Valine May Be the First Limiting Branched-Chain Amino Acid in Egg Protein in Men[1,2]

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ABSTRACT Recently, we defined an estimate for total branched-chain amino acids (BCAA) using the indicator amino acid oxidation technique in men fed the three BCAA (leucine, isoleucine and valine) in the proportion present in egg protein. Although egg protein is regarded as a high quality dietary protein source, it is not known whether the proportions of the three BCAA are optimal. Five men with known total BCAA requirements were restudied. Each men was studied with isoleucine, leucine or valine held constant at that individual's requirement level while the intake of the other two BCAA was reduced; one BCAA was held constant and the intake of the other two was reduced by 10 and 20% in random order. The label appearance from the oxidation of L-[^{13}C]-phenylalanine to $^{13}CO_2$ ($F^{13}CO_2$) in breath was monitored in response to the change in amino acid intake. When either isoleucine or leucine was held constant, and the other two BCAA reduced by 20% (valine and leucine, or valine and isoleucine, respectively) $F^{13}CO_2$ increased ($P = 0.007$, $P = 0.038$, respectively). We conclude that valine may be the first limiting BCAA in egg protein. J. Nutr. 133: 3533–3539, 2003.

KEY WORDS: • *branched-chain amino acids* • *egg protein* • *limiting amino acid* • *indicator amino acid oxidation* • *phenylalanine*

The branched chain amino acids (BCAA),[4] leucine (Leu), isoleucine (Ile) and valine (Val), differ from most of the other dietary indispensable amino acids. They are similar in structure and share common enzymes for their transamination and oxidative decarboxylation (1,2). They are also metabolized mainly by peripheral tissues such as muscle (1–3). Much of the nutritional research on BCAA has dealt with their metabolism and the effect of excessive intake of individual BCAA, especially Leu.

Considerable interaction has been reported in humans and animals in response to disproportionate intakes of the BCAA (2,4–6). Oral or intravenous administration of BCAA to humans elicited different responses among the three amino acids (2,6–8). The addition of excessive quantities of Leu to a low protein diet depressed growth and food intake and depleted body pools of Ile and Val (2,4,9). These adverse effects were ameliorated by supplementing the diet with small quantities of Ile and Val (2,4,9). High intakes of Leu by humans or animals enhanced the activity of branched-chain keto acid dehydrogenase (EC 1.2.4.4) in various tissues (4,10), thereby decreasing Val and Ile concentrations in blood and tissue. The excess of Leu increased the oxidation of Ile and Val, which limits their availability to synthesize protein (2,4–6).

However, in most cases, relatively large excesses of BCAA were used. More recently (10,11), the effects of excess Leu on Val oxidation and vice versa were studied in men. Within the ranges studied, excess Leu intake had no effect on Val oxidation and its requirement estimate, or excess Val on Leu requirement, by direct amino acid oxidation.

From a practical and nutritional point of view, it is important to know the effect of small or moderate changes in BCAA intake because changing the test indispensable amino acid in requirement studies will alter the overall pattern of indispensable amino acids in the mixture. It was suggested that this may affect the requirement estimates (12).

We recently defined the total BCAA requirement using the indicator amino acid oxidation (IAAO) technique in healthy adult men fed the three BCAA in the proportion present in egg protein to reduce the possibility of interactions among these amino acids and imbalances in the mixture affecting the estimate of requirements (13). Having defined the total BCAA requirement, it was important for us to know whether the BCAA balance that was used in the mixture of total BCAA was optimal. Therefore, our objective was to investi-

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[4] Abbreviations used: BCAA, branched-chain amino acid; $F^{13}CO_2$, rate of release of $^{13}CO_2$ from ^{13}C-phenylalanine oxidation; IAAO, indicator amino acid oxidation; KIV, α-ketoisovalerate; RMR, resting metabolic rate.

