Branched-Chain Amino Acid Requirements in School-Aged Children Determined by Indicator Amino Acid Oxidation (IAAO)\textsuperscript{1,2}

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ABSTRACT The current WHO/FAO/UNU recommendations for BCAA requirements in school-aged children are based on nitrogen balance studies that have tended to produce lower estimates of amino acid requirements that those determined using stable isotope methodologies. The new dietary reference intake (DRI) recommendations for total BCAA requirements in children were determined using a factorial approach that included adult BCAA requirements plus the additional needs for growth. The purpose of this study was to determine directly total BCAA requirements in school children aged 6–10 y using indicator amino acid oxidation (IAAO). Five children (8.5 ± 1.2 y) were assigned randomly to receive 7 graded intakes of total BCAA. Individual BCAA in the test diet were provided in the same proportions as those present in egg protein to minimize the potential interactive effects of individual BCAA on assessment of requirement. Total BCAA requirement was determined by measuring the oxidation of L-[1-\textsuperscript{13}C] phenylalanine to \textsuperscript{13}CO\textsubscript{2} in \textsuperscript{12}C-phenylalanine oxidation; FFMs, fat-free mass; RMR, resting metabolic rate.

KEY WORDS: • indicator amino acid oxidation • amino acid requirements • children

The BCAA comprise 14% of the total amino acids present in skeletal muscle protein and differ from other indispensable amino acids in that they are oxidized primarily in the skeletal muscle by the BCAA dehydrogenase enzyme complex (1–4). All three of the individual BCAA (leucine, valine and isoleucine) share this common pathway and have been shown to exhibit antagonism in their respective catabolism at varying levels of intake (1–4).

The current data available on BCAA requirements include data from human and animal models (5). The WHO/FAO/UNU recommendations for BCAA requirements are available for adults, infants, children aged ~2 y of age and for children 10–12 y of age (6). Data for leucine and valine requirements based on plasma amino acid concentrations, direct amino acid oxidation and 24-h amino acid balance studies are also available for adults (7–11). These data indicate that leucine and valine requirements are two- to threefold higher than requirements based on the WHO/FAO/UNU recommendations (6). Reexamination of these stable isotope studies by the dietary reference intake (DRI) committee resulted in increases in the estimated average requirements (EAR) for leucine and valine (12). There are no isotope data for isoleucine requirements. Recently, our group conducted a study using indicator amino acid oxidation (IAAO) to determine total BCAA requirements in children aged 6–10 y using indicator amino acid oxidation (IAAO). Five children (8.5 ± 1.2 y) were assigned randomly to receive 7 graded intakes of total BCAA. Individual BCAA in the test diet were provided in the same proportions as those present in egg protein to minimize the potential interactive effects of individual BCAA on assessment of requirement. Total BCAA requirement was determined by measuring the oxidation of L-[1-\textsuperscript{13}C] phenylalanine to \textsuperscript{13}CO\textsubscript{2} in \textsuperscript{12}C-phenylalanine oxidation; FFMs, fat-free mass; RMR, resting metabolic rate.

6 Abbreviations used: \(BPhe\), phenylalanine released from endogenous proteolysis; DRI, dietary reference intake; EAR, estimated average requirement: \(\delta^{13}CO\textsubscript{2}\), rate of release of \(^{13}CO\textsubscript{2}\) from \(^{13}C\)-phenylalanine oxidation; FFMs, fat-free mass; HFB, N-heptafuropyrutobuty; HSC, Hospital for Sick Children; IAAO, indicator amino acid oxidation; LBM, lean body mass; NOPD, nonoxidative phenylalanine disposal; RMR, resting metabolic rate.
measurement of nitrogen balance in the childhood studies (14,15).

The current DRI recommendations for the mean level of intake for total BCAA intake for school age children is 99 mg/(kg·d) (12). The childhood DRI amino acid requirements were based on adult amino acid requirements plus that required for growth. An assumption was made that the maintenance requirements for dietary essential amino acids is the same for children as it is for adults. The adult essential amino acid needs were based on carbon oxidation and balance techniques. The growth component was based on changes in body composition, specifically deposition of body protein during growth (12).

