Protein requirement of healthy school-age children determined by the indicator amino acid oxidation method

Rajavel Elango, Mohammad A Humayun, Ronald O Ball, and Paul B Pencharz

ABSTRACT

Background: The current Dietary Reference Intake (DRI) recommendations for protein requirements in children are based on a factorial estimate and have not been directly determined.

Objective: The objective of the current study was to determine the protein requirement in healthy, school-age children by measuring the oxidation of L-[1-13C]-phenylalanine to 13CO2 [label tracer oxidation (F13CO2)] in response to graded intakes of protein.

Design: Seven healthy children (6–11 y old) each randomly received a minimum of 7 protein intakes (range: 0.1–2.56 g · kg⁻¹ · d⁻¹) for a total of 56 studies. The diets provided energy at 1.7 times the resting energy expenditure and were made isocaloric by using carbohydrate. Protein was given as an amino acid mixture on the basis of the egg-protein pattern, except for phenylalanine and tyrosine intakes, which were maintained constant across intakes. The mean protein requirement was determined by applying a 2-phase linear regression crossover analysis on F13CO2 data, which identified a breakpoint (requirement) at minimal F13CO2 in response to graded amounts of protein intake.

Results: Mean and population-safe (upper 95% CI) protein requirements were determined to be 1.3 and 1.55 g · kg⁻¹ · d⁻¹, respectively. These results are significantly higher than the mean and population-safe protein requirements currently recommended by the DRI 2005 for macronutrients (0.76 and 0.95 g · kg⁻¹ · d⁻¹, respectively). These results are significantly higher than the mean and population-safe protein requirements currently recommended by the DRI 2005 for macronutrients (0.76 and 0.95 g · kg⁻¹ · d⁻¹, respectively). The IAAO-determined values were 41% and 50% higher than current DRI recommendations of 0.66 and 0.80 g protein · kg⁻¹ · d⁻¹, respectively, in adults (11). On the basis of adult IAAO protein-requirement data, we hypothesized that the current DRI recommendations for young children are underestimated. The IAAO method has previously been successfully applied to identify amino acid requirements in adult humans by us (12–15) and others (16, 17) and in children (18–22) and neonates (23–25). The minimally invasive IAAO method has several advantages (26–28) in the determination of requirements for amino acids and protein in vulnerable populations, such as in children because the isotope is delivered orally (13), enrichment of 13C is measured in breath and urine.

INTRODUCTION

Current recommendations for protein requirements in children (6–10 y old) are based on the recent DRIs published in 2005 (1). The mean (EAR) and population-safe (RDA) recommendations for good-quality protein were set at 0.76 and 0.95 g · kg⁻¹ · d⁻¹, respectively. The more-recent FAO 2007 recommendations (2) were also similar. These recommendations were derived by using a factorial calculation in which the mean requirement was maintenance needs plus an additional component for growth, which was estimated from the rate of protein deposition and efficiency of protein use (3). Maintenance needs were based on adult protein requirements derived from nitrogen-balance studies. Nitrogen balance has various methodologic flaws, including the overestimation of nitrogen intakes, underestimation of nitrogen excretion, and, hence, an overall underestimation of nitrogen balance, and their limitations are well documented (4–8). Furthermore, nitrogen-balance studies require a minimum 7-d test-diet adaptation for the dietary change to be reflected in urinary nitrogen excretion (9). Because of ethical reasons, such prolonged periods of adaptation to deficient protein intakes are not possible in young children. Hence, there is a need to develop a valid and minimally invasive technique to directly determine protein requirements in children.

Ball and Bayley (10) developed a novel method to determine protein requirements in young pigs by using the IAAO method. We applied the IAAO method to determine total protein requirements in adult humans (11). The mean and population-safe protein requirements were determined to be 0.93 and 1.2 g protein · kg⁻¹ · d⁻¹, respectively. The IAAO-determined values were 41% and 50% higher than current DRI recommendations of 0.66 and 0.80 g protein · kg⁻¹ · d⁻¹, respectively, in adults (11). On the basis of adult IAAO protein-requirement data, we hypothesized that the current DRI recommendations for young children are underestimated. The IAAO method has previously been successfully applied to identify amino acid requirements in adult humans by us (12–15) and others (16, 17) and in children (18–22) and neonates (23–25). The minimally invasive IAAO method has several advantages (26–28) in the determination of requirements for amino acids and protein in vulnerable populations, such as in children because the isotope is delivered orally (13), enrichment of 13C is measured in breath and urine.

