Reevaluation of the protein requirement in young men with the indicator amino acid oxidation technique\textsuperscript{1–3}

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

ABSTRACT

Background: The current estimated protein requirements are based on the nitrogen balance method, which has many limitations. An alternate approach is needed to permit a reevaluation of protein requirements.

Objective: The objective was to determine protein requirements in men by using the indicator amino acid oxidation technique.

Design: Eight healthy men randomly received graded protein intakes (0.10, 0.30, 0.60, 0.90, 1.2, 1.5 and 1.8 g kg\textsuperscript{-1} \cdot d\textsuperscript{-1}) as a crystalline amino acid mixture along with L-[1-\textsuperscript{13}C]phenylalanine. The mean protein requirement was determined by applying a biphasic linear regression crossover analysis on \textsuperscript{13}CO\textsubscript{2} data, which identified a breakpoint at the minimal rate of appearance of \textsuperscript{13}CO\textsubscript{2} to graded protein intakes.

Results: The mean and population-safe (recommended dietary allowance; RDA) protein requirements were found to be 0.93 and 1.2 g kg\textsuperscript{-1} \cdot d\textsuperscript{-1}, respectively. These requirements are comparable with those estimated by the application of a biphasic linear regression model to the data from nitrogen balance studies (0.91 and 1.0 g kg\textsuperscript{-1} \cdot d\textsuperscript{-1}, respectively). These requirements are 41% and 50% higher than the current recommendations for the estimated average requirement (EAR) of 0.66 g kg\textsuperscript{-1} \cdot d\textsuperscript{-1} and the RDA of 0.80 g kg\textsuperscript{-1} \cdot d\textsuperscript{-1}, as determined by applying a linear regression model where it intersects the zero balance line.

Conclusion: The indicator amino acid oxidation technique defined a protein requirement that is comparable with that estimated by the application of a biphasic linear regression model to nitrogen balance data in the literature. Our data and the reanalysis of the preexisting nitrogen balance data suggest that the current recommended protein requirements are too low and require reassessment. Am J Clin Nutr 2007;86:995–1002.

KEY WORDS Protein requirement, indicator amino acid oxidation, nitrogen balance, men

INTRODUCTION

In 2002, the Food and Nutrition Board (1) proposed average and “safe” intakes of 0.66 and 0.80 g kg\textsuperscript{-1} \cdot d\textsuperscript{-1} of good quality protein, respectively. These recommendations were based on a meta-analysis of nitrogen balance studies (2), in which protein requirements were estimated by fitting a linear regression analysis model to the data and determining where it intersected the zero balance line. However, the physiologic response relation between nitrogen intake and balance is not linear because of a decreased efficiency of protein utilization as zero balance is approached (3, 4). Because the physiologic response relation is curvilinear, the 2-phase linear regression model (5) or a smooth nonlinear model (4, 6) was proposed to be a more realistic biological analysis to determine protein requirements (1). However, these models were not adopted in the current report on Dietary Reference Intakes (DRI) (1) because it was perceived that more data points on each individual were needed than were available in published studies. Furthermore, a high intake of protein in adults does not result in further protein accretion. Therefore, at some point the slope of nitrogen balance versus protein intake must equal zero, which further supports the idea that simple linear regression is not appropriate.

Although nitrogen balance is a gold standard, it has many limitations (7). Nitrogen balance is a relatively small value, which is obtained by subtracting the large value of all nitrogen losses from a (similarly) large value of all nitrogen intakes. This results in considerable error in the prediction of balance (8, 9) because the nitrogen intake tends to be overestimated and nitrogen excretion tends to be underestimated. Overestimation of nitrogen intake and underestimation of nitrogen excretion falsely results in positive nitrogen balance (10); in adults this is biologically implausible.

Considering the inherent problems associated with the nitrogen balance technique and the application of a single linear regression model on nitrogen balance data, we believe that the current protein requirements are underestimated. To test our hypothesis, we applied an alternative and more direct approach, the indicator amino acid oxidation (IAAO) technique, and determined the protein requirements by feeding graded protein intakes and measuring changes in oxidation of orally administered L-[1-\textsuperscript{13}C]phenylalanine. The IAAO is a robust technique that has been successfully used previously by our group to

\textsuperscript{1}From the Research Institute, The Hospital for Sick Children, Toronto, Canada (MAH, RE, and PBP); the Department of Nutritional Sciences, University of Toronto, Toronto, Canada (PBP and ROB); and the Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Canada (ROB and PBP).

