Phenylalanine Requirement in Children with Classical Phenylketonuria

Determined by Indicator Amino Acid Oxidation.

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ABSTRACT

Dietary restriction of phenylalanine is the main treatment for Phenylketonuria (PKU), and current estimates of requirements are based on plasma phenylalanine concentration and growth. The present study is to determine more precisely phenylalanine requirements in patients with the disease using Indicator Amino Acid Oxidation (IAAO), with L-[1-\(^{13}\)C]lysine as the indicator. Breath \(^{13}\)CO\(_2\) production (F\(^{13}\)CO\(_2\)) was used as the endpoint. Finger-prick blood samples were also collected, for measurement of phenylalanine, in order to relate phenylalanine intake to blood phenylalanine levels. The mean phenylalanine requirement estimated using a two-phase linear regression crossover analysis, was 14mg·kg\(^{-1}\)·day\(^{-1}\) and the safe population intake (upper 95% confidence interval of the mean), was found to be 19.5 mg·kg\(^{-1}\)·day\(^{-1}\). A balance between phenylalanine intake and the difference between fed and fasted blood phenylalanine concentration was observed at an intake of 20 mg·kg\(^{-1}\)·day\(^{-1}\). The similarity between these two values (19.5 and 20 mg·kg\(^{-1}\)·d\(^{-1}\)) suggests that the maximal phenylalanine intake for children with PKU should be no higher than 20 mg·kg\(^{-1}\)·d\(^{-1}\).

Keywords: phenylketonuria, amino acid requirements, lysine
INTRODUCTION

Dietary phenylalanine restriction has been the mainstay of treatment of PKU for over 40 years (8). Its main aim is to maintain phenylalanine intakes that will allow optimum growth and brain development by supplying adequate energy, protein and other nutrients, while restricting phenylalanine. Implementation soon after birth usually prevents most of the overt clinical manifestations of PKU. Nevertheless, there is a considerable body of evidence, which suggests that neuropsychological and cognitive functions are not entirely normalized in individuals with PKU receiving current treatment regimens (28, 30, 31, 32).

Although dietary phenylalanine restriction remains the main treatment in PKU, present estimates of phenylalanine requirements are based on plasma phenylalanine levels and growth rate in relation to dietary intake, and not on direct and sensitive measurements of amino acid metabolism. This study is the second in a two part series, from the same laboratory, estimating aromatic amino acid (phenylalanine and tyrosine) requirements in children with PKU more sensitively and accurately by isotope tracer methods (20, 38, 39).

The indicator amino acid oxidation technique, which is used to determine amino acid requirements, involves feeding the subjects at levels above and below the predicted requirement break-point (20, 38). Since patients with classical PKU have negligible or very minimal capacity to oxidize phenylalanine (33, 34), we reasoned that their dietary phenylalanine requirements will be lower than healthy children, by an amount equal to the obligatory losses of phenylalanine. Dietary requirements for phenylalanine in children have yet to
be defined. So we turned to our previous study using indicator amino oxidation, which showed that the mean tyrosine requirement of children with PKU was 19.2 mg·kg⁻¹·day⁻¹ (3). Next we used a ratio between phenylalanine and tyrosine in the tissues of humans and animal of 55:45 (16, 24, 37, 39) which when multiplied with the tyrosine mean requirements predicts a mean phenylalanine requirement, for healthy children, of approximately 23.5 mg·kg⁻¹·day⁻¹. The requirement for phenylalanine or any other indispensable amino acid is the sum total of that needed for protein synthesis plus irreversible losses (10). Since there are no data in children we had to turn to a study of phenylalanine requirement in adult males (39), in which obligatory oxidation was estimated to be approximately 26%. Using the predicted phenylalanine requirement for children, and the estimated obligatory oxidation for phenylalanine, the obligatory loss was calculated to be approximately 6.1 mg·kg⁻¹·day⁻¹. Subtracting the estimate for obligatory loss from the predicted mean requirement of 23.5 mg·kg⁻¹·day⁻¹, the resulting value of 17.4 mg·kg⁻¹·day⁻¹ was the mean phenylalanine requirement predicted for children with PKU.

The objectives of this study were, to determine the phenylalanine requirement of children with classical PKU using the technique of indicator amino acid oxidation, and to compare the results to our previously estimated tyrosine requirement obtained using the same technique.
METHODS

Study Subjects

Five children (mean ± SEM age, 10.2 ± 1.2 yr) with classical PKU, treated by the PKU Clinic at the Hospital for Sick Children, Toronto, ON, participated in this study. All subjects were studied on an outpatient basis. Each subject was selected for study based on the following criteria: plasma phenylalanine concentration ≥ 1200 µM at diagnosis, prepubertal males or females 5-13 years old in good health, treated by dietary phenylalanine restriction from early infancy, willingness to participate in the study. Subjects were excluded if they were taking 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 and energy metabolism, or significant weight changes (> 10% of body weight) in the three months before the study.

