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Daily requirement for and splanchnic uptake of leucine in healthy adult Indians[1–4]

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ABSTRACT

Background: The 1985 FAO/WHO/UNU requirement for leucine is too low according to tracer-derived estimates of leucine oxidation and balance in adults from developed regions.

Objective: The leucine requirement in populations in developing countries was assessed with use of the 24-h tracer balance method and on the basis of nitrogen balances.

Design: Twenty healthy Indian men were studied during their consumption for 6 d of 2 L-amino acid diets that supplied either 14 and 30 (n = 10) or 22 and 40 (n = 10) mg leucine·kg^{-1}·d^{-1} in random order. At 1800 on day 7, a 24-h constant intravenous [^{13}C]leucine tracer-infusion protocol was conducted to determine leucine oxidation and daily leucine balance. During the intake of 40 mg leucine/d, [$^{2}H_{3}$]leucine was given orally to assess the splanchnic uptake of leucine.

Results: Mean 24-h leucine oxidation rates were 29.8, 30.6, 33.6, and 39.3 mg·kg^{-1}·d^{-1} at leucine intakes of 14, 22, 30, and 40 mg·kg^{-1}·d^{-1}, respectively; daily leucine balances were −16.5, −9.0, −3.3, and 0.5 mg·kg^{-1}·d^{-1}, respectively. Mixed-models linear regression of balance against leucine intake resulted in a zero balance at a leucine intake of 37.3 mg·kg^{-1}·d^{-1}. Nitrogen balances were −12.7, −17.9, −3.9, and 1.0 mg·kg^{-1}·d^{-1} at leucine intakes of 14, 22, 30, and 40 mg·kg^{-1}·d^{-1}. Regression of nitrogen balance against intake resulted in a zero balance at a leucine intake of 37.6 mg·kg^{-1}·d^{-1}. The first-pass splanchnic uptake of leucine was 45.7% and 33.9% in the fasted and fed periods, respectively.

Conclusion: A tentative mean leucine requirement of 40 mg·kg^{-1}·d^{-1} is proposed for healthy Indian adults, as it is for Western subjects. *Am J Clin Nutr* 2001;74:747–55.

KEY WORDS Indian adults, leucine requirement, amino acid oxidation, amino acid balance, splanchnic uptake, India

INTRODUCTION

There is an expanding body of evidence recommending for adult humans the use of revised indispensable amino acid requirements (1, 2), which are ≈2–3 times higher than the current international recommendations (3). The former requirements were initially based on results of short-term tracer studies (4, 5) and predicted obligatory losses (2). For leucine, short-term tracer balance studies showed that the requirement for body leucine equilibrium was greater than the 1985 FAO/WHO/UNU value of 14 mg·kg^{-1}·d^{-1} (3) and in the range of ≈40 mg·kg^{-1}·d^{-1} (6, 7). These short-term tracer balance findings were confirmed in longer-term 24-h tracer balance studies in Western subjects (8, 9), in whom leucine equilibrium was achieved at a leucine intake of 40 mg·kg^{-1}·d^{-1} but not at an intake of 14 mg·kg^{-1}·d^{-1}. In these studies, estimated leucine oxidation rates and, therefore, the tracer balance technique, were validated against measures of nitrogen excretion (8) made at the actual time of the tracer study. Millward (10) proposed a leucine requirement of 26 mg·kg^{-1}·d^{-1}. This requirement is based on his recomputation of the regression equation of Hegsted (11) and was derived by using a value of 5 mg N·kg^{-1}·d^{-1} for integumental and increased losses and assuming a body weight of 60 kg. The requirement estimated originally by Hegsted (11) was 43 mg leucine·kg^{-1}·d^{-1}.

These findings of a higher leucine requirement for adult humans have not been validated with use of the same techniques in populations around the world. Thus, these findings need to be validated in populations from developing countries, where there may be physiologic adaptations to chronically lower intakes than are usual for Western subjects, resulting in lower physiologic requirements. Therefore, the first aim of this study was to apply the 24-h leucine tracer balance technique in a healthy population from a developing country, studied for 1 wk, at intakes of leucine that ranged from the FAO/WHO/UNU recommendation (3) to the tentative requirement (40 mg·kg^{-1}·d^{-1}) proposed at the Massachusetts Institute of Technology (MIT) (12), which was predicted from obligatory oxidative amino acid losses (13) or estimated with use of the tracer balance approach. Thus, intermediate leucine intakes of 22 and 30 mg·kg^{-1}·d^{-1} were used in the present study in an attempt to assess an inflection point on

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TABLE 1
Characteristics of healthy Indian men studied to determine their leucine requirements[1]

Characteristic	Value
Age (y)	21.35 ± 1.11
Weight (kg)	59.48 ± 8.05
Height (m)	1.72 ± 0.06
BMI (kg/m^2)	20.05 ± 1.50
Midupper arm circumference (cm)	25.10 ± 2.27
Percentage body fat (%)	15.70 ± 4.44
Fat-free mass (kg)	49.71 ± 6.49

[1] $\bar{x}$ ± SD; n = 20.

the leucine balance versus leucine intake curve. Nitrogen balances were also measured on the last 4 d of the 1-wk experimental feeding period.

Studies in which the lysine requirement was estimated with use of the indicator (tracer leucine) amino acid oxidation balance technique (14, 15) also suggest that the first-pass splanchnic uptake of leucine might be higher than that for Western subjects at a generous intake of leucine. It was therefore of interest to measure the splanchnic uptake of leucine in Indian subjects because the uptake may have a bearing on whether leucine oxidation rates were measured with acceptable accuracy. Hence, a second but less extensive aim of the present study was to measure the splanchnic extraction of leucine at the highest leucine intake (40 mg · kg^{-1} · d^{-1}) after the simultaneous administration of leucine tracers orally and intravenously.