\textsuperscript{2}Supported by grant MT 10321 from the Canadian Institutes for Health Research. Mead Johnson Nutritional (Canada) donated the protein-free powder for the experimental diets.

\textsuperscript{3}Reprints not available. Address correspondence to PB Pencharz, Division of Gastroenterology, Hepatology and Nutrition, The Hospital for Sick Children, 555 University Avenue, Toronto, ON, Canada M5G 1X8. E-mail: paul.pencharz@sickkids.on.ca..

Received January 8, 2007.
Accepted for publication May 15, 2007.
determine protein requirements in pigs (11) and by our group (12–18) and others (19–21) to determine amino acid requirements in adults and children.

SUBJECTS AND METHODS

Subjects

Eight healthy adult men were studied (on an outpatient basis) in the Clinical Investigation Unit at the Hospital for Sick Children (HSC), Toronto, Canada. The subjects reflected the ethnic diversity of Toronto: 2 were South Asian, 3 were East Asian, 1 was African, and 2 were white. Subject characteristics, body composition, and energy intakes are described in Table 1. None of the subjects had a history of recent weight loss or illness, and none was using any medication at the time of entry into the study. The Research Ethics Board of the HSC approved all procedures. Informed written consent was obtained from the participating subjects. The subjects received financial compensation for their inconvenience.

Experimental design and tracer protocol

The study design was based on the minimally invasive IAAO model (22) used in healthy adults (16, 18) and children (17, 23). Two days before the study day, subjects consumed a maintenance diet supplying 1.0 g protein · kg⁻¹ · d⁻¹ and 1.7 × resting energy expenditure (REE). On the study day, after the subjects had fasted for 12 h, they randomly received 1 of 7 dietary protein intakes (0.10, 0.30, 0.60, 0.90, 1.2, 1.5, and 1.8 g · kg⁻¹ · d⁻¹) as a crystalline amino acid mixture and an energy intake of 1.5 × REE. The study day diet was consumed as 8 isocaloric hourly meals, each meal representing one-twelfth of the subject’s total daily energy requirement. Subjects were not allowed to eat or drink anything else except for water. The study days were separated by ≥1 wk; all subjects completed all 7 studies within 3 mo.

The tracer protocol was started with the fifth meal to measure phenylalanine kinetics with the use of L-[1-¹³C]phenylalanine [99 atom% excess (APE); Cambridge Isotope laboratories, Woburn, MA], Oral priming doses of 0.176 mg/kg NaH¹³CO₃ (99 APE; Cambridge Isotope laboratories) and 0.66 mg/kg L-[1-¹³C]phenylalanine were given with the fifth hourly meal. An hourly oral dosing protocol of L-[1-¹³C]phenylalanine (1.2 mg · kg⁻¹ · d⁻¹) was commenced simultaneously (with the fifth meal) and continued for the remaining 3 h of the study. The amount of L-[1-¹³C]phenylalanine given during the study day was subtracted from the dietary provision of phenylalanine such that the total intake of phenylalanine was 30.5 mg · kg⁻¹ · d⁻¹ with a tyrosine intake of 40 mg · kg⁻¹ · d⁻¹ (to ensure an excess of tyrosine).

Study diets

The maintenance diet (energy: REE × 1.7 and protein: 1.0 g · kg⁻¹ · d⁻¹) for the 2 d before the study day for all the 7 studies was provided in the form of milk shakes (Scandipharm; Scandipharm, Birmingham, AL), which were weighed in daily portions for each subject and supplemented with additional protein (Promod; Ross laboratories, Columbus, OH) and energy (Caloreen; Nestle Clinical Nutrition, North York, Canada), depending on each subject’s requirement (1.7 × individual’s resting metabolic rate). Subjects were instructed to add a predetermined volume of homogenized milk (measuring cup provided) containing 3.25% fat to their daily portion of milk shakes and to drink the milk shakes at regular times throughout the day. REE was measured by open-circuit indirect calorimetry (2900 Computerized Energy measurement System; Sensormedics, Yorba Linda, CA).