(29, 30), and the length of test intake adaptation is 8 h (31). In addition, requirements for protein and amino acid intakes in growing pigs determined by using IAAO were not different from those determined by growth experiments (10, 32), which thus validated the approach as an accurate method for the determination of requirements in children. The objective in the current study was to determine the total protein requirement in school-age children (6–10 y old) by using the IAAO method.

SUBJECTS AND METHODS

Subjects

Seven healthy school-age children (5 boys and 2 girls) participated in the study beginning in May 2007 at the Clinical Investigation Unit, The Hospital for Sick Children, Toronto, Canada. Subject characteristics, body composition, and energy intakes are summarized in Table 1. The children who participated in the study did not have any recent history of weight loss or illness, and none of the children used any medication during the study period. Informed written consent was obtained from the parent or guardian, and the assent of participating children was also obtained. The parent or guardian of each participating child received financial compensation for costs incurred by participating in the studies. All procedures in the study were approved by the Research Ethics Board at the Hospital for Sick Children.

Experimental design

The experimental design was based on the minimally invasive IAAO model developed in adults to determine protein requirements (11). Before the studies commenced, all children were fasted overnight (~12 h) and were brought to the Clinical Investigation Unit for body composition analysis and REE measurements. Body composition was measured by bioelectrical impedance analysis (BIA model 101A; RJL Systems). REE was measured by continuous, open-circuit indirect calorimetry (Vmax Encore, Metabolic cart; VIASYS). Two days before the study day, the children were prescribed a standardized diet on the basis of 3-d food records and provided energy at 1.7 times the REE and 1.5 g protein · kg⁻¹ · d⁻¹. Menu plans were provided that consisted of typical foods consumed by the children, and 2-d food records were collected before each study to ensure consistency of dietary intakes. On the study day, after a 12-h fast, the children were randomly assigned to receive a test protein intake (range: 0.1–2.56 g · kg⁻¹ · d⁻¹). Multiple test intakes that ranged from deficient to excess provided the best estimate of the slope of the decrease in IAAO with the increase in the test protein and amino acid intakes, as shown recently in our neonatal studies of amino acid requirements (23–25). All subjects participated in a minimum of 7 test intakes, which included ≥3 intakes ≤0.9 g protein · kg⁻¹ · d⁻¹ and 3 intakes ≥0.9 g protein · kg⁻¹ · d⁻¹ for a total of 56 studies. Each subject’s study day was separated by ≥1 wk.

Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>8.4 ± 1.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>31.9 ± 11.4</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>132.6 ± 19.8</td>
</tr>
<tr>
<td>Percentage IBW (%)</td>
<td>98.5 ± 11</td>
</tr>
<tr>
<td>LBM (kg)</td>
<td>24.5 ± 6.6</td>
</tr>
<tr>
<td>REE (kcal/d)²</td>
<td>1147 ± 289</td>
</tr>
<tr>
<td>Energy intake (kcal/d)²</td>
<td>1950 ± 492</td>
</tr>
</tbody>
</table>

1 All values are means ± SDs. IBW, ideal body weight; LBM, lean body mass; REE, resting energy expenditure.
2 Determined by bioelectrical impedance analysis.
3 Determined by open-circuit indirect calorimetry.

Study diets

The study-day diet was consumed as 8 hourly isocaloric meals, with each meal representing one-twelfth of the daily energy requirement. The experimental diet consisted of a protein-free liquid formula made with protein-free powder (PFD1; Mead Johnson), flavored drink crystals (Tang and Kool-Aid; Kraft Foods), corn oil, and protein-free cookies. Energy was provided at 1.7 times the REE on the basis of each subject’s measured REE after a 12-h fast, as previously described. Protein was given as a crystalline l-amino acid mixture on the basis of the egg-protein pattern, except for phenylalanine and tyrosine intakes, which were maintained constant across intakes. Amino acid compositions of selected test protein intakes are provided in Table 2. The carbohydrate intake was adjusted with varying protein intakes to maintain an isocaloric diet. The diet provided 33% of the energy as fat, 48–66% of the energy as carbohydrates, and 1–19% of the energy as protein. Subjects also consumed a daily multivitamin supplement (Centrum Junior; Whitehall-Robins) for the duration of all studies. During the study in the Clinical Investigation Unit, the children were not allowed to consume anything else except water.