SUBJECTS AND METHODS

Subjects

Twenty young adult men recruited from the student population of St John's Medical College, Bangalore, India, participated in this experiment. The physical characteristics of the subjects are given in **Table 1**. The body mass indexes (BMIs; in kg/m^2) of the subjects ranged from 18.60 to 23.53. All subjects were in good health as determined by medical history, physical examination, blood cell count, routine blood biochemical profile, and urinalysis, and their mean habitual leucine intake was estimated to be <70 mg · kg^{-1} · d^{-1} on the basis of a 3-d weighed dietary intake record. Mean (±SD) estimated habitual energy and protein intakes were 154 ± 21 kJ · kg^{-1} · d^{-1} and 0.9 ± 0.1 g · kg^{-1} · d^{-1}, respectively. Subjects who smoked cigarettes, consumed ≥5 alcoholic drinks/wk, or drank >6 cups of caffeinated beverages/d were excluded from participation. The purpose of the study and the potential risks involved were explained to each subject. Written consent was obtained from each subject and the research protocol was approved by the Human Ethical Approval Committee of St John's Medical College.

Anthropometric measurements

Anthropometric and skinfold-thickness measurements were made on day 0. Subjects were weighed while wearing minimal clothing with the use of a digital scale (Soehnle-Waagen GmbH & Co, Murrhardt, Germany) that had a precision of 0.1 kg. All weights were measured twice and the mean was expressed to the nearest 0.1 kg. The heights of shoeless subjects were recorded with the use of a vertically mobile scale (Holtain Ltd, Crymych, United Kingdom) and are expressed to the nearest centimeter. Biceps, triceps, subscapular, and suprailiac skinfold-thickness measurements were made in duplicate while subjects were standing, and the mean of each was used for additional calculations. All skinfold-thickness measurements were standardized (16) and carried out to the nearest 0.2 mm with the use of skinfold calipers (Holtain Ltd). The logarithm of the sum of the 4 skinfold thicknesses was used in age- and sex-specific equations (17) to obtain an estimate of body density, from which percentage body fat was determined (18).

Diet and experimental design

The 24-h [^{13}C]leucine tracer experiment was carried out after a 6-d period in which the experimental diet was consumed. Each group of 10 subjects was studied during 2 separate diet periods, during which they consumed a weight-maintaining diet based on an L-amino acid mixture providing different daily lysine intakes (**Table 2**). Daily energy intakes were designed to maintain body weight, and the energy requirement was calculated to be ≈1.6 × basal metabolic rate (BMR) from days 1 to 6 and ≈1.35 × BMR on day 7 (tracer study day). The subjects were encouraged to maintain their customary physical activity levels but were asked to refrain from excessive or competitive exercise. The major energy supply was given in the form of a sugar-oil formula and protein-free, wheat-starch cookies (**Table 3**). Nonprotein energy was provided as fat (≈43% of energy) and carbohydrate (≈56% of energy). The main source of carbohydrate was beet sugar and wheat starch, to attain a low ^{13}C content in the diet and a relatively steady background in breath $^{13}CO_2$ enrichment over the 24-h period.

TABLE 2
Composition of amino acid mixtures used to supply 4 leucine intakes daily

Amino acid	Leucine intake (mg · kg^{-1} · d^{-1})			
	14	22	30	40
	mg/g mixture			
L-Tryptophan	15.77	15.74	15.70	15.66
L-Threonine	47.61	47.51	47.41	47.28
L-Isoleucine	63.52	63.39	63.25	63.08
L-Leucine[1]	4.30	11.59	18.67	27.62
L-Lysine · HCl	84.56	84.38	84.20	83.98
L-Methionine	30.00	29.94	29.88	29.80
L-Cystine	22.25	22.20	22.16	22.10
L-Phenylalanine	55.26	55.14	55.03	54.88
L-Tyrosine	41.18	41.09	41.01	40.90
L-Valine	71.03	70.88	70.73	70.54
L-Histidine · HCl	31.00	30.94	30.87	30.79
L-Arginine · HCl	76.41	76.25	76.09	75.88
L-Alanine	193.58	193.16	192.75	192.24
L-Aspartic acid	12.06	12.04	12.01	11.98
L-Glutamic acid	29.83	29.77	29.70	29.62
Glycine	99.37	94.02	88.82	82.25
L-Proline	40.75	40.66	40.58	40.47
L-Serine	81.51	81.33	81.16	80.94
Total[2]	1000.00	1000.00	1000.00	1000.00

[1] 9.25 mg leucine · kg^{-1} · d^{-1} was added to each mix every day, except on the infusion day, when this amount of leucine was infused as a tracer. If 2 tracers were infused ([$^2H_3$]- and [^{13}C]leucine), appropriate reductions in unlabeled leucine intake were made in the diet.

[2] 1.11 g mixture · kg^{-1} · d^{-1} was given to subjects and provided 160 mg N · kg^{-1} · d^{-1}.

