Lysine requirement of the enterally fed term infant in the first month of life 1–3

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ABSTRACT

Background: Infant nutrition has a major impact on child growth and functional development. Low and high intakes of protein or amino acids could have a detrimental effect.

Objective: The objective of the study was to determine the lysine requirement of enterally fed term neonates by using the indicator amino acid oxidation (IAAO) method. L-[1-13C]phenylalanine was used as an indicator amino acid.

Design: Twenty-one neonates were randomly assigned to lysine intakes that ranged from 15 to 240 mg · kg⁻¹ · d⁻¹. Breath, urine, and blood samples were collected at baseline and during the plateau. The mean lysine requirement was determined by using biphasic linear regression crossover analysis on the fraction of 13CO2 recovery from L-[1-13C]phenylalanine oxidation (F13CO2) and phenylalanine oxidation rates calculated from the L-[1-13C]phenylalanine enrichment of urine and plasma.

Results: The mean (±SD) phenylalanine flux calculated from urine and plasma L-[1-13C]phenylalanine enrichment data were 88.3 ± 6.9 and 84.5 ± 7.4 μmol · kg⁻¹ · h⁻¹, respectively. Graded intakes of lysine had no effect on phenylalanine fluxes. The mean lysine requirement determined by F13CO2 was 130 mg · kg⁻¹ · d⁻¹ (upper and lower CIs: 183.7 and 76.3 mg · kg⁻¹ · d⁻¹, respectively). The mean requirement was identical to the requirement determined by using phenylalanine oxidation rates in urine and plasma.

Conclusions: The mean lysine requirement of enterally fed term neonates was determined by using F13CO2 and phenylalanine oxidation rates calculated from the L-[1-13C]phenylalanine enrichment of urine and plasma. These methods yielded a similar result of 130 mg lysine · kg⁻¹ · d⁻¹. This study demonstrates that sampling of 13CO2 in expired air is sufficient to estimate the lysine requirement by using the IAAO method in infants. This trial was registered at www.trialregister.nl as NTR1610.

INTRODUCTION

Lysine is an essential amino acid that is primarily used for protein synthesis (1). In addition, lysine, together with methionine, is required for the biosynthesis of carnitine, which is essential for fatty acid metabolism (2). Lysine is the first limiting amino acid in the all cereal–based diet consumed by a large proportion of the world’s population (3). A deficiency in the intake of lysine limits protein synthesis and causes weight loss in infants (4). In contrast, excess lysine intake also reduces the growth rate of animals caused by an imbalanced diet (5, 6). Thus, the dietary intake of amino acids is important for the rate of protein synthesis and growth.

Only a few studies have been performed in infants to determine enteral lysine requirements (4, 7). The criteria for the adequacy of a diet were the nitrogen balance and growth rates, which may not be the most sensitive methods. Thereby, the number of infants (n = 6–13) studied was relatively small. Because breast milk is considered to be the optimal nutrition for infants ≤6 mo of age, the joint WHO/FAO/United Nations University expert consultation (8) recommended a lysine intake of 119 mg · kg⁻¹ · d⁻¹ on the basis of the average intake of exclusively breastfed infants rather than on the available experimental evidence. Recently, the IAAO4 method has been developed to estimate essential amino acid requirements (9).

Our aim was to determine the lysine requirement of enterally fed neonates by using the IAAO method. Furthermore, we aimed to test whether requirement estimates on the basis of F13CO2 yielded similar results compared with the phenylalanine oxidation rates measured in urine and plasma. In addition, to shorten our study protocol, we compared the lysine requirement derived from F13CO2 data from a short-term (420-min) tracer infusion protocol with the results derived from a 900-min infusion protocol.

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4 Abbreviations used: APE, atom percent excess; F13CO2, fraction of 13CO2 recovery from L-[1-13C]phenylalanine oxidation; IAAO, indicator amino acid oxidation; MPE, mole percent excess.