Tracer protocol

On each study day, subjects consumed 4 hourly meals before the oral tracer infusion protocol. A priming dose of 0.176 mg NaH¹³CO₃/kg (99 atom percent excess; Cambridge Isotope Laboratories, Woburn, MA) and 3.048 mg/kg of L-[¹³C]phenylalanine (99 atom percent excess; Cambridge Isotope Laboratories) was given at the fifth meal. Hourly doses of L-[¹³C] phenylalanine (1.958 mg · kg⁻¹ · h⁻¹) were given with subsequent meals until the end of the study. The quantity of phenylalanine supplied as l-[¹³C]phenylalanine during the last 4 h of the study was subtracted from the diet to provide a total intake of 30.5 mg phenylalanine · kg⁻¹ · d⁻¹. Tyrosine was provided at 61 mg · kg⁻¹ · d⁻¹ to ensure an excess of tyrosine. Metabolic channeling of phenylalanine metabolism occurs in hepatocytes, such that the intracellular partitioning of tyrosine is determined by the tyrosine concentration. Specifically, if extracellular tyrosine is low and phenylalanine is at a normal plasma concentration, tyrosine use in protein synthesis takes precedence over tyrosine degradation or export (33). Therefore, tyrosine should be present in relative excess to sensitively partition the carboxyl carbon of phenylalanine between the incorporation into protein or oxidation. The amounts of phenylalanine and tyrosine in the current study were comparable to earlier childhood amino acid IAAO–requirement studies (18–22) and were shown to
TABLE 2
Amino acid composition of reference protein and selected test protein intakes

<table>
<thead>
<tr>
<th>Reference protein¹</th>
<th>0.1 g protein/kg</th>
<th>0.52 g protein/kg</th>
<th>0.9 g protein/kg</th>
<th>1.3 g protein/kg</th>
<th>1.72 g protein/kg</th>
<th>2.1 g protein/kg</th>
<th>2.5 g protein/kg</th>
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</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>61.5</td>
<td>6.1</td>
<td>32.0</td>
<td>55.3</td>
<td>79.9</td>
<td>105.7</td>
<td>129.1</td>
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<tr>
<td>L-Arginine–HCl²</td>
<td>75.1</td>
<td>7.5</td>
<td>39.1</td>
<td>67.6</td>
<td>97.6</td>
<td>129.2</td>
<td>157.7</td>
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<tr>
<td>L-Asparagine</td>
<td>33.3</td>
<td>3.3</td>
<td>17.3</td>
<td>29.9</td>
<td>43.2</td>
<td>57.2</td>
<td>69.8</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>33.3</td>
<td>3.3</td>
<td>17.3</td>
<td>29.9</td>
<td>43.2</td>
<td>57.2</td>
<td>69.8</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>22.1</td>
<td>2.2</td>
<td>11.5</td>
<td>19.9</td>
<td>28.7</td>
<td>38.0</td>
<td>46.4</td>
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<tr>
<td>L-Glutamine</td>
<td>56.6</td>
<td>5.7</td>
<td>29.4</td>
<td>51.0</td>
<td>73.6</td>
<td>97.4</td>
<td>118.9</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>56.6</td>
<td>5.7</td>
<td>29.4</td>
<td>51.0</td>
<td>73.6</td>
<td>97.4</td>
<td>118.9</td>
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<tr>
<td>L-Glycine</td>
<td>33.3</td>
<td>3.3</td>
<td>17.3</td>
<td>29.9</td>
<td>43.2</td>
<td>57.2</td>
<td>69.8</td>
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<td>L-Histidine</td>
<td>22.7</td>
<td>2.3</td>
<td>11.8</td>
<td>20.4</td>
<td>29.5</td>
<td>39.0</td>
<td>47.7</td>
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<td>L-Isoleucine</td>
<td>62.8</td>
<td>6.3</td>
<td>32.7</td>
<td>56.5</td>
<td>81.7</td>
<td>108.1</td>
<td>131.9</td>
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<td>L-Leucine</td>
<td>83.3</td>
<td>8.3</td>
<td>43.3</td>
<td>74.9</td>
<td>108.3</td>
<td>143.2</td>
<td>174.9</td>
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<tr>
<td>L-Lysine–HCl³</td>
<td>75.7</td>
<td>7.6</td>
<td>39.4</td>
<td>68.1</td>
<td>98.4</td>
<td>130.2</td>
<td>159.0</td>
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<tr>
<td>L-Methionine</td>
<td>29.6</td>
<td>3.0</td>
<td>15.4</td>
<td>26.7</td>
<td>38.5</td>
<td>51.0</td>
<td>62.2</td>
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<tr>
<td>L-Phenylalanine⁴</td>
<td>54.7</td>
<td>30.5</td>
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<tr>
<td>L-Proline</td>
<td>41.9</td>
<td>4.2</td>
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<td>37.7</td>
<td>54.5</td>
<td>72.1</td>
<td>88.1</td>
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<td>L-Serine</td>
<td>83.9</td>
<td>8.4</td>
<td>43.6</td>
<td>75.5</td>
<td>109.0</td>
<td>144.3</td>
<td>176.1</td>
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<tr>
<td>L-Threonine</td>
<td>47.1</td>
<td>4.7</td>
<td>24.5</td>
<td>42.4</td>
<td>61.2</td>
<td>81.0</td>
<td>98.9</td>
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<td>L-Tryptophan</td>
<td>15.6</td>
<td>1.6</td>
<td>8.1</td>
<td>14.0</td>
<td>20.3</td>
<td>26.8</td>
<td>32.8</td>
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<tr>
<td>L-Tyrosine</td>
<td>40.7</td>
<td>6.10</td>
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<td>61.0</td>
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<td>L-Valine</td>
<td>70.3</td>
<td>7.0</td>
<td>36.5</td>
<td>63.2</td>
<td>91.3</td>
<td>120.8</td>
<td>147.5</td>
</tr>
</tbody>
</table>

