 2 hour visits to the Clinical Nutrition Laboratory of the Hospital for Sick Children. You may be present with your child during the entire study protocol. On each study day, your child will have to fast (no food or drink, except water) from 8 PM the evening before the test until the testing is complete. We will start by measuring your child's height and weight. Body fat will be determined by measuring skin thickness from the arm, waist and back using a caliper. After this we will measure the amount of muscle in the body by placing four small stickers on the right arm and leg. These stickers are connected to a machine by wires. The machine computes the amount of muscle in the body by passing a small current through the body. The current used in this test is extremely small and the body does not feel anything. The body fat and muscle measurements are completely safe and painless and will be done on both study days.

Your child will then lie down on a bed and rest for 30 minutes during which time your child can watch TV, read or listen to music. A clear plastic hood will be placed over your child’s head for another 30 minutes. Your child will be able to breathe air normally. From the oxygen your child breathes in and the carbon dioxide breathed out, we will calculate how many calories your child uses while resting.

One of the study days will take place on the same day as a clinic visit. We will need one teaspoon (5 ml) of blood to determine the amount of neurotransmitter present. This blood will be taken by the PKU nurse along with the blood usually taken during a clinic visit.
Your child will also be asked to collect all the urine that is passed over 24 hours prior to the second study day. A container will be provided for this purpose. The urine will be used to measure certain hormones.

**Potential Harms:**
All of the tests used in this protocol are harmless.

**Potential Benefits:**
Your child will benefit directly from participating in this study. The results of the study will enable us to determine your child's individual energy requirements which can lead to improved dietary treatment. The results of the study will also be beneficial to other individuals with PKU since they may help us to understand why some individuals with PKU are overweight.

**Confidentiality:**
Confidentiality will be respected and no information that discloses the identity of your child will be released or published without consent. For your information, the research consent form will be inserted in the patient's health record.

**Participation:**
Participation in research must be voluntary. If you choose not to participate, your child and other family members will continue to have access to quality care at HSC. You can withdraw your child anytime during the study and again your family will continue to have access to quality care at HSC. You may ask any questions now or during the study.
ENERGY REQUIREMENTS IN PATIENTS WITH PKU

Consent

I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at The Hospital for Sick Children for my child and other members of my family. As well the potential harms and discomforts have been explained to me and I understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that the records relating to my child's care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I hereby consent for my child_______________________________to participate.

________________________________________________________________________

Name of Parent

________________________________________________________________________

Signature

The person who may be contacted about the research is________________________

Who may be reached at telephone #__________________________________________

________________________________________________________________________

Name of person who obtained consent

________________________________________________________________________

Signature

________________________________________________________________________

Date
Title: ENERGY REQUIREMENTS IN PATIENTS WITH MSUD

Investigators:

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Dr. P. Pencharz Division of Gastroenterology/Nutrition Ph: 813-6176
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Ms. W. Schoonheydt Division of Clinical Genetics Ph: 813-6356

Purpose of the Research:
A balance between the amount of food we eat and the energy (calories) we burn is essential for good health. Energy requirements in patients with MSUD are not well described because they have not been previously measured. We want to see if individuals with MSUD burn energy slower because of changes in certain hormones and neurotransmitters (chemicals in the brain) that occur. A better understanding of the way energy is burned will help us to develop better dietary treatment for MSUD.

Description of the Research:
If you decide to enter the study, we will be measuring the amount of energy that you burn while resting. This will involve one 2 hour test period which will take place at a location close to where you live. On the study day, you will have to fast (no food or drink, except water) from 8 PM the evening before the test until the testing is complete. We will start by measuring your height and weight. Body fat will be determined by measuring skin thickness from the arm, waist and back using a caliper. After this we will measure the amount of muscle in your body by placing four small stickers on the right arm and leg. These stickers are connected to a machine by wires. The machine computes the amount of muscle in the body by passing a small current through the body. The current used in this test is extremely small and the body does not feel anything. The body fat and muscle measurements are completely safe and painless.

You will then lie down on a bed or sit in a chair and rest for 30 minutes during which time you can read. A clear plastic hood or mask will be placed over your head for another 30 minutes. You will be able to breathe air normally. From the oxygen you breathe in and the carbon dioxide you breathe out, we will calculate how many calories you use while resting.