The study day diet consisted of a protein-free liquid formula containing protein-free powder (Product 80056; Mead Johnson, Evansville, IN), flavoring crystals (Tang and Koolaid; Kraft, Don Mills, Canada), corn oil, the crystalline amino acid mixture (representing various protein intake levels) (Table 2), and protein-free cookies. The carbohydrate content of the meal was adjusted according to the level of protein intake to give isocaloric diets. The study diet provided energy at 1.5 × REE with 33% of energy from fat and variable energy from carbohydrate (48–66%) and protein (1–19%). Feeding graded protein intakes in the form of an amino acid mixture provides graded levels of both indispensable amino acids and nitrogen to determine protein requirements.

Sample collection and analysis

Breath and urine samples were collected as described previously (16). Breath samples were stored at room temperature until analyzed. Urine samples were stored at −20 °C. During each study day, open-circuit indirect calorimetry (2900 Computerized Energy measurement System) was performed for 20 min to measure the rate of carbon dioxide production (V̇CO₂).

Enrichment of¹³C in breath was analyzed by continuous-flow isotope ratio mass spectrometry (20/20 isotope analyzer; PDZ Europa Ltd, Cheshire, United Kingdom). All analyses were performed in triplicate. Enrichments were expressed as APE compared with a reference standard of compressed carbon dioxide gas. L-[1-¹³C]Phenylalanine enrichment in urine samples was analyzed with a triple quadrupole mass analyzer (API 4000; Applied Biosystems/MDS SCIEX, Concord, Canada) coupled to an HPLC system (Agilent 1100; Agilent, Mississauga, Canada) as described previously (17). Isotopic enrichment was expressed as molecule % excess and was calculated from peak area ratios at isotopic steady state at plateau and baseline.

Tracer kinetics

Kinetics were calculated according to the stochastic model of Matthews et al (24), as previously used by Zello et al (12). Isotopic steady state in the tracer enrichment at baseline and plateau was represented by unchanging values of [1-¹³C]phenylalanine in urine and ¹³CO₂ in breath. At plateau,
The APE was calculated by subtracting the mean \(^{13}\)CO\(_2\) enrichments of the 3 baseline samples from the 4 plateau samples.

Phenylalanine flux (\(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\)) was calculated from the dilution of orally administered \(L-\text{[\(^{13}\)C]phenylalanine}\) into the metabolic pool (at steady state) by using enrichments of \(L-\text{[\(^{13}\)C]phenylalanine}\) in urine (12, 24). The rate of appearance of \(^{13}\)CO\(_2\) in breath (\(F^{13}\)CO\(_2\) \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\)) after the oxidation of ingested \(L-\text{[\(^{13}\)C]phenylalanine}\) was calculated according to the model of Matthews et al (24) by using a factor of 0.82 to account for the retention of \(^{13}\)CO\(_2\) in the bicarbonate pool of the body in the fed state (25). The rate of phenylalanine oxidation (\(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\)) was calculated from \(F^{13}\)CO\(_2\) and urinary \(L-\text{[\(^{13}\)C]phenylalanine}\) enrichment (12, 24).

### Meta-analysis of nitrogen balance studies

The current EAR and RDA for protein are based on the approach in which single linear regression was applied to the data from a meta-analysis of 19 nitrogen balance studies (6) and the estimated requirement was where the regression line intersects zero balance. Applying single linear regression on the nitrogen balance data underestimated the protein requirements because 1) the physiologic response relation between nitrogen intake and balance is curvilinear because the efficiency of protein utilization decreases as zero balance is approached, and 2) the nitrogen balance values are overestimated because of inherent methodologic problems (1–4). As suggested by the current DRI report (5), we agree that the better way for determining protein requirement is the application of the biphasic linear model on the nitrogen balance data. On the basis of the above argument, we applied biphasic linear regression analysis to 28 nitrogen balance studies (20–53), including the 19 studies used previously (6), to estimate the current EAR and RDA using linear regression analysis (Table 3). The selection criteria for the studies included the 1) use of repeated measures within the same subject (minimum number of 3 intakes per subject), 2) adaptation of subjects to each level of intake for \(\geq 6\) d, and 3) use of standard nitrogen balance techniques (\(\geq 3\) d of balance and inclusion of urine and feces in excretion measurements). Data for nitrogen intakes, nitrogen balances, number of subjects, and the type of protein source used were collected from these studies (Table 3). Data were uniformly converted into units of \(\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}\) at all protein intakes.

### Statistical analysis

Data were analyzed by using PROC MIXED (SAS version 8.2; SAS Institute Inc, Cary, NC). Repeated-measures analysis of variance (ANOVA) was performed on primary and derived variables to assess the effects of protein intake, subject, and interactions. Tukey’s test was used for post hoc analysis of the ANOVA results. Results are expressed as means ± SEs. Statistical significance was assumed at the 5% level of significance (\(P < 0.05\)).