¹ Selected test protein intakes and their amino acid compositions. Subjects each received an individual protein intake test amount that ranged from 0.1 to 2.5 g protein · kg⁻¹ · d⁻¹.
² Represents the egg-protein composition.
³ Actual amounts of amino acids were as follows: 62.1 mg arginine/g and 60.6 mg lysine/g.
⁴ L-Phenylalanine intake was kept constant at 30.5 mg · kg⁻¹ · d⁻¹.
⁵ L-Tyrosine intake was kept constant at 60.1 mg · kg⁻¹ · d⁻¹.

Sample collection and analysis

Breath and urine samples were collected on all study days. Previously, during the development of the minimally invasive IAAO model, Bross et al (29) showed that at isotopic steady state urinary and plasma enrichments of L-[1-¹³C]phenylalanine are similar. Three baseline breath samples were collected 45, 30, and 15 min, and 2 baseline urine samples were collected 45 and 15 min before the tracer protocol began. Four plateau breath and urine samples were collected at isotopic steady state every 30 min beginning 2.5 h after start of the tracer protocol. CVs between the 4 plateau values of enrichment were <5% and between 5% and 8% in breath and urine enrichments, respectively. Breath samples were collected in disposable Exetainer tubes (Labco Ltd) with a collection mechanism that permitted the removal of dead-space air. Breath samples were stored at room temperature, and urine samples were stored at −20°C until analyzed. During each study day, the rate of carbon dioxide production (VCO₂) was measured immediately after the fifth meal for a period of 20 min with an indirect calorimeter (Vmax Encore, Metabolic cart; VIASYS).

Expired ¹³CO₂ enrichment was measured with a continuous-flow isotope-ratio mass spectrometer (CF-IRMS20/20 isotope analyzer; PDZ Europa Ltd). Enrichments were expressed as atom percent excess compared with a reference standard of compressed CO₂ gas. Urinary L-[1-¹³C]phenylalanine enrichment was analyzed by an API 4000 triple quadrupole mass spectrometer (Applied Biosystems-MDS Sciex) that was operated in positive electrospray ionization mode. This was coupled to an Agilent 1100 HPLC system (Agilent Technologies Canada Inc). All aspects of the system operation and data acquisition were controlled with Analyst NT software (version 1.4.1; Applied Biosystems). Briefly, a 62.5-μL urine aliquot was deproteinized with 200 μL methanol and centrifuged at 7000 × g for 5 min. The supernatant fluid was freeze-dried and reconstituted in 1 mL 0.1% formic acid. Chiral separations were performed with a Chirobiotic T (teicoplanin) HPLC column (25 cm × 4.6 mm, 5 μm; Astec) by using an isocratic gradient with 95.5 (10 mmol/L NH₄Ac; pH = 4.1):(2:1 MeOH:0.1% formic acid in acetonitrile) buffer at 700 μL/min (34). Selected ion chromatograms were obtained by monitoring the m/z product ions of 165 and 166 for [1- ¹³C]phenylalanine, which corresponded to the unenriched (M) and enriched (M+1) peaks, respectively. The areas under the peaks were integrated by Analyst NT software (version 1.4.1; Applied Biosystems). Iso- topic enrichment was expressed as molar percent excess and calculated from peak area ratios at isotopic steady state at baseline and the plateau.