The standard dietary management of subjects consists of a phenylalanine free medical food fed at a level which provided the recommended dietary protein for age, for healthy children (9). In addition low protein foods are fed which provide additional protein in an amount which is 50% of the recommended protein intake for age for healthy children (9). Dietary compliance is monitored regularly (on average about every 8 weeks) by an experienced clinical dietitian and by measuring blood phenylalanine, using the same technique as was used in the present study (see below).
Subject characteristics at the beginning of the study are summarized in Table 1. The purpose of the study, study procedures, benefits and potential risks were explained to the study participants and their parents. Written consent was obtained from each parent and assent was obtained separately from each child. All procedures used in the study were approved by the Research Ethics Board, of The Hospital for Sick Children.

Study Design

Each subject was studied on six non-consecutive days over a three month period, at each of 6 phenylalanine intake levels, in random order, to allow an estimation of each individual’s needs as well as the population requirement. The levels were 0, 10, 15, 20, 25 and 35 mg·kg\(^{-1}\)·day\(^{-1}\). A total of 30 oxidation studies were conducted.

Experimental Diet

A flavored liquid formula (Protein-Free Powder, Product 80056, Mead Johnson, Evansville, IN) and protein free cookies (40) developed for use in amino acid kinetic studies supplied the energy in the diet. The diet was prepared and weighed (Sartorius Balance, model BP110 S, Sartorius Canada Inc.) in the research kitchen at The Hospital for Sick Children. The diet was administered on the study day as eight isocaloric, isonitrogenous, hourly meals. Each meal represented 1/8\(^{th}\) of the subject’s total daily requirements. The macronutrient composition of the experimental diet, expressed as a percentage
of dietary energy was approximately 37% fat, 52% carbohydrate and 11% protein (40).

The nitrogen content of the diet was provided as a crystalline amino acid mixture and based on the amino acid composition of egg protein. The amino acid mixture (protein) was provided to each child on each study day at a level of 1.5 g·kg⁻¹·day⁻¹. This level was chosen because it met and exceeded the recommended level (12) and was similar to the subjects’ habitual protein intakes (3).

Total energy intakes were based on each subject’s resting energy expenditure (REE) measured by indirect calorimetry (Vmax 29, SensorMedics Corporation, Yorba Linda, CA) and multiplied by an activity factor of 1.5 (3,12).

Body weight was measured to the nearest 0.1 kg on a balance scale (Toledo Scale, model 2020, Windsor, ON, Canada) with subjects wearing light clothing and no shoes. Standing height was measured without shoes to the nearest 0.1 cm, using a wall-mounted stadiometer. Protein and energy requirements were calculated for each study day using the weight and height measurements from the previous study day. Mean energy intakes for each child over the 6 study days are presented in Table 1.

Tyrosine was provided at an intake of 32 mg·kg⁻¹·day⁻¹. This represented the 95% safe population estimate as determined by Bross, (3) plus an additional 20% to ensure no subject would receive a deficient intake of tyrosine. Lysine, which was used as the indicator amino acid, was provided at 64 mg·kg⁻¹·day⁻¹ (3).
Phenylalanine was provided at graded intakes of 0, 10, 15, 20, 25, and 35 mg·kg⁻¹·day⁻¹. The 10 and 15 mg·kg⁻¹·day⁻¹ levels represent 66% and 100% respectively of the minimum recommended daily intake for children with PKU (8). The current recommended intake range is from 15 to 35 and 15 to 30 mg·kg⁻¹·day⁻¹ for children aged 4 to 7 and 5 to 15 years respectively (8). The intake of 20 mg·kg⁻¹·day⁻¹ represents an intake similar to the tyrosine requirement determined by Bross, (3) of 19.2 mg·kg⁻¹·d⁻¹ and 25 mg·kg⁻¹·day⁻¹ represents the level of phenylalanine equal to 55% of the estimated total aromatic amino acid requirement. Finally, 35 mg·kg⁻¹·day⁻¹ represents the upper limit of the phenylalanine recommendation for children with PKU (8). Since there was uncertainty with regard to where the oxidation break-point would occur, subjects were randomly started on either the lowest (0 mg·kg⁻¹·day⁻¹) or highest 35 mg·kg⁻¹·day⁻¹ phenylalanine intake and then the predicted break-point of around 17 mg·kg⁻¹·day⁻¹ was bracketed by alternately using the next highest phenylalanine test intake (starting from zero) and the next lowest phenylalanine intake (starting with 35 mg·kg⁻¹·day⁻¹).

**Body Composition Measurements**

Body composition, (fat and fat free mass) was determined using bioelectrical impedance analysis (BIA) and multiple skinfold thickness measurements. BIA was performed at the beginning of each study day prior to meal ingestion. Resistance (R) and reactance (Xc) measurements were done using a 4-terminal bioimpedance analyzer (RJL Systems, Model 101A, Detroit,
while the subject lay in a supine position on a hospital bed with all four limbs apart. Two detector 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 bisecting the ulnar tubercle, and between the medial and lateral malleoli, with the proximal edges 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. Three readings for both reactants and resistance (in Ohms) were taken for each subject and the mean of the three readings was used to determine fat free mass (FFM).

