Total branched-chain amino acids requirement in patients with maple syrup urine disease by use of indicator amino acid oxidation with L-[1,13C]phenylalanine

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Riazi, Roya, Mahroukh Rafii, Joe T. R. Clarke, Linda J. Wykes, Ronald O. Ball, and Paul B. Pencharz. Total branched-chain amino acids requirement in patients with maple syrup urine disease by use of indicator amino acid oxidation with l-[1,13C]phenylalanine. Am J Physiol Endocrinol Metab 287: E142–E149, 2004.—Maple syrup urine disease (MSUD) is an autosomal recessive disorder caused by defects in the mitochondrial multienzyme complex branched-chain α-keto acid dehydrogenase (BCKD; EC 1.2.4.4), responsible for the oxidative decarboxylation of the branched-chain ketoacids (BCKA) derived from the branched-chain amino acids (BCAA) leucine, valine, and isoleucine. Deficiency of the enzyme results in increased concentrations of the BCAA and BCKA in body cells and fluids. The treatment of the disease is aimed at keeping the concentration of BCAA below the toxic concentrations, primarily by dietary restriction of BCAA intake. The objective of this study was to determine the total BCAA requirements of patients with classical MSUD caused by marked deficiency of BCKD by use of the indicator amino acid oxidation (IAAO) technique. Five MSUD patients from the MSUD clinic of The Hospital for Sick Children participated in the study. Each was randomly assigned to different intakes of BCAA mixture (0, 20, 30, 50, 60, 70, 90, 110, and 130 mg·kg⁻¹·day⁻¹), in which the relative proportion of BCAA was the same as that in egg protein. Total BCAA requirement was determined by measuring the oxidation of l-[1,13C]phenylalanine to 13CO₂. The mean total BCAA requirement was estimated using a two-phase linear regression cross-over analysis, which showed that the mean total BCAA requirement was 45 mg·kg⁻¹·day⁻¹, with the safe level of intake (upper 95% confidence interval) at 62 mg·kg⁻¹·day⁻¹. This is the first time BCAA requirements in patients with MSUD have been determined directly.

indicator amino acid oxidation; amino acid requirements

MAPLE SYRUP URINE DISEASE (MSUD) is an autosomal recessive disorder, first described by Menkes et al. (24) in 1954. MSUD is caused by deficiency of multienzyme complex branched-chain α-keto acid dehydrogenase (BCKD; EC 1.2.4.4), responsible for the oxidative decarboxylation of the keto acids derived from the branched-chain amino acids (BCAA) leucine, valine, and isoleucine. The defect results in neurotoxic accumulation of leucine, valine, isoleucine, and their respective α-keto acids (α-ketoisocaproatate, α-ketoisovalerate, and α-keto-β-methylvalerate) in cells and body fluids (11, 28).

The BCAA account for 40% of the essential amino acids in healthy subjects. The main metabolic fate of dietary BCAA is incorporation into body protein. The first step in the catabolism of these amino acids is a reversible transamination to the corresponding keto acids, followed by irreversible oxidative decarboxylation by BCKD to the corresponding α-ketoacyl-CoA (6, 17, 23).

MSUD presents a heterogenous clinical phenotype. The disease ranges from severe classic forms to mild variant types and is usually classified by an indirect parameter, such as residual BCKD enzyme activity, onset of the symptoms, and dietary leucine tolerance (11, 28). Long-term therapy is based on dietary restriction of BCAA intake designed to maintain plasma levels within a range (11, 15, 31) that is accepted to be nontoxic and supports optimal growth and development (11, 15, 31). Determination of the daily requirements of BCAA in the face of marked deficiency of oxidative decarboxylation is generally determined indirectly by monitoring the effect of dietary treatment on growth (11, 15). BCAA requirements in patients with MSUD have never before been measured directly.

The indicator amino acid oxidation (IAAO) technique provides a functional approach for determining indispensable amino acid requirements. It is a safe and noninvasive method that can be used in vulnerable groups, such as subjects with inborn error of metabolism [e.g., phenylketonuria (PKU) and MSUD]. Recently this technique has been used to determine the requirement for tyrosine (4) and phenylalanine (10) in children with the classical form of PKU. The method involves feeding the subjects the amino acid of interest at intakes below and above the predicted requirement (breakpoint) (10, 20, 38). The requirement for any dietary indispensable amino acid is the sum of the total of that amino acid needed for protein synthesis and irreversible losses, of which the main part is obligatory oxidation (16). Because in classic forms of the disease the activity of the oxidation pathway is almost negligible (7, 11), the total BCAA requirement for patients with the classical form of MSUD would be expected to be less than the total BCAA requirement for healthy adults by an amount equal to the obligatory oxidative losses of the amino acids.