No data are currently available in children for the assessment of BCAA requirements using stable isotope methodology. The purpose of this study was to determine directly total BCAA requirements in healthy school-aged children using the minimally invasive IAAO method. Due to concerns regarding the potential interactive effects of individual BCAA, the model used in these studies included feeding dietary BCAA in the same proportions as those present in egg protein (18–22). This is the same approach we used in adults (13).

SUBJECTS AND METHODS

Determination of total BCAA requirements in healthy school-aged children. Healthy school-aged children (n = 5) between the ages of 6.8 and 10 y participated in this study. Subject characteristics, body composition and energy needs are summarized in Table 1. All subjects were studied on an outpatient basis in the Clinical Investigation Unit at the Hospital for Sick Children (HSC). None of the children participating in the study had a recent history of weight loss or recent illness. Subjects were excluded if they were taking medications that alter protein or energy metabolism (e.g., corticosteroid therapy) or were diagnosed with any endocrine/metabolic disorders. Written consent and/or assent was obtained from study participants and their responsible caregivers. The purpose of these studies and potential risks were explained before obtaining written consent/assent. All study procedures were approved by the Research Ethics Review Board, at the HSC. Study participants and their responsible caregivers were provided with financial compensation for costs incurred in participating in these studies.

Experimental design. The study design was based on the adapted, noninvasive IAAO model of Bross et al. (16,17,23,24). The IAAO is based on the premise that the partitioning of an indispensable amino acid between protein oxidation and protein synthesis is sensitive to the intake of the most limiting amino acid (16). The IAAO entails the administration of oral stable isotope tracers and the collection of urine and breath samples for assessment of isotope enrichment. In this study, L-[1-13C] phenylalanine was used as the indicator, and a mixture of BCAA (based on the profile of egg protein) was used as the test amino acid. Each subject received seven dietary intakes of the total BCAA 75, 85, 100, 125, 150, 200 and 225 mg/(kg·d) over 7 different study days. These levels were based upon a reanalysis of the original nitrogen balance studies of the BCAA in children by Nakagawa et al. (14,15). Corrections for miscellaneous nitrogen losses of 8 mg/(kg·d) were made, and the data reanalyzed using nonlinear regression to determine an EAR level for total BCAA. This was calculated to be 133 mg/(kg·d). Total BCAA levels of 75–225 mg/(kg·d) provided in the diet represented intake levels in excess of 2 SD of the EAR based on this reanalysis.

Each study day was preceded by a 2-d adaptation period in which study participants were adapted to a dietary protein intake of 1.5 g protein/(kg·d) and was followed by a single study day on which phenylalanine kinetics were measured with the use of L-[1-13C] phenylalanine. This level was chosen because it met and exceeded the recommended protein requirement (12,18). In addition, the level of protein and energy provided in this diet approximated the subjects habitual protein and energy intake. Menu plans were provided by the investigator consisting of typical foods consumed by the child; food records were collected to ensure consistency of dietary intake before each study day. We used this length of preadaptation of protein intake to study phenylalanine and tyrosine requirements in children with phenylketonuria previously (17,25,26). Zello et al. (27) also showed that phenylalanine flux and oxidation were similar in subjects consuming phenylalanine over a 6-h period after being adapted for 3, 6 and 9 d to two different levels of phenylalanine intake. Hence, preadaptation to a level of amino acid intake is not necessary before conducting oxidation studies. The dietary study periods were separated by ≥1 wk; all subjects completed all study days within 2 mo.

Dietary protein and energy intakes. Energy needs of study participants were determined by measuring resting metabolic rate (RMR) after a 12-h overnight fast, using, open-circuit indirect calorimetry (2900 Computerized Energy Measurement System; Sensormedics, Yorba Linda, CA). The RMR was multiplied by an activity factor of 1.7 to ensure age-appropriate growth for study participants over the course of the study (Table 1). All of the children maintained their typical patterns of activity over the course of the study period.