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TABLE 1

Characteristics and energy intake of the men who participated in the study

Subject	Age	Weight	Height	BMI	RMR[1]	Intake[2]	LBM[3]	FFM[4]
	y	*kg*	*cm*	*kg/m²*	*kJ/d*		*kg*	
1	27	70.9	177.7	22.5	5813	9883	50.9	49.8
2	38	78.5	170.3	27.1	7211	12260	53.5	57.4
3	22	68.5	174.6	22.5	7308	12424	52.2	53.3
4	27	77.0	168.0	27.3	7518	12781	52.9	57.7
5	20	82.4	183.3	24.5	8589	14603	66.9	66.2
Mean ± SD	26.8 ± 6.7	75.5 ± 5.7	174.8 ± 6.1	24.8 ± 2.4	7287 ± 991	12386 ± 1684	55.2 ± 6.6	56.9 ± 6.1

[1] Resting metabolic rate, measured by indirect calorimetry.
[2] Calculated as RMR × 1.7.
[3] Lean body mass determined from bioelectrical impedance analysis (20).
[4] Fat-free mass, determined from the sum of skinfold thickness (18).

gate the limiting BCAA, by changing the balance of the three BCAA in the mixture.

SUBJECTS AND METHODS

IAAO is a functional method based on the concept that the amount of the limiting amino acid governs the partition of the other indispensable amino acids between retention for protein synthesis or oxidation. Our indicator amino acid was L-[1-^{13}C]-phenylalanine, in the presence of an excess of tyrosine, and our test amino acid was a mixture of the three BCAA.

Subjects. Five healthy adult young men volunteers who had participated in our first study to determine the total BCAA requirement (mean age ± SD = 26.8 ± 6.7 y) and whose individual total BCAA requirements were known, received six levels of intake of a BCAA mixture in random order. Characteristics of the men who participated in the study are summarized in **Table 1**. There was no history of weight loss, unusual dietary habits, endocrine disorders or use of any kind of medication or hormonal treatment. Each man was told the purpose of the study and the possible risks involved and written consent was obtained. All men received financial compensation for their participation in the study. All procedures used in the study were approved by the University of Toronto Human Experimental Committee and the Human Subjects Review Committee of The Hospital for Sick Children (HSC).

Experimental design. We determined the visual breakpoint (requirement) for each subject from our first experiment. Each man received randomly 10 and 20% less than their requirement for each individual BCAA. For clarification, this is illustrated by a specific example (**Fig. 1**). If a man's requirement for total BCAA from our previous study was 80 mg/(kg · d), based on our BCAA mixture, Ile would be 23.2 mg/(kg · d), Leu would be 30.8 mg/(kg · d) and Val would be 26 mg/(kg · d), as shown in Figure 1. We kept Ile at this level [23.2 mg/(kg · d)] and changed the other two amino acid stepwise, from 10% less than the total BCAA requirement to 20% less than the total BCAA requirement. The same procedure was carried out for the other two BCAA. Therefore each man was assigned to six levels of test intake.

Diet and energy intakes. The energy intake for men was determined by measuring their 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 the individual's weight maintenance for short-term amino acid oxidation studies (Table 1) (14,15). The men maintained their ordinary levels of activity throughout the study periods.

Dietary intake during the 2-d adaptation period was a milkshake diet (Scandishake; Scandipharm, Birmingham, AL), supplemented with additional protein (Promod; Ross Laboratories, Columbus, OH) and energy (Caloreen; Nestle Clinical Nutrition, North York, Canada) to meet each subject's requirements. All men were told to add a predetermined volume of homogenized milk containing 3.25% fat to their daily portions and drink the milkshake at regular meal times during the day. No other food or beverages, including products containing artificial sweeteners, were consumed during the adaptation period.

Our BCAA mixture was based on the proportion of these amino acids in egg protein, in which Leu is 38.5% (8.5 g/100 g), Val is 32.5% (7.2 g/100 g) and Ile is 29% (6.4 g/100 g) of the total BCAA content (22 g/100 g). The experimental diet was based on an amino acid mixture developed for amino acid kinetic studies (16). The nitrogen content of the diet was supplied at the level of 1 g protein/(kg · d) and provided as an L-amino acid mixture based on egg protein. The experimental diet on the study day included 15 mg/(kg · d) phenylalanine to ensure adequate dietary phenylalanine, as previously determined by amino acid oxidation studies when tyrosine was present in relative excess at ~40 mg/(kg · d) (17). The main

