Phenylalanine requirement in children with classical PKU determined by indicator amino acid oxidation

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Courtney-Martin, Glenda, Rachelle Bross, Mahroukh Raffi, Joe T. R. Clarke, Ronald O. Ball, and Paul B. Pencharz. Phenylalanine requirement in children with classical phenylketonuria (PKU), and current estimates of requirements are based on plasma phenylalanine concentration and growth. The present study aimed to determine more precisely the phenylalanine requirements in patients with the disease by use of indicator amino acid oxidation, with L-[1-13C]lysine as the indicator. Breath 13CO2 production (F13CO2) was used as the end point. Finger-prick blood samples were also collected for measurement of phenylalanine to relate phenylalanine intake to blood phenylalanine levels. The mean phenylalanine requirement, estimated using a two-phase linear regression crossover analysis, was 14 mg/kg/day.

Dietary phenylalanine restriction has been the mainstay of treatment of phenylketonuria (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 suggesting that neuropsychological and cognitive functions are not entirely normalized in individuals with PKU receiving present treatment regimens (28, 30–32).

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 more sensitively and accurately the aromatic amino acid (phenylalanine and tyrosine) requirements in children with PKU by use of isotope tracer methods (20, 38, 40).

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, 40). Because patients with classical PKU have a negligible or very minimal capacity to oxidize phenylalanine (33, 34), we reasoned that their dietary phenylalanine requirements would be lower than those of healthy children by an amount equal to the obligatory losses of phenylalanine. Dietary requirements for phenylalanine in children have yet to be defined. Therefore, we turned to our previous study (3) using indicator amino acid oxidation, which showed that the mean tyrosine requirement of children with PKU was 19.2 mg·kg−1·day−1. Next, we used a ratio between phenylalanine and tyrosine in the tissues of humans and animals of 55:45 (16, 24, 37, 38), which, when multiplied by the tyrosine mean requirements, predicts a mean phenylalanine requirement for healthy children of ~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). Because there are no data in children, we had to turn to a study of phenylalanine requirement in adult males (38), in which obligatory oxidation was estimated to be ~26%. By use of the predicted phenylalanine requirement for children and the estimated obligatory oxidation for phenylalanine, the obligatory loss was calculated to be ~6.1 mg·kg−1·day−1. When the

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estimate of obligatory loss from the predicted mean requirement of 23.5 mg·kg⁻¹·day⁻¹ was subtracted, 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 by use of the technique of indicator amino acid oxidation and to compare the results with our previously estimated tyrosine requirement obtained using the same technique.

METHODS

Study subjects. Five children (mean ± SE age, 10.2 ± 1.2 yr) with classical PKU, treated by the PKU Clinic at the Hospital for Sick Children (Toronto, ON, Canada), participated in this study. All subjects were studied on an outpatient basis. Each subject was selected for study on the basis of the following criteria: having a plasma phenylalanine concentration of ≥1,200 μM at diagnosis, being prepubertal males or females 5–13 yr old and in good health, being treated by dietary phenylalanine restriction from early infancy, and being willing to participate in the study. Subjects were excluded if they were taking medication that might alter protein or energy metabolism or if they had recent illness, IQ <80, history of endocrine disease or any other medical condition that might alter protein and energy metabolism, or significant weight changes (>10% body wt) in the 3 mo before the study. The standard dietary management of subjects consists of a phenylalanine-free medical food fed at a level that provides the age-specific recommended dietary protein for healthy children (9). In addition, low-protein foods are fed that provide additional protein in an amount that is 50% of the age-specific recommended protein intake for healthy children (9). Dietary compliance is monitored regularly (on average about every 8 wk) by an experienced clinical dietician and by measuring blood phenylalanine by use of the same technique as that used in the present study (see Blood collection and analysis).