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TABLE 3
Composition of the experimental diet used to supply adequate energy with 1 of 4 test leucine intakes

Diet	
Protein-free cookies[1]	
Carbohydrate content (% of energy)	56
Fat content (% of energy)	43
Flavored drink[2]	
Carbohydrate content (% of energy)	56
Fat content (% of energy)	43
Total diet	
Carbohydrate content (% of energy)	56
Fat content (% of energy)	43
Supplement	
Multivitamin-multimineral capsule[3]	1
Sodium chloride (g/d)[4]	6
Calcium (mg/d)[5]	1000
Potassium (mg/d)[6]	1260
Dietary fiber (g/d)[7]	20
Choline (mg/d)[8]	500

[1] Made from protein-free wheat starch, butter, and beet sugar.
[2] Unsweetened flavored beverage (Kool-Aid; Kraft General Foods, Inc, White Plains, NY) with beet sugar, sunflower oil (Flora; Brooke Bond Lipton India Ltd, Calcutta), and flavoring (Vivonex; Sandoz Nutrition Corp, Minneapolis).
[3] Becadexamine tablet (Glaxo India Ltd, Ahmedabad, India). Each tablet contained 5000 IU vitamin A, 400 IU cholecalciferol, 15 mg α-tocopheryl acetate, 5 mg thiamine, 5 mg riboflavin, 2 mg vitamin B-6, 45 mg nicotinamide, 5 mg D-panthenol, 1000 μg folic acid, 5 μg vitamin B-12, 75 mg vitamin C, 50 mg ferrous fumarate, 70 mg dibasic calcium phosphate, 0.1 mg copper sulfate, 0.01 mg magnesium sulfate, 50 mg zinc sulfate, 0.025 mg potassium iodide, and 0.15 mg magnesium oxide.
[4] Captain Cook iodized salt; International Best Foods, Ltd, Bombay, India.
[5] Shelcal-250; Elder Pharmaceuticals Ltd, Bombay, India.
[6] K-Guard; Wockhardt Ltd, Aurangabad, India.
[7] Sat-Isabgol; Charak Pharmaceuticals Ltd, Gujarat, India.
[8] Choline chloride; Rolex Chemical Industry, Bombay, India.

The subjects were randomly assigned to receive 2 experimental diets providing either 14 and 30 or 22 and 40 mg leucine $\cdot$ kg^{-1} $\cdot$ d^{-1} (Table 2). The second diet period began within 1–4 wk of the end of the first 24-h diet period. During this interval, subjects consumed their free-choice diets.

During the experimental dietary period, all other nutrients were provided in adequate amounts (Table 3). A choline supplement of 500 mg was given daily and dietary fiber was provided as 20 g ispagul (Charak Pharmaceuticals Ltd, Gujarat, India) when requested by the subject. The total daily food intake was consumed as 3 isoenergetic, isonitrogenous meals (at 0800, 1300, and 2000). Each morning, body weight was measured and vital signs were monitored. All of the subjects' meals were consumed at the kitchen of the Nutrition Research Center, under supervision of the dietary staff.

24-h Tracer-infusion protocol

The primed 24-h tracer-infusion protocol was conducted in all subjects according to a standard design, as previously described (8, 14, 15). After the subjects consumed their last meal at 1500 on day 6, the tracer administration began at 1800 and ended at 1800 on day 7. Subjects received 10 small isoenergetic, isonitrogenous meals at hourly intervals beginning at 0600 and ending at 1500; together, these meals provided the equivalent of the 24-h dietary intake for that day. Indirect calorimetry was performed hourly, and blood was withdrawn half-hourly for measurement of [$^2H_3$]leucine, [^{13}C]leucine, [$^2H_3$]α-ketoisocaproic acid (KIC), and [^{13}C]KIC enrichments. Throughout the 24-h study, the subjects remained in bed in a reclining position, except during sleep when they lay supine. Thus, the 24-h study was divided into two 12-h metabolic periods (fasted and fed).

The primed, intravenous administration of 1-[^{13}C]leucine (99.3 atom%; MassTrace, Woburn, MA) was given at a known rate of ≈2.8 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1}; the prime was ≈4.2 μmol/kg and was administered as a bolus at the start of the experiment. The bicarbonate pool was primed intravenously with 0.8 μmol [^{13}C]sodium bicarbonate/kg (99.9 atom%; MassTrace). In subjects who received the highest leucine intake (40 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}; $n = 10$), the splanchnic uptake of leucine was studied after a primed, intermittent (hourly) oral dose of 2.8 μmol [$^2H_3$]leucine $\cdot$ kg^{-1} $\cdot$ h^{-1}; the prime was ≈4.2 μmol/kg. The tracers were prepared in physiologic saline, under sterile conditions.

Recovery of $^{13}CO_2$ and the contribution of dietary ^{13}C to breath $^{13}CO_2$

Because the diets used contained low amounts of ^{13}C-enriched carbohydrate, the contribution to breath $^{13}CO_2$ from the experimental diet was expected to be low, although a correction was made for this small contribution of endogenous ^{13}C substrate oxidation over the 24-h study period, as previously described (8). The recovery of breath $^{13}CO_2$ was calculated for every 30-min interval as previously described (8). Values at each time point were used to correct each 30-min estimate of $^{13}CO_2$ production from [^{13}C]leucine oxidation (*see* below).

Indirect calorimetry

Minute-to-minute total carbon dioxide production ($\dot{V}CO_2$) and oxygen consumption ($\dot{V}O_2$) were determined with an open-circuit indirect calorimeter with a ventilated hood, as previously described (14, 19). Whole-system calibration was verified by combustion of pure ethanol; the observed difference between measured and predicted total $\dot{V}CO_2$ was <3% and the average respiratory quotient was between 0.64 and 0.68. Measurements of respiratory exchange were made during alternate hours throughout the entire 24-h period.

Collection and analysis of breath samples

Three baseline breath samples were collected at −30, −15, and −5 min before the 24-h tracer infusion started and then at half-hourly intervals throughout the 24-h study, except between 0000 and 0600, when samples were collected hourly. Breath gas was collected in a specially designed bag that permitted the removal of dead space air and was transferred into three 10-mL non-silicon-coated glass tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) with a thin needle (PrecisionGlide, 24G; Becton Dickinson) that was attached to the bag by means of a 3-way tap. When the breath-sample collection coincided with hourly meals or isotope administration, the breath sample was collected first. The samples were stored at room temperature until isotope ratio mass spectrometry (Europa Scientific, Crewe, United Kingdom) was used to analyze the ratio of $^{13}CO_2$ to $^{12}CO_2$ as previously described (14). The increase in breath enrichment after isotope administration was expressed as atom percent excess (APE). The APE was calculated by taking the

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arithmetic difference between enrichment of each breath sample and the predose basal breath sample.

