Lysine requirement of adult males is not affected by decreasing dietary protein1–3

Alison M Duncan, Ronald O Ball, and Paul B Pencharz

ABSTRACT Recently, we reported the lysine requirement to be 41.2 mg·kg⁻¹·d⁻¹ using indicator amino acid oxidation (IAAO) in healthy adult males fed a protein intake of 1.0 g·kg⁻¹·d⁻¹. A lower protein intake has been hypothesized to significantly lower the requirement estimate. We tested this hypothesis using IAAO by estimating the lysine requirement at a protein intake of 0.8 g·kg⁻¹·d⁻¹ in five healthy adult males. Lysine requirement was determined from the rate of appearance of ¹³C₄O₂ in breath (F¹³CO₂) by using a primed 4-h continuous infusion of L-[l-¹³C]phenylalanine. Phenylalanine flux was not affected by graded increases in dietary lysine. Phenylalanine oxidation and F¹³CO₂ decreased linearly (P < 0.05) as lysine intake increased to a breakpoint, after which the rates were not significantly different. Two-phase linear regression was used to analyze this breakpoint (mean lysine requirement) to occur at a dietary lysine intake of 45.0 mg·kg⁻¹·d⁻¹. We conclude that no reduction appears in the lysine requirement with a lower protein intake. Thus, lysine requirement appears not to be altered by protein intakes within the lower end of the protein requirement range. Am J Clin Nutr 1996;64:718–25.

KEY WORDS Indicator oxidation, amino acids, lysine requirement, lysine, stable isotopes

INTRODUCTION

Despite years of research, amino acid requirements remain a controversial issue. The current adult requirements for indispensable amino acids (IDAAs) are based on estimates derived from nitrogen balance studies (1). These nitrogen balance studies started with the classical work of Rose (2) in young men followed by the work of Leverton (3) in young women. Recent literature contains clear messages that these requirements are underestimated as a result of methodologic limitations (4–8). Experiments have been conducted addressing the issue of amino acid requirements with more sensitive measures, such as direct amino acid oxidation (DAAO) (6, 9).

The DAAO method involves the measurement of flux and oxidation of an amino acid at graded dietary concentrations of that same amino acid. The amino acids that have been studied include leucine (10), valine (11), threonine (12), lysine (13), and phenylalanine (9). These studies suggest that current amino acid requirements (1) have been underestimated by a factor of two to three. However, DAAO is not without its own constraints, adding to the current controversy about determining accurate amino acid requirements (14, 15).

Most recently, the indicator amino acid oxidation (IAAO) technique was developed and validated in the piglet model as an independent method for determining amino acid requirements (16, 17) and then subsequently used to determine IDAA requirements in adult humans (8, 18). IAAO is based on the concept that the amount of the limiting amino acid governs the partition of any IDAA between retention for protein synthesis and oxidation. Therefore, when one IDAA is limiting for protein synthesis, the other amino acids will be oxidized (because they are present in relative excess). As the dietary amount of the limiting amino acid increases, the uptake of the other dietary amino acids for protein synthesis will also increase, thereby decreasing their oxidation. This will occur until the required amount of the limiting amino acid is reached, at which point further additions of the test amino acid will have no effect on the metabolism of the other IDAAs (8, 16, 17).

Although IAAO has been able to overcome many of the limitations of DAAO (8), it was unknown whether adaptation to a lower protein intake would result in a lower estimate of the IDAA requirement (14, 15). To study this question, we chose to compare our earlier estimate of lysine requirement determined in subjects receiving a protein intake of 1 g·kg⁻¹·d⁻¹ using IAAO (18), with an estimate of lysine requirement determined in similar subjects receiving a protein intake of 0.8 g·kg⁻¹·d⁻¹. We chose the lower protein intake because of earlier amino acid oxidation studies in which we showed that a protein intake of 0.8 g·kg⁻¹·d⁻¹ was just sufficient to meet the needs of all subjects (19). We judged that this 20% difference in protein intake was sufficient to study the hypothesis (14, 15) that adaptation to a lower protein intake would result in a lower estimate of lysine requirement.

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SUBJECTS AND METHODS

Subjects

Five healthy adult male volunteers (mean age: 27 ± 5.7 y) participated in the study as outpatients in the Clinical Investigation Unit at The Hospital for Sick Children (HSC), Toronto. None of the subjects had a history of recent weight loss, unusual dietary practices, or endocrine disorders, or used medication. Subject characteristics are summarized in Table 1. The purpose of the study and the potential risks involved were explained to each subject and written consent was obtained. The subjects were financially compensated for their participation. All procedures used in the study were approved by the University of Toronto Human Experimentation Committee and the Human Subjects Review Committee of HSC.