Multiple skinfold thickness measurements were taken from 4 different sites on the subject’s non-dominant side; triceps, biceps, sub-scapula and suprailliac. Each measurement was taken by the same individual. A total of 3 measurements from each site were taken using a skin-fold caliper (British Indicators Ltd., St. Albans, England , U.K. ) and the average value was used in the equation. Body density was derived from the sum of the 4 skin-folds using an age specific equation (2,7) and another equation was used to predict percentage of body fat ,from body density ( 27).

Oral Isotope Infusion Studies
L-[1-\textsuperscript{13}C]lysine·HCl, with an enrichment of 99% (Mass Trace, Woburn, MA), was used in this study. Quality control tests were performed by the manufacturer. Chemical purity, isotope enrichment and position were confirmed by GCMS and a second confirmation was performed by nuclear magnetic resonance (NMR). Optical isomer purity (< 0.2% D-isomer) was confirmed by chiral HPLC. A stock solution of 10 mg·mL\textsuperscript{-1} was prepared using sterile water. From the stock solution, the priming and continuous bolus doses were dispensed into multiple vials. Each subject was given a priming oral dose of L-[1-\textsuperscript{13}C]lysine·HCl in the amount of 2.5 mg·kg\textsuperscript{-1} (13.6\mu M·kg\textsuperscript{-1}) and eight subsequent oral bolus doses of equal amounts; 1.4 mg·kg\textsuperscript{-1}·h\textsuperscript{-1} (7.62\mu M·kg\textsuperscript{-1}·h\textsuperscript{-1}) (3).

Details of the isotope and meal consumption protocol have been previously described (3). Methods of urine collection and storage are also identical to those previously described (3). Briefly, eight hourly, isocaloric, isonitrogenous meals were consumed beginning 4 hours before the start of the oral isotope infusion. The amount of lysine in each meal was held constant. This was achieved by reducing the amount of dietary lysine in the last four meals by the same amount corresponding to the [\textsuperscript{13}C] lysine administered. The amount of phenylalanine in each meal was dependent on the test level being studied on that particular study day. Because the amount of phenylalanine in the diet was manipulated, L-alanine was adjusted to keep the nitrogen content of the diet constant.

Breath samples were collected into vacuum tubes while the subjects stood or sat in an upright position. The instrument used for breath collection
was The EasySampler, (QuinTron, Milwaukee, Wisconsin, USA). Subjects were instructed to take a normal breath and exhale normally into a bag with their mouths fitted over a mouthpiece. The subjects were instructed to breathe until the bag was filled with air (about 250 mL air) representing the dead space air. While the subjects continued to breathe, an Exetainer® (Labco Limited, Buckinghamshire, England) was pushed into the needle holder at the bottom of the mouthpiece until the rubber stopper was punctured. The sample was collected into the Exetainer while the subject kept their mouths tightly closed over the mouthpieces. The Exetainers were removed from the needle holder after the subjects stopped breathing. Thereafter subjects were asked to remove their mouth from the mouthpiece. All breath samples were kept at room temperature and analyzed within one week of each study day.

The rate of production of CO₂ (VCO₂) was measured by indirect calorimetry (Vmax 29, SensorMedics Corporation, Yorba Linda, CA); where CO₂ production rate is the product of flow, measured by a mass flow analyzer, and CO₂ concentration, measured by infrared spectroscopy. Each measurement was performed while the subject lay comfortably in a supine position on a hospital bed. On each study day, prior to VCO₂ measurement, the gas analyzers were calibrated with standard gases (Tank 1: 4% CO₂, 16% O₂, balanced with N₂, Tank 2: 26% O₂ balanced with N₂, PraxAir Inc, Brampton ON). VCO₂ measurements were corrected to standard temperature and pressure.

Blood Collection and Analysis
Capillary blood samples were obtained from a finger prick incision made to the index finger of the non-dominant hand (Softlick Blood letting Device, Boehringer, Laval, Quebec). This was done in order to relate phenylalanine intake to blood phenylalanine levels because diet therapy in PKU is mainly managed by monitoring blood phenylalanine in response to dietary intake. In order to ensure arterialized blood, the hand was heated inside a thermostatic chamber maintained at 60°C for at least 15 minutes before the blood was sampled (38). While holding the finger, approximately 5 drops (< 1 mL) of blood was gently spotted on Guthrie filter paper (Ministry of Health, Ontario, Canada, Newborn Screening Program) to totally saturate an area of about 1cm in diameter. Blood was collected at the beginning of each study day, prior to meal ingestion with subjects still in the fasted state and again at the end of the study day after each subject had received and consumed all eight meals. At each time, two blood spots were collected. The blood spots were left to air-dry overnight, then analyzed for phenylalanine concentration according to the method of Dooley (4). This method is used for the quantitative determination of blood phenylalanine and is based on the NAD-dependent oxidative deamination of phenylalanine in the presence of excess phenylalanine dehydrogenase. Phenylalanine reacts with the enzyme phenylalanine dehydrogenase and in the process converts NAD to NADH. The NADH was measured by reaction with indonitrotetrazolium chloride catalyzed by diaphorase, which forms a coloured formazan product.
Analytical Procedures