Estimation of isotope kinetics

The whole-body phenylalanine flux was calculated from the dilution of L-[1-¹³C]phenylalanine in the body amino acid pool at isotopic steady state by using the following equation (13, 35):

\[
\text{Flux} = \frac{\text{dilution} \times \text{pool size}}{\text{dilution time}}
\]

where dilution is the fractional increase in concentration, and pool size is the weight of the body.
where $Q$ is phenylalanine flux (µmol · kg$^{-1}$ · h$^{-1}$); $i$ is the rate of $l$-[1$^{13}$C]phenylalanine infused (µmol · kg$^{-1}$ · h$^{-1}$), and $E_i$ and $E_u$ are the isotopic enrichments as mole fractions (mole percent excess) of the infused and urinary phenylalanine, respectively, at isotopic plateau.

The rate of phenylalanine oxidation was calculated as follows:

$$O = F^{13}CO_2(1/E_u - 1/E_i) \times 100$$

where $O$ represents phenylalanine oxidation (µmol · kg$^{-1}$ · h$^{-1}$), and $F^{13}CO_2$ represents the rate of $^{13}$CO$_2$ released by phenylalanine tracer oxidation (µmol · kg$^{-1}$ · h$^{-1}$) calculated by the following equation:

$$F^{13}CO_2 = (FCO_2)/ECO_2(44.6)/(W)(0.82)(100)$$

where $FCO_2$ is the CO$_2$ production rate (mL/min), $ECO_2$ is the $^{13}$CO$_2$ enrichment in expired breath at isotopic steady state (atom percent excess); $W$ is the weight (kg) of the subject. The constants 44.6 (µmol/mL) and 60 (min/h) were used to convert $FCO_2$ to micromoles per hour. The factor 0.82 is the correction for CO$_2$ retained in the body because of bicarbonate fixation (36), and the factor 100 changes the atom percent excess to a fraction.

**Statistical analysis**

Results are expressed as means ± SDs. A mixed linear model with the subject as a random variable by using Proc Mixed program (SAS/STAT version 8.2; SAS Institute) was used to analyze the effects of amounts of protein intake on $F^{13}CO_2$, phenylalanine flux and oxidation. When warranted, a post hoc analysis was performed by using Tukey-Kramer's multiple-comparisons test. Differences were considered significant at $P < 0.05$.

Estimates of the mean protein requirement for children were derived by breakpoint analysis by using a 2-phase linear regression crossover model (27, 37) in SAS/STAT (version 8.2; SAS Institute). A general outline for the procedure is as follows. The method selected the model with a minimum residual SE in a stepwise partitioning of protein-intake values ($x$) between 2 regression lines. The lines were estimated for each candidate breakpoint by using mixed models to account for repeated measures within the subject. With the use of $I$ as an indicator variable equal to 0 for $x$ values left of the breakpoint and equal to 1 for $x$ values to the right of the breakpoint, the model is

$$Y = \beta_0 + \beta_1x + \beta_2I + \beta_3Ix$$

where $Y = F^{13}CO_2$ or phenylalanine oxidation, $x = $ protein intake, $\beta_0 = $ left-line intercept, $\beta_0 + \beta_2 = $ right-line intercept, $\beta_1 = $ left-line slope, and $\beta_1 + \beta_3 = $ right-line slope. Therefore, $Y = \beta_0 + \beta_1x$ for the left line and $Y = (\beta_0 + \beta_2) + (\beta_1 + \beta_3)x$ for the right line. Equating these, $\beta_0 + \beta_1x = (\beta_0 + \beta_2) + (\beta_1 + \beta_3)x$, and solving for $x$ yields the breakpoint at $x = -(\beta_2 + \beta_3)$. The SE for the breakpoint estimate was provided by Fieller’s Theorem (37), which uses SEs and a covariance of $\beta_2$ and $\beta_3$ estimates. The safe intake of protein was estimated as the upper limit of the 95% CI, which was calculated as the breakpoint + $t$ × SE.

