Evidence That Phenylalanine May Not Provide the Full Needs for Aromatic Amino Acids in Children

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ABSTRACT: Phenylalanine is nutritionally classified as an indispensable amino acid and can be converted to tyrosine by phenylalanine hydroxylation. The initial goal of the present study was to determine the aromatic amino acid (phenylalanine plus tyrosine) requirements in healthy children fed a diet without tyrosine by using the indicator amino acid oxidation (IAAO) method using lysine as the indicator amino acid. Healthy school-age children ($n = 5$) were fed in random order a diet with eight graded intakes of phenylalanine without tyrosine. The requirement was determined by the rate of recovery of $^{13}CO_2$ from L-[1-^{13}C]lysine oxidation (F$^{13}CO_2$). Phenylalanine (total aromatic amino acid) requirement, in the absence of tyrosine, for children was determined to be 28 mg/kg/d, which was only 64% of the adult requirement, which is biologically absurd. A possible reason for the lower estimate of phenylalanine requirement could be lower phenylalanine hydroxylation rate in children, which is supported by the finding of lower urinary tyrosine/phenylalanine ratios in children compared with adults. In conclusion, this study indicates that phenylalanine may not provide the total needs for aromatic amino acids in children fed an amino acid–based diet without tyrosine. (*Pediatr Res* **61:** 361–365, 2007)

Phenylalanine, an indispensable amino acid (1), either incorporates into protein or is converted to tyrosine. In addition to protein synthesis, tyrosine is also a precursor for catecholamine synthesis and serves as a substrate for thyroid hormone and melanin synthesis. Phenylalanine hydroxylation is an important step for both eliminating excess phenylalanine and providing tyrosine in the body. Tyrosine is considered a conditionally indispensable amino acid in preterm infants (2–4).

The current dietary reference intake (DRI) recommendation for the estimated average requirement (EAR) for total aromatic amino acids (phenylalanine + tyrosine) for school-age children is 33 mg/kg/d, based on adult amino acid requirements plus an estimate of that required for growth (5). The EAR for total aromatic amino acids for the healthy adult was

estimated from the average of two very different estimates (15 and 39 mg/kg/d) in the current literature using stable isotope techniques. Therefore, both the adult EAR and the factorially calculated requirement for children are questionable. We have recently published studies, using lysine (6) and leucine (7) as indicator amino acids, and showed that adult aromatic amino acid requirements are 42–44 mg/kg/d. Furthermore, we have shown that the maintenance requirements for both the branched-chain amino acids and the sulfur amino acids are similar in children and adults (8,9). If this relationship is the same for aromatic amino acids, the requirement for children would be predicted to be in the range of 42–44 mg/kg/d.

The only data in the current literature regarding aromatic amino acid requirements determined by stable isotope technique in children were the requirements for children with classic phenylketonuria (PKU) (10,11). Phenylalanine and tyrosine requirements for children with classic PKU, determined by the IAAO technique, were estimated to be 14 and 19 mg/kg/d, respectively (10,11) with sum of 33 mg/kg/d. Clearly, these various estimates disagree substantially.

No data are currently available in healthy children for aromatic amino acid requirements using stable isotope techniques. The initial goal of this study was to estimate the total aromatic acid requirement in healthy children fed a diet without tyrosine, using the IAAO technique. The IAAO technique reflects the partitioning of a dietary essential amino acid (the indicator) between incorporation into protein (protein synthesis) and degradation (3). In an earlier study in adults, when dietary phenylalanine was held at 9.1 mg/kg/d, we were able to demonstrate a "requirement break-point for tyrosine" of 6 mg/kg/d and hence a total aromatic amino acid requirement of 15.1 mg/kg/d (5). This value is considerably less than our current estimates for total aromatic amino acid needs of 42–44 mg/kg/d (8,9), and we interpreted the difference as being due to the limited amount of phenylalanine (9.1 mg/kg/d) fed. Therefore, the IAAO method allows determination of the requirement of the test amino acid under the biologic and dietary conditions in force during the study. The current study was designed to determine total aromatic