The $^{13}$CO$_2$ enrichment in expired CO$_2$ was measured with a continuous flow isotopic ratio mass spectrometer (CF-IRMS, Model ANCA GSL) (Europa Scientific Inc. Crewe, UK). Each set of eight samples was separated by two reference samples (5% CO$_2$), which were previously calibrated to an international reference standard (NBS-20), (National Institute for Standards and Technology, Gaithersburg, MD). The results represent the absolute $^{13}$C enrichment present in that sample. Plateau enrichment was calculated as the difference in isotopic abundance at plateau and natural (baseline) isotopic abundance, and were expressed as atom percent excess (APE).

The amino acids in 500 µL of urine were derivatized to their N-heptafluorobutyrl-$n$-propyl esters, by the method of Patterson et al (23). Isotopic enrichment for urinary free lysine was measured by GCMS [Hewlett-Packard model 5890 Series II GC (Mississauga, ON) VG Trio-2 quadrupole mass spectrometer system]. Details of the method have been previously described by Bross et al (3).

Data Analysis

A stochastic model was used to calculate lysine kinetics (36). Isotopic steady state of lysine in urine and CO$_2$ breath were defined as a coefficient of variation of less than 5% between sampling time points and the absence of a significant slope. The difference between the mean breath CO$_2$ isotope enrichment values of the 3 baseline and 5 plateau samples was expressed as
atom percent excess (APE) above baseline at isotopic steady state. Also, the
difference between the mean ratio of the enriched peak (m + 1) to the
unenriched (m) peak of lysine in urine for baseline and plateau samples was
expressed as molecule percent excess (MPE). Typical $^{13}$CO$_2$ and $[^{13}$C] lysine
enrichment in breath and urine respectively, at baseline and plateau for an
individual study have been previously presented by Bross et al (3).

Lysine kinetics were estimated from breath and urine enrichment data
using standard equations (22). The equations used to calculate flux, oxidation,
and rate of release of $^{13}$CO$_2$ in breath in response to lysine oxidation have been
described previously by Bross et al (3). Briefly apparent lysine flux (Q) was
calculated using the following equation:

$$Q = I \left[\left(\frac{E_I}{E_p}\right) - 1\right]$$  \hfill (Eq. 1)

Where I is the mass of the isotope; E$_I$ is the enrichment of the isotope; and E$_p$ is
the enrichment of the $[^{13}$C] lysine at plateau. The rate of $^{13}$CO$_2$ released in
breath ($F^{13}$CO$_2$) from oxidation of the $[^{13}$C] lysine tracer was calculated as follows

$$F^{13}$CO$_2 = \frac{FCO_2(EO_2)(44.6(60))/W(0.82)(100)}{1}$$  \hfill (Eq 2)

Where FCO$_2$ is the carbon dioxide production rate measured by indirect
calorimetry; ECO$_2$ is the $^{13}$CO$_2$ enrichment in breath at the isotopic steady state
(APE); and W is the weight of the subject (kg). The constants are; 44.6. to
convert gas volumes to moles; 60, to show time per hour; 0.82 to allow for delay of excretion into breath of the label from the bicarbonate pool; and 100, to convert APE to a fraction.

The mean requirement for phenylalanine was determined by breakpoint analysis, using a two-phase linear regression crossover model (26) as previously described (6). The upper 95% confidence limits, which represent the safe population requirement, were determined using Fieller’s theorem (26). Statistical analyses were performed on primary and derived values. Repeated measures analysis of variance (SAS Institute, Cary NC, 1996, release 6.12) was used to assess the relationship between apparent lysine flux, lysine oxidation, lysine $^{13}$CO$_2$ and blood phenylalanine concentration to the experimental variables: phenylalanine intake and subject. The Least Square Difference multiple range test was used to test the significance of specific differences between variables grouped according to phenylalanine test intakes. Results were considered to be statistically significant at $P \leq 0.05$.

RESULTS

Subject characteristics are summarized in Table 1. Their body composition determined by skin-fold measurements gave group values that were very similar to those obtained by BIA. The group fat free mass was 81% and fat mass 19% of total body weight. Energy intake on the six study days was established as resting metabolic rate times 1.5 since the children activity was
limited during the 8 to 9 hours of the study days and the individual values are also shown in Table 1, ranging from 1,505 to 2,139 kcal.kg\(^{-1}\).d\(^{-1}\).

Phenylalanine intake had no effect on apparent lysine flux (P = 0.79) Table 2. However, significant differences were observed between apparent lysine flux for the individual subjects (P = 0.02).