Collection and analysis of blood and urine samples

Blood samples were collected at 30-min intervals between 0000 and 2400 of the tracer infusion period. Three baseline samples at −30, −15, and −5 min were taken before administration of the [^{13}C]leucine tracer. Blood sampling (≈5 mL per sample) was performed through a 20-gauge, 5-cm catheter placed into a superficial vein of the dorsal hand or wrist on the nondominant side. The catheter was introduced in an antiflow position to facilitate blood withdrawal while the hand was in a custom-made warming box that was maintained at 65°C for 15 min before withdrawal of each sample to achieve arterialization of venous blood. The arterialization of the blood sample was checked earlier by measuring hemoglobin saturation in the withdrawn blood; saturation was >90%. The patency of the vein was maintained by slow infusion of normal saline. Blood samples were drawn into 5-mL syringes and transferred into anticoagulant tubes and centrifuged for 15 min at 1200 × *g* in a refrigerated centrifuge (4°C). The plasma was removed and the samples were stored at −80°C until analyzed.

The method for measuring [$^2H_3$]leucine, [^{13}C]leucine, [$^2H_3$]KIC, and [^{13}C]KIC enrichments in plasma was previously described (8, 14), except that we used a Varian 2000 ITD mass spectrometer coupled to a Varian 3800 gas chromatograph (Varian, Palo Alto, CA). Enrichments were measured against calibration graphs prepared from standard mixtures ranging from 0% to 4% molar fractions of the isotope being analyzed. Standard mixtures of known molar ratios of both [^{13}C]KIC and [$^2H_3$]KIC against unlabeled KIC were analyzed to determine the response of the peak area ratios of these ions: either 260:259 (when only [^{13}C]leucine was infused) or 260:259 and 262:259 (when both [^{13}C]leucine and [$^2H_3$]leucine were infused). For leucine, the parent ion was 302, and the peak area ratios 303:302 ([^{13}C]leucine) and 305:302 ([$^2H_3$]leucine) were measured. The correlation coefficient was always >0.99. This procedure also corrected for the small cross contribution of one tracer to the primary ion of the other when a mixture of 2 isotope species was analyzed, when [^{13}C]leucine and [$^2H_3$]leucine were infused. For each plasma sample, the measured peak area ratios were solved for the 2 unknowns ([^{13}C] and [$^2H_3$] enrichments) with the use of response factors determined that day from the standards. The CVs for repeated measures of single-sample enrichment were 2.1% and 2.9% for [^{13}C]KIC and [$^2H_3$]KIC, respectively, and were 1.8% and 1.6% for [^{13}C]leucine and [$^2H_3$]leucine, respectively. The CV for 6 repeated measurements of a quality-control plasma sample enriched with [^{13}C]KIC over 3 mo was 0.5%. The isotopic abundance of plasma [^{13}C]KIC was considered to represent the enrichment of the intracellular leucine pool that was undergoing leucine oxidation (8, 20).

Complete 24-h urine collections were made throughout the study and the daily fecal output was homogenized with known quantities of distilled water; the samples were kept at −20°C until analyzed. The urine and fecal samples were analyzed for their total nitrogen content with use of the micro-Kjeldahl method.

Leucine oxidation

Leucine oxidation was computed for consecutive half-hourly intervals to improve the accuracy of the 24-h leucine oxidation value because there was a variable rate of leucine oxidation throughout the 24-h period. For each half-hourly interval, leucine oxidation was computed as follows:

$$\text{Leucine oxidation } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\text{ min}^{-1}) = {}^{13}CO_2 \text{ production } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\text{ min}^{-1})/{}^{13}\text{C[KIC] enrichment (APE/100)} \quad (1)$$

where ^{13}C[KIC] enrichment is the average of the 2 enrichments determining the specific half-hourly interval and where

$$^{13}CO_2 \text{ production } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\text{ min}^{-1}) = \dot{V}CO_2 \ (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\text{ min}^{-1}) \times {}^{13}CO_2 \text{ enrichment} \times 1/R \quad (2)$$

where $^{13}CO_2$ enrichment = (APE/100) and R is the recovery of $^{13}CO_2$ computed for each time interval as previously described (12, 13).

In addition, within each metabolic period, $\dot{V}CO_2$ over the time interval when it was not directly measured was derived as the arithmetic average of $\dot{V}CO_2$ measured just before and after this interval.

Leucine balance

The 24-h leucine balance (input − measured output) was computed as follows:

$$\text{Input } (\mu\text{mol/kg}) = \text{dietary leucine} + \text{intravenous tracer} \quad (3)$$

$$\text{Output } (\mu\text{mol/kg}) = \text{sum of determined oxidation for the 48 half-hourly intervals} \quad (4)$$

Splanchnic uptake of leucine

The splanchnic uptake of leucine was calculated for the 12-h fasted and fed periods as follows:

$$\text{Splanchnic uptake } (\%) = 1 - ([^2H_3]\text{leucine enrichment}/[^2H_3]\text{leucine infusion rate})/([^{13}\text{C}]\text{leucine enrichment}/[^{13}\text{C}]\text{leucine infusion rate}) \quad (5)$$

where leucine enrichment refers to the mean plasma enrichment during the 12 h of fasting and feeding.

Nitrogen balance

Nitrogen balance was computed as follows:

$$\text{Nitrogen balance } (\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}) = \text{nitrogen intake} - (\text{urinary total nitrogen} + \text{fecal nitrogen} + 8) \quad (6)$$

where all nitrogen values were for a 24-h period and the factor of 8 mg·kg^{-1}·d^{-1} refers to miscellaneous losses of nitrogen, including that in skin, hair, and nails (3).