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 nonconsecutive days over a 3-mo period, at each of six 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 80065, Mead Johnson, Evansville, IN) and protein-free cookies (39) 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, Mississauga, ON, Canada) 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 one-eighth of the subject’s total daily requirements. The macronutrient composition of the experimental diet, expressed as a percentage of dietary energy, was ~37% fat, 52% carbohydrate, and 11% protein (39).

The nitrogen content of the diet was provided as a crystaline amino acid mixture and was 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 subject’s habitual protein intakes (3).

Total energy intakes were based on each subject’s resting energy expenditure (REE), measured by indirect calorimetry (Vmax 29, SensorMedics, Yorba Linda, CA) 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 by means of a wall-mounted stadiometer. Protein and energy requirements were calculated for each study day by 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 et al. (3), plus an additional 20% to ensure that 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 from 15 to 30 mg·kg⁻¹·day⁻¹ for children aged 4–7 and 5–15 yr, respectively (8). The intake of 20 mg·kg⁻¹·day⁻¹ represents an intake similar to the tyrosine requirement.

Table 1. Subject characteristics of children with PKU who participated in the phenylalanine requirement study

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age, yr</th>
<th>Weight, kg</th>
<th>Height, cm</th>
<th>% IBW</th>
<th>FFM-BIA, kg</th>
<th>FFM-SF, kg</th>
<th>Energy Intake, kcal/day (mean ± SE)</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>2,129 ± 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>1,505 ± 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>33.9</td>
<td>29.2</td>
<td>1,239 ± 5</td>
</tr>
<tr>
<td>BS</td>
<td>M</td>
<td>12</td>
<td>40.5</td>
<td>154.1</td>
<td>92.1</td>
<td>33.2</td>
<td>33.6</td>
<td>2,069 ± 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>1,993 ± 7</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>10.2 ± 1.2</td>
<td>35.8 ± 3.4</td>
<td>143.1 ± 6.2</td>
<td>94.9 ± 3.6</td>
<td>28.9 ± 2.6</td>
<td>29.0 ± 2.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PKU, phenylketonuria; IBW, ideal body weight, determined by matching weight percentile to actual height percentile and dividing actual weight by matched weight × 100%; FFM-BIA, fat-free mass determined by bioelectrical impedance analysis; FFM-SF, fat-free mass determined by multiple skinfold thickness.
determined by Bross et al. (3) of 19.2 mg·kg⁻¹·day⁻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). Because 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 −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 (FFM)] was determined using bioelectrical impedance analysis (BIA) and multiple skinfold thickness measurements. BIA was performed at the beginning of each study day before meal ingestion. Resistance and reactance measurements were done using a four-terminal bioimpedance analyzer (model 101A, RJL Systems, Detroit, MI) while the subject lay in a supine position on a hospital bed with all four limbs apart. Two detector electrodes were placed on the lateral malleoli, with the proximal edges dissecting the medial malleoli, and the proximal edges dissecting 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 reactance and resistance were taken for each subject, and the mean of the three readings was used to determine FFM.

Multiple skinfold thickness measurements were taken from four different sites on the subject’s nondominant side: triceps, biceps, subscapula, and suprailiac. Each measurement was taken by the same individual. A total of three measurements from each site were taken using a skinfold caliper (British Indicators, St. Albans, UK), and the average value was used in the equation. Body density was derived from the sum of the four skinfolds by use of an age-specific equation (2, 7), and another equation was used to predict the percentage of body fat from body density (27).

Oral isotope infusion studies. L-[1-13C]lysine·HCl, with an enrichment of 99% (MassTrace, Woburn, MA), was used in this study. Quality control tests were performed by the manufacturer. Chemical purity, isotope enrichment, and position were confirmed by GC-MS, 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 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-[1-13C]lysine·HCl in the amount of 2.5 mg/kg (13.6 µM/kg) and eight subsequent oral bolus doses of equal amounts: 1.4 mg·kg⁻¹·h⁻¹ (7.62 µM·kg⁻¹·h⁻¹) (3).