Breath carbon dioxide production rates were constant within each subject across the 6 test intakes of phenylalanine (data not shown) but ranged from 189 to 235 mL/min between the five subjects (p < 0.001). The effect of phenylalanine intake on individual rate of \(^{13}\)CO\(_2\) (F\(^{13}\)CO\(_2\)) release is shown in Table 3. For every subject, F\(^{13}\)CO\(_2\) decreased with increasing phenylalanine intakes up to a specific phenylalanine intake after which F\(^{13}\)CO\(_2\) increased. The individual subject (P = 0.008) as well as phenylalanine intake (P = 0.004) had a significant effect on F\(^{13}\)CO\(_2\). Figure 1 shows the mean breakpoint in the F\(^{13}\)CO\(_2\) data. Using a two-phase linear regression crossover model, a breakpoint of 14 mg phenylalanine·kg\(^{-1}\)·day\(^{-1}\) was found. The upper 95% confidence limit of the breakpoint, which represents the safe population intake, was determined to be 19.5-mg phenylalanine·kg\(^{-1}\)·day\(^{-1}\). The individual F\(^{13}\)CO\(_2\) data is shown in Figure 2. From this data individual phenylalanine requirement estimates can be obtained by visual inspection and the data ranged from 13 to 20 mg.kg\(^{-1}\).d\(^{-1}\) with an average of 15.2 mg.kg\(^{-1}\).d\(^{-1}\). Despite the approximate nature of the visual estimates, the average value and range are comparable to those obtained by two-phase linear cross-over regression analysis. The pattern of lysine oxidation
mirrored the F\textsuperscript{13}CO\textsubscript{2} data but was not significantly affected by phenylalanine intake (Table 2).

Mean fasted and fed state blood phenylalanine concentrations are presented in Figure 2. In the fasted state, there was no difference in the mean blood phenylalanine concentration at any of the intake levels. In the fed state, there was no difference in mean blood phenylalanine concentration at phenylalanine intakes from 0 to 25-mg phenylalanine·kg\textsuperscript{-1}·day\textsuperscript{-1}. However blood phenylalanine concentration at an intake of 35 mg of phenylalanine·kg\textsuperscript{-1}·day\textsuperscript{-1}, was increased and significantly higher than at all other phenylalanine intake levels.

Figure 3 presents the relationship between the mean differences between fed and fasted blood phenylalanine concentrations in response to varying phenylalanine intake levels. Correlation analysis showed that there was a direct relationship between phenylalanine intake and difference in (fed minus fasted) blood phenylalanine concentration, with 98% of the difference in blood phenylalanine concentration between fed and fasted states being accounted for by phenylalanine intake ($r^2 = 0.98$), $P < 0.0001$. The individual data for each subject share a similar pattern to that of the group as a whole. From Figure 3, at intakes of 20 mg of phenylalanine kg\textsuperscript{-1}·day\textsuperscript{-1} or less, blood phenylalanine concentration is less than or similar to the fasted concentration while at intakes of 25 and 35 mg of phenylalanine·kg\textsuperscript{-1}·day\textsuperscript{-1} the fed blood phenylalanine concentration was higher than fasted concentration. At an intake of about 20mg
of phenylalanine·kg⁻¹·day⁻¹ there was a balance between phenylalanine intake and the difference in (fed minus fasted) blood phenylalanine concentration.

DISCUSSION

The results of the present indicator amino oxidation study show that the mean phenylalanine requirement estimated by a two-phase linear regression crossover model is 14mg·kg⁻¹·day⁻¹ for prepubertal children between the ages of 6 to 13 years with classical PKU (Figure 1). Holt & Snyderman, (15), using growth and nitrogen balance, estimated the phenylalanine requirement (in the presence of dietary tyrosine) for 27 children with PKU. Their requirement estimates covered a broad range: 55 to 90 mg·kg⁻¹·day⁻¹ at 2 months (mean 70 mg·kg⁻¹·day⁻¹) to 25 to 80 mg·kg⁻¹·day⁻¹ at 12 months (mean 35 mg·kg⁻¹·day⁻¹).

Based on those results Holt & Snyderman, (15) concluded that the phenylalanine requirement of children with PKU is no different than that of normal children. However, these authors made no attempt to differentiate between classical or variant PKU in their study. In addition, when the requirement estimates from Holt & Snyderman (15) were used to treat children with PKU from birth, the children had prolonged periods of high plasma phenylalanine (1).