The protein content of the experimental diet was provided as an L-amino acid mixture based on the amino acid composition of egg protein. BCAA were provided in the same proportion as in egg protein: 38.5% leucine, 29% isoleucine and 32.5% valine. This was done to minimize potential interactive effects of the BCAA in determination of requirement (19–22). The experimental diet included 25 mg/(kg·d) phenylalanine to ensure adequacy of dietary intake. This was provided in the presence of excess tyrosine [40 mg/(kg·d)]. This level of tyrosine in the diet was shown to minimize the conversion of phenylalanine to tyrosine, which results in the channeling of phenylalanine oxidation when intake is higher than is needed for protein synthesis (27). Diets were kept isonitrogenous by

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (y)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>%IBW</th>
<th>FFM (kg)</th>
<th>LBM (kg)</th>
<th>RMR (kJ/d)</th>
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<tr>
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<td>28.6</td>
<td>26.6</td>
<td>5775</td>
<td>9820</td>
</tr>
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</table>

1 Tanner stage (mean ± SD): 1.2 ± 0.4 (range 1–2), n = 4 girls, 1 boy.
2 IBW, ideal body weight. FFM, fat-free mass was determined from the sum of skinfold thickness (28–32).
3 LBM, lean-body mass was determined from bioelectrical impedance analysis (27–32).
4 RMR, resting metabolic rate was measured by open-circuit indirect calorimetry.
5 Calculated RMR × 1.7 (activity factor).
varying the levels of serine and glycine. Protein in the experimental diet was provided in the crystalline amino acid form to avoid issues potentially affecting the measurement of amino acid oxidation. This includes the effect of decreased bioavailability of amino acids associated with digestion and absorption on amino acid oxidation, which could potentially result in altered determinations of the breakpoint (28). The experimental diet consisted of a flavored protein-free liquid formula (Protein-Free Powder, product 80056; Mead Johnson, Evansville, IN; Tang and Kool-Aid, Kraft Foods, Toronto, Canada), the crystalline L-amino acid study mixture and protein-free cookies (23–27). The experimental diet provided ~10% of total energy from protein, 53% from carbohydrate, and 37% from fat. All diets were prepared and weighed (scale model PE2000; Mettler, Nanikon, Switzerland) in the HSC research kitchen.

Experimental diets were provided in nine isonitrogenous, isoenergetic hourly meals that provided 75% of daily energy and protein needs. Each experimental meal consisted of the crystalline amino acid mixture (containing varying amounts of total BCAA) added to the flavored protein-free liquid and two protein-free cookies. Multivitamin supplements (Centrum; Whitehall-Robins, Mississauga, Canada) were given to study participants throughout the course of the study to ensure adequacy of micronutrient status.

**Body composition.** Children were weighed on each study day on a balance scale (model 2020, Toledo Scale, Windsor, Canada) to the nearest 0.1 kg after voiding. Standing height was measured to the nearest 0.1 cm with a wall-mounted stadiometer on each study day. Multiple skinfold thicknesses (triceps, biceps, subcapular and suprailiac) were measured before each study day to the nearest 1 mm with Harpenden calipers (British Indicators, St Albans, UK) to estimate fat mass and fat-free mass (FFM), by subtraction from body weight (29–36). Bioelectrical impedance analysis was performed in fasting subjects on each study day using a mixed-frequency analyzer (50 kHz) (BIA, model 101A; RJL Systems, Detroit, MI). Resistance (R) and reactance (Xc) measurements were made using a four-terminal bioelectrical impedance analyzer. The mean of three readings for R and Xc (Ω) taken for each child was used to determine lean body mass (LBW) (30,36).

**Isotope infusion studies.** The stable isotope tracers used in these studies were as follows: NaH13CO3 (Cambridge Isotope Laboratories, Woburn, MA) and L-[1-13C]phenylalanine (Mass Trace, Woburn, MA) with 99% atom enrichment. Isotopic and optical purity of L-[1-13C]phenylalanine was verified by the manufacturer using GC-MS and NMR. The enrichment and enantiomeric purity of the L-[1-13C]phenylalanine was reconfirmed by GC-MS of the N-heptadecanobutryl (HFB) N-propyl ester derivative using a chiral column (ChirasolvVal, R symbol, Alltech Associates, Deerfield, IL). The measured fractional molar abundance of 1-[1-13C]phenylalanine was 97.5%. This value was used in the calculation of phenylalanine turnover. Tracer solutions were prepared in deionized water and stored at -20°C.

