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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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 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.

industrial amino acid oxidation; amino acid requirements

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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-ketoisocaproate, 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.
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 over a period of 2–3 mo. 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 cookies. 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, 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).

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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>1,905</td>
<td>30.7</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SD

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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Body composition measurements. Subjects were weighed on a balance scale (model 2020; Toledo Scale, Windsor, ON, Canada) to determine the lean body mass (LBM) (21). 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 body sites daily requirement.2 Priming doses of L-[^1-13]Cphenylalanine were given at the 5th meal, and then a continuous dose of L-[^1-13]Cphenylalanine was started and given throughout the remaining 4 h.3 Three baseline urine and breath samples were collected at 15, 30, and 45 min before start of isotope protocol. Five plateaux urine and breath samples were collected at isotopic steady state every 30 min during the period of 150–270 min after initiation of isotope protocol.4 Carbon dioxide production rate (VCO₂) was measured with indirect calorimetry after 4 h of experimental diet consumption.

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>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>0.607</td>
</tr>
<tr>
<td>2</td>
<td>0.549</td>
</tr>
<tr>
<td>3</td>
<td>0.456</td>
</tr>
<tr>
<td>4</td>
<td>0.586</td>
</tr>
<tr>
<td>5</td>
<td>0.684</td>
</tr>
</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-[^1-13]Cphenylalanine oxidation expressed in pmol·kg⁻¹·h⁻¹; BCAA, branched-chain amino acids.
hourly meals beginning in the morning of the study day. With the fifth meal, the subjects were given an oral prime dose of NaH\(^{13}\)CO\(_3\) in the amount of 0.176 mg/kg body wt (2.07 μmol/kg body wt) and an oral prime dose of L-[\(^{1-13}\)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-[\(^{1-13}\)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-[\(^{1-13}\)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 state. A 5-ml blood sample was also taken from each subject. A 5-ml blood sample was also taken from each subject. A fasted state. A 5-ml blood sample was also taken from each subject.

Analytical procedures. The enrichment of \(^{13}\)C in breath CO\(_2\) 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 \(13°\)C). This was coupled to an Agilent 1100 HPLC system. All plasma samples were taken on each level and at the end of each study. A 5-ml blood sample was taken only once on a prestudy day, and the fed state. A 5-ml blood sample was also taken from each subject. A fasted state. A 5-ml blood sample was also taken from each subject.

The rate of CO\(_2\) production (V\(_{\text{CO}_2}\)) 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\(_{\text{CO}_2}\) measurements were corrected to standard temperature and pressure.

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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 \(\pm\) [\(^{1-13}\)C]phenylalanine in urine and \(^{13}\)CO\(_2\) 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 \(^{13}\)CO\(_2\) 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 molar ratio of the \(^{13}\)CO\(_2\) enrichment (m + 1) to the unenriched (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 \(^{13}\)CO\(_2\) in breath in response to phenylalanine oxidation have been described previously (38).

Phenylalanine flux (μmol·kg\(^{-1}\)·h\(^{-1}\)) was calculated using the equation
\[
Q = I[\left(\frac{E_I}{E_p}\right) - 1]
\]
where \(I\) is the mass of the isotope, \(E_I\) is the enrichment of the isotope, and \(E_p\) is the enrichment of the L-[\(^{1-13}\)C]phenylalanine at plateau. The rate of \(^{13}\)CO\(_2\) release in breath (F\(^{13}\)CO\(_2\)) from oxidation of the L-[\(^{1-13}\)C]phenylalanine tracer (F\(^{13}\)CO\(_2\) in μmol \(^{13}\)CO\(_2\)·kg\(^{-1}\)·h\(^{-1}\)) was calculated as
\[
F^{13}\text{CO}_2 = (F_{\text{CO}_2}(F^{13}\text{CO}_2)(44.6)/(60)/(W)\times0.82)\times100
\]
where F\(^{13}\)CO\(_2\) is the CO\(_2\) production rate measured by indirect calorimetry, E\(_{\text{CO}_2}\) is the \(^{13}\)CO\(_2\) 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 \(^{13}\)CO\(_2\) 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-[\(^{1-13}\)C]phenylalanine oxidation (O, μmol·kg\(^{-1}\)·h\(^{-1}\)) was calculated from F\(^{13}\)CO\(_2\) and urinary free phenylalanine enrichment (22, 38)
\[
O = F^{13}\text{CO}_2/\left(1 - E_p/ E_I\right) \times 100
\]

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\(^{13}\)CO\(_2\), 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/Statistical Analysis System, Cary, NC).