**RESULTS**

**Subject characteristics**

Seven healthy, school-aged children (8.4 ± 1.4 y old) completed the study. Subject anthropometric measures (Table 1) were within the normal range for age (38) and did not change during the course of the study. Similarly, energy intakes of subjects were adequate. According to self- and parent-rated Tanner staging (39), all subjects were either in Tanner stage I or II.

**Phenylalanine flux and oxidation**

Phenylalanine flux was not affected ($P > 0.05$) within each individual by different protein intakes as required by the IAAO method (Table 3). Phenylalanine oxidation declined in response to graded increases in protein intakes until 1.2–1.3 g · kg$^{-1}$ · d$^{-1}$, after which further increases in protein intakes did not influence phenylalanine oxidation (Figure 1). Two-phase linear

### TABLE 3

<table>
<thead>
<tr>
<th>Subject</th>
<th>Test protein intakes</th>
<th>Phenylalanine flux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g · kg$^{-1}$ · d$^{-1}$</td>
<td>µmol · kg$^{-1}$ · h$^{-1}$</td>
</tr>
<tr>
<td>1</td>
<td>0.16, 0.52, 0.94, 1.24, 1.54, 1.66, 1.9, 2.08, 2.44</td>
<td>38.7 ± 3.8$^a$</td>
</tr>
<tr>
<td>2</td>
<td>0.2, 0.6, 0.9, 1.2, 1.5, 1.8, 2.2</td>
<td>39.7 ± 3.9$^a$</td>
</tr>
<tr>
<td>3</td>
<td>0.34, 0.64, 0.7, 1.12, 1.36, 1.84, 1.96, 2.26, 2.38</td>
<td>39.1 ± 3.4$^a$</td>
</tr>
<tr>
<td>4</td>
<td>0.1, 0.58, 0.82, 1.06, 1.3, 1.6, 2.02</td>
<td>37.4 ± 6.7$^{ab}$</td>
</tr>
<tr>
<td>5</td>
<td>0.22, 0.46, 0.76, 1.0, 1.42, 1.78, 2.2, 2.32, 2.5</td>
<td>35.8 ± 3.8$^{ab}$</td>
</tr>
<tr>
<td>6</td>
<td>0.24, 0.42, 0.86, 1.1, 1.62, 2.1, 2.52</td>
<td>44.1 ± 3.8$^a$</td>
</tr>
<tr>
<td>7</td>
<td>0.28, 0.4, 0.88, 1.18, 1.48, 1.72, 2.14, 2.56</td>
<td>31.0 ± 2.7$^a$</td>
</tr>
</tbody>
</table>

1 Subjects participated in a range of protein intakes (0.1–2.56 g · kg$^{-1}$ · d$^{-1}$); each subject participated in a minimum of 7 test intakes, which included ≥3 intakes of <0.9 g · kg$^{-1}$ · d$^{-1}$ and 3 intakes of >0.9 g · kg$^{-1}$ · d$^{-1}$ in a total of 56 studies.

2 Values are means ± SDs. No significant differences ($P > 0.05$) in phenylalanine flux were observed within each subject because of various test protein intakes. Values with different superscript letters within the column were significantly different ($P < 0.05$). Post hoc analysis was performed by using Tukey-Kramer’s multiple-comparison tests.
regression crossover analysis identified a breakpoint and upper 95% CI for in the relation between protein intake and phenylalanine oxidation to be 1.25 g · kg⁻¹ · d⁻¹ and 1.50 g · kg⁻¹ · d⁻¹ (r² = 0.75), respectively.