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We have previously determined the estimated average requirement of total BCAA by IAAO technique in seven healthy adult men to be 144 mg·kg⁻¹·day⁻¹ (27). On the basis of the proportion of each individual BCAA in the mixture used, which was patterned after egg protein, the leucine was estimated to be 55 mg·kg⁻¹·day⁻¹, valine, 47 mg·kg⁻¹·day⁻¹, and isoleucine, 42 mg·kg⁻¹·day⁻¹. Zello et al. (39) showed that when a subject was given 1 g protein·kg body wt⁻¹, day⁻¹, the oxidation of leucine was estimated to be 41% of the intake of the amino acid. There are no studies that estimated obligatory oxidation for the other BCAA in healthy subjects. We assumed that the estimate of obligatory oxidation for leucine in healthy subjects in this study was also applicable to all BCAA. Deducing the percentage (41%) of oxidation estimated in healthy subjects from the total BCAA requirement determined for healthy adults from our previous study, the total BCAA requirement in patients with MSUD can be predicted to be 85 mg·kg⁻¹·day⁻¹. The objective of this study was to determine directly, by use of the IAAO technique, what the total BCAA requirements are in patients with classical MSUD.

METHODS

IAAO is a functional method based on the concept that the amount of the limiting amino acid governs the partition of any indispensable amino acids between retention for protein synthesis and oxidation. When an indispensable amino acid is limiting in the diet for protein synthesis, all of the other amino acids are in relative excess and therefore must be oxidized (40). As the dietary level of the limiting amino acid is increased in graded amounts, the uptake of the other amino acids for protein synthesis increases, and the oxidation decreases. The decrease in oxidation continues until the requirements are met, after which further increase of the limiting amino acid (or test amino acid) will have no effect on the uptake of the other indispensable amino acids for protein synthesis or oxidation (37).

In the current study, the indicator amino acid used was L-[1-¹³C]phenylalanine, and our test amino acid was a mixture of the three BCAA.

Study subjects. Five MSUD patients (mean age ± SD = 20.8 ± 6.1) from the MSUD clinic of The Hospital for Sick Children (HSC) participated in the study. All subjects were studied on an outpatient basis. Each subject was selected for the study on the basis of the following criteria: early clinical onset of the disease (<1 mo of age), a plasma leucine concentration of 200 μM on an unrestricted diet, good general health, treatment with BCAA-restricted diets from early infancy, and willingness to participate in the study. Subjects were excluded if they had recent illness, an IQ <80, or history of recent significant weight changes (>10% body wt). Subject characteristics are summarized in Table 1. The purpose of the study, study procedures, benefits, and potential risks were explained to the study participants, and written consent was obtained. All procedures used in the study were approved by the Research Ethics Board of the Hospital for Sick Children.

**Study design.** The study design was based on the IAAO noninvasive technique model of Bross et al. (5). Each subject was studied at each of seven total BCAA intakes, in random order, on 7 nonconsecutive days. The diet provided 1 g protein·kg⁻¹·day⁻¹. The proportion of BCAA in the mixture was based on the proportion of the BCAA in egg protein. Initially, subjects received dietary intake levels of the BCAA mixture of 0, 30, 50, 70, 90, 110, and 130 mg·kg⁻¹·day⁻¹. However, analysis of ¹³CO₂ enrichment in breath samples and the BCAA concentrations in plasma (especially leucine) that were obtained from some of our subjects suggested a need to add intake levels of 20 and 60 mg·kg⁻¹·day⁻¹ for three of our subjects and to delete the high levels of 110 and 130 mg·kg⁻¹·day⁻¹. Accordingly, the design was adjusted so that all subjects were studied at seven levels of intake, with the exception of our subject 1, who was studied at nine levels of intake. The distribution of the levels of intake among the subjects is shown in Table 2. The study day protocol was also depicted in Fig. 1.

To avoid the effect of the dietary BCAA intake levels on BCAA plasma concentrations (especially in case of higher intake levels), patients were studied with a 5- to 7-day interval period between study days. Our previous IAAO studies showed that the carryover effect of the isotope will not affect the background enrichment after 2 days (37), so the minimum interval level was 3 days; therefore, the minimum interval between the studies could be 3 days.

**Experimental diet.** The energy intake for subjects was determined by open-circuit indirect calorimetry (Sensormedics 2900; Sensormedics, Anaheim, CA) to measure their resting metabolic rate after a 5-h fast. The resting metabolic rate was multiplied by an activity factor of 1.5 to ensure weight maintenance for short-term amino acid oxidation studies (Table 1) (2, 38).