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SUBJECTS AND METHODS

Subjects

Twenty-one neonates admitted to the Neonatal Ward in the Children’s Hospital of Fudan University participated in the study. Each subject was selected for study by using the following criteria: fully enterally fed infants with a gestational age of ≥37 wk, birth weight ≥2500 g, and clinically stable with a weight-gain rate ≥5 g · kg⁻¹ · d⁻¹ in the preceding 3 d. Subjects were excluded if they had congenital anomalies, gastro-intestinal pathology, or sepsis.

The study was approved by the Institutional Review Boards of the Children’s Hospital of Fudan University, and a statement of no objection was obtained from the Erasmus Medical Centre–Sophia Children’s Hospital. Written consent was obtained from at least one of the parents of each subject by a Chinese-speaking researcher.

Study formula

The study formula used was an elemental formula that was based on free amino acids. The composition was the same as Neocate (SHS International) except for the lysine and phenylalanine content. Lysine, which was completely withdrawn from the study formula, was separately added in the form of L-lysine to obtain different amounts of intake. The phenylalanine intake was kept constant during the study by separately adding L-phenylalanine during the 24-h adaptation period to obtain the same amount as in the Neocate (SHS International), and this amount of phenylalanine was given as stable isotope L-[1-13C] phenylalanine on the tracer infusion day. The phenylalanine intake during the study was 166 mg · kg⁻¹ · d⁻¹, which was above the recommended amount of 72 mg · kg⁻¹ · d⁻¹ (8). A generous amount of tyrosine (166 mg · kg⁻¹ · d⁻¹) was provided to ensure that the newly formed [1-13C]tyrosine hydroxylated from [1-13C]phenylalanine would be directly channeled to oxidation into 13CO₂, which can be measured in expired air (10).

This amount of tyrosine was almost twice the amount ingested by exclusively breastfed infants (8). The nitrogen intake was kept constant for all subjects by the substitution of L-alanine for the lysine that was withdrawn. The caloric intake was kept constant during the study period in all infants.

Experimental design

The study was designed to determine the lysine requirement of term neonates by using the minimally invasive IAAO method (9). The IAAO method is based on the concept that, when the test amino acid intake is insufficient to meet the requirement, protein synthesis will be limited and all of the amino acid will be oxidized, including the indicator amino acid, which is labeled with a stable isotope. As the dietary intake of the test amino acid increases, the oxidation rate of the indicator amino acid will decrease until the requirement of the test amino acid is met. Once the requirement of the test amino acid is met, an additional increase in its intake will have no further influence on the oxidation rate of the indicator amino acid. The oxidation of the indicator amino acid can be measured in expired air as 13CO₂. To use the IAAO method in infants and children, the study protocol must be noninvasive. Initially, the IAAO method was used in adults to determine amino acid requirements by measuring the amino acid kinetics in plasma and the rate of release of 13CO₂ from the oxidation of the indicator amino acid in expired air (11–13). Because a good correlation between [1-13C]phenylalanine enrichment in urine and plasma has been shown in adults (14, 15) and in neonates (16), the IAAO method has been used in vulnerable populations, such as parenterally fed neonates (17–19).

During the study, all infants received a fluid intake of ~150 mL · kg⁻¹ · d⁻¹, a calorific intake of 108 kcal · kg⁻¹ · d⁻¹, and an amino acid intake equal to the protein intake of ~2.96 g · kg⁻¹ · d⁻¹. Infants were randomly assigned to one of the graded test intakes of lysine, which ranged from 15 to 240 mg · kg⁻¹ · d⁻¹.

Each study took place over a 39-h period whereby the study formula was fed to neonates. After 24 h of study-formula consumption, tracers were administered on day 2 for 15 h. Infants were bottle fed every 3 h during the adaptation period. Subsequently, the feeding regimen changed to an hourly bottle feeding during the tracer infusion until the end of the study. On the tracer day, a nasogastric tube was placed for tracer infusion. Infants received a primed (14 μmol/kg) continuous (9 μmol · kg⁻¹ · h⁻¹) enteral infusion of [13C]bicarbonate (sterile, pyrogen free, 99% 13C APE; Cambridge Isotopes) for 3 h to quantify individual CO₂ production rates. Phenylalanine was used as the indicator amino acid. After the [13C]bicarbonate infusion was stopped, a primed (34 μmol/kg) continuous (27 μmol · kg⁻¹ · h⁻¹) enteral infusion of [1-13C]phenylalanine (99% 13C APE; Cambridge Isotopes) was started and lasted for 12 h. The duration of [1,13C]phenylalanine infusion was 12 h to ensure achievement of the steady state in urine and to ensure adequate urine-sample collection during the steady state. Syringes were weighted before and after the study to determine the exact amount of tracers that were given to the infants. The tracer infusion day is depicted in Figure 1.