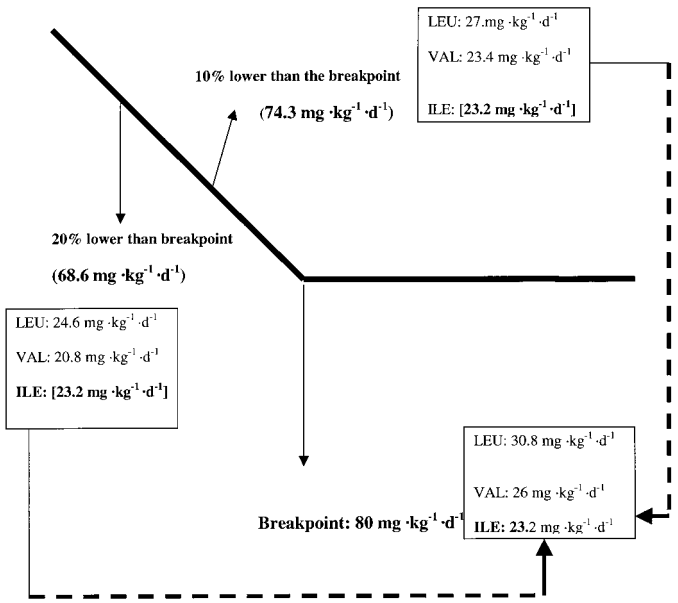

FIGURE 1 Example of the experimental design. If a subject's visual breakpoint (requirement) was 80 mg/(kg · d) on the basis of the branched-chain amino acid (BCAA) proportion in our mixture, isoleucine (Ile) intake would be 23.2 mg/(kg · d), leucine (Leu) intake would be 30.8 mg/(kg · d) and valine (Val) intake would be 26 mg/(kg · d). In this example, we kept Ile at the requirement level [23.2 mg/(kg · d)] and changed the other two amino acid stepwise, from 10% less than their value at requirement to 20% less than their value at requirement. The same procedure was carried out for the other two BCAA. Therefore, each subject participated in 6 levels of test intake.

source of energy in the experimental diet was a flavored liquid protein-free formula (Protein-Free Powder, product 80056; Mead Johnson, Evansville, IN; Tang and Kool-Aid; Kraft Foods, Toronto, Canada). The rest of the energy requirement was met by protein-free cookies (HSC Research Kitchen) (16). The experimental diet provided 53% of 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 consisted of 9 hourly isonitrogenous, isoenergetic meals that differed in total BCAA content on each study day. Serine and glycine were used to keep the meals isonitrogenous at different total BCAA intakes. Each meal contained a bottle of flavored protein-free formula to which the amino acids mixture was added, and 2 protein-free cookies. Although the protein-free formula had a complete mixture of vitamins and minerals, an additional multivitamin supplement (Centrum; Whitehall-Robins, Mississauga, Canada) was given for the period of all studies to ensure adequate vitamin B intake.

Body composition measurements. Men were weighed after voiding each morning on a balance scale (model 2020, Toledo Scale, Windsor, Canada) to the nearest 0.1 kg. Standing heights were measured to the nearest 0.1 cm with the wall-mounted stadiometer on the prestudy day. Multiple skinfold thicknesses (triceps, biceps, subscapular and suprailiac) were measured before the study day to the nearest 1 mm with Harpenden calipers (British Indicators, St. Albans, UK) to estimate fat mass and fat-free mass by subtraction from body weight (18). Bioelectrical impedance analysis (19,20) was performed during fasting on the prestudy day by using a fixed-frequency analyzer (50 kHz) (BIA, model 101A; RJL Systems, Detroit, MI). Resistance (R) and reactance (X_C) measurements were made by using a four-terminal bioelectrical impedance analyzer. Three readings of both R and X_C (in Ω) were taken for each individual and equations were used to determine the lean body mass (20).