The main source of energy in the experimental diet was a flavored liquid protein-free formula (Protein-Free Powder, product 80056; Mead Johnson, Evansville, IN) and protein-free cooked food. The protein content of the experimental diet was provided in the form of an amino acid mixture developed specifically for amino acid kinetic studies (36). The experimental diet provided 53% of total energy as carbohydrate, 10% as protein, and 37% as fat, with the protein-free formula providing 65% and the cookies ~25% of total daily energy intake. All of the diets were prepared and weighed (scale model PE2000; Mettler, Nanikon, Switzerland) in the HSC research kitchen and were dispensed in the form of nine hourly isonitrogenous, isocaloric meals. The study protocol on each IAAO is depicted in Fig. 1.

Nitrogen content of the diet was made up of 1 g protein·kg⁻¹·day⁻¹ and was provided as an l-amino acid mixture based on egg protein. The experimental diet included 15 mg·kg⁻¹·day⁻¹ of phenylalanine to ensure adequate dietary phenylalanine, as previously determined by amino acid oxidation studies with tyrosine present in

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**Table 1. Characteristics and energy intakes of patients who participated in the study**

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Gender</th>
<th>Age, yr</th>
<th>Weight, kg</th>
<th>Height, cm</th>
<th>BMI, kg/m²</th>
<th>RMR, kcal</th>
<th>Intake, kcal</th>
<th>FFM, kg</th>
<th>LBM, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>20</td>
<td>48</td>
<td>155.6</td>
<td>19.8</td>
<td>1,389</td>
<td>2,083</td>
<td>33.8</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>14</td>
<td>46.8</td>
<td>164.8</td>
<td>17.2</td>
<td>1,645</td>
<td>2,796</td>
<td>39.7</td>
<td>40.5</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>16</td>
<td>53</td>
<td>146.5</td>
<td>24.7</td>
<td>1,338</td>
<td>2,007</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>27</td>
<td>50.3</td>
<td>158</td>
<td>20.1</td>
<td>1,500</td>
<td>2,550</td>
<td>42.3</td>
<td>40.4</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>27</td>
<td>52.5</td>
<td>152.5</td>
<td>22.6</td>
<td>1,310</td>
<td>1,965</td>
<td>30.7</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SD

20.8 ± 6.1  50.1 ± 2.7  155.6 ± 6.8  20.9 ± 2.9  1,437 ± 138.5  2,280 ± 371.4  38.6 ± 4.4  34.8 ± 5.2

BMI, body mass index; RMR, resting metabolic rate, measured by indirect calorimetry; Intake, calculated as RMR × 1.7 or 1.5; FFM, fat-free mass, determined from the sum of skinfold thicknesses (12); LBM, lean body mass, determined from bioelectrical impedance analysis (21). *We were not able to measure skinfold thickness of 2 subjects due to technical difficulties.

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relative excess (37). The diet contained a generous amount of tyrosine (40 mg·kg⁻¹·day⁻¹).

The BCAA mixture used to provide BCAA in experimental diets was based on the proportion of these amino acids in egg protein: 38.5, 32.5, and 29% for leucine, valine, and isoleucine, respectively. Serine and glycine were given to ensure that the meals were isonitrogenous at different total BCAA intakes. Each meal contained a bottle of flavored protein-free formula to which the amino acid mixture was added, plus two protein-free cookies.

**Body composition measurements.** Subjects were weighed on a balance scale (model 2020; Toledo Scale, Windsor, ON, Canada) to the nearest 0.1 kg after they had voided on the morning of the prestudy day and on all experimental study days. Standing heights were measured to the nearest 0.1 cm with the wall-mounted stadiometer on the prestudy day.

Body composition (fat and fat-free mass) was determined in the fasted state on a prestudy day by use of skinfold thickness and bioelectrical impedance analysis (BIA). Multiple skinfold thickness measurements (to the nearest 1 mm) were made from four different sites (triceps, biceps, subscapular, and suprailiac) on the subject’s nondominant side before the study day. Each measurement was made by the same individual. A total of three measurements from each site was made with a Harpenden calliper (British Indicators; St. Albans, UK), and the average value was used in the equation. Body density was derived from the sum of the four skinfolds by use of an age-specific equation (12), and another equation was used to predict the percentage of body fat from body density (30).

BIA (1, 21, 26) was performed with a fixed-frequency analyzer (50 KHz; model 101A; RJL Systems, Detroit, MI). Resistance (R) and reactance (Xc) measurements were made with a four-terminal BIA analyzer while the subject lay in a supine position on a hospital bed with all four limbs apart. Three readings of both R and Xc (in Ω) were taken for each subject, and the mean of the three readings was used to determine the lean body mass (LBM) (21).