Breath samples were obtained by using the direct nasopharyngeal sampling method described by van der Schoor et al (22). Briefly, a 6F gastric tube (6 CH Argyle; Sherwood Medical) was placed 1–1.5 cm into the nasopharynx, and the end-tidal breath was taken slowly with a syringe. Collected air was transferred into 12-mL sterile, nonsilicon-coated evacuated glass tubes (Vacutainer; Becton Dickinson). Briefly, a 6F gastric tube (6 CH Argyle; Sherwood Medical) was placed 1–1.5 cm into the nasopharynx, and the end-tidal breath was taken slowly with a syringe. Collected air was transferred into 12-mL sterile, nonsilicon-coated evacuated glass tubes (Van Loenen Instruments) and was stored at room temperature until analysis. Baseline breath samples were collected before the start of tracer infusion. Duplicated breath samples were obtained at

FIGURE 1. Schematic overview of tracer infusion day. Arrows indicate times that breath, urine, and plasma samples were taken.
15-min intervals during the period of 105–180 min after the tracer infusion, and duplicated samples were obtained at 10-min intervals during the period of 360–420 min (the first plateau period). Another set of duplicated samples were obtained at 10-min intervals during the last hour of L-[1-13C]phenylalanine infusion (the second plateau period). To validate the short-term study protocol, the requirement estimated during the first 13CO2 enrichment plateau was compared with the requirement estimated during the second plateau. The period of 360–420 min was chosen because the isotopic steady state of L-[1-13C]phenylalanine in expired air was obtained after 360 min of tracer infusion in our pilot study, which was 180 min after L-[1-13C]phenylalanine infusion.

Urine samples were collected by using urine bags. One urine sample (1 mL per sample) was collected at baseline, and 4–10 samples were collected depending on the void frequencies of the infants from 360 min onward until the end of the study. Urine samples were kept at −80°C until analysis.

Blood samples (0.5 mL per sample) were collected by venipuncture. One blood sample was taken at the baseline, and one blood sample was taken at the end of the study. Blood samples were collected in anticoagulant tubes and were immediately centrifuged; the plasma was stored at −80°C until analysis.

Analytic procedures

13CO2 isotopic enrichment in breath samples was analyzed by an infrared isotope analysis technique (Helifan; Analytic Fischer Instruments). The 13C enrichment was expressed as the APE above baseline.

Urine and plasma enrichment of L-[1-13C]phenylalanine were measured by gas chromatography–mass spectrometry (MSD 5975C Agilent GCMS; Agilent Technologies) as their ethyl chloroformate ester derivatives. Briefly, amino acid fractions in 50 μL urine and 30 μL plasma were isolated by a Dowex cation-exchange resin column (AG 50W-X8, hydrogen form; Bio-Rad Laboratories) and were eluted with 0.7 mL 6 M NH4OH. The eluate was evaporated under vacuum at room temperature in a speedvac (GeneVac miVac; GeneVac Ltd). Ethyl chloroformate derivatization of the samples was performed according to a modified procedure of Hušek (23). A CP-Chirasil L-Val GC column (25 m × 0.25 mm id, 0.12 μm film thickness; Varian) was used for the separation of d-[1-13C]phenylalanine and L-[1-13C]phenylalanine. An enrichment calibration curve was made for the measurement of L-[1-13C]phenylalanine in urine and plasma. Samples were measured by using a selected ion-monitoring mode method by using the mass fragments with an m/z of 176 for the unenriched (M) and an m/z 177 for the enriched (M + 1) L-phenylalanine. Each sample was analyzed in triplicate by using gas chromatography–mass spectrometry. Enrichments were calculated from the mean of the 3 analyses.