Abbreviations: DRI, dietary reference intake; **EAR,** estimated average requirement; **F^{13}CO$_2$,** rate of $^{13}CO_2$ release from ^{13}C-lysine; **IAAO,** indicator amino acid oxidation; **LBM,** lean body mass; **PKU,** phenylketonuria; **RMR,** resting metabolic rate

amino acids in children, and we expected to find a value comparable with that determined in adults; as we have shown for the branched-chain amino acids and for the sulfur amino acids. However, when we found a biologically absurd value of 28 mg/kg/d, which was only 64% of the comparable value in adult of 42–44 mg/kg/d, we sought to find an explanation for this low value. Having considered all options, we came to the conclusion that the rate of phenylalanine hydroxylation in the children fed a tyrosine-free crystalline amino acid–based diet was limited so that protein synthesis was optimized at a level that was only 64% of that seen in adults. The clinical conclusion is that children receiving amino acid–based diets should always receive amino acid mixtures containing tyrosine.

SUBJECTS AND METHODS

Subjects. Five healthy children participated in the study on an outpatient basis in the Clinical Investigation Unit at The Hospital for Sick Children, Toronto, Ontario, Canada. Subject characteristics are described in Table 1. Subjects were excluded from the study if they had an inborn error of metabolism, a chronic disease, an endocrine disorder, unusual dietary practice, recent weight loss, pharmacologic therapy, or hormone treatment. The purpose of the study and the potential risks involved were explained in detail to each subject and their responsible caregivers, and written consent and assent were obtained from all participants and their responsible caregivers. All procedures used in the study were approved by the Research Ethics Board of The Hospital for Sick Children. All caregivers received financial compensation for transportation costs and time.

Experimental design. In the study, each subject received eight graded levels of phenylalanine intake (5, 10, 20, 30, 40, 50, 60, and 70 mg/kg/d) in random order on the study day. No tyrosine was supplied on the study day based on the assumption that phenylalanine can provide the entire requirement of tyrosine (12). Before the study, multiple skinfold thicknesses (triceps, biceps, subscapular, and suprailiac) were measured and bioelectrical impedance analysis was performed. Lean body mass (LBM) was calculated from reactance and resistance (13), measured by bioelectrical impedance analysis (BIA, model 101A; RJL Systems, Detroit, MI), and fat-free mass was calculated from density derived from the four-skinfold thickness (14), measured by a skinfold caliper (British Indicator, St. Albans, UK). Two days before each study day, subjects consumed a diet that provided adequate energy and protein intake at 1.5 g/kg/d. Menu plans were provided according to typical food consumed by the subjects. Food records were collected to ensure consistency of dietary intake before the study day. Subjects' weight and height were measured on each IAAO study day. During each IAAO study day, the experiment lasted for 8 h.

Table 1. *Subject characteristics of the healthy children participating in the study*

	Value*
Age, y	9.1 ± 1.4
Weight, kg	28.3 ± 5.1
Height, cm	134.1 ± 10.2
%IBW†	93.3 ± 13.7
Lean body mass‡ (kg)	23.7 ± 3.6
Fat free mass§ (kg)	22.8 ± 3.2
Resting metabolic rate¶ (MJ/d)	4.92 ± 0.55
Energy intake‖ (MJ/d)	8.36 ± 0.94

* Values are means ± SD (standard deviation), $n = 5$.

† IBW, ideal body weight, determined by actual body weight divided by matched weight (to actual height percentile) × 100.

‡ Determined from bioelectrical impedance analysis (13).

§ Calculated from multiple skinfold thickness measurements (14).

¶ Measured using continuous, computerized, open-circuit indirect calorimetry.

‖ Calculated from resting metabolic rate multiplied by an activity factor of 1.7.

Dietary intake and experimental diet. The experimental diet during each IAAO study provided energy (1.7 times resting metabolic rate) with 37% from fat, 52% from carbohydrate, and 10% from protein. During the course of the study, the energy was given at the level of multiplication of the resting metabolic rate (RMR) and an activity factor of 1.7 to ensure age-appropriate growth of the participating children. The subjects maintained their normal pattern of activity over the course of the study period. The RMR was determined after a 10- to 12-h overnight fast by continuous, open-circuit indirect calorimetry (2900 Computerized Energy Measurement System-Paramagnetic; Sensormedics, Yorba Linda, CA). Subjects consumed the isocaloric isonitrogenous hourly meals in a liquid formula (Protein-Free Powder, Product 80056, Mead Johnson, Evansville, IN; Tang and Koolaid, Kraft Foods, Toronto, Canada) with protein-free cookies, as previously described (15), for 8 h, and each meal represented 1/12 of the daily intake. The protein content was provided at a level of 1.5 g/kg/d, given as a crystalline L-amino acid mixture based on the amino acid profile of egg protein. The lysine intake level on the study day was fixed at a level of 64 mg/kg/d. Alanine was in various amounts to keep the meals isonitrogenous at different phenylalanine intakes.