**Tracer protocol.** The stable isotopes used in these studies were NaH¹³CO₃ (Cambridge Isotope Laboratories, Woburn, MA) and L-[¹-¹³C]phenylalanine (MassTrace, Woburn, MA) with a 99 atom %. Isotopic and optical purity of L-[¹-¹³C]phenylalanine was verified by the manufacturer with gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance. We confirmed the enrichment and enantiomeric purity of the L-[¹-¹³C]phenylalanine tracer by GC-MS, and of the N-heptfluorobutyryl n-propyl ester derivative (25) with a chiral column (ChirasilVal, R symbol; Alltech Associates, Deerfield, IL). The measured fractional molar abundance of L-[¹-¹³C]phenylalanine was 97.5%. Isotope solutions were prepared in deionized water and stored at −20 °C. The subjects consumed four

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### Table 2. Individual F¹³CO₂ data at all total BCAA intake levels for all patient participants

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Total BCAA Intake, mg·kg⁻¹·day⁻¹</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td></td>
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<tr>
<td>2</td>
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<td>3</td>
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</tbody>
</table>

Mean±SD 0.576±0.08 0.481±0.16 0.400±0.14 0.339±0.07 0.399±0.007 0.319±0.08 0.332±0.12 0.328±0.06 0.386±0.18

F¹³CO₂, rate of release of ¹³CO₂ from L-[¹-¹³C]phenylalanine oxidation expressed in μmol·kg⁻¹·h⁻¹; BCAA, branched-chain amino acids.

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**Fig. 1.** Study day protocol on each indicator amino acid oxidation (IAAO) study day to determine total branched-chain amino acid (BCAA) requirement in patients with maple syrup urine disease (MSUD). Experimental diet was a liquid formula (crystalline amino acid with graded levels of total BCAA intake at 0, 20, 30, 50, 60, 70, 90, 110, and 130 mg·kg⁻¹·day⁻¹, given in random order) and protein-free cookies. Diet was provided hourly for 9 h. Each meal was isonitrogenous, isocaloric, and represented one-twelfth of each subject’s daily requirement. Priming doses of L-[¹-¹³C]phenylalanine and NaH¹³CO₃ were given at the 5th meal, and then a continuous dose of L-[¹-¹³C]phenylalanine was started and given throughout the remaining 4 h. Three baseline urine and breath samples were collected at 15, 30, and 45 min before start of isolate protocol. Five plateau urine and breath samples were collected at isotopic steady state every 30 min during the period of 150–270 min after initiation of isolate protocol. Carbon dioxide production rate (VCO₂) was measured with indirect calorimetry after 4 h of experimental diet consumption.
hourly meals beginning in the morning of the study day. With the fifth meal, the subjects were given an oral prime dose of NaH<sup>13</sup>CO<sub>3</sub> in the amount of 0.176 mg/kg body wt (2.07 μmol/kg body wt) and an oral prime dose of L-[1-<sup>13</sup>C]phenylalanine in the amount of 0.664 mg/kg body wt (3.99 μmol/kg body wt), with a constant oral dose of L-[1-<sup>13</sup>C]phenylalanine in an equal amount of 1.2 mg/kg body wt (7.23 μmol/kg body wt) thereafter with each hourly meal until the end of the study. The amount of dietary phenylalanine in the last five meals was reduced by an amount that corresponded to the amount of L-[1-<sup>13</sup>C]phenylalanine given orally during the tracer infusion, so that the total phenylalanine intake remained unchanged.

**Sample collection and analysis.** It has previously been shown in our lab that isotopic enrichment of urinary amino acids reflects enrichment of amino acids in plasma from heparinized blood (5, 32, 35). Three baseline samples of breath and urine were collected, at 60, 45, and 30 min before the isotope was given orally. A background isotopic steady state was achieved within 4 h of the start of the feeding. From 2 h after the oral infusion of the isotope, breath and urine samples were collected every 30 min during isotopic steady state. A 5-ml blood sample was also taken from each subject. A fasted blood sample was taken only once on a prestudy day, and the fed plasma samples were taken on each level and at the end of each study day to determine the profile of the amino acids in the plasma. The blood samples were kept on ice until centrifugation at 1,200 g for 10 min at 4°C. Plasma was stored at −20°C until analyzed by HPLC (Dionex Summit HPLC System; Oakville, ON, Canada). Urine samples (to be stored at −20°C) and breath samples were collected in disposable Haldene-Priestley tubes (Venoject; Terumo Medical, Elkton, MD) with a collection mechanism that allows the removal of dead-air space (13). Samples were stored at room temperature until analyzed.