Experimental design

The total duration of the experiment was 18 d, broken up into two 9-d dietary periods. These two periods were separated by ≥ 2 wk but ≤ 1 mo to allow for a break from the experimental diet. On days 3, 6, and 9 of each dietary period the subjects received a test intake of dietary lysine as 10, 20, 30, 40, or 60 mg · kg⁻¹ · d⁻¹ assigned in random order. Each subject was studied at all six lysine intakes, thereby ensuring sufficient test lysine intakes for individual estimates of requirement.

Diet and energy intakes

The experimental diet was based on an amino acid mixture developed for amino acid kinetic studies (20). The dietary amino acid content was calculated as g amino acid · kg⁻¹ · d⁻¹ to ensure that each subject received the same amount of nitrogen per kilogram body weight. The sole source of amino nitrogen came from a crystalline amino acid mixture based on intact egg protein, consumed at 0.8 g · kg⁻¹ · d⁻¹. The experimental diet included 14 mg phenylalanine · kg⁻¹ · d⁻¹ to ensure adequate dietary phenylalanine, as previously determined by amino acid oxidation when tyrosine was present in relative excess (9). The diet also contained generous amounts of tyrosine and lysine (40 and 60 mg · kg⁻¹ · d⁻¹, respectively). The intake of dietary phenylalanine was set at just above the 95% CI for phenylalanine requirement (9), because the indicator amino acid is most sensitive to the intake of the test amino acid (in this case lysine) when set at a level just above its requirement (8,17). Phenylalanine intakes in the present and an earlier study (18) were identical, notwithstanding the 20% differences in total protein (amino acid) intakes.

The main source of energy in the experimental diet came from a flavored liquid formula (Protein-Free Powder, product 80056; Mead Johnson, Evansville, IN) containing no amino nitrogen, with the remaining energy coming from protein-free cookies (HSC Research Kitchen). Overall, the proportions of energy in the experimental diet were ~53% carbohydrate, 37% fat, and 10% protein. The diets were prepared and weighed (model PE 2000; Mettler Scale, Greifensee, Switzerland) in the Research Kitchen of HSC and portioned into four isonitrogenous, isonitrogenous meals that were consumed daily at 0800, 1200, 1600, and 2000 on the nonstudy days. The energy intake was based on each subject’s basal metabolic rate, as calculated from a nomogram (1), multiplied by an activity factor of 1.7. This method for determining energy requirement has been shown to result in maintenance of subjects’ weight during short-term amino acid oxidation studies (18, 21). The subjects were encouraged to maintain their usual physical activity level and were provided with forms to record all activities throughout the study. The subjects were weighed (model 2020; Toledo Scale, Windsor, Canada) in the morning before each dietary period and on all experimental study days.

Body-composition measurements

Body composition was determined by using bioelectrical impedance analysis (BIA; 22) and multiple skinfold-thickness measurements. This was done to monitor any changes in body composition resulting from the experimental diet and to relate body composition to lysine requirement. BIA was performed once at the beginning of each study period and before meal 1 on each infusion study day. Reactance (R) and resistance (Xc) measurements were made by using a four-terminal bioimpedance analyzer (model 101A; RJL Systems, Detroit). As previously described, three readings for both R and Xc (in Ω) were taken for each subject and equations were used to predict lean body mass (LBM; 23).

Multiple skinfold-thickness measurements were taken both before and on completion of each study period. Skinfold-thickness measurements were taken at four sites including the triceps, biceps, suprailium, and subscapula on the subject’s nondominant side. All measurements were performed by the same individual and taken to the nearest 0.1 mm by using a skinfold caliper (British Indicators, Ltd., St Albans, United