Conversely, our present data are consistent with those of a retrospective study, which examined phenylalanine requirements in children with PKU who had normal rates of growth (18). Those children were followed from birth to 2
years. Plasma phenylalanine over the 2 year period averaged 345 ± 285 µM therefore those children were fairly well controlled. The authors found that between the ages of 0 to 4, 4 to 12 and 12 to 24 months, phenylalanine intake needed to maintain a normal or moderately elevated plasma phenylalanine was approximately 68, 58 and 50 %, respectively, of the requirement of normal children (15). There was a separation of the children into classical or variant PKU groups. When the authors compared their data with those of Holt & Snyderman, (15) they found that the lowest estimate corresponded with the intakes of the classical PKU children in their study, whereas the higher estimates from Holt & Snyderman corresponded to the intakes of the variant PKU children. In addition to defining the mean phenylalanine requirement in the present study, a safe level of intake necessary to meet the requirement of 95% of the population (the 95% confidence limit) was estimated at 19.5 mg·kg^{-1}·day^{-1}. This was necessary, because by definition, a requirement should be broad enough to cover the needs of almost all individuals (95%) within a given population. However, in a disease state like PKU the implications of setting a phenylalanine requirement at the 95% confidence level are unclear. All individuals were studied at each of the 6 phenylalanine intake levels; therefore estimation of individual requirement was possible and ranged from 13 to 20 mg·kg^{-1}·day^{-1}. The 95% confidence limit of 19.5 mg·kg^{-1}·day^{-1}, is 4.5 to 6.5 mg·kg^{-1}·day^{-1} higher than the individual estimate for 4 out of the 5 children studied. In the present study however, there was no change in mean blood phenylalanine concentration between phenylalanine intakes of 0 to 25 mg·kg^{-1}·day^{-1} (Figure 4). This is
evidence that the indicator method is more sensitive than measurement of blood 
phenylalanine, and suggests that at such small differences in intake above the 
mean requirement (4.5 to 6.5 mg·kg\(^{-1}\)·day\(^{-1}\)), no significant changes would be 
detected in blood phenylalanine concentration. On the other hand, there are 
many studies in which the consequence of inadequate intakes of phenylalanine 
in a PKU population have been described (11,13,14). Severe mental and growth 
retardation have been reported. Because the impact on blood phenylalanine is 
negligible, with intakes in such a small excess of requirements, and because we 
have evidence of the consequences of intakes that are inadequate to meet 
requirements in children, we propose that the estimated 95% confidence limit of 
19.5 mg·kg\(^{-1}\)·day\(^{-1}\), be accepted as the recommended phenylalanine intake in 
prepubertal children with classical PKU between the ages of 6 to 13 years old. 

There was a very high degree of inter-individual, as well as intra-individual 
variability between the study days in the baseline blood phenylalanine 
concentrations in the present study (data not shown). However, when this was 
controlled for, by subtracting the fasting levels from the fed levels, a very clear 
picture emerged (Figure 4). These data showed that 98% of the change in blood 
phenylalanine concentrations with feeding was accounted for by phenylalanine 
intake alone. These data also lend support for the mean and 95% confidence 
requirement estimate determined from the indicator tracer studies. The least 
difference in blood phenylalanine occurred at phenylalanine intakes between 15 
and 20 mg·kg\(^{-1}\)·day\(^{-1}\) (Figure 4). The fact that, no change from baseline was 
detected in the mean blood data at phenylalanine intakes from 0 to 25 mg·kg\(^{-1}\).
day\(^{-1}\) (Figure 3), is evidence that blood levels of an amino acid (even in a
disease such as PKU with the absence of catabolic enzyme activity), are a
relatively insensitive measure, compared to oxidation, measured by \(^{13}\)CO\(_2\), and
should not by used as a sole measure of requirement.

In every indicator study we have previously performed (3, 6, 20, 38) to
determine amino acid requirements the expected pattern of indicator amino acid
oxidation has been observed. Namely, a decrease in the oxidation of the
indicator amino acid as the level of the test amino acid is increased in the diet,
until the mean requirement level (breakpoint) is reached; after which increases
in the test amino acid has no further effect on the oxidation of the indicator
amino acid. This pattern was also observed by Bross (3), in her study on
tyrosine requirement in a similar population of children with PKU. In the present
study however, an increase in the oxidation of the indicator was observed
beyond the \(^{13}\)CO\(_2\) breakpoint (mean requirement) (Figure 1). A similar pattern
was observed for all individuals studied (Table 3 and Figure 2). Since, indicator
amino acid oxidation is a reflection of the partitioning of the essential amino
acids between incorporation into protein (synthesis) versus oxidation, this
suggests that beyond mean requirement, further increase in the intake of
phenylalanine in PKU, results in a decrease in whole body protein synthesis.

The present data does not permit an explanation for the decreased whole
body protein synthesis when phenylalanine intakes increase above the break-
point. There are however data in the literature which show that elevated plasma
phenylalanine levels interfere with the metabolism of other essential amino
acids. Wapnir & Lifshitz, (35) have shown that plasma tryptophan levels in PKU are lower than controls even after the implementation of a low phenylalanine diet. Lipovac et al, (21) have shown that amino acid catabolism was increased in the tissues of rats in which hyperphenylalaninemia was induced. Optimal protein synthesis is dependent upon an ideal balance of amino acids being present together with sufficient non-protein energy (5).

An alternative explanation of the $^{13}\text{CO}_2$ pattern is that the indicator amino acid oxidation model failed in part in the present study. Against this is the fact that the indicator model worked satisfactorily in a similar group of children with PKU, who were being studied to determine their tyrosine needs (3).