The protein requirement (breakpoint) was determined by applying a biphasic linear regression crossover model on \(F^{13}\)CO\(_2\) data for the current study and nitrogen balance data for the meta-analysis (12). This model selects for the minimum residual SE in a stepwise partitioning of data points between 2 regression lines. The first regression line has a slope and the second line is horizontal with minimal or no slope. The safe intake (upper 95% CI, equivalent to the RDA) was calculated by using Fieller’s theorem (54).

---

### TABLE 2

Amino acid composition of reference protein and various test protein intakes as amino acid mixture

<table>
<thead>
<tr>
<th>Reference protein(^1)</th>
<th>0.1 g/kg protein</th>
<th>0.3 g/kg protein</th>
<th>0.6 g/kg protein</th>
<th>0.9 g/kg protein</th>
<th>1.2 g/kg protein</th>
<th>1.5 g/kg protein</th>
<th>1.8 g/kg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{mg/g})</td>
<td>(\text{mg/0.1 g})</td>
<td>(\text{mg/0.3 g})</td>
<td>(\text{mg/0.6 g})</td>
<td>(\text{mg/0.9 g})</td>
<td>(\text{mg/1.2 g})</td>
<td>(\text{mg/1.5 g})</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>61.4</td>
<td>6.2</td>
<td>18.4</td>
<td>36.9</td>
<td>55.3</td>
<td>73.8</td>
<td>92.2</td>
</tr>
<tr>
<td>L-Arginine · HCl(^2)</td>
<td>75.1</td>
<td>7.5</td>
<td>22.5</td>
<td>45.1</td>
<td>67.6</td>
<td>90.1</td>
<td>112.7</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>33.3</td>
<td>3.3</td>
<td>10.0</td>
<td>20.0</td>
<td>30.0</td>
<td>39.9</td>
<td>49.9</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>33.3</td>
<td>3.3</td>
<td>10.0</td>
<td>20.0</td>
<td>30.0</td>
<td>39.9</td>
<td>49.9</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>22.1</td>
<td>2.2</td>
<td>6.6</td>
<td>13.3</td>
<td>19.9</td>
<td>26.5</td>
<td>33.2</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>56.6</td>
<td>5.7</td>
<td>17.0</td>
<td>34.0</td>
<td>51.0</td>
<td>68.0</td>
<td>85.0</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>56.6</td>
<td>5.7</td>
<td>17.0</td>
<td>34.0</td>
<td>51.0</td>
<td>68.0</td>
<td>85.0</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>33.3</td>
<td>3.3</td>
<td>10.0</td>
<td>20.0</td>
<td>30.0</td>
<td>39.9</td>
<td>49.9</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>22.7</td>
<td>2.3</td>
<td>6.8</td>
<td>13.6</td>
<td>20.4</td>
<td>27.2</td>
<td>34.1</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>62.8</td>
<td>6.3</td>
<td>18.9</td>
<td>37.7</td>
<td>56.6</td>
<td>75.4</td>
<td>94.3</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>83.3</td>
<td>8.3</td>
<td>25.0</td>
<td>50.0</td>
<td>75.0</td>
<td>99.9</td>
<td>124.9</td>
</tr>
<tr>
<td>L-Lysine · HCl(^3)</td>
<td>75.7</td>
<td>7.6</td>
<td>22.7</td>
<td>45.4</td>
<td>68.1</td>
<td>90.8</td>
<td>113.5</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>29.6</td>
<td>3.0</td>
<td>8.9</td>
<td>17.8</td>
<td>26.7</td>
<td>35.6</td>
<td>44.5</td>
</tr>
<tr>
<td>L-Phenylalanine(^4)</td>
<td>54.7</td>
<td>30.5</td>
<td>30.5</td>
<td>30.5</td>
<td>30.5</td>
<td>30.5</td>
<td>30.5</td>
</tr>
<tr>
<td>L-Proline</td>
<td>41.9</td>
<td>4.2</td>
<td>12.6</td>
<td>25.2</td>
<td>37.7</td>
<td>50.3</td>
<td>62.9</td>
</tr>
<tr>
<td>L-serine</td>
<td>83.9</td>
<td>8.4</td>
<td>25.2</td>
<td>50.3</td>
<td>75.5</td>
<td>100.7</td>
<td>125.8</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>47.1</td>
<td>4.7</td>
<td>14.1</td>
<td>28.3</td>
<td>42.4</td>
<td>56.5</td>
<td>70.6</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>15.6</td>
<td>1.6</td>
<td>4.7</td>
<td>9.4</td>
<td>14.0</td>
<td>18.7</td>
<td>23.4</td>
</tr>
<tr>
<td>L-Tyrosine(^5)</td>
<td>40.7</td>
<td>40.7</td>
<td>40.7</td>
<td>40.7</td>
<td>40.7</td>
<td>40.7</td>
<td>40.7</td>
</tr>
<tr>
<td>L-Valine</td>
<td>70.3</td>
<td>7.2</td>
<td>21.1</td>
<td>42.2</td>
<td>63.2</td>
<td>84.3</td>
<td>105.4</td>
</tr>
</tbody>
</table>