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>Energy intake (MJ/d)</th>
<th>LBM (kg)</th>
<th>FFM (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>186.0</td>
<td>83.3 ± 0.1</td>
<td>24.1 ± 0.3</td>
<td>14.0</td>
<td>62.8 ± 0.7</td>
<td>63.5 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>174.8</td>
<td>84.0 ± 1.0</td>
<td>27.5 ± 0.3</td>
<td>13.7</td>
<td>61.3 ± 0.7</td>
<td>61.7 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>185.5</td>
<td>78.4 ± 1.4</td>
<td>22.1 ± 0.4</td>
<td>12.9</td>
<td>64.9 ± 1.4</td>
<td>64.7 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>168.9</td>
<td>73.5 ± 0.4</td>
<td>25.8 ± 0.1</td>
<td>12.9</td>
<td>55.6 ± 0.3</td>
<td>55.1 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>170.7</td>
<td>78.6 ± 0.2</td>
<td>26.9 ± 0.1</td>
<td>13.4</td>
<td>63.2 ± 0.5</td>
<td>62.4 ± 0.8</td>
</tr>
<tr>
<td>x ± SD</td>
<td>27 ± 5.7</td>
<td>177.8 ± 9.0</td>
<td>79.6 ± 4.3</td>
<td>25.3 ± 2.2</td>
<td>13.4 ± 0.5</td>
<td>61.6 ± 3.6</td>
<td>61.5 ± 3.7</td>
</tr>
</tbody>
</table>

1 x ± SD over two 9-d dietary periods.
2 Calculated with 1985 FAO/WHO/UNU BMR equation with an activity factor of 1.7 (1).
3 Lean body mass determined from bioelectrical impedance analysis.
4 Fat-free mass determined from skinfold thicknesses.

TABLE 1

Subject characteristics

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Density was derived from the sum of the four skinfold thicknesses and an equation was used to predict fat-free mass (FFM; 24).

Isotope infusion studies

The stable isotopes used in this study included NaH\(^{13}\)CO\(_3\) with an enrichment of 99 atom percent (Merck, Sharp and Dohme, Montreal) and L-\([\text{1-}^{13}\text{C}]\)phenylalanine with an enrichment of 99 atom percent (Tracer Technologies, Somerville, MA). Optical rotation tests with a chiral column confirmed the absence of the \(\delta\)-isomer. The isotopes were sterilized by passage through a 0.22-\(\mu\)m filter (Millipore Corporation, Bedford, MA) under a laminar flow hood and then dispensed into single-dose vials in the pharmacy department at HSC. The solutions then underwent a bacteriologic test over 7 d in culture to ensure no bacterial growth. The Limulus Amebocyte Lysate (LAL) test was also performed to verify that the solutions were pyrogen-free (25). Infusions were conducted via a 21-gauge butterfly needle (Venisystems, Abbott Ireland Ltd, Dublin) inserted into the antecubital fossa vein of the subject’s left arm by using aseptic sterile procedures. Each subject received a priming dose of NaH\(^{13}\)CO\(_3\) (0.099 mg/kg) and a priming dose of L-\([\text{1-}^{13}\text{C}]\)phenylalanine (0.664 mg/kg) as described previously (18). A constant infusion of L-\([\text{1-}^{13}\text{C}]\)phenylalanine (1.2 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) h\(^{-1}\)) was then conducted for 4 h by using a calibrated syringe pump (IVAC Syringe Pump 700 Series; Eli Lilly Inc, San Diego).

A total of 30 flux and oxidation studies were carried out; each of the five subjects underwent six IAAO studies. On each study day (days 3, 6, and 9), the subjects were maintained in a temperature-controlled metabolic study facility at HSC. The 0800 and 1200 meals were divided into six equal portions, which the subject consumed hourly beginning 2 h before infusion of the isotope. This allowed a metabolic steady state to be achieved (20). The amount of dietary phenylalanine in the last four meals was reduced by an amount that corresponded to the amount of L-\([\text{1-}^{13}\text{C}]\)phenylalanine administered during the tracer infusion. Because lysine intake varied in the diet with each different test intake, L-alanine was used to keep the total amino acid content constant. On the study days, the test lysine intake and the nonlabeled phenylalanine were given to the subjects separately, thereby allowing the intake to be known precisely.

Three baseline samples of blood and breath were taken before the isotope infusion was initiated. Once the isotope infusion was started blood and breath samples were taken every 30 min. Blood was collected from a 21-gauge needle inserted into a superficial dorsal vein in the right hand. The line was kept patent by administering heparin between blood samplings. To arterialize the venous blood, the hand was heated inside a thermostatic chamber maintained at 60 °C for \(\approx\) 15 min before the blood was sampled (26). Three milliliters of the arterialized venous blood was drawn into heparinized syringes (Aspirator; Marquest Medical Products, Englewood, CO). The blood samples were kept on ice until centrifugation at 400 \(\times\) g for 5 min at 4 °C. The plasma was then frozen at −20 °C until analyzed.