Tracer protocol. The isotopic-labeled tracers used in the studies were $NaH^{13}CO_3$ with 99% enrichment and L-[1-^{13}C]lysine·2HCl with 99% enrichment (Cambridge Isotope Laboratories, Woburn, MA). Isotopic and optical purity of L-[1-^{13}C]lysine was verified by the manufacturer of the isotopes using chemical ionization gas chromatography–mass spectrometry and nuclear magnetic resonance. The enrichment of the L-[1-^{13}C]lysine tracer was reconfirmed by liquid chromatography mass spectrometry of the butanol derivative. The measured fractional molar abundance of L-[1-^{13}C]lysine was 99.5%. The oral tracer study started after a 4-h enteral intake adaptation period. A primed dose of $NaH^{13}CO_3$ (2.023 μmol/kg) and L-[1-^{13}C]lysine (16.305 μmol/kg) were given with the fifth meal. In addition, the L-[1-^{13}C]lysine isotope (8.153 μmol/kg) was given with hourly meals, starting with the fifth meal, for the next 4 h.

Sample collection. Breath and urine samples were collected on each IAAO study day. Three baseline breath samples were collected at 45, 30, and 15 min, and two urine samples were collected at 45 and 15 min before the tracer protocol began. Because isotopic plateau in CO_2 and urine are reached within 2 h after initiating isotope infusion (16), expired breath $^{13}CO_2$ and urine samples were collected every 30 min, beginning 2.5 h after the tracer protocol began. The same pattern also was seen in the present study, and the slope of the plateau enrichment in breath samples was not significantly different from zero. The coefficient of variation between the four plateau values of breath enrichment was 2% to 5%. Breath samples were collected in disposable Haldane-Priestley tubes (Venoject; Terumo Medical, Elkton, MD) using a collection mechanism that allowed the removal of dead-space air. The breath samples were stored at room temperature until ^{13}C enrichment analysis, and the urine samples were stored at −20°C before analysis.

Analytical procedures. Expired $^{13}CO_2$ enrichment was measured by a continuous-flow isotope ratio mass spectrometry (CF-IRMS20/20, PDZ Europa, Cheshire, UK) and was expressed as atom percent excess against a reference standard of compressed CO_2 gas.

Urinary L-[1-^{13}C]lysine enrichment was measured by a triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS SCIEX, Concord, ON, Canada) operated in positive ionization mode with TurbolonSpray ionization probe source (operated at 5800 V and 600°C), which was coupled with an Agilent 1100 HPLC system (Agilent Technologies Canada Inc., Mississauga, ON). The detailed procedure was described in a previous study (6).

Urinary free amino acid concentrations were determined by high-performance liquid chromatography (HPLC) analysis. The urine samples plus norleucine (as the internal standard), were extracted using a cation exchange column (Dowex 50 W-×8, 100–200 mesh H$^+$ form; Bio-Rad Laboratories, Hercules, CA). Urinary phenylalanine and tyrosine were derivatized with phenylisothiocyanate (adapted from PicoTag; Waters Corp., Milford, MA) (17–19), and their phenylisothiocyanate derivatives were separated and analyzed against a 18-amino-acid standard mix (Sigma Chemical Co., St. Louis, MO) by reversed-phase (C18, 2.9 mm × 300 mm Pico Tag column, Waters Corp.) column using HPLC (Dionex Summit HPLC System, Dionex, Sunnyvale, CA; operated under HPLC pump model P580A LPG and UV/VIS 170S). The areas under the peaks were integrated using the Chromeleon software (version 6.2; Dionex, Oakville, ON, Canada).