Statistical methods and data evaluation

Data are presented as means ± SDs. The metabolic variables were analyzed with the use of mixed-models analysis of variance (PROC MIXED, version 6.12; SAS Institute Inc, Cary, NC). The models for 12-h leucine oxidation and flux included factors for diet period, leucine intake (which is both a within-subject and between-subject factor), metabolic period (fasted or fed), and the interaction between leucine intake and metabolic period. For flux, if the interaction between leucine intake and metabolic period was significant, then model contrasts were used to make pairwise comparisons of interest and comparisons of 24-h flux between different lysine intakes. If the interaction was not significant and the main effect of leucine intake was, then model contrasts were used to compare means between

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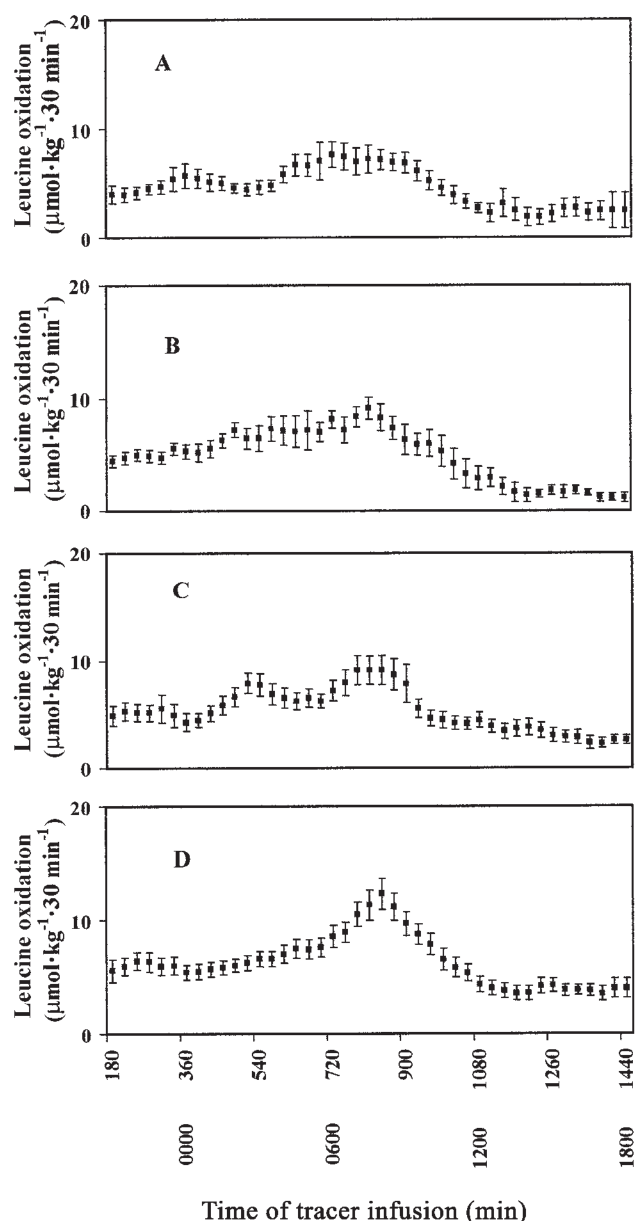

FIGURE 1. Mean (±SD) leucine oxidation measured for each 30-min interval throughout a 24-h tracer infusion of 12 (A), 20 (B), 28 (C), and 36 (D) mg L-[1-^{13}C]leucine·kg^{-1}·d^{-1}. Feeding began with small meals at 0600 to provide the required intake of leucine for that level of feeding (ie, 14, 22, 30, and 40 mg leucine·kg^{-1}·d^{-1}) and was adjusted for the amount of leucine that was administered with the tracer infusion. The feeding ended with the last meal at 1500.

leucine intakes regardless of metabolic period; these comparisons applied to the 24-h flux values. For oxidation, rather than generally assessing the interaction between leucine intakes and metabolic period and proceeding as described above, we fit the model with interaction and used model contrasts to test specific hypotheses: *1*) 12-h oxidation during the fed period would be higher at a leucine intake of 40 mg·kg^{-1}·d^{-1} than at the 3 lower intakes, but oxidation at the 3 lower leucine intakes would not differ significantly, and *2*) 12-h oxidation would be positive (ie, fed − fast > 0) at a leucine intake of 40 mg·kg^{-1}·d^{-1}, with either no difference or negative differences in oxidation at the 3 other leucine intakes. These hypotheses are based on the following observations: *1*) studies in growing rats showed that oxidation rates of leucine (21), threonine (22), and lysine (23) are low and relatively constant within the lower submaintenance range; *2*) oxidation increases with additional increases in intake beginning before the intake that maximizes growth, feed efficiency, or both is reached (21–23); *3*) leucine oxidation during the fed period is higher at a daily leucine intake of 38.3 mg·kg^{-1}·d^{-1} than at an intake of 14 mg·kg^{-1}·d^{-1}(9); and *4*) the known decline in the efficiency of dietary protein utilization at the upper end of the submaintenance range declines as the intake of good-quality protein just begins to reach the intake required for maintenance of nitrogen equilibrium (24). Because there was no a priori hypothesis about 12-h fasted oxidation, and therefore no a priori hypothesis about 24-h oxidation, the main effect of intake was assessed. If this test was significant, pairwise comparisons were made between oxidation values at the different intakes. For completeness, the test for the interaction is also reported.

For leucine and nitrogen balance, for which only 24-h values were analyzed, the models included only a factor for diet period and for leucine intake; if the main effect was significant, then model contrasts were used to compare means between intakes. Mean leucine or nitrogen balances were compared with the equilibrium state of zero balance, separately for each intake, within the respective model. For the zero balance intercept analysis, leucine intake was included in the models as a continuous covariate, and the best polynomial model (up to cubic) was selected for analysis. Corresponding 95% Fieller's CIs for zero balance leucine intake were calculated.