The rate of CO<sub>2</sub> production (V<sub>CO2</sub>) was measured for 30 min by indirect calorimetry (Sensormedics 2900; Sensormedics; Fig. 1). Each measurement was performed while the subject lay comfortably in a supine position on a hospital bed. V<sub>CO2</sub> measurements were corrected to standard temperature and pressure.

**Analytical procedures.** The enrichment of <sup>13</sup>C in breath CO<sub>2</sub> was measured on a continuous-flow isotope ratio mass spectrometer (PDZ Europa, Cheshire, UK). Breath samples from baseline and from those taken during the period that the isotope was given were expressed in atom percent excess (APE) <sup>13</sup>CO<sub>2</sub> over a reference standard of compressed CO<sub>2</sub> gas.

Urinary L-[1-<sup>13</sup>C]phenylalanine enrichment was measured by use of a bench top triple quadrupole mass spectrometer API 4000 [Applied Biosystems (AB)/MDS SCIEX; Concord, ON, Canada] operated in negative (chiral amino acid separation) ionization mode with the TurboIonSpray ionization probe source (operated at 5,800 V and at 3,500 °C). This was coupled to an Agilent 1100 HPLC system. All components were reconstituted in 1 ml of 2 mmol/l NH<sub>4</sub>Ac, pH 4.5. The blood samples were obtained by monitoring mass-to-charge ratios of 164 and 165 for L-[1-<sup>13</sup>C]phenylalanine corresponding to the unenriched (m) and enriched (m+1) peaks, respectively. The areas under the peaks were integrated by use of the quantitation method of Analyst software (version 1.2). Isotopic enrichment was expressed as molecule % excess.

Plasma BCAA, tyrosine, and phenylalanine concentrations were determined by reverse-phase HPLC technique with a precolumn derivatization with phenylisothiocyanate (PITC) (3, 8, 9). The derivatized amino acids were subjected to LC separation on a bonded phase column that contained dimethyldecysilslyl bonded amorphous silica (3.9 × 300-mm Pico.Tag column; Waters, Milford, MA). Eluents chosen were called eluent A with 70 mmol/l sodium acetate, pH 6.50, containing 2.5% acetonitrile (vol/vol), and eluent B with 45% acetonitrile, 15% methanol, and 40% water (9). The areas under the peaks were integrated using Chromelon software (version 6.2) provided by the Dionex Summit HPLC system (Dionex, Oakville, ON, Canada).

**Isotope kinetics.** The model used to evaluate phenylalanine kinetics was described by others (34, 37, 38) as a constant infusion approach to study amino acid oxidation. The isotopic steady state in the metabolic pool was represented by plateau in free L-[1-<sup>13</sup>C]phenylalanine in urine and <sup>13</sup>CO<sub>2</sub> in breath. An isotopic plateau was defined as the absence of a significant slope and a coefficient of variation (CV) <5%. The difference between mean breath <sup>13</sup>CO<sub>2</sub> enrichments of the three baseline and five plateau samples was used to determine APE above baseline at isotopic steady state. Also, the difference between the mean breath and the mean of the <sup>13</sup>CO<sub>2</sub> enrichment of the plateau samples was used to determine m peak of phenylalanine in urine for baseline and plateau samples was expressed as mole percent excess (MPE). Phenylalanine kinetics were estimated from breath and urine enrichment data by use of standard equations (22). The equations used to calculate the flux, oxidation, and rate of release of <sup>13</sup>CO<sub>2</sub> in breath in response to phenylalanine oxidation have been described previously (38).

Phenylalanine flux (μmol·kg<sup>−1</sup>·h<sup>−1</sup>) was calculated using the equation

\[
Q = I[E(1/E_p) − 1] \tag{1}
\]

where I is the mass of the isotope, E<sub>i</sub> is the enrichment of the isotope, and E<sub>p</sub> is the enrichment of the L-[1-<sup>13</sup>C]phenylalanine at plateau. The rate of <sup>13</sup>CO<sub>2</sub> release in breath (F<sub>13</sub>CO<sub>2</sub>) from oxidation of the L-[1-<sup>13</sup>C]phenylalanine tracer (F<sup>13</sup>CO<sub>2</sub> in μmol <sup>13</sup>CO<sub>2</sub>·kg<sup>−1</sup>·h<sup>−1</sup>) was calculated as

\[
F_{13}CO_2 = (F_{13}CO_2(44.6)(60)/W)(0.82)(100) \tag{2}
\]

where F<sub>13</sub>CO<sub>2</sub> is the CO<sub>2</sub> production rate measured by indirect calorimetry, E<sub>C</sub> is the <sup>13</sup>CO<sub>2</sub> enrichment in breath at the isotopic steady state (APE), and W is the weight of the subject (kg). The constants are 44.6 to convert gas volume to moles, 60 to show time per hour, 0.82 to correct for the <sup>13</sup>CO<sub>2</sub> retained in the body in the fed state due to bicarbonate fixation (18), and 100 to convert APE to a fraction.