Seven-minute breath samples were collected every 30 min during the 4-h infusion. While maintaining a reclined position, the subject breathed normally while wearing a ventilated face mask (Scott 802167–30; Sensormedics, Anaheim, CA). Once the subject’s air flow had stabilized (carbon dioxide between 0.5% and 0.8%), the breath was collected by using a vacuum extraction system (Pump VB0025, vortex blower; Spencer Turbine Company, Windsor, CT) combined with a gas flow meter. To trap the respiratory carbon dioxide the breath was bubbled at a rate of 500 mL/min through 10 mL 1 mol NaOH/L solution in a modified reflux condenser for 7 min. The resulting NaH\(^{13}\)CO\(_3\) solution was then injected (Monoject; Sherwood Medical, St Louis) into labeled evacuated glass tubes (Vacutainer Brand 6441, 100 \(\times\) 16 mm; Becton Dickinson Inc, Mississauga, Canada). The tubes were evacuated of any air introduced during the injection with the syringe and frozen at −20 °C until analyzed. To determine carbon dioxide volume, the carbon dioxide analyzer (Beckman Medical Gas Analyzer LB-2; Beckman, Fullerton, CA) was calibrated with a standardized gas (nitrogen; Linde Medical Gas, Union Carbide, Toronto) and a span gas (0.53% carbon dioxide, 20.88% oxygen, Linde Medical Gas). Barometric pressure and temperature were entered into the computer to ensure that carbon dioxide volumes were obtained at standard temperature and pressure. Carbon dioxide volume measurements were taken simultaneously with the breath samples: three baseline samples were taken before the isotope infusion and samples were taken every 30 min throughout the isotope infusion.

Analytic methods

The enrichment of \(^{13}\)C in breath carbon dioxide was measured on a dual-inlet isotope-ratio mass spectrometer (Micromass 602D; Vacuum Generator, Altringham, United Kingdom) by using techniques described by Jones et al (27). Breath \(^{13}\)CO\(_2\) enrichments were expressed as atoms percent excess (APE) over a reference standard of compressed carbon dioxide gas.

The isotope enrichment values for plasma free \([\text{1-}^{13}\text{C}]\)phenylalanine were measured by gas chromatography–selected ion monitoring–negative chemical ionization–mass spectrometry (model 5890, series II GC; Hewlett Packard, Mississauga, Canada; Trio-2 quadrupole MS system; Vacuum Generator). Amino acids in 100 \(\mu\)L plasma were derivatized according to the method described by Patterson et al (28) to their \(N\)-heptafluorobutyl \(n\)-propyl esters. Selected ion chromatographs were obtained by monitoring the mass-to-charge ratio of 383 and 384 for \([\text{1-}^{13}\text{C}]\)phenylalanine corresponding to the unenriched (M) and enriched (M+1) peaks, respectively. The areas under the peaks were integrated by a Digital DECp 450,LP computer (Digital Instruments, Santa Barbara, CA) using the LAB-BASE program (Vacuum Generator Biotech).

Estimation of isotope kinetics

The model used to study phenylalanine metabolism was a stochastic model used by others (6, 9, 18), which uses a constant infusion approach to study amino acid oxidation. Isotopic steady state in the metabolic pool was represented by plateaus in \([\text{1-}^{13}\text{C}]\)phenylalanine in plasma and \(^{13}\)CO\(_2\) in breath. A plateau was defined as a CV < 5% and absence of a significant slope. This was achieved in plasma \([\text{1-}^{13}\text{C}]\)phenylalanine and breath \(^{13}\)CO\(_2\) by 120 min from the start of the isotope infusion and was maintained to the end of the study at 240 min. The mean breath isotope enrichment values of the three baseline samples and the five plateau samples were used to determine APE above baseline at isotopic steady state. The mean ratio of the enriched peak (M+1) to the unenriched peak (M)
for both baseline and plateau samples was used to calculate molecules percent excess (MPE).

Flux was measured from the dilution of the L-[1-13]Cphenylalanine infused in the plasma metabolic pool once isotopic steady state was reached by using the following equation (29):

\[ Q = \bar{I}(E/E_0 - 1) \quad (I) \]

where \( i \) is the rate of \([1\text{C}]\)phenylalanine infused (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)), \( E \) is the enrichment of \([1\text{C}]\)phenylalanine infused (MPE), and \( E_0 \) is the enrichment of plasma phenylalanine at isotopic plateau (MPE). The \(-1\) removes the contribution of the infusion to the flux.