\(^1\) Represents egg protein composition.

\(^2\) Actual concentration of amino acids in HCl form in amino acid mixture: arginine, 62.1 mg/g; and lysine, 60.6 mg/g.

\(^3\) L-Phenylalanine intake was kept constant at 30.5 mg · kg\(^{-1} \cdot d\(^{-1}\) at all protein intakes.

\(^4\) L-Tyrosine intake was kept constant at 40.7 mg · kg\(^{-1} \cdot d\(^{-1}\) at all protein intakes.

\(^5\) Represents egg protein composition.
The study design for the current study included repeated tests within a subject (7 protein intakes per subject). The 8 subjects, providing a total of 56 data points, were predicted to be adequate to estimate the mean and population safe requirements of protein in adults by applying a 2-phase linear regression crossover analysis on the data, as determined previously in children and adults (17, 16).

**RESULTS**

**Phenylalanine flux and oxidation**

Phenylalanine flux was not affected by protein intake (Table 4), which provides evidence that the precursor pool for indicator oxidation did not change in size in response to the test protein intake. Phenylalanine oxidation (μmol·kg⁻¹·h⁻¹) declined in response to increases in protein intake. The phenylalanine oxidation values were significantly different only at the protein intake of 0.1 g·kg⁻¹·d⁻¹ compared with 0.6–1.8 g·kg⁻¹·d⁻¹ (P < 0.05), 0.3 g·kg⁻¹·d⁻¹ compared with 0.9–1.8 g·kg⁻¹·d⁻¹ (P < 0.05), and 0.6 g·kg⁻¹·d⁻¹ compared with 1.2–1.8 g·kg⁻¹·d⁻¹ (P < 0.05). Protein intakes between 0.9 and 1.8 g·kg⁻¹·d⁻¹ were not significantly different (P > 0.05) for phenylalanine oxidation.

**L[1-13C]Phenylalanine label oxidation**

Dietary protein intake reduced the oxidation of L-[1-13C]phenylalanine measured as the rate of label appearance...
TABLE 4
Phenylalanine flux and oxidation at 7 protein intakes in healthy men

<table>
<thead>
<tr>
<th>Protein intake (g·kg⁻¹·d⁻¹)</th>
<th>Phenylalanine flux ( μmol·kg⁻¹·h⁻¹ )</th>
<th>Phenylalanine oxidation ( μmol·kg⁻¹·h⁻¹ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>67.6 ± 4.4</td>
<td>13.0 ± 0.8*a</td>
</tr>
<tr>
<td>0.3</td>
<td>62.0 ± 3.0</td>
<td>10.5 ± 0.5*b</td>
</tr>
<tr>
<td>0.6</td>
<td>60.6 ± 5.9</td>
<td>8.3 ± 0.6*c</td>
</tr>
<tr>
<td>0.9</td>
<td>52.7 ± 6.6</td>
<td>7.2 ± 0.7*b,c</td>
</tr>
<tr>
<td>1.2</td>
<td>53.3 ± 6.8</td>
<td>4.9 ± 0.6*d</td>
</tr>
<tr>
<td>1.5</td>
<td>56.1 ± 2.7</td>
<td>5.2 ± 0.4*e</td>
</tr>
<tr>
<td>1.8</td>
<td>56.9 ± 5.5</td>
<td>4.8 ± 0.3*f</td>
</tr>
</tbody>
</table>

\( n = 8 \) per mean. Repeated-measures ANOVA was performed to assess the effects of protein intake on phenylalanine flux and oxidation with Tukey’s test for post hoc analysis. Means in a column with different superscript letters are significantly different, \( P < 0.05 \).