Isotope infusion studies. The stable isotopes used in these studies were as follows: $NaH^{13}CO_3$ (Cambridge Isotope Laboratories, Woburn, MA) and L-[1-^{13}C]phenylalanine (Mass Trace, Woburn, MA) with a 99 atom % excess. The isotopic and optical purity of L-[1-^{13}C]phenylalanine was verified by the manufacturer using GC-MS and NMR. The enrichment and enantiometric purity of the L-[1-^{13}C]phenylalanine were reconfirmed by GC-MS of the *n*-propyl heptofluorobutyramide derivative (21) using a chiral column (Chiralsil-Val, R symbol, Alltech Associates, Deerfield, IL). The fractional molar abundance of L-[1-^{13}C]phenylalanine was 97.5%. This value was used in our calculation of phenylalanine turnover. Isotope solutions were prepared in deionized water and stored at −20°C. The morning of each study day, the subjects consumed 4 hourly meals of the experimental diet. With meal 5, all men were given an oral prime dose of $NaH^{13}CO_2$ (2.07 μmol/kg body) and an oral prime dose of L-[1-^{13}C]phenylalanine (3.99 μmol/kg body) with a constant infusion of L-[^{13}C]phenylalanine (7.23 μmol/kg body) thereafter with each hourly meal until the end of the study. The amount of dietary phenylalanine in the last 5 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 phenylalanine intake remained unchanged.

Sample collection. It was shown previously in our laboratory that isotopic enrichment of urinary amino acids reflects enrichment of amino acids in plasma from heparinized blood (22–24). 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 in breath $^{13}CO_2$ within 4 h of the start of the feeding. Isotopic steady state was achieved in both breath and urine by 120 min after the initiation of the isotope protocol and was maintained to the end of the study at 270 min. Five plateau samples were collected by sampling every 30 min during the period from 150 to 270 min after the start of the isotope protocol.

A 5-mL blood sample was also taken from each individual at the end of each study day to determine the profile of the amino acids in plasma. The blood samples were kept on ice until centrifugation at 1200 × *g* for 10 min at 4°C. Plasma was stored at −20°C until analyzed by HPLC. Urine samples stored at −20°C and breath samples were collected in disposable Haldene-Priestly tubes (Venoject; Terumo Medical Corp, Elkton, MD) using a collection mechanism that allows the removal of dead-air space (25). Samples were stored at room temperature until analyzed. Indirect calorimetry (2900 Computerized Energy Measurement System; Sensormedics) was carried out to determine the carbon dioxide production rate for 30 min, after 4 h of consuming the experimental diet, on each study day.

Analytical procedure. Expired $^{13}CO_2$ enrichment was measured by continuous flow isotope ratio MS (model 20/20, PDZ Europa, Cheshire, UK) and was expressed as atom % excess against a reference standard of compressed CO_2 gas.

Urine samples (150 μL) were mixed with 400 μL methanol (MeOH) (for deproteination) and centrifuged at 7000 × *g* for 5 min. The supernatant was then transferred to derivatizing tubes and dried under N_2 at 45°C. The dried amino acids were reconstituted in the proper buffer, 5 mL of 50% acetonitrile + 0.1% formic acid.

Urinary L-[1-^{13}C]phenylalanine enrichment was measured by bench-top triple quadrupole MS API 4000 (Applied Biosystems/MDS SCIEX; Concord, Canada) operated in positive ionization mode with the TurboIonSpray ionization probe source (operated at 5800 V and at 600°C), which was coupled to an Agilent 1100 HPLC system (Agilent, Mississauga, Canada). The individual components were separated using a Waters (Milford, MA) Xterra MS C18 3.5 μm, 2.1 × 150 mm column (for phenylalanine separation) and were eluted with a binary LC gradient (20–40% aqueous acetonitrile containing 0.025% formic acid and 0.05% trifluoroacetic acid for all L-amino acids). Maximum sensitivity was achieved by means of product ion scans and performing multiple reaction monitoring from the fragmentation of the protonated $[M+H]^+$ molecule (for all L-amino acid analysis). All aspects of system operation and data acquisition were controlled using The Analyst NT v1.2 software (Applied Biosystems/MDS SCIEX).