The rate of \(^{13}\text{CO}_2\) released by phenylalanine tracer oxidation (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)) was calculated by the following equation (29):

\[ F^{13}\text{CO}_2 = (FCO_2)(ECO_2)(44.6)(60)(W)(0.82)(100) \quad (2) \]

where \( FCO_2 \) is the carbon dioxide production rate (\( \text{cm}^3/\text{min} \)), \( ECO_2 \) is the \(^{13}\text{CO}_2\) enrichment in expired breath at isotopic steady state (APE), and \( W \) is the weight of the subject (kg). The constants 44.6 \( \mu \text{mol/cm}^3 \) and 60 \( \text{min/h} \) convert \( FCO_2 \) to \( \mu \text{mol/h} \) and the factor 100 changed APE to a fraction. The factor 0.82 accounts for \(^{13}\text{CO}_2\) retained in the body because of bicarbonate fixation (30).

The rate of phenylalanine oxidation (\( O \)) (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)) was calculated as follows:

\[ O = F^{13}\text{CO}_2(1/E_0 - 1/E_p) \times 100 \quad (3) \]

where the second term eliminates the contribution of the oxidation of the \([1\text{C}]\)phenylalanine tracer to the phenylalanine oxidation rate.

**Data analysis**

A three-factor general linear model (GLM) (31) was used to assess the relation of \( F^{13}\text{CO}_2\), phenylalanine flux, and phenylalanine oxidation to the following experimental variables: 1) lysine test intakes, 2) order of lysine test intakes, 3) subject, and 4) interactions. Nonsignificant interactions (\( P \geq 0.1 \)) were dropped from the model. Results were considered to be statistically significant at \( P \) values \( \leq 0.05 \).

The mean requirement for lysine was determined by breakpoint analysis using a two-phase linear-regression crossover model (32) similar to the method described in animal studies using IAAO (16, 17). The 95% CIs for the mean lysine requirement were determined by using Fieller's theorem (32).

The lysine requirement for each individual subject was also estimated by visual inspection of the breakpoint from the phenylalanine oxidation curves.

An unpaired \( t \) test was conducted to compare the breakpoint obtained from this study, which used a protein intake of 0.8 g · kg\(^{-1}·\text{d}^{-1} \), and the breakpoint obtained from the study that used a protein intake of 1.0 g · kg\(^{-1}·\text{d}^{-1} \) (18). Significance was considered at a \( P \) value \( \leq 0.05 \).

LBM was calculated from the BIA measurements as described previously (23). FFM was calculated from the sum of the four skinfold-thickness measurements (24). One-factor repeated-measures analysis of variance (31) was used to assess the effect of the experimental diet on weight and LBM over the 9-d dietary period. A \( P \) value \( \leq 0.05 \) was considered significant. Correlation analysis (31) was used to evaluate the relation between the following parameters: LBM and FFM, LBM and lysine requirement, FFM and lysine requirement, and \( F^{13}\text{CO}_2 \) and LBM by lysine intake.

**RESULTS**

Subject weight, LBM, and FFM did not significantly change over the two 9-d dietary periods (Table 1), evidence that the subjects were in an isenergetic state throughout the study. Lysine intake had no significant effect on the estimate of phenylalanine flux (Table 2). Conversely, lysine intake significantly affected the release of \(^{13}\text{CO}_2\) from the indicator amino acid L-[1-13]Cphenylalanine (Table 2). Three-factor general linear modeling revealed that both subject (\( P = 0.009 \)) and lysine intake (\( P = 0.003 \)) had a significant effect on \(^{13}\text{CO}_2\). Although subjects had significant differences in their absolute rates of \(^{13}\text{CO}_2\), they all showed a similar pattern in which \(^{13}\text{CO}_2\) decreased with increasing lysine intakes to a certain point after which it remained relatively stable. The order in which lysine intakes were tested did not have a significant effect on \(^{13}\text{CO}_2 \) (\( P = 0.10 \)). The interaction between lysine intakes and the order of lysine intakes studied was also not significant (\( P = 0.19 \)).