Isotopic enrichment was calculated at the isotopic steady state and was expressed as the MPE.

Calculations

The isotopic steady state was represented by plateaus in 13CO2 and L-[1-13C]phenylalanine enrichments in urine. The last plasma sample was considered to be at an isotopic plateau. Plateaus were determined by visual inspection and were confirmed by regression analysis as a slope not significantly different from zero.

Phenylalanine flux (Q) was measured from the dilution of the administered L-[1-13C]phenylalanine into the amino acid pool by using enrichments of L-[1-13C]phenylalanine in urine or plasma once the isotopic steady state was reached by using the following equation:

\[ Q_{\text{urine or plasma}} = \frac{\left( I_{\text{PHE}} - I_{\text{urine or plasma}} \right)}{\left[ I_{\text{PHE}} \right]} - 1 \]  

where \( I_{\text{PHE}} \) is the infusion rate of [1-13C]phenylalanine in μmol·kg⁻¹·h⁻¹, and \( I_{\text{urine or plasma}} \) is the isotopic enrichment of L-[1-13C]phenylalanine in urine or plasma, respectively.

The estimated body CO2 production rate (mmol·kg⁻¹·h⁻¹) was calculated as follows (20):

\[ \text{Body CO}_2 \text{ production} = \frac{\left( I_{\text{urine}} + I_{\text{urine}} - 1 \right) \times \text{iB}}{1000} \]  

where \( I_{\text{urine}} \) is the 13C enrichment of [1-13C]bicarbonate in the infusate (APE), \( I_{\text{urine}} \) is the 13C isotopic enrichment in expired air during [1-13C]bicarbonate infusion (APE), \( iB \) is the infusion rate of [1-13C]bicarbonate (μmol·kg⁻¹·h⁻¹). This equation does not correct for the retention of labeled carbon within the body bicarbonate pool and will overestimate the CO2 production rate. However, the same correction factor has to be applied to quantify the phenylalanine oxidation rate with the assumption of a constant CO2 production rate during the [1-13C]bicarbonate infusion and during the L-[1-13C]phenylalanine infusion (24). Consequently, this correction factor can be diminished in the following equation, and there is no need to measure the exact CO2 production rate.

The fraction of 13CO2 recovery from L-[1-13C]phenylalanine oxidation in percentage (F13CO2) was calculated by using the following equation (24):

\[ F_{\text{13CO}_2} = \frac{\left( I_{\text{PHE}} \times iB \right)}{\left( I_{\text{PHE}} \times I_{\text{B}} \right)} = \frac{1}{100} \]  

where \( I_{\text{PHE}} \) is the 13C isotopic enrichment in expired air during [1-13C]phenylalanine infusion (APE), \( iB \) is the infusion rate of [1-13C]bicarbonate (μmol·kg⁻¹·h⁻¹), and \( I_{\text{B}} \) is the 13C isotopic enrichment in expired air during [1-13C]bicarbonate infusion.

Whole-body phenylalanine oxidation by using urinary L-[1-13C]phenylalanine enrichment or plasma L-[1-13C]phenylalanine enrichment was calculated as follows:

\[ \text{Whole-body phenylalanine oxidation} = \left( F_{13\text{CO}_2} \times 100 \right) \times \text{Q}_{\text{urine or plasma}} \]  

Statistical analysis

Descriptive data are expressed as means ± SDs. The effect of lysine intake on phenylalanine was tested with Pearson’s correlation coefficient analysis. The difference in L-[1-13C]phenylalanine enrichment of urine during the isotopic plateau and plasma at 900 min was evaluated by a paired t test. Bland and Altman analysis (25) was used to assess the agreement of L-[1-13C]phenylalanine enrichment of urine during the isotopic...
plateau and plasma at 900 min. The determination of the mean lysine requirement (ie, the breakpoint) was performed by using a biphasic linear regression crossover model (26). With the biphasic linear regression analysis, the regression equation was split into 2 parts. For the first part, an intercept and slope were estimated, whereas for the second part, the slope was restricted to zero. Therefore, the estimated intercept of the second line was equal to the breakpoint. The model with the best fit on the basis of the highest $r^2$ was selected. The 95% CIs were calculated. The analyses were performed in STATA (version 11; StataCorp LP). $P < 0.05$ was considered significant.