Subjects consumed 4 hourly meals on each study day before consuming the stable isotope tracers. At the fifth meal, the subjects were given a priming oral dose of NaH13CO3 (2.07 μmol/kg) and a priming oral dose of L-[1-13C]phenylalanine (6.55 μmol/kg). A constant oral dose of L-[1-13C]phenylalanine (11.8 μmol/kg) was given on an hourly basis commencing with the fifth meal, and was provided on an hourly basis with subsequent meals until the end of the study. Phenylalanine intake was kept constant by reducing phenylalanine in the last 5 meals. Label recovery of the 13C-labeled sodium bicarbonate was monitored by measuring VCO2 production because this has been shown to be tightly regulated by label recovery (37).

**Sample collection and analysis.** Breath and urine samples were collected for measurement of isotopic enrichment. This method was developed by Bross et al. (16,17) who demonstrated that urinary enrichment of isotope reflects plasma isotopic enrichment. Baseline samples of breath and urine were collected 30, 45 and 60 min before the first isotope dose to establish that background isotopic steady state had been achieved within 4 h of feeding. After the initiation of the oral isotope infusion, breath and urine samples were collected every 30 min between 150 and 270 min. Breath samples were collected in disposable Haldane-Priestley tubes (Venoject, Terumo Medical, Elkton, MD) with the use of a collection mechanism that enables the removal of dead air space (9). Breath samples were stored at room temperature. Urine samples were stored at -20°C. Indirect calorimetry (2900 Computerized Energy Measurement System; Sensormedics) was done to determine carbon dioxide production rate on each study day for 20–30 min after 5 h of consuming the experimental diet.

**Analytical procedures.** 13CO2 enrichment was measured in breath samples using a continuous flow isotope ratio MS (model 2020, PDZ Europa, Cheshire UK). 13CO2 enrichment was expressed as atom% excess, again a reference standard of compressed CO2. Amino acids in urine were isolated and derivatized before analysis of isotopic enrichment of L-[1-13C]phenylalanine based on Patterson et al. (38). Urine (1 mL) was deproteinized and acidified with 500 μL of 2.5 mol/L trichloroacetic acid and centrifuged at 7000 x g. The supernatant was eluted through a cation exchange column (Dowex 50 W-X8, 100–200 mesh H+ form; Bio-Rad Laboratories, Hercules, CA), then freeze-dried (Freezone 12L; Labconco, Kansas City, MO) before derivatization to its HFB N-propyl ester derivative. L-[1-13C]phenylalanine enrichment was measured using methane negative chemical ionization GC-MS (Hewlett Packard 5890 series; GC; Hewlett Packard 5988A MS system, Mississauga, Canada). A chiral fused-silica capillary column (Val-D R Symbol, Alltech Associates, Deerfield, IL) was used to separate optical isomers of phenylalanine. Selected ion chromatograms were obtained by monitoring ions m/z 383 and 384 for L-phenylalanine and L-[1-13C]phenylalanine, respectively. Isotope enrichment in mol% excess was calculated from peak area ratios at isotopic steady state and baseline. Isotopic steady state was considered to be achieved when breath 13CO2 reached a plateau (absence of a significant slope) with a CV of <5% (Fig. 1).

**Isotope kinetics.** A stochastic model was used to calculate phenylalanine kinetics (39) with a constant oral administration of isotope to study amino acid oxidation. Flux [μmol/(kg·h)] was calculated from isotope dilution of the infused tracer in the metabolic pool at steady state (urinary enrichment) using standard equations (39,40). F13CO2 was calculated and the rate of tracer oxidation [μmol/(kg·h)] calculated according to the model of Matthews et al. (41). The rate of L-[1-13C]phenylalanine oxidation [μmol/(kg·h)] was calculated from urinary phenylalanine enrichment and F13CO2 (27,40).