Details of the isotope and meal consumption protocol have been previously described (5). Methods of urine collection and storage were also identical to those previously described (3). Briefly, eight hourly, isocaloric, isonitrogenous meals were consumed beginning 4 h 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 correspond-

ing to the [12C]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, WI). Subjects were instructed to take a normal breath and exhale normally into a bag with their mouths fitted over mouthpieces. The subjects were instructed to breathe until the bag was filled with air (~250 ml of air) representing the dead space air. While the subjects continued to breathe, an Exeteran (Labco, Buckinghamshire, UK) was pushed into the needle holder at the bottom of the mouthpiece until the rubber stopper was punctured. The sample was collected into the Exeteran while the subjects kept their mouths tightly closed over the mouthpieces. The Exeterans were removed from the needle holder after the subjects stopped breathing into them. Thereafter, subjects were asked to remove their mouths from the mouthpieces. All breath samples were kept at room temperature and analyzed within 1 wk of each study day.

The rate of production of CO₂ (VCO₂) was measured by indirect calorimetry (Vmax 29), where the 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, before 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, Brampton, ON, Canada)]. 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 nondominant hand (Softick Bloodletting Device; Boehringer, Laval, QC, Canada). This was done to relate phenylalanine intake to blood phenylalanine levels, because diet therapy in PKU is managed mainly by monitoring blood phenylalanine in response to dietary intake. To ensure arterialized blood, the hand was heated inside a thermostatic chamber maintained at 60°C for ≥15 min before the blood was sampled (41). While the finger was held, about five drops (~1 ml) of blood were gently spotted onto Guthrie filter paper (Newborn Screening Program, Ministry of Health, Toronto, ON, Canada) to totally saturate an area of ~1 cm in diameter. Blood was collected at the beginning of each study day, before 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 and were 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 indocarboxylic chloride catalyzed by diaphorase, which forms a colored formazan product.

Analytical procedures. The 13C1CO₂ enrichment in expired CO₂ was measured with a continuous-flow isotopic ratio mass spectrometer (model ANCA GSL; Europa Scientific, Crewe, UK). Each set of eight samples was separated by two reference samples (5% CO₂), which were previously cali-
brated 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 was expressed as atom percent excess (APE).

The amino acids in 500 μl of urine were derivatized to their N-heptafluorobutanyl-n-propyl esters by the method of Patterson et al. (23). Isotopic enrichment for urinary free lysine was measured by GC-MS [Hewlett-Packard model 5890 Series II GC (Mississauga, ON, Canada) 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 values of lysine in urine and CO₂ breath were defined as a coefficient of variation of <5% between sampling time points and the absence of a significant slope. The difference between the mean breath CO₂ isotope enrichment values of the three baseline and five plateau samples was expressed as APE above baseline at isotopic steady state. Also, the difference between the mean ratio of the enriched peak ($m$) to the unenriched ($m$) peak of lysine in urine for baseline and plateau samples was expressed as mole percent excess (MPE). Typical $^{13}$CO₂ and [13C]lysine enrichments 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 by use of standard equations (22). The equations used to calculate flux, oxidation, and rate of release of $^{13}$CO₂ 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 equation

$$Q = I[(E_1/E_0) - 1]$$

where $I$ is the mass of the isotope; $E_1$ is the enrichment of the isotope; and $E_0$ is the enrichment of the [13C]lysine at plateau. The rate of $^{13}$CO₂ released in breath ($F^{13}$CO₂) from oxidation of the [13C]lysine tracer was calculated as

$$F^{13}$CO₂ = (FCO₂/W)(ECO₂/(44.6(60)/W)(0.82)(100)$$

where FCO₂ is the CO₂ production rate measured by indirect calorimetry, ECO₂ is the $^{13}$CO₂ 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 break-point analysis with the use of 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 $F^{13}$CO₂, 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 skinfold measurements, gave group values that were very similar to those obtained by BIA. The group FFM 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’s activity was limited during the 8–9 h of the study days, and the individual values are also shown in Table 1, ranging from 1,505 to 2,139 kcal·kg⁻¹·day⁻¹.