Furthermore, the data on the change in blood phenylalanine (Figure 4) in response to increasing intakes of phenylalanine is supportive of the value for the upper limit of phenylalanine intake, defined by the indicator model. While this issue needs further investigation, we feel the balance of the evidence supports our interpretation that protein synthesis is adversely affected above a phenylalanine intake of 20 mg·kg$^{-1}$·day$^{-1}$.

Although the lysine oxidation data (Table 2) followed the same pattern as the $^{13}\text{CO}_2$ data (Table 3), there was too much variance in the oxidation data, to show a significant effect of phenylalanine intake on lysine oxidation, (by analysis of variance). Further, we were unable to define a break-point, when performing two-phase linear cross-over analysis of the lysine oxidation data, in response to graded intakes of lysine (data not shown). We have made similar observations in studies of tryptophan requirement in women (20), and lysine requirement in
men (6). In those studies (6, 20) we were able to define a break-point in $^{13}$C-label oxidation ($F^{13}\text{CO}_2$), while not being able to define a break-point in the oxidation of the indicator amino acid. The latter is calculated from plasma $^{13}$C-lysine enrichments, in addition to $F^{13}\text{CO}_2$. We believe the failure to be able to show a break-point with oxidation of the indicator amino acid is due to the fact that plasma may not always be representative of the intra-cellular pool(s) from which amino acid oxidation takes place.

In the present study we chose to use an orally administered lysine tracer, in order to make the study minimally invasive and thereby suitable for studies in children (3). A consideration when using an oral tracer is that it may be taken up and oxidized in the gut, and indeed it has been shown that 35 to 53% of dietary lysine is taken up and oxidized, during first pass in the gut (29). This uptake of tracer within the gut results in a lower plasma enrichment and hence accounts for the higher estimates of apparent lysine flux, in the present study, compared with earlier work, in children, when the tracer was given intravenously (17). We have recently shown that, gut uptake of the tracer does not alter the break-point and hence does not alter the requirement estimate, of the test amino acid (19)

What is important is that changes in the test amino acid intake (phenylalanine) does not affect the flux of the indicator (lysine) which did not happen (Table 2). This condition is necessary since it means that indicator label flux is being partitioned between oxidation and whole body protein synthesis in response to changes in the test amino acid intake (38, 42).
This study provides further support for the suitability of the indicator amino acid technique, for the estimation of amino acid requirement in vulnerable groups of individuals. It also demonstrates the suitability of lysine as an indicator. This is underlined by the fact that phenylalanine intake had no effect on apparent lysine flux; a critical condition for any indicator study.

In conclusion, in light of the similarity of the results from the upper 95% confidence interval of the indicator study and the change in blood phenylalanine levels (fed minus fasting), children with PKU should not be fed more than 20 mg·kg\(^{-1}\)·day\(^{-1}\) of phenylalanine in their diet.
REFERENCES


23. **Patterson, B. W., D.L. Hachey, G. L. Cook, J. M. Amann and P. D.**


27. **Siri, W. E.** *Body composition from fluid spaces and density analysis of methods.* Donner Laboratory of Bioshysics and Medical Physics, California, 1956.


29. **Stoll, B., J, Henry, P.J. Reeds, H. Yu, F. Jahoor and D.G. Burrin.** Catabolism dominates the first-pass intestinal metabolism of dietary


31. **Thompson, A. J., I. Smith, B. E. Kendall, B.D. Youl and D. Brenton**


FIGURE CAPTIONS

**Figure 1** Effect of phenylalanine intake on mean rate of $^{13}$CO$_2$ release (F$^{13}$CO$_2$) in children with phenylketonuria. Mean ($\pm$ SEM) at 6 test phenylalanine intakes. Pooled data of all observations (n=30) and all subjects (n=5). The breakpoint estimates the mean phenylalanine requirement of the sample population: break-point value 14 mg.kg$^{-1}$.d$^{-1}$ (95% confidence limits 8.5 – 19.5); $r^2 = 0.4$, $p< 0.007$

**Figure 2** Effect of phenylalanine intake on F$^{13}$CO$_2$ shown as individual data

**Figure 3** Fasted (A) and fed-state (B) plasma phenylalanine concentration. Mean ($\pm$ SEM) in response to varying phenylalanine intakes. Pooled data of all observations (n=30) and all subjects (n=5).

**Figure 4** Relationship between phenylalanine intake and mean difference (fed minus fasted) in plasma phenylalanine concentration. Mean ($\pm$ SEM); Pooled data for all observations (n=30) and all subjects (n=5): $r^2 = 0.98$, $p< 0.0002$
Table 1. Subject Characteristics of Children with Phenylketonuria (PKU) who participated in the Phenylalanine Requirement Study