RESULTS

Subject characteristics

Twenty-one term neonates participated in the study. The neonates were studied at a lysine intake that ranged between 15 and 240 mg·kg⁻¹·d⁻¹. Subject characteristics are summarized in Table 1. All subjects were growing well before entering the study. The mean (±SD) weight-gain rate 3 d before the study was 9 ± 4 g·kg⁻¹·d⁻¹. The mean (±SD) energy intake was 109.1 ± 0.8 kcal·kg⁻¹·d⁻¹. The nitrogen intake was equivalent to a protein intake of 2.99 ± 0.02 g·kg⁻¹·d⁻¹. The infants were clinically stable and considered healthy because they were discharged on the study day or the day after. The primary reasons for admissions were unconjugated hyperbilirubinemia ($n = 15$), pneumonia ($n = 3$), infection suspicion ($n = 2$), and skin infection ($n = 1$). Intravenous antibiotics (penicillins and/or cephalosporins) were given to 15 of the 21 neonates.

Phenylalanine kinetics

Complete data sets of breath and urine samples were obtained from all but one subject. We could not obtain the last blood sample from the one infant.

The mean (±SD) phenylalanine flux calculated from urinary enrichment and plasma enrichment was 88.3 ± 6.9 and 84.5 ± 7.4 μmol·kg⁻¹·h⁻¹, respectively. There were no significant correlations between urinary phenylalanine flux and lysine intake ($P = 0.73$) or plasma phenylalanine flux and lysine intake ($P = 0.53$). The $^{13}$C enrichments in expired air of the first and second plateaus during $L$-[1-$^{13}$C]phenylalanine infusion are shown in Figure 3. The breakpoints in $F^{13}$CO₂ data as analyzed by bi-phasic linear regression crossover analysis from $^{13}$CO₂ isotopic enrichment of the first plateau (the period from 360 to 420 min) and the second plateau (the period from 840 to 900 min), are shown in Figure 3, A and B, respectively. For the first and second $F^{13}$CO₂-plateau data, a negative correlation was shown between lysine intake (if the intake increased to 130 mg·kg⁻¹·d⁻¹) and $F^{13}$CO₂; additional increases in lysine intake did not affect $F^{13}$CO₂. The breakpoint represented the mean lysine requirement, which was 130 mg·kg⁻¹·d⁻¹, with 95% upper and lower CIs of 183.7 and 76.3 mg·kg⁻¹·d⁻¹, respectively, for the first plateau ($P < 0.0001$; $r^2 = 0.46$). The breakpoint of the second plateau was also 130 mg·kg⁻¹·d⁻¹, with 95% upper and lower CIs of 183.7 and 76.3 mg·kg⁻¹·d⁻¹, respectively ($P < 0.0001$; $r^2 = 0.51$).

As illustrated in Figure 4, the urinary $L$-[1-$^{13}$C]phenylalanine enrichment was significantly different from the plasma $L$-[1-$^{13}$C]phenylalanine enrichment ($P = 0.04$, 2-tailed). From the Bland and Altman analysis (25), the mean (upper and lower 95% CIs) difference between urine and plasma enrichments was −0.72 (2.06, −3.51) MPE. There was a 5% probability that the measured enrichment by using urine and plasma differed more than this amount (Figure 5). Phenylalanine oxidation calculated from the urine and plasma enrichment data also decreased with increasing lysine intake to 130 mg·kg⁻¹·d⁻¹; an additional increase of lysine intake >130 mg·kg⁻¹·d⁻¹ did not result in an additional decrease of phenylalanine oxidation. The breakpoints in the urinary and plasma phenylalanine oxidation data are shown in Figure 6, A and B, respectively. Identical to the breakpoint determined by using $F^{13}$CO₂, the breakpoint determined by using phenylalanine oxidation rates in urine and plasma was 130 mg·kg⁻¹·d⁻¹ ($P < 0.0001$; $r^2 = 0.5$; and $P < 0.0001$, $r^2 = 0.49$, respectively). The 95% upper and lower CIs for urine were 183.2 and 76.8 mg·kg⁻¹·d⁻¹, respectively. The upper CI for plasma was 185.6 mg·kg⁻¹·d⁻¹, and the lower CI was 74.4 mg·kg⁻¹·d⁻¹.