We will need one teaspoon (5 ml) of blood to determine the amount of neurotransmitter present. This will be taken from your arm using a small needle.

You will also be asked to collect all the urine that is passed over 24 hours prior to your study day. A container will be provided for this purpose. The urine will be used to measure certain hormones.
**Potential Harms:**
There might be a small amount of bleeding when blood is taken from a vein and there might be slight discomfort and redness which usually disappears within a few days. All of the tests used in this protocol are harmless.

**Potential Benefits:**
You will benefit directly from participating in this study. The results of the study will enable us to determine your individual energy requirements which can lead to improved dietary treatment. The results of the study will also be beneficial since they will provide us with knowledge about the energy needs of other individuals with MSUD.

**Confidentiality:**
Confidentiality will be respected and no information that discloses your identity will be released or published without consent. For your information, the research consent form will be inserted in the patient's health record.

**Participation:**
Participation in research must be voluntary. If you choose not to participate, you and your family members will continue to have access to quality care at HSC. You can withdraw anytime during the study and again your family will continue to have access to quality care at HSC. You may ask any questions now or during the study.
Consent

I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at The Hospital for Sick Children for me and other members of my family. As well the potential harms and discomforts have been explained to me and I understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that the records relating to me and my care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I _____________________________ hereby consent to participate.

_____________________________
Name of Patient & age

_____________________________
Signature

The person who may be contacted about the research is______________________________

Who may be reached at telephone #______________________________

_____________________________
Name of person who obtained consent

_____________________________
Signature

_____________________________
Date
RESEARCH CONSENT FORM
(For patients less than 16 years)

Title: ENERGY REQUIREMENTS IN PATIENTS WITH MSUD

Investigators:
Rachelle Bross M.Sc., R.D. Division of Gastroenterology/Nutrition Ph: 813-6175
Dr. P. Pencharz Division of Gastroenterology/Nutrition Ph: 813-6176
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Purpose of the Research:
A balance between the amount of food we eat and the energy (calories) we burn is essential for good health. Energy requirements in patients with MSUD are not well described because they have not been previously measured. We want to see if individuals with MSUD burn energy slower because of changes in certain hormones and neurotransmitters (chemicals in the brain) that occur. A better understanding of the way energy is burned will help us to develop better dietary treatment for MSUD.

Description of the Research:
If you and your child decide that he/she will enter the study, we will be measuring the amount of energy that your child burns while resting. This will involve one 2 hour test period which will take place at a location close to where you live. You may be present with your child during the entire study period. On the study day, your child will have to fast (no food or drink, except water) from 8 PM the evening before the test until the testing is complete. We will start by measuring your child’s height and weight. Body fat will be determined by measuring skin thickness from the arm, waist and back using a caliper. After this we will measure the amount of muscle in the body by placing four small stickers on the right arm and leg. These stickers are connected to a machine by wires. The machine computes the amount of muscle in the body by passing a small current through the body. The current used in this test is extremely small and the body does not feel anything. The body fat and muscle measurements are completely safe and painless.

Your child will then lie down on a bed and rest for 30 minutes during which time your child can be read to. A clear plastic hood will be placed over your child’s head for another 30 minutes. Your child will be able to breathe air normally. From the oxygen your child breathes in and the carbon dioxide breathed out, we will calculate how many calories your child uses while resting.

We will need one teaspoon (5 ml) of blood to determine the amount of neurotransmitter present. This will be taken from your child’s arm using a small needle.

Your child will also be asked to collect all the urine that is passed over 24 hours prior to the study day. A container will be provided for this purpose. The urine will be used to measure certain hormones.
**Potential Harms:**
There might be a small amount of bleeding when blood is taken from a vein and there might be slight discomfort and redness which usually disappears within a few days. All of the tests used in this protocol are harmless.

**Potential Benefits:**
Your child will benefit directly from participating in this study. The results of the study will enable us to determine your child's individual energy requirements which can lead to improved dietary treatment. The results of the study will also be beneficial since they will provide us with knowledge about the energy needs of other individuals with MSUD.

**Confidentiality:**
Confidentiality will be respected and no information that discloses the identity of your child will be released or published without consent. For your information, the research consent form will be inserted in the patient's health record.