The mean \(^{13}\text{CO}_2\) and SE of the mean for all five subjects at lysine intakes from 10 to 60 mg · kg\(^{-1}·\text{d}^{-1} \) are shown in Figure 1. Regression analysis showed that phenylalanine assimilation, measured by \(^{13}\text{CO}_2\), decreased as lysine intake increased from 10 to 40 mg · kg\(^{-1}·\text{d}^{-1} \), and remained stable at lysine intakes between 40 and 60 mg · kg\(^{-1}·\text{d}^{-1} \). Using a two-phase linear-regression crossover model, a breakpoint in

**TABLE 2**

Effect of lysine intake on phenylalanine flux and oxidation, measured by the rate of \(^{13}\text{CO}_2\) release

<table>
<thead>
<tr>
<th>Lysine intake (mg · kg(^{-1}·\text{d}^{-1} ))</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine flux (( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} ))</td>
<td>35.2 ± 8.3(^2)</td>
<td>32.6 ± 4.7</td>
<td>32.4 ± 5.0</td>
<td>43.0 ± 8.6</td>
<td>31.2 ± 1.1</td>
<td>35.0 ± 4.8</td>
</tr>
<tr>
<td>Phenylalanine oxidation (( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} ))</td>
<td>0.426 ± 0.05(^p)</td>
<td>0.392 ± 0.05(^p)</td>
<td>0.350 ± 0.07(^p)</td>
<td>0.299 ± 0.08(^p)</td>
<td>0.293 ± 0.04(^p)</td>
<td>0.292 ± 0.06(^p)</td>
</tr>
<tr>
<td>Correlation coefficient (( r^2 ))</td>
<td>0.41</td>
<td>0.36</td>
<td>0.55</td>
<td>0.26</td>
<td>0.43</td>
<td>0.90</td>
</tr>
<tr>
<td>( P )</td>
<td>0.25</td>
<td>0.28</td>
<td>0.16</td>
<td>0.38</td>
<td>0.23</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\(^1\) Lysine intake had no significant effect on phenylalanine flux, but did have a significant effect on the rate of \(^{13}\text{CO}_2\) release from oxidation of L-[1-13]Cphenylalanine (\( P = 0.003 \)). Means with different superscript letters are significantly different, \( P = 0.05 \). Only at a lysine intake of 60 mg · kg\(^{-1}·\text{d}^{-1} \) was there a significant correlation between lean body mass and \(^{13}\text{CO}_2\).

\(^2\) \( x \) ± SD.
the $F^{13}CO_2$ response curve occurred at a dietary lysine intake of 45.0 mg·kg$^{-1}$·d$^{-1}$ (95% CIs: 23.0, 67.0). Given the fact that every subject was studied at all six dietary lysine intakes, individual requirements were estimated from visual inspection of the $F^{13}CO_2$ curves. Individual requirements varied from 30 to 50 mg lysine·kg$^{-1}$·d$^{-1}$ for all five subjects studied. Phenylalanine oxidation data showed the same pattern; however, because of the greater variability of plasma phenylalanine enrichment at a lysine intake of 40 mg·kg$^{-1}$·d$^{-1}$, it was not possible to calculate a breakpoint. A comparison of the pattern of phenylalanine catabolism, measured by $^{13}CO_2$, recovery in the subjects in the present study (with a protein intake of 0.8 g·kg$^{-1}$·d$^{-1}$), with that from our earlier study, when the subjects received a protein intake of 1.0 g·kg$^{-1}$·d$^{-1}$ is shown in Figure 2. The estimates of lysine requirement as defined by the breakpoint of 45.0 mg lysine·kg$^{-1}$·d$^{-1}$ for the present study and 41.2 mg lysine·kg$^{-1}$·d$^{-1}$ in the subjects receiving the higher protein intake, were not different.

No significant correlation was found between the lysine requirement estimates for the individual subjects and their LBM ($r^2 = 0.09, P = 0.61$). The relation between $F^{13}CO_2$ and LBM at each of the six lysine intakes studied is shown in Table 2. For lysine intakes of 10–50 mg·kg$^{-1}$·d$^{-1}$ there was no significant relation found between $F^{13}CO_2$ and LBM. However, at a lysine intake of 60 mg·kg$^{-1}$·d$^{-1}$ there was a significant correlation found between $F^{13}CO_2$ and LBM ($r^2 = 0.90, P = 0.01$), as illustrated in Figure 3.

**DISCUSSION**

This study explored the postulated limitation of IAAO that the amount of protein in the diet would affect the amino acid

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**FIGURE 1.** Effect of lysine intake on the production of labeled carbon dioxide in breath ($F^{13}CO_2$). $\bar{x} \pm$ SEM for $n = 5$ subjects at each of the six lysine intakes. Also shown is the breakpoint value determined by two-phase linear crossover (see text for details).

