Daily methionine requirements of healthy Indian men, measured by a 24-h indicator amino acid oxidation and balance technique[1–3]

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ABSTRACT

Background: The 1985 FAO/WHO/UNU upper requirement for the sulfur-containing amino acids in healthy adults, which was set at 13 $mg \cdot kg^{-1} \cdot d^{-1}$, is based on nitrogen balance studies in Western subjects. Short-term tracer-based studies also estimated a mean requirement of 13 $mg \cdot kg^{-1} \cdot d^{-1}$, but whether this estimate is applicable to healthy populations worldwide is unknown.

Objective: Using a 24-h indicator amino acid oxidation and balance method with 7 test methionine intakes (3, 6, 9, 13, 18, 21, and 24 $mg \cdot kg^{-1} \cdot d^{-1}$), we assessed methionine requirements in healthy, well-nourished Indians.

Design: Twenty-one healthy, well-nourished Indian men were studied during each of 3 randomly assigned 7-d diet periods in which methionine intakes (diet devoid of cysteine) were equally placed on either side of the putative mean methionine requirement of 13 $mg \cdot kg^{-1} \cdot d^{-1}$. Twenty-four–hour indicator amino acid oxidation and balance were measured on day 7 by using a 24-h [^{13}C]leucine tracer infusion. The breakpoint in the relation between these values and the methionine intake was determined.

Results: Two-phase linear regression of daily leucine oxidation against methionine intake estimated a breakpoint in the response curve at a methionine intake of 14 $mg \cdot kg^{-1} \cdot d^{-1}$ (95% CI: 11, 23 $mg \cdot kg^{-1} \cdot d^{-1}$). The breakpoint estimated from the leucine balance–methionine intake relation was 15 $mg \cdot kg^{-1} \cdot d^{-1}$ (95% CI: 11, 27 $mg \cdot kg^{-1} \cdot d^{-1}$).

Conclusions: From the 24-h indicator amino acid oxidation and balance approach, a mean methionine requirement, in the absence of cysteine intake, of 15 $mg \cdot kg^{-1} \cdot d^{-1}$ is proposed for healthy, well-nourished Indian adults. This requirement is similar to that established in Western adults. *Am J Clin Nutr* 2003;77:1198–205.

KEY WORDS Well-nourished Indian adults, methionine requirement, requirement for sulfur-containing amino acids, 24-h indicator amino acid oxidation, 24-h indicator amino acid balance

INTRODUCTION

The daily requirement for sulfur-containing amino acids (SAAs; ie, methionine and cysteine) has important implications for evaluations of the nutritional adequacy of vegetarian protein sources. The 1985 FAO/WHO/UNU Expert Consultation (1) set the *upper* dietary requirement for SAAs at 13 $mg \cdot kg^{-1} \cdot d^{-1}$ on the basis of nitrogen balance studies (2). However, the results of more recent short-term direct or indicator amino acid–based tracer studies (3–8) suggest that the *mean* methionine requirement is similar to or slightly higher than this value.

The nitrogen balance technique has several disadvantages (9), and the short-term direct amino acid balance (DAAB) approach also has potential problems. These problems include the facts that *1*) methionine balances are based on oxidation data from a few hours of measurement that are extrapolated to an estimate for 24 h, *2*) the methionine supplied by the intravenously administered tracer is not massless and thus may constitute a significant proportion of total methionine at very low methionine intakes, and *3*) intracellular methionine enrichment may be less than plasma methionine enrichment (10), meaning that the oxidation of methionine in the short-term DAAB studies (3–6) was underestimated and thus led to an underestimate of the requirement.