**Participation:**
Participation in research must be voluntary. If you choose not to participate, your child and other family members will continue to have access to quality care at HSC. You can withdraw your child anytime during the study and again your family will continue to have access to quality care at HSC. You may ask any questions now or during the study.
ENERGY REQUIREMENTS IN PATIENTS WITH MSUD

Consent

I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at The Hospital for Sick Children for my child and other members of my family. As well the potential harms and discomforts have been explained to me and I understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that the records relating to my child’s care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I hereby consent for my child_____________________________to participate.

________________________________________
Name of Parent

________________________________________
Signature

The person who may be contacted about the research is________________________

Who may be reached at telephone #________________________

________________________________________
Name of person who obtained consent

________________________________________
Signature

________________________________________
Date
RESEARCH INFORMATION FORM
(For patients 7 - 15 years)

Title: ENERGY REQUIREMENTS IN PATIENTS WITH PKU

Investigators:
Rachelle Bross M.Sc., R.D. Division of Gastroenterology/Nutrition Ph: 813-6175
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Dr. W. Hanley Division of Clinical Genetics Ph: 813-6635
Ms. W. Schoonheyt Division of Clinical Genetics Ph: 813-6356

Why are we doing this study? Children with PKU tend to be overweight on average and we do not know why. We want to see if children with PKU burn energy slower than children without PKU. This is important to know in order to help us know exactly how much food you need to eat.

What will happen during the study? We will start by measuring your height and weight. We will also measure the amount of fat and muscle in your body. This will only take a few minutes and is completely painless. You will then lie down on a bed for 30 minutes and rest. A clear plastic hood is then placed over your head for another 30 minutes. You can watch TV, listen to music or read during the test. From the oxygen that you breath in and the carbon dioxide that you breathe out, we will figure out how many calories you use while resting.

During your regular clinic visit, the PKU nurse will take an extra teaspoon of blood that we need to measure certain chemicals. You will also need to pee into a bottle everytime you go to the bathroom for a whole day and a whole night.

Are there good things and bad things about the study? All of the tests used in the study are harmless and when the study is over we will be able to tell you exactly how much food (calories) you need to eat.
Who will know about what I did in the study? If you are part of this study your name and address will not be given to anyone.

Can I decide if I want to be in the study? If you do not want to be part of this study that is O.K. No one will be upset. If you say yes now and change your mind, you can say no to the doctor or nurse and that is O.K. Your mother or father are also given this information. They will talk to you about it. Please ask the doctor or nurse any questions that you may have.

Assent

I was present when______________________________ read this form and gave his/her verbal assent.

______________________________________________
Name of person who obtained assent

______________________________________________
Signature Date
Title: ENERGY REQUIREMENTS IN PATIENTS WITH MSUD

Why are we doing this study? The food we eat is burned by our bodies to give us energy. We want to see if children with MSUD burn energy slower than children without MSUD. This is important in order to help us know exactly how much food you need to eat.

What will happen during the study? We will start by measuring your height and weight. We will also measure the amount of fat and muscle in your body. This will only take a few minutes and is completely painless. You will then lie down on a bed for 30 minutes and rest. A clear plastic hood is then placed over your head for another 30 minutes. You can read during the test. From the oxygen that you breathe in and the carbon dioxide that you breathe out, we will figure out how many calories you use while resting.

We will need to take some blood from your arm to measure certain chemicals. If you have had bloodwork done before this is exactly the same. You will also need to pee into a bottle everytime you go to the bathroom for a whole day and a whole night.

Are there good things and bad things about the study? All of the tests used in the study are harmless and when the study is over we will be able to tell you exactly how much food (calories) you need to eat.
Who will know about what I did in the study? If you are part of this study your name and address will not be given to anyone.

Can I decide if I want to be in the study? If you do not want to be part of this study that is O.K. No one will be upset. If you say yes now and change your mind, you can say no to the doctor or nurse and that is O.K. Your mother or father are also given this information. They will talk to you about it. Please ask the doctor or nurse any questions that you may have.

Assent

I was present when_________________________ read this form and gave his/her verbal assent.

_________________________
Name of person who obtained assent

_________________________ ______________________
Signature Date