Plasma enrichments and splanchnic uptake of leucine were estimated only at the 40-mg·kg^{-1}·d^{-1} leucine intake. The model for enrichments included factors for tracer (^{13}C and $^{2}H_3$), molecule (leucine and KIC), and metabolic period (fasted and fed) and their interactions; model contrasts were used to make pairwise comparisons of interest, as appropriate from the significant interactions. The model for splanchnic uptake of leucine included a factor for metabolic period.

All reported *P* values are two-sided, except for the comparisons of leucine and nitrogen balance with the equilibrium state of zero balance. In this instance the hypothesis is that balance is negative; therefore, one-sided tests were used. Post hoc pairwise comparisons were adjusted with the use of Tukey's method. *P* values ≤0.05 were considered significant.

RESULTS

Anthropometry

The mean anthropometric indexes of the subjects are summarized in Table 1. The subjects' mean BMI was 20.05 ± 1.50 and their mean percentage body fat was relatively low: 15.70 ± 4.44%. The characteristics of the subjects in the present study were similar to those of the subjects in our previous 24-h tracer studies (14, 15). There were no significant changes in weight during the 6-d experimental diet periods.

Leucine oxidation

The temporal pattern of leucine oxidation over the 24-h test period at all 4 leucine intakes is depicted in **Figure 1**. When leucine oxidation values were assessed by mixed-models ANOVA,

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TABLE 4
Summary of leucine oxidation and flux at 4 leucine intakes in healthy Indian men[1]

	Leucine intake ($mg \cdot kg^{-1} \cdot d^{-1}$)			
Leucine index	14	22	30	40
Oxidation (mg leucine $\cdot kg^{-1} \cdot d^{-1}$)				
12-h Fasted	15.7 ± 4.7	17.4 ± 7.1	17.8 ± 6.5	19.2 ± 6.5
12-h Fed	14.1 ± 6.3	13.2 ± 4.5	15.8 ± 5.5	20.1 ± 4.2
Total, 24 h	29.8 ± 10.1	30.6 ± 9.8	33.6 ± 9.9	39.3 ± 9.2
Total intake (mg leucine $\cdot kg^{-1} \cdot d^{-1}$)[2]	13.3 ± 0.9	21.6 ± 1.0	30.3 ± 0.8	41.0 ± 4.2
24-h Balance (mg leucine $\cdot kg^{-1} \cdot d^{-1}$)[3,4]	−16.5 ± 10.0[5] (−32 to 5)	−9.0 ± 9.4[5] (−24 to 6)	−3.3 ± 9.8[6] (−18 to 9)	0.5 ± 10.2[6] (−13 to 16)
Flux ($\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$)[7]				
12-h Fasted	45.1 ± 19.3	54.2 ± 15.4	50.5 ± 17.2	48.3 ± 10.2
12-h Fed	37.8 ± 11.4	39.9 ± 8.6	41.9 ± 10.1	48.8 ± 7.3
24-h	41.5 ± 12.1	47.1 ± 11.0	46.2 ± 12.0	48.5 ± 8.2

[1] $\bar{x}$ ± SD; range in parentheses. n = 10 per intake.
[2] Diet + tracer.
[3] Intake − oxidation.
[4] Significant effect of leucine intake, $P = 0.002$ (mixed-models ANOVA).
[5] Significantly different from 0, $P < 0.05$ (mixed-models ANOVA).
[6] Significantly different from 14 $mg \cdot kg^{-1} \cdot d^{-1}$, $P < 0.05$ (mixed-models ANOVA).
[7] There was no significant interaction between leucine intake and metabolic period, $P = 0.07$ (mixed-models ANOVA). Flux was significantly higher during the fasted period than during the fed period, $P = 0.011$.

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the interaction between leucine intake and metabolic period was not significant (**Table 4**). However, we tested our hypothesis as indicated previously. Thus, in the fed state, leucine oxidation either tended to be or was higher at a leucine intake of 40 $mg \cdot kg^{-1} \cdot d^{-1}$ than at the 2 lowest intakes ($P = 0.066$ compared with 14 mg; $P = 0.010$ compared with 22 mg) but did not differ significantly from oxidation at an intake of 30 mg leucine. During the fasted period, there were no significant differences in oxidation between the 4 leucine intakes. When combined as 24-h values, leucine oxidation tended to be higher at a leucine intake of 40 $mg \cdot kg^{-1} \cdot d^{-1}$ than at the 3 other intakes, but differences between the diets were not significant. Furthermore, although there was a positive difference between fed and fasted values (ie, 0.9 ± 1.9 $mg \cdot kg^{-1} \cdot d^{-1}$ at an intake of 40 mg leucine $\cdot kg^{-1} \cdot d^{-1}$), the difference was not significant. The difference between the 2 metabolic periods was negative at the 2 lowest leucine intakes, but was only significant at a leucine intake of 22 $mg \cdot kg^{-1} \cdot d^{-1}$ ($P = 0.038$).

Leucine balance

Daily leucine balance differed significantly between the 4 leucine intakes ($P = 0.002$) and progressively rose toward zero as the leucine intake increased (Table 4). Leucine balances at the 2 lowest leucine intakes were significantly different from zero balance: −16.5 ± 10.0 ($P < 0.001$; one-sided) and −9.0 ± 9.4 ($P < 0.01$; one-sided) $mg \cdot kg^{-1} \cdot d^{-1}$at 14 and 22 mg leucine $\cdot kg^{-1} \cdot d^{-1}$, respectively. The balances expressed as a percentage of leucine showed the same pattern and ranged between −124% and 0.6% of the leucine intake, from the lowest to the highest leucine intakes, respectively.