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<tr>
<th>Subject characteristics of infants who participated in the study ($n = 21$)</th>
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<tr>
<td>Birth weight (kg)</td>
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<td>Gestational age (wk)</td>
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<td>Age on study day (d)</td>
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<td>Weight on study day (kg)</td>
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<td>Weight gain before study (g·kg⁻¹·d⁻¹)</td>
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<td>Sex (F:M)</td>
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$^1$ Mean ± SD (all such values).

DISCUSSION

To our knowledge, this was the first study of the lysine requirement of fully enterally fed term neonates that used the IAAO method. The mean lysine requirement of enterally fed term neonates was estimated to be 130 mg·kg⁻¹·d⁻¹.

The experimental evidence for the lysine requirements of infants is very scarce. With the use of nitrogen balance and
weight gain, Holt and Snyderman (27) estimated lysine requirements of 6 infants of postnatal ages between 1 and 5 mo to be 90–105 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\). The difference in estimated requirements with our study might have been due to the ages of the infants studied, the small number of infants studied, and the use of the nitrogen-balance method, which may have underestimated the requirement. Fomon et al (7) observed adequate growth in 13 normal full-term female infants during the ages of 8 to 41 d with an average lysine intake of 114 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\), which was in the same range as our estimates. The infants in the study of Fomon et al (7) were fed ad libitum, which meant that the infants could regulate their own intakes, which resulted in a wide range of observed intakes.

Recently, Chapman et al (17) estimated the lysine requirement of parenterally fed postsurgical neonates by using the IAAO method to be 104.9 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\). Because the use of dietary essential amino acids by the intestine results in a lower systemic availability of these essential amino acids (28, 29), a higher amino acid requirement can be expected in fully enterally fed neonates. The first-pass lysine uptake in preterm infants with full enteral feeding was 18% (29). In our results, a requirement of 130 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\) fit perfectly in the parenteral requirement determined by Chapman et al (17) at a first-pass use of 20%.

The current recommended lysine intake is based on the human-milk composition (8). Human milk has huge variations in protein concentrations; the protein content declines from 23 g/L on the postpartum day to 3–14 g/L on day 28 (30, 31). This decline in protein content is accompanied by changes in the whey:casein ratio (32); consequently, the amino acid composition changes during the lactation period. However, the average lysine intake estimated in exclusively breastfed infants in the first month of life is 119 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\) (8), which is comparable with our estimated requirement. The gross amino acid composition of human milk may not necessarily reflect the requirement profile of infants who consume infant formula because protein and amino acid digestibility and bioavailability are different in human milk from that in formula. Our study provided scientific knowledge of the amino acid need of infants fed an infant formula.

![FIGURE 3](https://example.com/fig3.png)

**FIGURE 3.** A: \(\text{F}^{13}\text{CO}_2\) during the first isotopic plateau (period: 360–420 min) at different lysine intakes (\(n = 21\)). With the use of a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\) \((P < 0.0001, r^2 = 0.46)\). Upper and lower 95% CIs of the breakpoint estimate were 188.4 and 71.6 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\), respectively. B: \(\text{F}^{13}\text{CO}_2\) during the second isotopic plateau (period: 840–900 min) at different lysine intakes (\(n = 21\)). With the use of a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\) \((P < 0.0001, r^2 = 0.51)\). Upper and lower 95% CIs of the breakpoint estimate were 183.7 and 76.3 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\), respectively. \(\text{F}^{13}\text{CO}_2\), fraction of \(\text{^{13}CO}_2\) recovery from \(\text{L-[1-^{13}C]phenylalanine oxidation}\).
Raffii et al (33) showed that the change in phenylalanine hydroxylation, which is the first step in phenylalanine oxidation, was better represented by apolipoprotein B-100 instead of plasma phenylalanine. However, the requirement derived from F\(^{13}\)CO\(_2\) data in our study was identical to the requirement estimated from the urine and plasma L-[\(\text{\textsuperscript{13}C}\)]phenylalanine enrichment data. The reason for the same estimates might have been due to the contamination of D-[\(\text{\textsuperscript{13}C}\)]phenylalanine in the tracer. A recent study showed a significant confounding effect of D-phenylalanine in urine even when [\(\text{\textsuperscript{13}C}\)]phenylalanine was used with an optical purity of 0.1% in neonates (34). We used a chiral column for the separation of the D- and L-phenylalanine to overcome this problem.