Selected ion chromatographs were obtained by monitoring mass-to-charge ratios of 166 and 167 for L-[1-^{13}C]phenylalanine corresponding to the unenriched (M) and enriched (M+1) peaks, respectively. The areas under the peaks were integrated by use of quantitation method of Analyst software (version 1.2), provided by AB-MDS SCIEX. Isotopic enrichment was expressed as molecule % excess and calculated from the peak area ratios at isotopic steady state and baseline.

Plasma free amino acids were separated using a cation exchange column, as mentioned earlier, using norleucine as an internal standard. Plasma BCAA concentrations were determined by a reverse-phase HPLC technique (Dionex Summit HPLC System, Dionex, Sunnyvale, CA; operated under HPLC pump model P580A LPG and UV/VIS Detector UVD 170S) using a precolumn derivatization with phenylisothiocyanate (adapted from Pico Tag; Waters) (26–28). The areas under the peaks were integrated using Chromeleon software (version 6.2) provided by Dionex Summit HPLC system.

Isotope kinetics. The model used to evaluate phenylalanine kinetics was described by others (14,17,29) using a constant infusion approach to study amino acid oxidation. The isotopic steady state in the metabolic pool was represented by a plateau in free L-[1-^{13}C]phenylalanine in urine and $^{13}CO_2$ in breath. A plateau was defined as a CV < 5% and the absence of a significant slope. The difference between mean breath $^{13}CO_2$ enrichment of the three baseline and five plateau samples was used to determine atom % excess above baseline at isotopic steady state.

Phenylalanine flux [μmol/(kg · h)] was measured during isotopic steady state from the dilution of L-[1-^{13}C]phenylalanine infused into the metabolic pool with urinary enrichments of L-[1-^{13}C]phenylalanine (14,30). The rate of $^{13}CO_2$ release from L-[1-^{13}C]phenylalanine oxidation [$F^{13}CO_2$ in μmol $^{13}CO_2$/(kg · h)] was calculated using a factor of 0.82 to account for the $^{13}CO_2$ retained in the body in the fed state due to bicarbonate fixation (31). The rate of L-[1-^{13}C]phenylalanine oxidation (μmol/(kg · h) was calculated from $F^{13}CO_2$ and urinary free phenylalanine enrichment (14,30).

Statistical analysis. Results are expressed as the means ± SD. Repeated-measures ANOVA was performed to assess the relationship of phenylalanine flux, and BCAA plasma concentration to the following variables: BCAA intakes, subjects and interactions, using

TABLE 2

The effect of varying proportions of branched-chain amino acid (BCAA) in experimental diet on the plasma BCAA concentrations for five healthy adult men

		BCAA proportion					
		Ile constant		Val constant		Leu constant	
Amino acid[1]	Visual breakpoint	−10%	−20%	−10%	−20%	−10%	−20%
		μmol/L					
Leucine	119.6 ± 12.9[a]	108.6 ± 24.2[b]	99.4 ± 14.3[bc]	83.8 ± 8.7[d]	84.9 ± 8.3[d]	94.6 ± 21.5[dc]	92.1 ± 18.5[dc]
Isoleucine	97.8 ± 12.4[a]	101.5 ± 20.0[a]	104.1 ± 15.9[a]	67.5 ± 8.1[bc]	79.4 ± 22.8[b]	73.3 ± 18.1[bc]	57.1 ± 9.2[c]
Valine	256.4 ± 39.4[a]	233.7 ± 44.4[ab]	229.8 ± 46.3[ab]	233.2 ± 36.4[ab]	261.1 ± 56.8[a]	192.1 ± 33[bc]	174.2 ± 26.6[c]

[1] Values are means ± SD. Due to technical reasons, the data in this table is for 4 subjects. Means in a row without a common superscript differ, $P < 0.05$.

SAS statistical software (version 8.2; SAS Institute, Cary, NC). In all cases, differences were considered significant at $P < 0.05$. The effect of BCAA intake on $F^{13}CO_2$ and phenylalanine oxidation was analyzed by repeated-measures ANOVA to compare the visual breakpoint with a 10 and 20% reduction for each of the subjects. Student-Newman-Keuls was performed as a post-hoc test. To compare the difference between breakpoint values and a 10% reduction and breakpoint values and a 20% reduction, an orthogonal contrast was applied. A post-hoc power analysis was applied to $F^{13}CO_2$ and phenylalanine oxidation data.