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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 BCAC (F13 CO2) in patients with MSUD for all 5 subjects (n = 37 observations). Total BCAA intake between 2 regression lines represents mean total BCAA requirement of 45 mg·kg−1·day−1.

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 difference (fed minus fasted) plasma BCAA concentration, with 53% of the difference in plasma leucine concentration between fed and fasted states being accounted for by total BCAA intake (r² = 0.53, P = 0.03), and that the fewest changes occurred when the total BCAA intake was ~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 (r² = 0.85, P = 0.0004), and that the fewest changes occurred when the total BCAA intake was ~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 (r² = 0.90, P < 0.0001), and the fewest changes occurred when the intake of total BCAA was ~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 F13 CO2 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 ~17%. However, on an individual basis there was much higher variability, with the dietitian underestimating the BCAA needs of three of the subjects, thus resulting in an overestimation.
of the study was 9 h and phenylalanine intake was 7.2 µmol·kg⁻¹·h⁻¹.

Differences between fed and fasted individual plasma BCAA concentrations were accounted for by the total BCAA intake, and the fewest changes occurred when the 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 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 F¹³CO₂. 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>Bₑ₈₆</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>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>59.1 ± 0.61</td>
<td>4.1 ± 1.3</td>
<td>55.1 ± 17.4</td>
<td>51.9 ± 0.66</td>
<td>28.6 ± 11.8</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>57.5 ± 8.9</td>
<td>3.2 ± 1.2</td>
<td>55.6 ± 10.8</td>
<td>51.6 ± 11.4</td>
<td>35.1 ± 11.9</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>65.4 ± 8.5</td>
<td>3.1 ± 0.9</td>
<td>62.3 ± 6.8</td>
<td>38.2 ± 8.5</td>
<td>37.2 ± 7.9</td>
</tr>
<tr>
<td>60</td>
<td>3</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>
</tr>
<tr>
<td>70</td>
<td>5</td>
<td>58.2 ± 11.2</td>
<td>2.6 ± 0.5</td>
<td>55.6 ± 11.3</td>
<td>50.9 ± 11.3</td>
<td>41.9 ± 4.9</td>
</tr>
<tr>
<td>90</td>
<td>5</td>
<td>60.3 ± 7.5</td>
<td>2.9 ± 1.4</td>
<td>57.4 ± 6.3</td>
<td>51.5 ± 5.2</td>
<td>39.7 ± 10.7</td>
</tr>
<tr>
<td>110</td>
<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>
</tr>
<tr>
<td>130</td>
<td>3</td>
<td>64.9 ± 17.9</td>
<td>3.8 ± 2.4</td>
<td>64.1 ± 16.2</td>
<td>57.9 ± 18.1</td>
<td>31 ± 11.1</td>
</tr>
</tbody>
</table>

Values are means ± SD expressed in µmol·kg⁻¹·h⁻¹. NOPD, nonoxidative phenylalanine disposal; Bₑ₈₆, phenylalanine release from proteolysis. Duration of the study was 9 h and phenylalanine intake was 7.2 µmol·kg⁻¹·h⁻¹.

There was a high degree of variability among the subjects in their baseline (fasting) plasma BCAA concentrations. To control for these interindividual and intr individu al differences in 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 ~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 F¹³CO₂. However, the least difference in valine and isoleucine plasma concentrations with feeding occurred when the total BCAA intake was