1 All values are \( \bar{x} \) ± SE.

2 Protein had no statistically significant effect on phenylalanine flux, \( P > 0.05 \).

in breath \( \left(^{13}\text{C}\right)\text{CO}_2 \) in response to graded intakes of protein (Figure 1). As the protein intake increased, \( \left(^{13}\text{C}\right)\text{CO}_2 \) decreased steadily (representing increased incorporation of label into protein) until a breakpoint was reached (between an intake of 0.9 and 1.2 g protein/kg); there was no further decrease in \( \left(^{13}\text{C}\right)\text{CO}_2 \) with the increase in protein intake (representing no further change in the incorporation of label into protein). Application of a biphasic linear regression crossover analysis on the \( \left(^{13}\text{C}\right)\text{CO}_2 \) data resulted in the identification of a breakpoint (estimate of mean protein requirement equivalent to the EAR) at 0.93 g·kg⁻¹·d⁻¹ and safe level of intake (the upper 95% CI, equivalent to the RDA) at 1.2 g·kg⁻¹·d⁻¹.

FIGURE 1. Relation between various protein intakes and the rate of appearance of orally administered \( t\left[{\text{13}}\text{C}\right]\)phenylalanine as \( \left({\text{13}}\text{C}\right)\text{CO}_2 \) in 8 healthy men. Values are \( \bar{x} \) ± SD (\( n = 8 \) per mean). The breakpoint estimates the mean protein requirement. The breakpoint was determined by using biphasic linear regression crossover analysis to minimize the total sum of squares in error for the combined line. The mean protein requirement was estimated to be 0.93 g·kg⁻¹·d⁻¹. The upper 95% CI estimates the population-safe protein intake and was estimated to be 1.24 g·kg⁻¹·d⁻¹.

FIGURE 2. Relation between various nitrogen intakes and the mean nitrogen balances from 28 nitrogen balance studies (data from references quoted in Table 3). Values are \( \bar{x} \) ± SE. The breakpoint estimates the mean nitrogen requirement. The breakpoint was determined by using biphasic linear regression crossover analysis to minimize the total sum of squares in error for the combined line. The mean nitrogen (or protein) requirement was estimated to be 145.9 mg·kg⁻¹·d⁻¹ (or 0.91 g protein·kg⁻¹·d⁻¹). The upper 95% CI estimates the population-safe nitrogen (or protein) intakes and was estimated to be 158.3 mg·kg⁻¹·d⁻¹ (or 0.99 g protein·kg⁻¹·d⁻¹). Two very positive nitrogen balances on the nonsloping line were removed, and a second breakpoint analysis was done. The new values were not significantly different from those from the initial analysis. The mean nitrogen (or protein) requirement was estimated to be 143.6 mg·kg⁻¹·d⁻¹ (or 0.9 mg protein·kg⁻¹·d⁻¹). The upper 95% CI was estimated to be 155.6 mg·kg⁻¹·d⁻¹ (or 0.97 g protein·kg⁻¹·d⁻¹).

Meta-analysis of nitrogen balance studies

Application of the biphasic linear regression model to the nitrogen balance data from 28 studies (Table 3) resulted in the estimation of a breakpoint of 0.91 g·kg⁻¹·d⁻¹ protein and a safe level of intake (the upper 95% CI, equivalent to the RDA) of 0.99 g·kg⁻¹·d⁻¹ (Figure 2).

DISCUSSION

This is first study that used the IAAO technique to determine protein requirements in healthy adults. Previously, Ball and Bayley (11) used the IAAO technique and estimated protein requirements for growing piglets (240 g·kg⁻¹·d⁻¹) by feeding graded levels of protein (120, 160, 200, 240, 280, and 300 g·kg⁻¹·d⁻¹) as amino acid mixture) and measuring the oxidation of \( \left[{\text{13}}\text{C}\right]\)phenylalanine. The protein estimates determined by Ball and Bayley (11) were comparable with the estimates obtained from growth studies in piglets fed milk-based diets [240 g·kg⁻¹·d⁻¹ (55, 56); 250 g·kg⁻¹·d⁻¹ (57)]. Furthermore, the IAAO technique indicates amino acid catabolism over periods as short as several minutes (58, 59) and does not need longer periods of adaptation to protein or amino acid intakes (60–64). Hence, in the current study we adapted this IAAO method to humans to determine protein requirements.