The rate of L-[1-<sup>13</sup>C]phenylalanine oxidation (O, μmol·kg<sup>−1</sup>·h<sup>−1</sup>) was calculated from F<sub>13</sub>CO<sub>2</sub> and urine free phenylalanine enrichment (22, 38)

\[
O = F_{13}CO_2(1/E_p - 1/E_i) \times 100 \tag{3}
\]

**Statistical analysis.** Estimation of the means and safe total BCAA intakes for patients with MSUD were derived by breakpoint analysis using a two-phase linear regression crossover model similar to a previously described method in animal and human studies that used IAAO (38). The 95% confidence interval (CI) for the mean total BCAA requirement was determined by use of Fieller’s theorem (29). The requirement for each individual was determined by visual inspection of the break point from the phenylalanine oxidation curves.

Repeated-measures analysis of variance was performed to assess the relationship of F<sub>13</sub>CO<sub>2</sub>, phenylalanine flux, phenylalanine oxidation, and plasma BCAA concentration to the variables of total BCAA intakes and subjects, followed by the Student-Newman-Keuls post hoc test, with SAS statistical software (SAS, version 8.2; SAS Institute, Cary, NC).
Institute, Cary, NC). In all cases, results were considered significant at \( P < 0.05 \).

RESULTS

Subject characteristics, body composition, and energy intake are presented in Table 1. Weight, LBM, and fat-free mass were not different during the experimental periods. The distribution of subjects across the intake levels is given in Table 2. Individual data points are also shown in Fig. 2. The effect of total BCAA intake on \( \mathrm{L} - \{\mathrm{1}^{13}\mathrm{C}\}\)phenylalanine oxidation to breath \( ^{13}\mathrm{CO}_2 \) (\( \mathrm{F}^{13}\mathrm{CO}_2 \)) is presented in Fig. 3. Visual inspection of individual break points determined total BCAA requirements to be 50, 90, 30, 20, and 30 mg·kg\(^{-1}\)·day\(^{-1}\) for subjects \( 1, 2, 3, 4, \) and \( 5 \), respectively. The breakpoint analysis resulted in the determination of a mean total BCAA requirement of 44.8 mg·kg\(^{-1}\)·day\(^{-1}\), which was determined by statistical analysis with a two-phase linear regression crossover model, and a safe-population intake of total BCAA of 62 mg·kg\(^{-1}\)·day\(^{-1}\). The effect of total BCAA intakes on phenylalanine kinetics is presented in Table 3.

The difference between fed and fasted individual plasma BCAA concentrations in response to different total BCAA intakes is given in Table 4. The relation among the mean differences between fed and fasted plasma BCAA concentrations in response to varying total BCAA intakes is also depicted in Figs. 4, 5, and 6. Correlation analysis showed that there was a direct relationship between total BCAA intake and change in (fed minus fasted) plasma BCAA concentration, with 53% of the difference in plasma BCAA concentrations between fed and fasted states being accounted for by total BCAA intake \( (r^2 = 0.53, P = 0.03) \), and that the fewest changes occurred when the total BCAA intake was \( \approx 80 \) mg·kg\(^{-1}\)·day\(^{-1}\). On the other hand, 85% of the difference in plasma valine concentration between fed and fasted states was accounted for by total BCAA intake \( (r^2 = 0.85, P = 0.0004) \), and that the fewest changes occurred when the total BCAA intake was \( \approx 60–65 \) mg·kg\(^{-1}\)·day\(^{-1}\); 90% of the difference in plasma isoleucine concentration between fed and fasted states was accounted for by total BCAA intake \( (r^2 = 0.90, P < 0.0001) \), and the fewest changes occurred when the intake of total BCAA was \( \approx 70 \) mg·kg\(^{-1}\)·day\(^{-1}\).