**FIGURE 2.** Comparison of the effect of lysine intake on production of labeled carbon dioxide ($F^{13}CO_2$) in subjects consuming diets containing a protein content of either 0.8 g ($n = 5$) or 1.0 g·kg$^{-1}$·d$^{-1}$ ($n = 7$); data derived from reference 18.
requirement estimate (14, 15). The results revealed that this hypothesis is invalid over the range of protein intakes studied; i.e., near the requirement (0.8 mg/kg·d\(^{-1}\)) and 25% above the requirement (1.0 mg/kg·d\(^{-1}\)).

We showed earlier in a DAAO study (determining phenylalanine requirement), that prior adaptation for 2, 5, or 8 d to the amount of the test amino acid did not affect the requirement estimate (9). Similarly, in our IAAO studies we showed that the order in which the test amino acid was administered did not affect the oxidation estimate (8, 18). Thus, the oxidation estimate for a particular amount of the test amino acid was the same whether the subject was studied on day 3, 6, or 9 of the experimental diet. Nevertheless, it still remains to be determined whether subjects would show differences if they were adapted to lower intakes for a period of several weeks. Clearly, this needs to be studied in the future.

Other investigators have studied the question of whether shorter-term oxidation studies, such as were used in this study, or the earlier DAAO study of lysine requirement (13), are a valid representation of 24-h utilization, and have shown that these shorter-term studies are valid at least for leucine (33, 34). We also studied leucine oxidation in the fed compared with the fasted state (19) and showed that in the fasted state, over the range of protein intakes studied (0.6–1.0 mg/kg·d\(^{-1}\)), there were no differences in the oxidation of leucine. Conversely, leucine oxidation in the fed state rose in comparison with the rate determined in the fasted state between protein intakes of 0.6 and 0.8 mg/kg·d\(^{-1}\) (19). Clearly, it is highly desirable that similar 24-h and/or fed compared with fasting oxidation studies also be conducted with lysine to confirm that it is sufficient to only conduct amino acid requirement estimate studies in the fed state.

It is important to note that we have not studied subjects consuming a deficient amount of protein. Instead, we chose the low protein intake of 0.8 mg/kg·d\(^{-1}\), based on our earlier study in which we used the difference in leucine oxidation in the fed as compared with the fasted state (19). It was the lowest protein intake for which there was an increase in leucine oxidation during feeding. Furthermore, at a protein intake of 0.6 mg/kg·d\(^{-1}\), not all of the subjects were in nitrogen balance, whereas they all were at an intake of 0.8 mg/kg·d\(^{-1}\). Although it is true that we have not studied intermediate protein intakes, the current FAO/WHO/UNU safe estimate for protein is 0.75 mg/kg·d\(^{-1}\) (1). Hence, we thought that for a study based on amino acid oxidation it was appropriate to choose a lower protein intake based on amino acid oxidation criteria, especially because it was close to the nitrogen-balance based estimate. However, this study is a first step. It is a matter of some urgency to study the effects of adaptation to a lower and hence limiting protein intake.

Because each subject was studied at all six lysine test intakes, it was possible to estimate individual lysine requirements by visual inspection. Although \(^13\)CO\(_2\) was subject-dependent across lysine intakes, the overall pattern of \(^13\)CO\(_2\) was similar, such that a breakpoint in the curve was apparent. The range in lysine requirement for the five subjects studied was 30–50 mg/kg·d\(^{-1}\). Clearly, there was a large interindividual variation despite the homogeneous population and controlled experimental conditions. Zello et al (18) also found lysine requirement to be subject-dependent with a range of 30–50 mg/kg·d\(^{-1}\) across seven subjects. The fact that Zello et al (18) examined seven subjects and the current study examined five subjects increases the database for lysine requirements of young adult males. With sufficient data, it may be that a normal distribution would be attained, allowing a valuable comparison to be made with the results of individual studies. Assuming that additional data would result in a normal distribution, the population mean would be ±35–40 mg lysine·kg\(^{-1}\)·d\(^{-1}\) and the safe intake (\(x \pm 2 SD\)) would be ±60 mg lysine·kg\(^{-1}\)·d\(^{-1}\). This is similar to the mean lysine requirements of 41.2 and 45.0 mg/kg·d\(^{-1}\) and the safe lysine requirements of 59.6 and 67.0 mg/kg·d\(^{-1}\) obtained by Zello et al (18) and the current study. All of these estimates were obtained by using the IAAO method, which measures net incorporation of the test amino acid (in this case lysine) for protein synthesis (8). Therefore, the range in lysine requirement estimates has to be due to differences between individuals in their oxidation of lysine. Currently, it is not understood why individuals differ in their rate of lysine oxidation; we suspect that the differences are probably genetic and deserve future study. It is well known that individuals vary significantly in their nutrient requirements (35).