In the current IAAO study, the mean and population-safe protein requirements were estimated to be 0.93 and 1.2 g·kg⁻¹·d⁻¹, respectively. These results are indirectly supported by Tarnopolsky et al (65) who used \( t\left[{\text{13}}\text{C}\right]\)leucine and determined the protein requirements of young sedentary men and strength athletes by feeding 0.86, 1.40, and 2.40 g protein·kg⁻¹·d⁻¹. The results of that study showed that whole-body protein synthesis remained similar (≈140 mg·kg⁻¹·h⁻¹).
when the protein intake was increased from 0.86 to 1.40 and 2.40 g·kg\(^{-1}\)·d\(^{-1}\) in sedentary men. The authors of that study concluded that a diet containing 0.90 g·kg\(^{-1}\)·d\(^{-1}\) was at or above physiologic protein requirements for sedentary men. Similarly, Zello et al (53) and Motil et al (66) used the direct amino acid oxidation (DAAO) technique and studied the effect of 3 protein intakes (0.6, 0.8, and 1.0 g·kg\(^{-1}\)·d\(^{-1}\); 0.1, 0.6, and 1.5 g·kg\(^{-1}\)·d\(^{-1}\), respectively) on L-[\(^{13}\)C]leucine metabolism. The results of Zello et al (53) showed that as the protein and leucine intakes increased, the production of \(^{13}\)CO\(_2\) increased from 0.6 to 1.0 g protein·kg\(^{-1}\)·d\(^{-1}\); however, the differences were only statistically significant between protein intakes of 0.6 and 0.8. Similarly, the results of Motil et al (66) showed that the rates of leucine incorporation into protein synthesis (113.3 ± 6.7 compared with 102.4 ± 7.6 μmol·kg\(^{-1}\)·h\(^{-1}\)) and leucine oxidation (46.3 ± 3.8 compared with 21.6 ± 1.1 μmol·kg\(^{-1}\)·h\(^{-1}\)) were significantly higher at a protein intake of 1.5 g·kg\(^{-1}\)·d\(^{-1}\) than at a protein intake of 0.6 g·kg\(^{-1}\)·d\(^{-1}\). Because there were too few levels of protein tested below and above requirements (53, 66), the results of these studies (53, 66) are inconclusive in terms of indicating a breakpoint (hence requirement) at the protein intake at which the change in \(^{13}\)CO\(_2\) production occurred from constant to increasing pattern. The minimum level of points to satisfactorily define a line is 3.

The current EAR recommendation and RDA for protein are 0.66 and 0.80 g·kg\(^{-1}\)·d\(^{-1}\), respectively. We believe that these recommendations are tentative because no long-term studies have suggested that these values would maintain nitrogen balance along with lean body mass, muscle mass, serum protein concentrations, immunity, functional capacity etc. Previously, a series of long-term balance studies (67–69) showed that intake of the proposed safe allowance of 0.57 g (70) egg protein resulted in negative nitrogen balance, loss of lean body mass, and deteriorating serum protein and transferase values unless additional energy or nonessential nitrogen was supplied. Jackson et al (71) determined the effect of a proposed safe protein intake (0.75 g·kg\(^{-1}\)·d\(^{-1}\)) on erythrocyte glutathione synthesis rate in young men. The results of that study showed that the erythrocyte glutathione synthesis rate was significantly lower (\(P < 0.05\)) on days 3 and 10 of the diet at the proposed safe protein intake (0.75 g·kg\(^{-1}\)·d\(^{-1}\)) than at baseline at the habitual protein intake (1.13 g·kg\(^{-1}\)·d\(^{-1}\)). The authors of that study suggested that a reduced antioxidant capacity and possibly an increased susceptibility to oxidation stress occurred at a protein intake of 0.75 g·kg\(^{-1}\)·d\(^{-1}\). This raises concern that the safe protein intake of 0.75 g·kg\(^{-1}\)·d\(^{-1}\) may not be either adequate or safe.