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (years)</th>
<th>WT. (kg)</th>
<th>HT. (cm)</th>
<th>% IBW</th>
<th>FFM-BIA (kg)</th>
<th>FFM-SF (kg)</th>
<th>Energy Intake (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>F</td>
<td>10</td>
<td>39.5</td>
<td>148.2</td>
<td>85.9</td>
<td>32.0</td>
<td>30.8</td>
<td>2129 ± 15</td>
</tr>
<tr>
<td>DN</td>
<td>M</td>
<td>6</td>
<td>22.5</td>
<td>119.1</td>
<td>102.3</td>
<td>19.0</td>
<td>20.6</td>
<td>1505 ± 18</td>
</tr>
<tr>
<td>AS</td>
<td>F</td>
<td>10</td>
<td>39.4</td>
<td>148.6</td>
<td>90.0</td>
<td>31.2</td>
<td>29.2</td>
<td>2139 ± 5</td>
</tr>
<tr>
<td>BS</td>
<td>M</td>
<td>12</td>
<td>40.5</td>
<td>154</td>
<td>92.1</td>
<td>33.2</td>
<td>33.6</td>
<td>2099 ± 5</td>
</tr>
<tr>
<td>AP</td>
<td>M</td>
<td>13</td>
<td>36.9</td>
<td>145.4</td>
<td>104.2</td>
<td>28.9</td>
<td>30.6</td>
<td>1993 ± 7</td>
</tr>
<tr>
<td></td>
<td>Mean ±</td>
<td>10.2</td>
<td>35.8</td>
<td>143.1</td>
<td>94.9</td>
<td>28.9</td>
<td>29.0</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>± 1.2</td>
<td>± 3.4</td>
<td>± 6.2</td>
<td>± 3.6</td>
<td>± 2.6</td>
<td>± 2.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

3 FFM-SF = Fat free mass determined by multiple skinfold thickness

4 M = male, F = female

5 Energy in kilocalories (kcal) per day
Table 2. Effect of phenylalanine intake on mean apparent lysine flux and oxidation in children with PKU

<table>
<thead>
<tr>
<th>Phenylalanine Intake mg·kg⁻¹·day⁻¹</th>
<th>0</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>35</th>
<th>Pooled SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent Lys. Flux</td>
<td>312</td>
<td>295</td>
<td>261</td>
<td>263</td>
<td>302</td>
<td>317</td>
<td>43</td>
</tr>
<tr>
<td>Lys. Oxidation</td>
<td>44.8</td>
<td>36.5</td>
<td>27.0</td>
<td>32.0</td>
<td>37.3</td>
<td>40.3</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Apparent lysine flux and oxidation are expressed as µmol ·kg⁻¹ ·h⁻¹. By repeated measures analysis of variance, phenylalanine intake had no significant effect on apparent lysine flux P = 0.79 or lysine oxidation, P = 0.23.
Table 3. Effect of phenylalanine intake on the rate of $^{13}\text{CO}_2$ released from L-[1-$^{13}$C]lysine oxidation ($F^{13}\text{CO}_2$) in children with PKU

<table>
<thead>
<tr>
<th>Subject</th>
<th>0</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>1.23</td>
<td>0.93</td>
<td>0.81</td>
<td>1.02</td>
<td>0.86</td>
<td>0.90</td>
</tr>
<tr>
<td>DN</td>
<td>0.92</td>
<td>0.87</td>
<td>0.78</td>
<td>0.88</td>
<td>0.96</td>
<td>1.00</td>
</tr>
<tr>
<td>AS</td>
<td>1.14</td>
<td>0.89</td>
<td>0.87</td>
<td>0.75</td>
<td>0.91</td>
<td>1.12</td>
</tr>
<tr>
<td>BS</td>
<td>1.31</td>
<td>1.19</td>
<td>0.85</td>
<td>1.11</td>
<td>1.07</td>
<td>1.41</td>
</tr>
<tr>
<td>AP</td>
<td>1.20</td>
<td>0.70</td>
<td>0.61</td>
<td>0.87</td>
<td>1.08</td>
<td>0.75</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>1.16 ± 0.1$^a$</td>
<td>0.92 ± 0.1$^c$</td>
<td>0.79 ± 0.1$^d$</td>
<td>0.93 ± 0.1$^c$</td>
<td>0.98 ± 0.1$^{bc}$</td>
<td>1.04 ± 0.1$^b$</td>
</tr>
</tbody>
</table>

$^{13}\text{CO}_2$ released from L-[1-$^{13}$C]lysine oxidation ($F^{13}\text{CO}_2$) is expressed as µmol ·kg$^{-1}$ ·day$^{-1}$. By repeated measures analysis of variance, phenylalanine intake had a significant effect on $F^{13}\text{CO}_2$. Means with different superscripts are significantly different, $P = 0.004$. 
Breakpoint = 14 mg·kg⁻¹·day⁻¹

Rate of $^{13}$CO₂ release (µmol·kg⁻¹·h⁻¹)

Phenylalanine intake (mg·kg⁻¹·day⁻¹)
A

Plasma phenylalanine concentration (µM) vs. phenylalanine intake (mg.kg⁻¹.day⁻¹)

B

Plasma phenylalanine concentration (µM) vs. phenylalanine intake (mg.kg⁻¹.day⁻¹)
Plasma Phenylalanine concentration (µM) vs. Phenylalanine intake (mg·kg⁻¹·day⁻¹)