**Statistical analysis.** A three-factor general linear model ANOVA was performed to assess the relationship of F13CO2, phenylalanine flux, phenylalanine oxidation, nonoxidative phenylalanine disposal (NOPD) and phenylalanine released from endogenous proteolysis (Pbpd) to the following variables: total BCAA intake, order of intake, subject and potential interactions. Body weight and
TABLE 2

Individual $^{13}$CO$_2$ data at all total BCAA intake levels for children participating in the study$^1$

<table>
<thead>
<tr>
<th>BCAA, mg/(kg · d)</th>
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<tr>
<td>75</td>
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<tr>
<td>$^{13}$CO$_2$ (µmol/(kg · h))</td>
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<tr>
<td>Subject</td>
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<td>Mean ± SD</td>
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$^1$ $^{13}$CO$_2$ is the rate of release of $^{13}$CO$_2$ from $^{13}$C-phenylalanine oxidation.

RESULTS

Five children (8.5 ± 1.2 y) were assigned randomly to receive seven graded intakes of total BCAA. Body composition measures (weight, height, % ideal body weight, FFM and LBM) were all within the normal ranges for age (33,34) and did not change over the study period (Table 1). The mean Tanner stage of all study participants was 1.2 (range 1–2). Results of the L-[1-$^{13}$C]phenylalanine $^{13}$CO$_2$ for the individual subjects participating in this study are shown in Table 2. $^{13}$CO$_2$ production was related to total BCAA intake (P = 0.0137) and affected by individual study subjects (P = 0.012). The rate of the release of $^{13}$CO$_2$ varied among the subjects, although the pattern of response to varying intakes of the total BCAA was consistent (Fig. 2). Phenylalanine flux (P = 0.8786), phenylalanine oxidation (P = 0.2371), NOPD (P = 0.8676) and $B_{\text{phc}}$ (P = 0.55) were not affected by total BCAA intake or order of test (Table 3) but differed among study subjects (P < 0.0001). VCO$_2$ production was not affected by total BCAA intake (P = 0.88). Breakpoint analysis of $^{13}$CO$_2$ production using a two-phase linear crossover model indicated a mean total BCAA and safe level of intake (upper 95% CI) at 147.3 and 191.5 mg/(kg · d), respectively, in healthy children (Fig. 3). The two regression lines represent the partitioning of the data that provided the best fit of the data ($r^2 = 0.25$, se of the breakpoint = 22.6).

DISCUSSION

The current study demonstrated that the mean requirement of total BCAA, as determined by IAAO, in healthy school-aged children is 147 mg/(kg · d), which is 48% higher than the current DRI recommendations of 99 mg/(kg · d) (12). The childhood DRI amino acid requirements were based on a factorial analysis that included adult amino acid requirements (maintenance) plus that required for growth. Maintenance nitrogen requirements in children [110 mg/(kg · d)] are similar to requirements in adults, suggesting that requirements for amino acids in children should differ only by requirements for growth (~5–6% of total nitrogen needs) (12). A recent study by our group determined total BCAA requirements in healthy men, using IAAO, to be 144 mg/(kg · d), which is similar to the mean requirement of 147 mg/(kg · d) determined in the present study (13). Growth requirements of total BCAA in children aged 6–10 y are ~10 mg/(kg · d) (12). On the basis of the work of Riazi et al. (13), this suggests that the total BCAA requirement in school-aged children is 154 mg/(kg · d), which is not significantly different from the mean requirement for total BCAA measured in this study. Hence, the results of this study provide a first direct test of the hypothesis of the DRI approach that, in the absence of direct experimental data, calculation of the EAA requirements for children can be done using this factorial approach.

Currently, limited data exist regarding BCAA requirements in children. The available data are based on nitrogen balance studies in school-aged children (14,15). Leucine, valine and isoleucine requirements were determined to be 44, 25 and 28 mg/(kg · d), respectively or 97 mg/(kg · d) for total BCAA (14,15). These results are lower than total BCAA requirements determined in this study. Potential underestimation of the BCAA requirement using nitrogen balance is likely due to the absence of measurement of miscellaneous (skin, hair) nitrogen losses and use of linear regression to determine nitrogen balance. Rand and Young (44) demonstrated that assessment of nitrogen balance is more accurately reflected in curvilinear regression analysis due to decreased efficiency of nitrogen utilization as the requirement is reached (43). There
are also concerns regarding the accuracy of this technique because of the short preadaptation periods used before measurement of nitrogen balance (14,15). We recently recalculated average total BCAA requirements in school-aged children to be 133 mg/(kg·d) by making corrections for miscellaneous nitrogenous losses of 8 mg/kg·d and by applying curvilinear regression analysis to the original nitrogen balance data (14,15). This estimate is not significantly different from the EAR of the total BCAA requirements measured in this study, and further supports the contention that current recommendations for BCAA requirements are underestimated in school-aged children.