The results of the present study suggest that the current EAR recommendations (0.66 g·kg\(^{-1}\)·d\(^{-1}\)) and RDA (0.80 g·kg\(^{-1}\)·d\(^{-1}\)) for protein are underestimated at 29% and 33%, respectively. As outlined in detail in Subjects and Methods (meta-analysis of nitrogen balance studies), we propose that these underestimations are the result of applying single linear regression on the nitrogen balance data. We argue that the breakpoint (protein requirement) determined by the biphase linear regression is more reliable because it remains the same for both true and apparent (overestimated) nitrogen balance values. This argument is illustrated in Figure 3, in which the effect on the estimate of protein requirements using both statistical methods on hypothetical data representing either a 10% overestimation of nitrogen balance values (apparent nitrogen balance) or true nitrogen balance is shown. As shown in the figure, a 10% overestimation of nitrogen balance resulted in a 20% underestimation of the protein requirement when linear regression analysis was applied to determine the zero balance value. On the other hand, similar protein requirement values were obtained when biphase linear regression analysis was applied to both the apparent and true nitrogen balance values. Furthermore, the application of both linear and biphase regression analysis on true nitrogen values yields the same estimates of protein requirements. However, because the true nitrogen balance values are very difficult to obtain and are seldom measured, it is prudent to apply biphase linear regression analysis on nitrogen balance data.

On the basis of the above argument, we applied biphase linear regression analysis to 28 nitrogen balance studies (Table 3) and estimated a breakpoint of 0.91 g protein·kg\(^{-1}\)·d\(^{-1}\) (Figure 2). This new protein requirement, derived from nitrogen balance studies, supports the protein requirement determined in the present study by IAAO (0.93 g·kg\(^{-1}\)·d\(^{-1}\)) and suggests that the previous application of linear regression analysis of nitrogen balance data resulted in underestimation of protein requirements.

The RDA of 1.2 g protein·kg\(^{-1}\)·d\(^{-1}\) corresponds to 17% of energy intake from protein for a 70-kg person consuming a diet providing 2856 kcal/d [1 kcal·kg\(^{-1}\)·h\(^{-1}\)·24 h × 1.7 (activity factor)]. This value is well within the recommended range for protein intake of 10–35% of total energy intake as recommended by the new DRI report (1) and does not exceed the value of 2.0 g protein·kg\(^{-1}\)·d\(^{-1}\), the upper limit of protein currently consumed safely by well-nourished populations (72). On the other hand, the current RDA (5) for protein for a 70-kg person is 56 g·kg\(^{-1}\)·d\(^{-1}\), which corresponds to 8% of energy intake for a diet providing 2856 kcal/d. This value of 8% is about one-half (15.5%) that found in Western diets in general (73) and that suggested by the current study (17%) and does not appear to be adequate for meeting the requirement of the entire population.

In summary, the mean and population-safe protein requirements of 0.93 and 1.2 g·kg\(^{-1}\)·d\(^{-1}\), respectively, as determined

![FIGURE 3. Hypothetical example of the relation between various protein intakes and nitrogen balances (true and 10% overestimated). Application of linear regression analysis on both overestimated and true nitrogen balance values resulted in nitrogen requirements of 100 and 120 mg·kg\(^{-1}\)·d\(^{-1}\), respectively (0.63 and 0.75 g·kg\(^{-1}\)·d\(^{-1}\) protein, respectively). Application of biphase linear regression analysis on both overestimated and true nitrogen balance values resulted in a nitrogen requirement of 120 mg·kg\(^{-1}\)·d\(^{-1}\). Application of linear regression analysis underestimated nitrogen requirements by 20% when the nitrogen balance values were overestimated by 10%.

Downloaded from https://academic.oup.com/ajcn/article-abstract/86/4/995/4649413 by guest on 24 April 2018
in the current study suggest that the EAR and RDA for protein requirements estimated by zero balance were underestimated by 29% and 33%, respectively. The results of the present study are comparable with those produced by the application of biphasic linear regression to data from nitrogen balance studies and previous stable-isotope studies. In conclusion, our data and the re-analysis of the preexisting nitrogen balance data suggest that the current recommendations for protein requirement are too low and require reassessment. The IAAO technique has advantages over the classic nitrogen balance technique because it \( J \) does not determine balance, the determination of which is technically demanding; 2) allows study of the same individual over an entire range of protein intakes, thus decreasing between-individual variability; and 3) is noninvasive and can be used to determine accurate protein requirements in healthy individuals and in patients with different diseases and conditions.

We thank the subjects who participated in the study and Linda Chow in the Department of Nutrition and Food Services (HSC) for preparing the protein-free cookies.

The authors’ responsibilities were as follows—MAH and RE: study design, data collection, sample and data analysis, and manuscript writing; ROB and PBP: study design, data analysis, and manuscript writing. The authors had no conflicts of interest.

REFERENCES