Statistical analysis. The mean requirements for total aromatic amino acids were determined using a two-phase linear regression crossover model. This model allows a partition of the data points between two separate regression lines that minimize the residual error. This results in a crossover value that is termed the breakpoint. This breakpoint estimates the amino acid requirement of a sample population. Estimates of the mean aromatic amino acid requirement were derived by breakpoint analysis of the rate of release of $^{13}CO_2$ ($F^{13}CO_2$) data using the mixed procedure of SAS (version 8.2, SAS Institute,
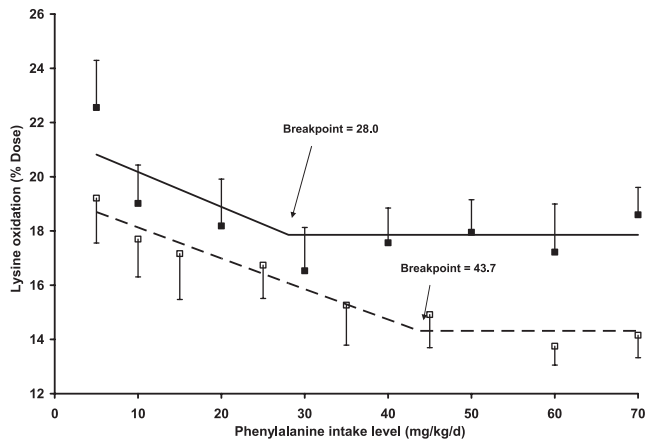
Figure 1. Effect of phenylalanine intake on lysine oxidation expressed as a percentage of dose in child (■) and adult (□) studies (6). Values are mean ± standard error of the mean (SEM) at all tested phenylalanine intake levels, $n = 5$. The intersection of two regression lines (breakpoint) represents the mean total aromatic amino acid requirement of 28.0 mg/kg/d for children and 43.7 mg/kg/d for adults (6). The breakpoint for children is significantly different from that for adults ($p < 0.0001$).
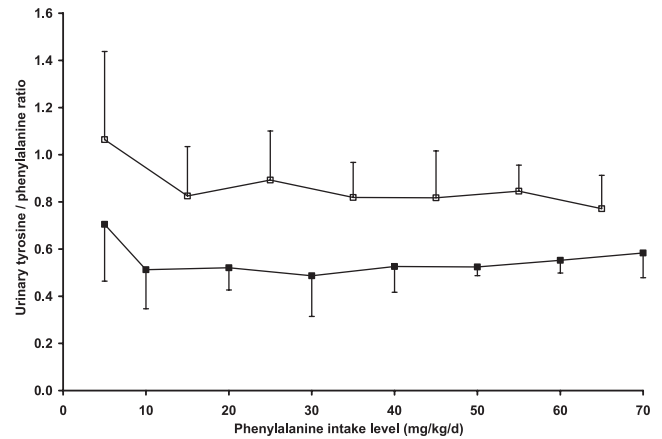

Figure 2. Effect of phenylalanine intake on urinary tyrosine/phenylalanine concentration ratio in child (■) and adult (□) studies. Values are mean ± SD at all tested phenylalanine intake levels, $n = 5$. Tyrosine/phenylalanine ratios of children in urine were significantly lower than those of adults ($p < 0.0001$).

Cary, NC) (20) as described previously (21) (subject as a random effect) (Fig. 1). PROC MIXED was used to determine the effects of phenylalanine intake level on $F^{13}CO_2$ (Table 2). Subject was included as a random effect in the model, and the p value was adjusted to the Tukey test for multiple comparisons. The statistical difference between the breakpoints of children and adults (6) was calculated *post hoc* using the comparison of a two-sample t procedure (22,23). The differences in the urinary tyrosine/phenylalanine concentration ratios between children and adults were analyzed by modeling urine as a function of intake and age group (adult *versus* child) using the mixed procedure of SAS. Group, group × intake, and group × intake (2) were included as fixed effects in the model, and the intercept was as a random effect. Differences were considered significant at $p < 0.05$.