There were some limitations in our study design. The study was performed by using an amino acid formula. Metges et al (35) have shown that leucine oxidation is higher and non-oxidative leucine disposal is lower when an amino acid diet is used compared with when a casein diet is used. These results suggest that leucine derived from an amino acid diet has a lower rate of use. Their findings were supported by the study of Dangin et al (36), which demonstrated that the protein digestion rate of use is an independent factor of protein retention. The effect of the decreased rate of use of amino acids by consuming an amino acid diet could result in higher requirement estimates compared with consumption of a protein diet. Therefore, our determined lysine requirement could have been an overestimation. Future studies with an intrinsically labeled protein that is the closest simulation to a normal dietary amino acid intake are required to evaluate this issue.

Another limitation of our study was the antibiotic used in our study population. Antibiotics are extensively prescribed to children who are admitted to children’s hospitals in China (37). As a result of this practice, 15 of 21 infants in our study received intravenous antibiotics. Antibiotic treatment has a major impact on the bacterial flora in the gastrointestinal tract (38), and it has been shown that microbial lysine can be made available to the human host (39, 40). Previous studies did not clarify the issue of whether microbial lysine contributes to the dietary amino acid–requirement estimates (41). To our knowledge, there are no data in the literature on antibiotic use and its effect on essential amino acid requirements.

In conclusion, this study was the first in a series of studies designed to determine the essential amino acid requirements of enterally fed neonates by using the adapted minimal invasive IAAO method. Under the conditions of this study, the lysine protocol. Both protocols yielded identical requirement estimates. Therefore, we concluded that a short (and, thus, less invasive) IAAO protocol is valid for enterally fed infants.

We showed a small but significant difference of L-[\(\text{\textsuperscript{13}C}\)]phenylalanine enrichment in urine compared with in plasma. Amino acid enrichment in urine is assumed to reflect the enrichment in arterialized blood. The difference might be because urine samples represent average enrichment values during the collection period, whereas plasma samples represent enrichment at a specific time and site of sampling. In our study, urine samples were collected in the period before the collection of the venous blood sample from the hand or foot. Another explanation might be that isotopic steady state had not yet been reached in the urine of neonates who had relative long voiding intervals, which resulted in few urine samples at steady state. The lower urinary L-[\(\text{\textsuperscript{13}C}\)]phenylalanine enrichment compared with in plasma was also shown in the studies by Zello et al (15) and Bross et al (14) in adults. A possible explanation is the short tracer-infusion time (4 h), which resulted in nonsteady states. The lack of significance in the study by Bross et al (14) was possibly the consequence of a small number of subjects (\(n = 4\)). Wykes et al (16) observed a higher enrichment in urine compared with in plasma. This observation might have been due to the contamination of D-[\(\text{\textsuperscript{13}C}\)]phenylalanine in the tracer. A recent study showed a significant confounding effect of D-phenylalanine in urine even when [\(\text{\textsuperscript{13}C}\)]phenylalanine was used with an optical purity of 0.1% in neonates (34). We used a chiral column for the separation of the D- and L-phenylalanine to overcome this problem.
requirement of enteraly fed term neonates was 130 mg · kg\(^{-1}\) · d\(^{-1}\). Current term formulas provide an excess of lysine (172–256 mg · kg\(^{-1}\) · d\(^{-1}\) ) according to our estimated mean requirement (42, 43). The lack of knowledge with regard to the optimal amino acid pattern in formula feeding is a reason to perform additional studies on the amino acid requirements of enteraly fed infants to optimize the nutrition for (preterm) infants.

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