1-[1-13C]Phenylalanine oxidation

The rate of 13CO₂ released from the oxidation of 1-[1-13C] phenylalanine (F₁3CO₂) declined in children with increasing protein intakes up to 1.3 g · kg⁻¹ · d⁻¹ (Figure 2). Additional increases in protein intakes did not result in changes in individual F₁3CO₂ values, which indicated no additional increases in the incorporation of label for protein synthesis. Two-phase linear regression crossover analysis of F₁3CO₂ data resulted in the identification of a breakpoint for the mean protein requirement of 1.3 g · kg⁻¹ · d⁻¹ (r² = 0.85). The mean protein requirement represents the estimated EAR. The upper 95% CI, which represents the population-safe or RDA, was determined to be 1.55 g protein · kg⁻¹ · d⁻¹. Breakpoint measurements by using 13C₄CO₂ have been recently shown to be very similar to breakpoint measurements for phenylalanine hydroxylation by using enrichments in apolipoprotein B-100, which is a hepatic export protein (30). Thus, measurements in breath are representative of the intracellular enrichment of phenylalanine at the site of protein synthesis in the liver and are preferable to the rate of phenylalanine oxidation calculated from plasma or urine enrichments. Therefore, mean and population-safe requirements for the protein intake determined by using F₁³CO₂ data are more appropriate to make recommendations.

**DISCUSSION**

The requirement for mean and population-safe protein requirements in healthy school-age children by using the IAAO technique was determined to be 1.3 and 1.55 g protein · kg⁻¹ · d⁻¹, respectively (Table 4). These results are 71% and 63% higher than the current DRI recommendations of 0.76 and 0.95 g protein · kg⁻¹ · d⁻¹, respectively. With the use of the IAAO method, we showed that adult protein requirements are underestimated by 29–33% (11). Mean and population-safe protein requirements for adults were determined to be 0.93 and 1.2 g · kg⁻¹ · d⁻¹, respectively, compared with the DRI recommendations of 0.66 and 0.8 g · kg⁻¹ · d⁻¹, respectively (11). Furthermore, in the same article, we reported on a reanalysis of the existing nitrogen-balance data in adults by using nonlinear regression and obtained a mean requirement for protein that was not different from the requirement by the IAAO method (11).

In the DRI (2005) report (1) and the recent FAO (2007) recommendations (2), a paucity of data on protein requirements in school-age children was noted. Gattas et al (40) conducted the only other direct study to estimate protein requirements in children of similar age (8–10 y old), by using the nitrogen balance. Eight healthy children in Chile each received 0.6, 0.8, 1.0, and 1.2 g protein · kg⁻¹ · d⁻¹ as a mixed diet for 10 d. A mean intake of 0.94 g protein · kg⁻¹ · d⁻¹ for satisfactory nitrogen retention by using single linear regression analysis and a population-safe intake of 1.2 g protein · kg⁻¹ · d⁻¹ were determined (40). These nitrogen-balance estimates are 38% and 29% lower than the mean and population-safe IAAO requirements of 1.3 and 1.55 g protein · kg⁻¹ · d⁻¹, respectively. However we believe that some of the differences could be explained by the choice of test-protein intakes as well as the method of data analysis. The highest intake tested was 1.2 g protein · kg⁻¹ · d⁻¹, which, thus, made it impossible to test for a response to a greater protein intake. Also, the choice of fitting a linear regression analysis model to determine the zero-nitrogen balance was not appropriate because the physiologic response relation between the nitrogen intake and balance is not linear; a decreased efficiency of protein use occurs as the zero balance approaches (1). This observation was also noted by Gattas et al (40) on the basis of the authors’ quote as follows: “the relationship was not linear at the highest amounts of intake for 2 of the 8 subjects.” A 2-phase linear regression analysis is more appropriate as shown previously in our reanalysis of existing

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**FIGURE 1.** Influence of dietary protein intake on Phe oxidation in healthy school-age children (n = 56 observations). Individual values for 5 boys and 2 girls (girls represented by symbols X, ●) who consumed a range of protein amounts (0.1–2.56 g · kg⁻¹ · d⁻¹) are shown. A 2-phase linear regression crossover analysis identified a breakpoint and an upper 95% CI for in the relation between protein intake and Phe oxidation to be 1.25 and 1.50 g · kg⁻¹ · d⁻¹ (r² = 0.75), respectively.