RESULTS

Body composition measures (weight, height, percentage of IBW, fat-free mass, and LBM) of the five children did not change over the study period (Table 1). Mean enrichment of $^{13}CO_2$ from L-[1-^{13}C]lysine oxidation ($F^{13}CO_2$) was affected by phenylalanine intake levels ($p = 0.01$) (Table 2). Phenylalanine intake did not affect lysine flux (data not shown). The mean total aromatic amino acid requirement determined by the two-phase linear regression crossover model was estimated to be 28.0 mg/kg/d, which was significantly lower than the requirement for adults (44 mg/kg/d) (6,7) (Fig. 2; $p < 0.0001$). The reason for this lower estimate in children was not clear. Unfortunately, due to ethical constraints, we were not

able to collect blood samples from the children. However, we were able to compare urinary concentration for phenylalanine and tyrosine in our children with those derived from adults used in our previous experiment (7). Urinary tyrosine/ phenylalanine concentration ratios for children were significantly lower than those for adults (Fig. 2; $p < 0.0001$).

DISCUSSION

This is the first study using stable isotope techniques to estimate aromatic amino acid requirements in healthy children. The total aromatic amino acid requirement in this study was estimated to be 28 mg/kg/d. This value is only 64% of the recently estimated adult requirements (42–44 mg/kg/d) using either leucine or lysine as the indicator amino acid (6,7). The children used in this study had a lower percentage of body fat compared with the adults used in the previous study (6). However, when the mean aromatic amino acid requirement was expressed as per unit of LBM, the requirement for children (34 mg/kg LBM/d) was still 48% lower than that for adults (55 mg/kg LBM/d) ($p < 0.001$). When compared with our estimated total aromatic amino acid requirement of 33 mg/kg/d for PKU children (10,11), the present estimate is more similar, but still 15% lower. A lower requirement for aromatic amino acids for children compared with adults is biologically implausible.

Table 2. *Individual $F^{13}CO_2$ data at all phenylalanine intake levels for healthy children participating in the study*

	Phenylalanine intake, mg/kg/d							
Subject	5	10	20	30	40	50	60	70
				μmol/kg/h				
1	1.61	1.29	0.97	0.91	1.30	1.14	1.18	1.25
2	1.89	1.47	1.52	1.44	1.40	1.52	1.58	1.41
3	1.45	1.40	1.49	1.33	1.14	1.57	1.32	1.61
4	2.24	1.63	1.84	1.72	1.71	1.37	1.87	1.58
5	2.01	1.96	1.59	1.34	1.62	1.72	1.06	1.72
Mean ± SD*	1.84 ± 0.32†	1.55 ± 0.26†§	1.48 ± 0.32†§	1.35 ± 0.29‡§	1.43 ± 0.23‡§	1.46 ± 0.22†§	1.40 ± 0.32‡§	1.52 ± 0.18†§

* Mean with different superscript symbols are significantly different, $p < 0.05$. By analysis of variance, phenylalanine intake significantly affects $F^{13}CO_2$ ($p = 0.01$).

To date, our laboratory has published studies of the requirements in adults and children for the branched-chain amino acids (8,24) and for the total sulfur amino acids (9,25). In both cases, the estimate in children was the same as in adults, lending support to the concept that the maintenance component of essential amino acid requirements in children is the same as it is in adults. Hence, we were expecting the aromatic amino acid requirements in children to be similar to those in adults.

There are few possible explanations for the lower requirement estimate in children determined in the present study. The requirement for phenylalanine is the sum of the amount needed for protein synthesis plus the irreversible oxidative losses (26). The phenylalanine requirement in children with PKU represents only the amount of phenylalanine needed for protein synthesis. Because there are no data on obligatory oxidation for phenylalanine in children, we calculated the obligatory loss using the adult data (26%) (27), which was approximately 4 mg/kg/d. Tyrosine requirement for children with classic PKU was determined to be 19 mg/kg/d (10). Hence, the total aromatic amino acid (phenylalanine + tyrosine) requirements for healthy children were calculated to be approximately 37 mg/kg/d, which was the sum of phenylalanine requirement for children with classic PKU (14 mg/kg/d) (11) plus obligatory losses (4 mg/kg/d) plus 19 mg/kg/d for tyrosine (10). This calculated estimate (37 mg/kg/d) is comparable with the reported estimate for adults (42–44 mg/kg/d) (6,7).