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>Subject No.</th>
<th>Plasma BCAA Concentration µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Leu 87.05</td>
<td>1</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td>Val -6.79</td>
<td>2</td>
<td>-142.27</td>
</tr>
<tr>
<td>20</td>
<td>Leu -13.59</td>
<td>3</td>
<td>-243.13</td>
</tr>
<tr>
<td></td>
<td>Val -46.04</td>
<td>4</td>
<td>-167.21</td>
</tr>
<tr>
<td>30</td>
<td>Leu 154.27</td>
<td>5</td>
<td>-218.34</td>
</tr>
<tr>
<td></td>
<td>Val 30.24</td>
<td>6</td>
<td>-226.62</td>
</tr>
<tr>
<td>50</td>
<td>Leu -0.2</td>
<td>7</td>
<td>-270.57</td>
</tr>
<tr>
<td></td>
<td>Val -63.18</td>
<td>8</td>
<td>-89.80</td>
</tr>
<tr>
<td>60</td>
<td>Leu 127.3</td>
<td>9</td>
<td>-230.73</td>
</tr>
<tr>
<td></td>
<td>Val 59.05</td>
<td>10</td>
<td>-102.78</td>
</tr>
<tr>
<td>70</td>
<td>Leu -6.67</td>
<td>11</td>
<td>-320.9</td>
</tr>
<tr>
<td></td>
<td>Val 15.52</td>
<td>12</td>
<td>-89.16</td>
</tr>
<tr>
<td>90</td>
<td>Leu 17.74</td>
<td>13</td>
<td>-354.78</td>
</tr>
<tr>
<td></td>
<td>Val 26.68</td>
<td>14</td>
<td>-94.86</td>
</tr>
<tr>
<td>110</td>
<td>Leu 189.54</td>
<td>15</td>
<td>-158.26</td>
</tr>
<tr>
<td></td>
<td>Val 300.9</td>
<td>16</td>
<td>-77.83</td>
</tr>
<tr>
<td>130</td>
<td>Leu 154.18</td>
<td>17</td>
<td>-84.15</td>
</tr>
<tr>
<td></td>
<td>Val 222.76</td>
<td>18</td>
<td>-64.84</td>
</tr>
</tbody>
</table>

Subjects (in one case by 50%) and overestimating for the remaining two subjects.

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the F \textsuperscript{13} CO \textsubscript{2} data, because calculation of phenylalanine oxidation, is more correct.

However, they can be used to calculate the mean requirement for 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 \( \sim 55 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) (45 + 10) for children 0–2 yr, and \( \sim 48 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) (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 \( > 60–70 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) of total dietary BCAA.

The changes in total BCAA intake (test amino acid) did not affect the flux of the indicator amino acid (L-[1-\textsuperscript{13}C]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-\textsuperscript{13}C]phenylalanine oxidation data and that from the F\textsuperscript{13}CO\textsubscript{2} 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 \( \sim 20\% \) 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 \textsuperscript{13}CO\textsubscript{2} (F\textsuperscript{13}CO\textsubscript{2}), which is an end product of the intracellular [\textsuperscript{13}C]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 \( \sim 5–7\% \) in 6–7-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 \( \sim 55 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) (45 + 10) for children 0–2 yr, and \( \sim 48 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) (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 \( > 60–70 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) of total dietary BCAA.

![Fig. 4. Relationship between total BCAA intake and mean difference (fed minus fasted) of plasma leucine concentration. Data are means (●) ± SE from all observations (n = 37); \( r^2 = 0.53, P = 0.03 \). Data show that 59% of changes in plasma leucine concentration with feeding were accounted for by total BCAA intake, and the fewest no. of changes in plasma leucine concentration occurred when intake of total BCAA was \( \sim 80 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \).

![Fig. 5. Relationship between total BCAA intake and mean difference (fed minus fasted) of plasma valine concentration. Data are means (●) ± SE from all observations (n = 37); \( r^2 = 0.85, P = 0.0004 \). Data show that 85% of the changes in plasma valine concentration with feeding were accounted for by total BCAA intake and the fewest no. of changes in plasma valine concentration occurred when total BCAA intake was \( \sim 60–65 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \).

![Fig. 6. Relationship between total BCAA intake and mean difference (fed minus fasted) of plasma isoleucine concentration. Data are means (●) ± SE from all observations (n = 37); \( r^2 = 0.90, P < 0.0001 \). Data show that 90% of the changes in plasma isoleucine concentration with feeding were accounted for by total BCAA intake and the fewest no. of changes in plasma isoleucine concentration occurred when intake of total BCAA was \( \sim 70 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \).}
ACKNOWLEDGMENTS

We thank Karen Chapman for coordinating the activity in the Clinical Investigation Unit of The Hospital for Sick Children (HSC), and Linda Chow (Department of Nutrition and Food Services, HSC) for preparing the protein-free cookies. Special thanks go to the patients who participated in this study. We are also grateful to Mead Johnson Nutritionals (Canada) for providing the protein-free powder for the experimental diets.

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REFERENCES