Leucine flux

For leucine flux, there was no significant interaction between leucine intake and metabolic period (Table 4). Without regard to leucine intake, flux was significantly higher in the fasted period than in the fed period ($P = 0.011$). The effect of leucine intake was not significant; thus, there were no significant differences in 24-h fluxes between leucine intakes.

Nitrogen balance

Nitrogen balances of 9 subjects from each group were estimated for the last 4 d of the 7-d experimental period and computed as the sum of the total daily urinary and fecal nitrogen output. A factor of 8 $mg \cdot kg^{-1} \cdot d^{-1}$ was added to the daily nitrogen output to account for miscellaneous nitrogen losses (3). Thus, nitrogen balances were −12.7 ± 14.8 (range: −48 to −1), −17.9 ± 18.9 (−40 to 7), −3.9 ± 26.3 (−32 to 49), and 1.0 ± 22.3 (−30 to 44) $mg \cdot kg^{-1} \cdot d^{-1}$ at leucine intakes of 14, 22, 30, and 40 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively. There was a tendency of an effect of intake ($P = 0.060$); nitrogen balances at the 2 lowest leucine intakes of 14 and 22 $mg \cdot kg^{-1} \cdot d^{-1}$ tended to be different or were significantly different from zero ($P = 0.080$ and $P = 0.017$, respectively).

Zero balance intercept analysis

A mixed-models linear regression model of the leucine balance data against leucine intake resulted in a zero leucine balance at a leucine intake of 37.3 $mg \cdot kg^{-1} \cdot d^{-1}$ ($P < 0.001$ for the intercept; 95% CI: 32, 50 $mg \cdot kg^{-1} \cdot d^{-1}$). A linear model of the nitrogen balance data against the leucine intake resulted in zero nitrogen balance at a leucine intake of 37.6 $mg \cdot kg^{-1} \cdot d^{-1}$ ($P = 0.002$ for the intercept; 95% CI not determined).

Splanchnic uptake of leucine

The plasma enrichments of [^{13}C]leucine, [$^{2}H_3$]leucine, [^{13}C]KIC, and [$^{2}H_3$]KIC in the fasted and fed periods are shown in **Table 5**. There was a significant interaction between tracer route and metabolic period; there were no significant differences between the fed and fasted periods with the ^{13}C tracer, whereas the difference was significant ($P = 0.019$) with the $^{2}H_3$ tracer. There was a significant interaction between tracer route and labeled molecular form (leucine or KIC). The plasma [^{13}C]KIC enrichment was significantly lower than the plasma [^{13}C]leucine enrichment ($P = 0.008$), regardless of metabolic

TABLE 5
Summary of tracer enrichments in plasma in the fasted and fed periods in the subjects who were administered [^{13}C]leucine intravenously and [$^{2}H_3$]leucine orally at a leucine intake of 40 mg · kg^{-1} · d^{-1} [1]

Tracer	Fasted	Fed	24 h
[^{13}C]Leucine	3.31 ± 0.56	3.25 ± 0.55	3.28 ± 0.43[2]
[$^{2}H_3$]Leucine	1.75 ± 0.20	2.12 ± 0.47[3]	1.94 ± 0.32
[^{13}C]KIC	3.03 ± 0.61	2.96 ± 0.45	3.00 ± 0.50
[$^{2}H_3$]KIC	1.83 ± 0.17	2.04 ± 0.27[3]	1.93 ± 0.20
Splanchnic uptake (%)[4]	45.7 ± 17.8	33.9 ± 19.2	39.8 ± 17.1

[1] $\bar{x}$ ± SD; n = 10. KIC, α-ketoisocaproic acid. There was a significant interaction between tracer route (^{13}C compared with $^{2}H_3$) and metabolic period, P = 0.035 (mixed-models ANOVA). There was a significant interaction between tracer route and molecular form (leucine compared with KIC), P = 0.002 (mixed-models ANOVA).

[2] Significantly different from [^{13}C]KIC, without regard to metabolic period, P = 0.008.

[3] Significantly different from fasted, without regard to molecular form, P = 0.019.

[4] Significant difference in splanchnic uptake between metabolic periods, P = 0.032 (mixed-models ANOVA).

period; however, [$^{2}H_3$]KIC and [$^{2}H_3$]leucine enrichments did not differ significantly.

The splanchnic uptakes of leucine, calculated from the plasma leucine enrichments for the 2 tracers, were 45.7 ± 17.8% and 33.9 ± 19.2% in the fasted and fed periods, respectively. The difference between the fasted and fed periods was significant. Averaged over the 24-h period, the splanchnic uptake of leucine was 39.8 ± 17.1%.

DISCUSSION

The findings in the present study add to the growing body of tracer-derived data that we have generated to quantify adult human amino acid requirements (1, 2, 8, 25). Our findings contributed to the recommendation by an FAO/WHO Expert Consultation (26) that the amino acid requirement pattern for preschool children be used for assessing dietary protein quality in adult nutrition (3). This pattern is similar to the proposed MIT amino acid requirement pattern (2) derived from our studies in adults and that we recommend be used for the assessment of dietary amino acid adequacy or in the planning of adequate indispensable amino acid intakes.