Differences in the diets for children and adults do not explain the difference in estimated aromatic amino acid requirement. All the indispensable amino acids were provided above adequate intakes in all the studies except the test amino acids, phenylalanine and tyrosine. The only sources of tyrosine on the study day were either from phenylalanine hydroxylation or tyrosine released by protein breakdown. There were differences between the children's and adult's intakes of energy (RMR $\times$ 1.7 *versus* RMR $\times$ 1.5, respectively) and total protein (1.5 g/kg/d and 1 g/kg/d, respectively). Excess energy and protein intake are not believed to affect the estimation of aromatic amino acid requirements because the previously estimated branched-chain amino acid and total sulfur amino acid requirements for children and adults, by the IAAO technique, were comparable (8,9,24,25).

Lysine oxidation (as percentage of dose) was higher for children than for adults at all phenylalanine intake levels (Fig. 2). The baseline (plateau) lysine oxidation in children was higher by 24%. However, the higher baseline lysine oxidation cannot be ascribed to a higher total lysine intake (28) because these were not different. In addition, a difference in baseline does not affect the breakpoint estimate of requirement (23) unless a new amino acid becomes limiting after the phenylalanine requirement has been satisfied.

The tyrosine-to-phenylalanine concentration ratio in urine was measured to assess whether tyrosine could have become a limiting amino acid for protein synthesis, which changes inversely to indicator oxidation: the rates were lower in children than in adults (Fig. 2, $p < 0.001$). A lower ratio indicates a lower rate of phenylalanine hydroxylation in children, and

thus a lower rate of tyrosine synthesis. From this evidence, we suggest that tyrosine may have been limiting in the children in the current study.

Hydroxylation of phenylalanine to tyrosine is the first and rate-limiting step in phenylalanine catabolic pathway. It is an important step for both eliminating excess phenylalanine and providing tyrosine to the body tissues when tyrosine dietary intake is low. Phenylalanine hydroxylase activity increases with age in rats and reaches a maximum in the young adult at an age of 50–60 d (29). Unfortunately, the rate of phenylalanine hydroxylation in healthy children has not been reported.

Phenylalanine hydroxylase activities have been found in human fetal liver at an early stage of development (30). Phenylalanine hydroxylation has been suggested to be limiting in the preterm infant (2,4). In contrast, Kilani *et al.* (31) showed that neonates were capable of converting phenylalanine to tyrosine. The rate of phenylalanine hydroxylation in the fasting state in infants with mean gestation age of 28–30 wk (6.0 μmol/kg/h) (31) was similar to that in adults (5.8 μmol/kg/h) (32). The phenylalanine hydroxylation rates in neonates receiving a moderate parenteral phenylalanine intake ranged from 11 to 22 μmol/kg/h (33–35). When neonates received an increased parenteral phenylalanine intake, the rate of phenylalanine hydroxylation increased to greater than 35 μmol/kg/h (35–37), and alternate catabolites of phenylalanine and tyrosine were found in urine in neonates receiving the higher phenylalanine intakes (35). The rates need to be interpreted carefully because at high phenylalanine intakes, this tracer model of phenylalanine hydroxylation may overestimate actual hydroxylation rate (38). Because these neonates were capable of converting phenylalanine to tyrosine by phenylalanine hydroxylation, we must assume that children are capable of converting phenylalanine to tyrosine. Unfortunately, there are no data in the literature for hydroxylation rate in children receiving graded phenylalanine intakes.

In summary, the mean phenylalanine requirement, in the absence of tyrosine, for healthy children was determined to be 28.0 mg/kg/d, which was 64% of that for adults, which is biologically implausible. We explored various explanations, and the only one with any evidence to support it is that phenylalanine hydroxylation may be limiting in children fed a tyrosine-free diet. The lower urinary tyrosine/phenylalanine ratios in children at graded phenylalanine intakes implied that phenylalanine hydroxylation may be limited. In conclusion, this study indicates that phenylalanine alone may not provide the needs of aromatic amino acids in children fed a diet without tyrosine. Further investigation of effects of phenylalanine intakes on phenylalanine hydroxylation rates in children is needed. A clinical consequence of this study is that children receiving amino acid–based diets should always receive tyrosine containing amino acid mixtures.

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