DISCUSSION

From the results of the present study, we estimated the average requirement of total BCAA to be 45 mg·kg\(^{-1}\)·day\(^{-1}\). On the basis of proportion of the BCAA in the mixture (i.e., egg protein proportion), the leucine requirement would be 17.3 mg·kg\(^{-1}\)·day\(^{-1}\), the valine requirement would be 14.6 mg·kg\(^{-1}\)·day\(^{-1}\), and the isoleucine requirement would be 13.1 mg·kg\(^{-1}\)·day\(^{-1}\). On the basis of the generally accepted assumption that any indispensable amino acid requirement is the amount needed for protein synthesis plus the irreversible losses resulting from obligatory oxidative losses (16), and with consideration that the obligatory leucine oxidation was estimated to be 41% in healthy subjects (39), we predicted that the mean total BCAA requirement would be 85 mg·kg\(^{-1}\)·day\(^{-1}\). This prediction was based on the assumption that the obligatory oxidation for leucine can be extrapolated to the other two BCAA. Therefore, the observed difference between what we predicted to be the total BCAA requirement and what we measured might be due to the above-mentioned extrapolation, as well as to a high degree of variability in the baseline BCAA plasma concentrations in subjects, thus resulting in an overestimation, that is, a predicted total BCAA requirement greater than the measured requirement.

For each of the five subjects, the treating dietitian had determined a prescribed level of BCAA intake primarily on the basis of his plasma BCAA concentrations. The mean prescribed BCAA intake was 36.6 mg·kg\(^{-1}\)·day\(^{-1}\). In addition, a visual estimate was obtained for the BCAA requirement for each patient on the basis of his \( \mathrm{F}^{13}\mathrm{CO}_2 \) data; the mean of these five visual breakpoints was 44 mg·kg\(^{-1}\)·day\(^{-1}\). For the group, the treating dietitian, who used plasma amino acid values, underestimated their BCAA requirements by \( \approx 17\% \). However, on an individual basis there was much higher variability, with the dietitian underestimating the BCAA needs of three of the

![Fig. 2. Effect of total BCAA intake on oxidation of \( \mathrm{L} - \{\mathrm{1}^{13}\mathrm{C}\}\)phenylalanine determined from the rate of release of \( \mathrm{^{13}CO}_2 \) (\( \mathrm{F}^{13}\mathrm{CO}_2 \)) at each of 9 levels of total BCAA intakes for all 5 subjects (\( n = 37 \) observations). Symbols represent data points for each individual subject.](image)
subjects (in one case by 50%) and overestimating for the remaining two subjects.

There was a high degree of variability among the subjects in their baseline (fasting) plasma BCAA concentrations. To control for these interindividual and intra-individual variabilities (in fed-state plasma BCAA concentrations), we subtracted the fed-state plasma BCAA concentrations from the fasting plasma BCAA concentrations. This is analogous to a similar calculation in children with PKU (10). These data showed that 53% of the changes in plasma leucine concentrations with feeding were accounted for by total BCAA intake, and the fewest differences in leucine plasma concentrations occurred when the total BCAA intake was ~80 mg·kg⁻¹·day⁻¹. On the other hand, 85% of the changes in plasma valine concentrations with feeding were accounted for by the total BCAA intake, and the fewest differences in valine plasma concentrations occurred when the intake of total BCAA intake was ~60–65 mg·kg⁻¹·day⁻¹. 90% of the changes in plasma isoleucine concentrations with feeding were accounted for by the total BCAA intake, and the fewest changes occurred when the intake of total BCAA intake was ~70 mg·kg⁻¹·day⁻¹. This indicates that leucine plasma concentrations are less sensitive than valine and isoleucine plasma concentrations. The difference in the plasma concentrations of the three BCAA with feeding also shows that plasma amino acid levels are relatively insensitive indicators of amino acid requirement compared with a more direct measurement, such as label oxidation, and in this regard, the IAAO as measured by ¹⁴C·CO₂. However, the least difference in valine and isoleucine plasma concentrations with feeding occurred when the total BCAA intake was

Table 3. Effect of total BCAA intake on phenylalanine kinetics for patient study participants

<table>
<thead>
<tr>
<th>Total BCAA Intake, mg·kg⁻¹·day⁻¹</th>
<th>No. of Subjects</th>
<th>Phenylalanine Flux</th>
<th>Phenylalanine Oxidation</th>
<th>NOPD</th>
<th>Bₘₑ</th>
<th>Phenylalanine Balance During Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>55.9±18.9</td>
<td>4.8±2.1</td>
<td>51.2±16.9</td>
<td>48.7±18.9</td>
<td>22.3±18.9</td>
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<tr>
<td>20</td>
<td>3</td>
<td>59.1±0.61</td>
<td>4.1±1.3</td>
<td>55.1±1.7</td>
<td>51.6±0.6</td>
<td>28.6±11.8</td>
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<tr>
<td>50</td>
<td>5</td>
<td>57.5±8.9</td>
<td>3.2±1.2</td>
<td>55.5±10.8</td>
<td>51.6±11.4</td>
<td>35.1±11.7</td>
</tr>
<tr>
<td>60</td>
<td>3</td>
<td>65.4±8.5</td>
<td>3.1±0.9</td>
<td>62±3.8</td>
<td>58.2±8.5</td>
<td>37.2±7.9</td>
</tr>
<tr>
<td>70</td>
<td>5</td>
<td>55.9±3.4</td>
<td>3.2±0.7</td>
<td>52.8±2.8</td>
<td>48.7±3.3</td>
<td>36.5±5.8</td>
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<tr>
<td>90</td>
<td>5</td>
<td>58.2±11.2</td>
<td>2.6±0.5</td>
<td>55.6±1.3</td>
<td>50.9±11.3</td>
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<td>2.9±1.4</td>
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<td>39.7±10.7</td>
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<tr>
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<td>3</td>
<td>71.5±7.2</td>
<td>3.4±0.9</td>
<td>68.1±6.2</td>
<td>64.3±7.2</td>
<td>34.7±8.2</td>
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Table 4. Difference between fed and fasted individual plasma BCAA concentrations in response to varying total BCAA intake