RESULTS

Characteristics and energy intake of men participating in the study are presented in Table 1. Weight, lean body mass and fat-free mass did not differ during the experimental periods.

BCAA intake affected plasma concentrations as follows (**Table 2**): when Val was kept constant at the requirement level and the other two BCAA were changed, plasma Leu and Ile decreased ($P < 0.0001$) compared with the corresponding values at the requirement level (or visual breakpoint). When Ile was kept constant, Leu concentration decreased ($P < 0.0001$) compared with the Leu concentration at the requirement level. When Leu was kept constant at the requirement level, Leu, Ile and Val plasma concentrations decreased ($P < 0.0001$), compared with the values at the requirement level.

Varying BCAA affected the proportions of ($F^{13}CO_2$) label oxidation (**Table 3**). Visual inspection of individual breakpoints from our previous study determined the total BCAA requirement to be 80, 100, 140, 120 and 140 mg/(kg · d) for subjects 1, 2, 3, 4 and 5, respectively. Changing Leu and Val while Ile was kept constant at the breakpoint level (requirement) affected L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) ($P = 0.02$); however, changing Ile and Leu (while Val was kept constant at the breakpoint level) or changing Ile and Val (while Leu was kept constant at the breakpoint level) did not affect L-[1-^{13}C]phenylalanine oxidation or $F^{13}CO_2$ ($P = 0.36$ and $P = 0.07$, respectively). In fact, when Ile was kept constant, $F^{13}CO_2$ tended to increase after both the 10 ($P > 0.058$) and 20% ($P = 0.007$) reductions compared with the breakpoint. $F^{13}CO_2$ did not differ between the two levels (−10 vs. −20%) ($P = 0.27$). When Val was kept constant, the 10% reduction ($P = 0.25$) or 20% reduction ($P = 0.199$) did not differ from the visual breakpoint, and there was no difference ($P = 0.88$) between the levels (–10% and −20%) . When Leu was held constant, the 10% reduction tended to differ com-

TABLE 3

The effect of varying the proportions of branched chain amino acid (BCAA) intake on $F^{13}CO_2$ data for five healthy adult men[1]

		BCAA proportion[2]					
		Ile constant		Val constant		Leu constant	
Subject	Visual breakpoint	−10%	−20%	−10%	−20%	−10%	−20%
		μmol/(kg · h)					
1	0.46	0.49	0.53	0.50	0.51	0.72	0.70
2	0.45	0.55	0.62	0.52	0.46	0.46	0.50
3	0.46	0.48	0.66	0.50	0.51	0.58	0.50
4	0.31	0.65	0.74	0.50	0.51	0.63	0.79
5	0.59	0.72	0.69	0.87	0.97	0.59	0.58
Mean ± SD	0.45 ± 0.099	0.58 ± 0.10	0.65 ± 0.08	0.58 ± 0.16	0.59 ± 0.21	0.60 ± 0.09	0.61 ± 0.13
Mean difference from the breakpoint, %		27.3	42.7	27.3	30.4	31.3	35.2

[1] $F^{13}CO_2$ is the rate of the release of $^{13}CO_2$ from ^{13}C-phenylalanine oxidation.
[2] $F^{13}CO_2$ values for determination of total BCAA requirement for each subject ($n = 35$ observations).

pared with the visual breakpoint ($P = 0.059$), and the the 20% reduction differed ($P = 0.038$) from the visual breakpoint; the 10 and 20% reductions did not differ ($P = 0.81$).