In the present study, lysine requirement was determined from the rate of \(^13\)CO\(_2\) release in breath from oxidation of the tracer \(\text{L-}\{1,13\text{C}\}\text{phenylalanine. This is supported by Zello et al (18), who showed that it was possible to estimate amino acid requirements by using IAAO without sampling the plasma pool. They found close agreement between breakpoints derived from \(^13\)CO\(_2\) data and phenylalanine oxidation data. Use of labeled carbon dioxide in breath as the measure of requirement was also validated in our earlier animal IAAO studies (16, 17). We do not have a complete explanation for the high variability in plasma phenylalanine enrichment at a lysine intake of 40 mg/kg·d\(^{-1}\), but have consistently noted higher variability between individual subjects in the metabolism of the indicator amino acid around the requirement breakpoint.}
Assessment of body composition also allowed for the possibility to express lysine requirement per kilogram of LBM and FFM. In the healthy population, LBM can vary among individuals of the same weight (36). Therefore, expressing amino acid requirement data on a per kilogram LBM basis for the entire population would be more precise than doing so on a body weight basis. Furthermore, because there are differences in body composition at the same body weight across sex, population groups, and in various disease states, expressing amino acid requirement per kilogram LBM would make comparisons more feasible.

A higher LBM is associated with a higher need for amino acids in animals (37). Clark et al (38) found a significant relation between lysine need and body surface area in humans. However, in the present study significant correlations were not found between LBM and individual lysine requirement. The small number of subjects \(n = 5\) and narrow range in LBM (55.6–64.9 kg), may not have been sufficient to allow a statistical relation to be evident. This hypothesis needs to be explored further.

Of interest was the relation between LBM and \(F^{13}\)CO\(_2\) at each lysine intake (Table 2). No significant relation was found below a lysine intake of 60 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\). At a lysine intake of 60 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\), however, a very significant correlation between \(F^{13}\)CO\(_2\) and LBM was observed \((r^2 = 0.90, P = 0.01)\) (Figure 3). This observation suggests that at a lysine intake of 60 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\), the differences in \(F^{13}\)CO\(_2\) among the five subjects were mainly due to differences in LBM. The fact that there were no relations seen below 60 mg lysine \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) may be explained by the fact that all subjects had not yet reached their individual lysine requirement. At a lysine intake of 60 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\), however, all subjects were receiving excess lysine and were oxidizing phenylalanine at their basal rate, allowing the relation to be shown. This result is consistent with our estimate for a safe lysine intake of \(\approx 60\) mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\).

The lysine requirement obtained in this IAAO study substantiates the contention that the current IDAA requirement estimates as published in the FAO/WHO/UNU report are too low (1). When compared with the current safe nitrogen balance estimate of 12 mg lysine \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\), the mean lysine requirement estimate of 45.0 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) found in this study is more than three times higher. Moreover, the lysine requirement calculated for 95% of the population of 67.0 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) is more than five times higher. Similar differences were shown previously in both an IAAO study (18) and a DAAO study (13) of lysine requirement with a similar population of young adult males. The fact that there is such clear reproducibility within this population group cannot be easily ignored. In a recent publication, we reviewed the newer methods for assessing dietary amino acid requirements (8); we refer the reader to this review for a more detailed consideration of methods for determining IDAA requirements.

The significance of a change in the current estimate of lysine requirement could have implications for the normal, healthy adult population. This is because the amount of lysine consumed daily is \(\approx 60\) mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) (39). Although this intake is much higher than the old nitrogen balance requirement estimate, it is similar to the safe requirement estimate determined by current oxidation studies (6). Therefore, lysine intakes may not be in considerable excess of requirements, as was assumed previously. Assuming a normal distribution in lysine requirement and lysine intake, advocating lower protein or amino acid intakes could place a portion of the population at risk of deficiency (35). Beaton (35) discusses this issue of the criteria of an adequate diet and the risks of deficiency. We conclude that the amount of protein in the diet within the range of 0.8–1.0 g \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) does not alter lysine utilization for protein synthesis and therefore does not affect the requirement estimate as determined by IAAO.

REFERENCES