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

Breath $V^{13}$CO₂ rates were constant within each subject across the six test intakes of phenylalanine (data not shown) but ranged from 189 to 235 ml/min among the five subjects ($P < 0.001$). The effect of phenylalanine intake on individual rate of $^{13}$CO₂ ($F^{13}$CO₂) release is shown in Table 3. For every subject, $F^{13}$CO₂ decreased with increasing phenylalanine intakes up to a specific phenylalanine intake, after which $F^{13}$CO₂ increased.

The individual subject ($P = 0.008$) as well as phenylalanine intake ($P = 0.004$) had a significant effect on $F^{13}$CO₂. Figure 1 shows the mean break point in the $F^{13}$CO₂ data. With the use of a two-phase linear regression crossover model, a break point of 14 mg phenylalanine·kg⁻¹·day⁻¹ was found. The upper 95% confidence limit of the break point, which represents the safe population intake, was determined to be 19.5 mg phenylalanine·kg⁻¹·day⁻¹. The individual $F^{13}$CO₂ data are shown in Fig. 2. From these data, individual phenylalanine requirement estimates can be obtained by visual inspection, and the data ranged from 13 to 20 mg·kg⁻¹·day⁻¹, with an average of 15.2 mg·kg⁻¹·day⁻¹. Despite the approximate nature of the visual estimates, the average value and range are comparable to those obtained by two-phase linear crossover regression analysis. The pattern of lysine

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**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 lysine 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>Lysine 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 ANOVA, phenylalanine intake had no significant effect on apparent lysine flux ($P = 0.79$) or lysine oxidation ($P = 0.23$).
oxidation mirrored the \( F^{13}\text{CO}_2 \) data but was not significantly affected by phenylalanine intake (Table 2).

Mean fasted- and fed-state blood phenylalanine concentrations are presented in Fig. 3. 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\( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \). However, blood phenylalanine concentration at an intake of 35 mg of phenylalanine\( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) was increased and was significantly higher than at all other phenylalanine intake levels.

Figure 4 presents the relation among 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 the difference in (fed minus fasted) blood phenylalanine concentration. At an intake of 20 mg of phenylalanine\( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \), 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 acid oxidation study show that the mean phenylalanine requirement estimated by a two-phase linear regression crossover model is 14 mg\( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) for prepubertal children between the ages of 6 and 13 yr with classical PKU (Fig. 1). Holt and 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: 55–90 mg\( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) at 2 mo (mean, 70 mg\( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \)) to 25–80 mg\( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) at

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**Table 3. Effect of phenylalanine intake on the rate of \( ^{13}\text{CO}_2 \) released from L-[1-\( ^{13}\text{C} \)]lysine oxidation in children with PKU**

<table>
<thead>
<tr>
<th>Phenylalanine Intake, mg( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} )</th>
<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>
<td></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>
<td></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>
<td></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>
<td></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>
<td></td>
</tr>
</tbody>
</table>

Mean ± SE: 1.16 ± 0.1\( * \) 0.92 ± 0.1\( ‡ \) 0.79 ± 0.1\( § \) 0.93 ± 0.1\( ‡ \) 0.98 ± 0.1\( ‡ \) 1.04 ± 0.1\( † \)