Total BCAA requirements in children were determined to avoid potential confounding interactions of individual dietary BCAA on the assessment of requirement. This is important because variations in dietary intake among the individual BCAA have been shown to affect plasma and BCAA metabolic pools of the other individual BCAA, making it difficult to assess individual BCAA requirements (19,20). This methodology was used previously by our group to determine total BCAA requirements in healthy adults (13). We believe that estimation of total BCAA requirements using a model that provides dietary BCAA in the same proportions as those present in egg protein should minimize the potential effect of significant interactions of the individual BCAA on assessment of requirement (19–22). This model assumes that the proportion of BCAA present in egg protein is optimal for protein synthesis in healthy children (6,13). If this is not the case, it is possible that an overestimation of the resulting requirements of the individual BCAA may have occurred. Further research should be done to determine whether these proportions are optimal for protein synthesis.

This study provides further support for the suitability of the IAAO technique for assessment of amino acid requirements in vulnerable populations. The modified version of the IAAO was developed by Bross et al. (16,17) for use in vulnerable populations such as children and pregnant women. This model enables measurement of isotopic enrichment in urine as a marker of plasma enrichment (16,17) and breath 13CO2 as a direct marker of change in oxidation of the isotope tracer in response to graded intakes of the test amino acid (BCAA). Breakpoint analysis using phenylalanine oxidation was not done in this study because phenylalanine oxidation was not significantly related to total BCAA intake (P = 0.23). Although several studies using the IAAO have shown changes in stable isotope oxidation in response to varying levels of intake of the test amino acid (13), others have failed to show a consistent pattern or change (25). This is likely due to the fact that plasma is not the true precursor pool and has a higher intrasubject variability. Several studies have shown that F13CO2 production represents the appropriate metabolic end point for measurement of phenylalanine oxidation (9–11,13,17,25). Hence, the use of this biological end point to determine total BCAA requirements is an appropriate tool.

Each child in this study was fed seven graded levels of intake of the total BCAA, which enabled repeated measurements of the change in oxidation of the stable isotope tracer (l-[1-13C]phenylalanine) in response to changes in intake of total BCAA (from deficient to sufficient). The use of repeated measurements within a single subject minimizes the potential for large intrasubject variation. Intrasubject variation has been shown to be responsible for the major source of variability in amino acid oxidation studies (16,37) and to be a potential source of error in estimation of amino acid requirements in humans. The sample size of 35 used to determine the breakpoint of the total BCAA in this study is similar to other studies using the IAAO for determination of amino acid requirements (13,17,23). Determination of leucine requirements in adults using carbon oxidation techniques has typically been done at varying levels of leucine intake (4–7 different levels of intake) with similar numbers of subjects (9–12). Use of fed state kinetics in this study also enables determination of amino acid requirements in children because it minimizes the invasiveness of the technique. The use of more invasive and lengthy studies used in 24-h indicator amino acid balance studies are not possible in children for these reasons. Because those 24-h
studies did not show differences in estimations of amino acid requirements between the 12-h fed IAAO method used in this study and the 24-h indicator balance studies (9,11,16), it is appropriate to study amino acid requirements in children using this minimally invasive fed state model (16). In addition, the major variables influencing the ability to study amino acid requirements in children include the level of invasiveness of the technique employed, the high costs associated with the use of these techniques and difficulty in subject recruitment.

The mean requirement and safe level of intake for total BCAA in healthy school-aged children were determined to be 147 and 192 mg/(kg · d), respectively. These estimates are not different from estimates determined using the IAAO in adult men. In conclusion, total BCAA requirements in healthy school-aged children, as estimated by IAAO, are significantly higher than the FAO/WHO/UNU and DRI recommendations.

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LITERATURE CITED