Flux and oxidation of L-[1-^{13}C]phenylalanine are given in **Table 4**. Phenylalinine flux was not affected by total BCAA intake ($P = 0.96$). Flux ($P = 0.081$) and oxidation rates ($P = 0.23$) did not differ among the individuals. However, changing the proportion of BCAA affected L-[1-^{13}C]phenylalanine oxidation ($P = 0.037$) when Ile was kept constant and the other two amino acids were decreased to 20 and 10% less than the requirement. When Ile was held constant, phenylalanine oxidation was not affected ($P = 0.158$) by the 10% reduction compared with the visual breakpoint; however, phenylalanine oxidation increased ($P = 0.012$) with the 20% reduction and there was no difference ($P = 0.223$) between the levels (−10 vs. –20%). When Val was held constant, phenylalanine oxidation did not differ from the 10% ($P = 0.176$) or 20% reduction ($P = 0.144$) compared with the breakpoint, and there was no difference ($P = 0.909$) between the levels (−10 vs. –20%). When Leu was held constant, the 10% reduction did not differ ($P = 0.125$) from the visual breakpoint; however, the 20% reduction increased ($P = 0.018$) compared with the visual breakpoint, and their was no difference ($P = 0.358$) between the levels (−10 vs. –20%).

DISCUSSION

We previously studied the mean requirement for total BCAA of young healthy adult men with the IAAO technique, using a mixture of BCAA based on the proportion of these amino acids in egg protein and determined the mean requirement for total BCAA to be 144 mg/(kg · d) (13). The concept of IAAO technique is that the partitioning of any indispensable amino acid between oxidation and protein synthesis is sensitive to the level of the most limiting amino acid in the diet. Therefore, when the limiting amino acid is provided, protein synthesis will increase and the oxidation of the amino acids and the indicator will decrease.

The present study is an extension of an earlier work to examine the limiting BCAA in our mixture. We recruited five men who had also participated in the first study for the requirement of total BCAA; therefore, we knew each individual's total BCAA requirement. There were two options in designing this study: *1*) to change two BCAA at the same time, while keeping one constant at the requirement level; or *2*) to change one BCAA and keeping the other two constant at the requirement level. We chose the first option because changes in the total BCAA between each level of intake (10% lower and 20% lower than requirement) were larger than in the second option. Therefore it was easier to monitor the changes in the plasma concentrations as well as other variables.

When Val intake (Val/Leu or Val/Ile) was reduced in the mixture, and the third BCAA was held constant, oxidation of the indicator amino acid was increased. That is, when Ile was held constant and Val and Leu reduced by 10% and 20% in the mixture, oxidation of the indicator amino acid increased significantly as indicated by $F^{13}CO_2$ and phenylalanine oxidation. Our next step was to investigate whether Val or Leu is limiting in the mixture. Therefore, we then kept Val constant and decreased the other two BCAA (Leu and Ile); oxidation of the indicator amino acid did not differ. We then decided to keep Leu at the requirement level and reduce Val and Ile 10% and 20% than the requirement. The oxidation of the indicator amino acid ($F^{13}CO_2$ and phenylalanine oxidation) increased significantly when the two amino acids were reduced 20%.

Keeping Ile at the requirement level and decreasing Val and Leu by 20% increased the $F^{13}CO_2$ and phenylalanine oxidation by ~43 and 71%, respectively. Keeping Leu at the requirement level and decreasing Val and Ile by 20% increased the $F^{13}CO_2$ and phenylalanine oxidation by 35 and 66%, respectively. When Val was held at the requirement level and Leu and Ile decreased by 20%, the $F^{13}CO_2$ and phenylalanine oxidation increased 30 and 39%, respectively.

The number of subjects in this study was limited by the fact that we had to define each individual's total BCAA requirement before entry into the study. Therefore, we felt that it was appropriate to analyze $F^{13}CO_2$ data (Table 3) and phenylalanine oxidation data (Table 4) in more detail. Beyond repeated-measures ANOVA, we compared the difference between breakpoint values and the 10% reduction and breakpoint values and the 20% reduction, even when differences were not present. In none of the studies did a 10% reduction result in an increase in $F^{13}CO_2$ or phenylalanine oxidation. However, with a 20% reduction, when either Ile or Leu was held constant, oxidation of the indicator increased significantly. In both of these studies, Val was reduced, whereas the other amino acid was either Ile or Leu; therefore, the common

TABLE 4

Effect of varying branched-chain amino acid (BCAA) proportions on phenylalanine kinetics of five healthy adult men[1]