In the present study, the leucine requirement was assessed principally by 2 methods: leucine carbon and nitrogen balances. Both methods showed agreement, with a significant relation between leucine balance and intake. Leucine balance can also be compared with nitrogen balance by using factors of 8% leucine (27) and 16% nitrogen in mixed-body proteins. With this method, the leucine balances converted into nitrogen balance values of −33, −18, −6.6, and 1.0 mg · kg^{-1} · d^{-1} at leucine intakes of 14, 22, 30, and 40 mg · kg^{-1} · d^{-1}, respectively. These values compare reasonably well with the mean measured nitrogen balance values of −12.7, −17.9, −3.9, and 1.0 mg · kg^{-1} · d^{-1} at the same leucine intakes. Both approaches suggest that the mean leucine requirement of our subjects is considerably higher than the FAO/WHO/UNU recommended intake of 14 mg · kg^{-1} · d^{-1} (3). From the regression analysis, the mean value was close to the estimate we proposed previously for Western subjects: 40 mg · kg^{-1} · d^{-1} (2). Note that the subjects in the present study were leaner (mean BMI: ≈23) than those subjects studied at MIT. Thus, when expressed on the basis of body cell mass, the leucine requirement of Indian subjects might be lower than that of Western subjects. This possibility is speculative and will require additional study to establish conclusively.

The 24-h leucine oxidation rates at leucine intakes of 14, 22, and 30 mg · kg^{-1} · d^{-1} approximate the predicted rate of obligatory oxidative amino acid loss (12), which is an approach we validated previously using isotopic tracer studies (28). The total oxidation rate at a leucine intake of 40 mg, on the other hand, is slightly higher than the obligatory oxidative amino acid loss and equal to that which we had predicted from the obligatory loss assuming an overall retention efficiency of ≈70% (12). Thus, these findings also support the conclusion that the mean physiologic requirement of leucine is ≈40 mg · kg^{-1} · d^{-1}. It would be difficult to argue that a leucine intake of 30 mg · kg^{-1} · d^{-1} would be a more prudent choice as the requirement on the basis of the observations in the present study. Despite the complexity and size of the present study, we appreciate that there is considerable variability in mean estimated lysine requirements; therefore, a requirement of 40 mg lysine · kg^{-1} · d^{-1} should be considered only tentative at this time. However, the fact that this mean estimated lysine requirement was derived from both leucine and nitrogen balances provides additional support for its choice as the tentative mean requirement.

The quantity of leucine taken up by the splanchnic region was reported to be ≈10–30% of the intake in the postabsorptive and postprandial states (29–33). Our studies suggest that only a small proportion of the leucine taken up by the splanchnic region is oxidized (34, 35), although leucine oxidation within the splanchnic region appears to increase in the fed state (36) and this might also be dependent on the leucine intake, as suggested from a comparison of our own recent findings (35) and those of Boirie et al (37). Because of a relatively wide range of estimates reported for splanchnic leucine uptake, close comparison with published values is difficult. However, it is reasonable to compare the mean value of 33.9% found in the present study in the fed state with that of Cortiella et al (7), who reported a first-pass splanchnic uptake of 21 ± 6% in subjects who also consumed an L-amino acid diet supplying a daily leucine intake of 40 mg · kg^{-1} · d^{-1}. This suggests that the fractional first-pass uptake of leucine may not be greatly different between healthy Western and Indian adults, although more extensive studies are needed to more firmly establish this point.

Nevertheless, a relatively small change in the proportional fate of dietary leucine during its passage toward the peripheral circulation, in terms of its conversion to and release as KIC or its oxidation, could affect the precision with which an intravenous [^{13}C]leucine tracer model can determine whole-body leucine oxidation. In a recent study (AV Kurpad, VR Young, unpublished observations, 2000) in which we administered [^{13}C]leucine orally, but in which the experimental and dietary conditions were the same as in a previous 24-h intravenous tracer study (15), the estimate of whole-body leucine oxidation increased by ≈11%. Although these findings might be explained by between-subject variation, it is also reasonable to propose that the higher rate of whole-body leucine oxidation was due to the oxidation of tracer and, therefore, of dietary leucine within the splanchnic region. If correct, and, in conjunction with the present estimate of the splanchnic uptake of leucine (24-h uptake: 39%), this would mean that ≈25–30% of dietary leucine taken up by the splanchnic

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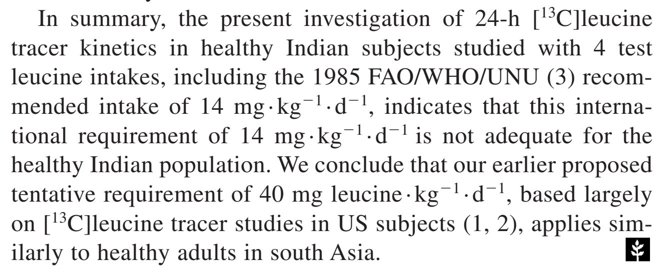
region was oxidized locally. We note that the estimate of an increment of 10% leucine oxidation (oral compared with intravenous administration) was obtained with a generous leucine intake of ≈100 $mg \cdot kg^{-1} \cdot d^{-1}$; in the present study, subjects received 40 mg $leucine \cdot kg^{-1} \cdot d^{-1}$. On the other hand, in earlier studies in Western subjects (28, 35) there were no significant differences in rates of whole-body leucine oxidation when we compared [^{13}C]leucine oxidation after the administration of intragastric and intravenous tracers. Whether Indian subjects differ from Western subjects in their immediate processing of leucine absorbed from the intestinal tract remains an important question for additional research. This point is strengthened by recent evidence indicating the quantitative significance of the splanchnic region and, particularly, the intestinal tissues (38) in the catabolism of dietary amino acids.



In summary, the present investigation of 24-h [^{13}C]leucine tracer kinetics in healthy Indian subjects studied with 4 test leucine intakes, including the 1985 FAO/WHO/UNU (3) recommended intake of 14 $mg \cdot kg^{-1} \cdot d^{-1}$, indicates that this international requirement of 14 $mg \cdot kg^{-1} \cdot d^{-1}$ is not adequate for the healthy Indian population. We conclude that our earlier proposed tentative requirement of 40 mg $leucine \cdot kg^{-1} \cdot d^{-1}$, based largely on [^{13}C]leucine tracer studies in US subjects (1, 2), applies similarly to healthy adults in south Asia.

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