\( ^{13}\text{CO}_2 \) released from L-[1-\( ^{13}\text{C} \)]lysine oxidation (\( F^{13}\text{CO}_2 \)) is expressed as \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). By repeated-measures ANOVA, phenylalanine intake had a significant effect on \( F^{13}\text{CO}_2 \). Means with different symbols are significantly different, \( P = 0.004 \).
12 mo (mean, 35 mg·kg⁻¹·day⁻¹). On the basis of those results, Holt and Snyderman concluded that the phenylalanine requirement of children with PKU is no different from that of normal children. However, these authors made no attempt to differentiate between classical and variant PKU in their study. In addition, when the requirement estimates from Holt and Snyderman 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 that examined phenylalanine requirements in children with PKU who had normal rates of growth (18). Those children were followed from birth to 2 yr of age. Plasma phenylalanine over the 2-yr period averaged 345 ± 285 μM; therefore, those children were fairly well controlled. The authors found that between the ages of 0 and 4, 4 and 12, and 12 and 24 mo, the phenylalanine intake needed to maintain a normal or moderately elevated plasma phenylalanine was ~68, 58, and 50%, respectively, of the requirement of normal children (18). There was a separation of the children into classical and variant PKU groups. When the authors compared their data with those of Holt and Snyderman (15), they found that the lowest estimate corresponded to the intakes of the classical PKU children in their study, whereas the higher estimates from Holt and Snyderman corresponded to the intakes of the variant PKU children.

In addition to the mean phenylalanine requirement being defined 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 six 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 4.5–6.5 mg·kg⁻¹·day⁻¹ higher than the individual estimate for four of the five children studied. In the present study, however, there was no change in mean blood phenylalanine concentration between phenylalanine intakes of 0–25 mg·kg⁻¹·day⁻¹ (Fig. 3). 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 (4.5–6.5 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 consequences of inadequate intakes of phenylalanine in a PKU population have been described (11, 13, 14). Severe mental retardation 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 and 13 yr.

There was a very high degree of interindividual as well as intraindividual 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 (Fig. 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 (Fig. 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 (Fig. 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 with oxidation, measured by F¹³CO₂, and should not be used as a sole measure of requirement.

In every indicator study that we have previously performed (3, 6, 20, 40) to determine amino acid requirements, the expected pattern of indicator amino acid oxidation has been observed, namely that 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 (break point) is reached, after which increase in the test amino acid has no further effect on the oxidation of the indicator amino acid. This pattern was also observed by Bross et al. (3) in a study on tyrosine requirement in a similar amino acid. This pattern was also observed by Bross et al. (3) in a 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 F¹³CO₂ break point (mean requirement; Fig. 1). A similar pattern was observed for all individuals studied (Table 3 and Fig. 2). Because indicator amino acid oxidation is a reflection of the partitioning of the essential amino acids between incorporation into protein (synthesis) and oxidation, this suggests that, beyond the mean requirement, a further increase in the intake of phenylalanine in PKU results in a decrease in whole body protein synthesis.

The present data do 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 that show that elevated plasma phenylalanine levels interfere with the metabolism of other essential amino acids. Wapnir and Lifshitz (35) have shown that plasma tryptophan levels in PKU are lower than in 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 on an ideal balance of amino acids being present together with sufficient nonprotein energy (5).

An alternative explanation of the F¹³CO₂ 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 (Fig. 4) in response to increasing intakes of phenylalanine are supportive of the value for the upper limit of phenylalanine intake, defined by the indicator model. Although this issue needs further investigation, we believe that 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 F¹³CO₂ 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). Furthermore, we were unable to define a break point when performing two-phase linear crossover 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, we were able to define a break point in ¹³C-label oxidation (F¹³CO₂), although we were not able to define a break point in the oxidation of the indicator amino acid. The latter is calculated from plasma [1-¹³C]lysine enrichments in addition to F¹³CO₂. We believe that 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 intracellular pool(s) from which amino acid oxidation takes place.

In the present study, we chose to use an orally administered lysine tracer to make the study minimally invasive and thereby suitable for studies in children (3). A consideration when an oral tracer is used is that it may be taken up and oxidized in the gut; indeed, it has been shown that 35–53% 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 (19) 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) do not affect the flux of the indicator (lysine) (see Table 2). This condition is necessary because 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 (40, 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.

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