BCAA proportion	Phenylalanine flux[2]	Phenylalanine oxidation	Mean difference from the breakpoint
	μmol/(kg · h)		%
Visual breakpoint (or the requirement)	47.91 ± 19.96	2.89 ± 1.14	
Ile constant (at the requirement level)			
(−10%)	49.73 ± 11.42	3.98 ± 0.94	37.7
(−20%)	54.86 ± 16.3	4.93 ± 1.11	70.5
Val constant (at the requirement level)			
(−10%)	51.02 ± 23.6	3.94 ± 1.53	36.3
(−20%)	50.51 ± 9.93	4.02 ± 0.70	39
Leu constant (at the requirement level)			
(−10%)	46.22 ± 15.71	4.08 ± 1.70	41.2
(−20%)	55.03 ± 9.40	4.79 ± 1.23	65.7

[1] Values are means ± SD, $n = 35$ observations.
[2] Phenylalanine flux was not affected by changes in BCAA proportions.

factor was a reduction in Val, supporting our interpretation that Val is the first limiting BCAA in the mixture.

In practical terms, this study was possible only because we retained 5 of the 7 men who participated in our previous experiment (13), which allowed us to know each individual's requirement. A post-hoc power analysis showed that the number of subjects was sufficient to detect the change in oxidation of the indicator amino acid when Ile was held constant (0.87) and when Leu was kept constant (0.93); when Val was held constant, the power was <0.8 (0.76). Hence the two studies, which collectively showed that Val is the most limiting BCAA, had sufficient power.

Although Leu metabolism in humans has been studied extensively (10,25,30,32–40), knowledge of Val and Ile metabolism (8,11,41–43) is scarce. Although Leu and Val have similar patterns of metabolism, it has been shown that Val catabolism is slower than that of Leu (8,43). In a study by Staten et al. (8), plasma Val concentration was found to be twice the plasma Leu concentration, whereas the α-ketoisovalerate (KIV) level was about half the α-ketoisocaproate level in normal men. Therefore the transamination equilibrium seems to favor Leu deamination, on the one hand, and KIV reamination on the other. This results in a less favorable transamination equilibrium for Val, and may require the body to maintain Val at a higher free concentration than Leu for similar overall rates of oxidation (8). Staten et al. (8) also suggested that although the decarboxylation step may limit the catabolism of the BCAA, the transamination step may regulate the plasma levels of the individual BCAA.

In this study, maintaining Val intake at the requirement level and changing the intakes of Ile and Leu to 10 and 20% less than the requirement did not change L-[1-^{13}C]phenylalanine oxidation. This may also indicate that Val is required at a higher concentration in the diet so that it can be available for protein synthesis and oxidation.

The pattern of BCAA plasma concentrations demonstrated a possible interaction among the three BCAA. Especially when Leu was held constant, their concentration decreased. We can speculate that when Leu was held constant while the other two BCAA were reduced, there was a relative excess of Leu and stimulation of branched-chain keto acid dehydrogenase activity (4). Conversely, we cannot explain why the Val concentration did not fall significantly (although it fell numerically) when Ile was held constant and Val and Leu were reduced.

From the nutritional and dietary standpoints, the profile of an amino acid in a food is important to improve protein synthesis. A notable example in this regard is lysine, which is a limiting amino acid in cereals. Although the BCAA are not limiting in any food items because there are known interaction among these amino acids, their balance in the diet and food items is of importance to promote protein synthesis in the body. The major finding of this study is that a lower ratio of Val to Ile and Leu may be limiting for protein synthesis.

As mentioned previously in an earlier work (13), we estimated the mean total BCAA requirement using a mixture of BCAA based on the proportion tested in this study. Therefore the individual BCAA requirement, which was calculated on the basis of this proportion, may have overestimated Leu and Ile requirements by ~10%. Future studies should focus on establishing the exact requirements for Leu and Ile. That is, while keeping Val constant at the requirement level, decrease Leu or Ile, one at a time, in the range of 10–20% to define the lowest amount of these BCAA for protein synthesis.

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