**FIGURE 2.** Influence of dietary protein intake on production of 13CO₂ from the oxidation of orally administered 1-[1-13C]-phenylalanine (F₁3CO₂) in healthy school-age children (n = 56 observations). Individual values for 5 boys and 2 girls (girls represented by symbols X, ●) who consumed a range of protein amounts (0.1–2.56 g · kg⁻¹ · d⁻¹) are shown. Each child participated in a minimum of 7 intakes in which intakes were randomly assigned to ensure 3 intakes <0.9 g · kg⁻¹ · d⁻¹ and 3 intakes >0.9 g · kg⁻¹ · d⁻¹. The breakpoint estimated the mean protein requirement. The breakpoint was determined by using 2-phase linear regression crossover analysis to minimize the total sum of squares in error for the combined lines. The mean protein requirement was estimated to be 1.3 g · kg⁻¹ · d⁻¹ (r² = 0.85). The upper 95% CI estimated the population-safe intakes and was estimated to be 1.55 g · kg⁻¹ · d⁻¹. F₁³CO₂, label tracer oxidation.
determine whether recommended intakes will maintain adequate health and or meet the body’s needs for various physiologic and metabolic functions. In adult humans, a series of long-term balance studies conducted previously (51–53) showed that the FAO 1973 (54) proposed safe allowance of 0.57 g egg protein resulted in a negative nitrogen balance, loss of lean body mass, and deteriorating serum protein and hepatic transferase values unless additional energy or nonessential nitrogen was provided. Young men who consumed 0.6–0.75 g protein · kg⁻¹ · d⁻¹ for 10 d had a significantly \((P < 0.05)\) lower erythrocyte glutathione synthesis rate, whole-body protein turnover, albumin synthesis rates, and nutrient transport proteins compared with baseline values on a habitual protein intake \((\sim 1.13 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})\) (55–57). These results increase the probability that the current RDA protein intake of 0.95 g · kg⁻¹ · d⁻¹ in children, for which the physiologic and metabolic adequacy has not been examined, is not adequate or safe.

The results from the current study suggest that an RDA of 1.55 g protein · kg⁻¹ · d⁻¹, which corresponds to 8.7% of energy intake from protein for a 26-kg child [reference weight for 6–10-y-olds (1)] who consumed a 1919 kcal/d diet [average estimated energy requirement for 6–10-y-old children (1)]. This value \((\sim 9\%)\) is well within the recommended range for a protein intake of 5–20% of total energy intake for young children (1). On a gram-per-day basis, the RDA on the basis of the current study corresponded to 40.3 g protein/d, which was below reported intakes of protein in North America for 4–8-y-olds [66.1 ± 13.7 g/d (58)] and in Europe for 7–10-y-olds [53–85 g/d (59)]. The Dietary Reference Intakes for protein \((0.95 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})\) corresponds to 24.7 g protein/d, which may not be adequate to meet the needs for young children worldwide. Protein-energy malnutrition remains one of the major forms of malnutrition globally, and \sim 32% of children in developing countries are stunted (60). Because it is well established in animal science (61) that lean mass and linear growth are determined in large part by a sufficient protein intake, it is conceivable that the current high prevalence of stunting in the poor in developing countries is due to inadequate protein intakes. The consequences of stunting include increased morbidity and mortality, poor physical and mental development and school performance, and reduced adult body size and capacity for physical work (62). Thus, there is an urgent need to reassess current protein-intake recommendations in children.

In conclusion, the mean and population-safe protein requirements for school-age children (6–10 y of age) were determined to be 1.3 and 1.55 g · kg⁻¹ · d⁻¹, respectively. These results suggest

### TABLE 4
Comparison of protein requirements in school-age children (6–10 y old)

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<tr>
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<td>0.94</td>
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<td>RDA</td>
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¹ DRI, Dietary Reference Intake; EAR, estimated average requirement; IAAO, indicator amino acid oxidation; RDA, Recommended Dietary Allowance.

² A 20% increase in the nitrogen-balance estimate derived by using single linear regression analysis by Gattas et al (40) was applied. In our previous study of adult protein requirements (11), we showed that nitrogen-balance data that were analyzed by using linear regression resulted in an overestimate of the zero-nitrogen balance by \(\geq 10\%\), which led to a 20% underestimate of protein requirements.
that the DRI (1) recommendations for an EAR and RDA of 0.76 and 0.95 g protein · kg⁻¹ · d⁻¹, which were estimated by factorial calculations, are underestimated by 71% and 63%, respectively. The current results are similar to our earlier demonstration in which we used the IAAO technique in adults that protein intake recommendations are underestimated by ~30% (11). To our knowledge, the current study was the first to directly estimate protein requirements in children by using stable isotopes and indicates that the DRI (1) and FAO (2) recommendations are severely underestimated.

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The authors’ responsibilities were as follows—RE and MAH: study design, data collection, sample and data analyses, and manuscript writing; and ROB and PBP: study design, data analyses, and manuscript writing. None of the authors had a conflict of interest.

REFERENCES