<table>
<thead>
<tr>
<th>Total BCAA intake, mg·kg⁻¹·day⁻¹</th>
<th>Fed-Fasted</th>
<th>Plasma BCAA Concentration μM</th>
<th>Subject No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Mean ± SE</th>
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<tr>
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<td>Ile</td>
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<td>82.8±76.1</td>
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<tr>
<td></td>
<td>Val</td>
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<tr>
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<td>112.5±35.8</td>
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</table>
the F13 CO2 data, because calculation of phenylalanine oxidation and the fewest no. of changes in plasma leucine concentration occurred when intake of total BCAA was ~80 mg·kg⁻¹·day⁻¹.

~60–70 mg·kg⁻¹·day⁻¹. This is very similar to our upper 95% CI estimated from the 13C label oxidation (F13 CO2) of 62 mg·kg⁻¹·day⁻¹. A similar relationship between changes in phenylalanine concentration and intake and the upper 95% CI of the break point for phenylalanine requirement was also observed in our studies of the phenylalanine requirement of children with PKU (10). This consistency between the two different inborn errors of amino acid metabolism is of interest and lends support for the estimate of the safe level of intake for the general population.

The changes in total BCAA intake (test amino acid) did not affect the flux of the inhibitor amino acid (L-1-[1-13C]phenylalanine), which is necessary for the IAAO technique. As we mentioned in our previous study (27), there is a difference between the average requirement of total BCAA determined from the L-[1-13C]phenylalanine oxidation data and that from the F13 CO2 data, because calculation of phenylalanine oxidation depends on the assumption that plasma phenylalanine accurately reflects intracellular enrichment. However, plasma α-ketoisocaproate enrichment is well recognized to be a better reflection of intracellular leucine enrichment than is plasma leucine enrichment (19). Plasma leucine enrichment is ~20% higher than enrichment of intracellular leucine, which would result in an underestimation of leucine oxidation. Although there are no data from studies in humans for phenylalanine, on the basis of the observations in this study, we think there is an underestimation of the requirement when plasma phenylalanine enrichment is used; therefore, the estimate of the requirement for total BCAA based on the rate of release of 13CO2 (F13 CO2), which is an end product of the intracellular [13C]phenylalanine oxidation, is more correct.

Our results apply directly to young adults with MSUD. However, they can be used to calculate the mean requirement for total BCAA for children of a younger age, because the requirement is equal to the amount needed for maintenance and the amount needed for growth in children (14). It has been estimated that, for children aged 0–2 yr, the requirement for maintenance accounts for 80–90% of the total protein or amino acid requirements (14, 33). Therefore, the growth components would be 20–10% of the total requirements. As the child grows older, the growth component will decrease to be ~5–7% in 6- to 7-yr-old children. Therefore, if the requirement for growth is considered to be 20% of the total amino acid requirement in children 0–2 yr, according to our estimate of requirement for adult MSUD patients from the present study, the mean requirement for total BCAA would be ~55 mg·kg⁻¹·day⁻¹ (45+10) for children 0–2 yr, and ~48 mg·kg⁻¹·day⁻¹ (45+3) for children 6–12 yr.

Our study demonstrates once more (4, 10) the suitability of the IAAO method for determining amino acid requirement in vulnerable groups. We conclude that the total BCAA requirement for patients with MSUD is 45 mg·kg⁻¹·day⁻¹. On the basis of the upper 95% CI of the BCAA requirement according to the indicator amino acid study and the changes in plasma valine and isoleucine concentrations, patients with MSUD should not require >60–70 mg·kg⁻¹·day⁻¹ of total dietary BCAA.
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GRANTS

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REFERENCES