The indicator amino acid oxidation and balance (IAAO and IAAB, respectively) method offers the possibility of assessing the SAA requirement without the problems alluded to above, because the oxidation and balance of an independent amino acid for which the kinetics are well-characterized are used in plotting a response curve to graded intakes of the test amino acid (in this case, methionine). The breakpoint on this curve is taken to represent the requirement for dietary methionine. The IAAO method has been used in short-term experiments using [^{13}C]phenylalanine as the indicator amino acid, breath $^{13}CO_2$ measurements as the response output, and a range of methionine intakes (with a diet devoid of cysteine); the mean requirement for methionine was found to be 12.6 $mg \cdot kg^{-1} \cdot d^{-1}$ (7). However, these studies were conducted in adults who were not adapted to their experimental diet before the tracer study, which comprised measurements for a few hours in the fed state only. We refined the IAAO technique to include a 7-d adaptation period to the experimental diet and a 24-h measurement of IAAO and IAAB to assess requirements for lysine and threonine in adult Indians (11–14). This approach may be regarded as the best current method for measuring amino acid requirements in adults.

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[2] Supported by the Nestlé Research Foundation, Lausanne, Switzerland, and by NIH grant DK42101.

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Received August 20, 2002.

Accepted for publication November 25, 2002.

TABLE 1
Characteristics of well-nourished Indian men studied for their methionine requirements[1]

	Value
Age (y)	20.6 ± 1.5
Weight (kg)	62.3 ± 5.7
Height (m)	1.7 ± 0.1
BMI (kg/m^2)	21.4 ± 1.3
MUAC (cm)	27.5 ± 2.3
Percentage body fat (%)	20.4 ± 4.8
FFM (kg)	49.6 ± 4.3

[1] $\bar{x}$ ± SD. MUAC, midupper arm circumference; FFM, fat-free mass.

Furthermore, there are also concerns that estimates of amino acid requirements obtained with white and US subjects may not be representative of global human amino acid requirements, particularly for those populations who consume diets based predominantly on cereals and legumes. Therefore, this study was designed to assess the methionine requirements, in the absence of dietary cystine, of healthy, young Indian men by using a 7-d dietary adaptation period and a 24-h IAAO and IAAB approach with [^{13}C]leucine as the indicator amino acid.

SUBJECTS AND METHODS

Subjects

Twenty-one healthy men participated in this experiment. The subjects were weighed to the nearest 0.1 kg, and their height was measured to the nearest 0.1 cm. The logarithm of the sum of 4 skinfold thicknesses (biceps, triceps, subscapular, and suprailiac) was used in age- and sex-specific equations (15) to obtain an estimate of body density, from which percentage body fat and fat-free mass were determined (16, **Table 1**). The purpose of the study and the potential risks involved were explained to each subject, and the Human Ethical Review Board of St John's Medical College approved the research protocol.

Diet and experimental design

Each subject was studied during 3 separate 6-d experimental diet periods, during which he consumed a weight-maintaining diet based on an L-amino acid mixture as previously described (11–13, **Table 2**). The diet was supplemented with 0.5 g choline/d and was devoid of cystine. The test methionine intakes were 3, 6, 9, 13, 18, 21, and 24 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}. Each subject was randomly assigned to 1 of 7 combinations of 3 methionine intakes, and the 3 intakes were distributed around a putative required intake of 13 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}. The order in which the subjects consumed the 3 intake amounts was randomized. Nine subjects were studied at each methionine intake. The subjects received their daily dietary intake as 3 isoenergetic, isonitrogenous meals (at 0800, 1300, and 2000), except on days 6 and 7 (*see* below).

Twenty-four–hour tracer-infusion protocol and sample collection

The primed 24-h intravenous [^{13}C]leucine approach was used, with the protocol of indirect calorimetry and blood and breath sampling as previously described (11–14). Briefly, [1-^{13}C]leucine (99.3 atom%; MassTrace, Woburn, MA) was given as a primed, constant intravenous infusion at a known rate of ≈2.8 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1} (the prime was ≈4.2 μmol/kg) into an antecubital vein. The bicarbonate pool was primed with 0.8 μmol [^{13}C]$NaHCO_3$ (99.9 atom%; MassTrace)/kg. The tracer administration began at 1700 on day 6, with subjects having consumed their last meal of that day at 1500, and lasted until 1800 on day 7. Therefore, the tracer infusion was given for 25 h, although only the data from the last 24 h were used in the calculation of daily

TABLE 2
Compositions of the amino acid mixtures used to supply 7 daily methionine intakes

	Methionine intake (mg $\cdot$ kg^{-1} $\cdot$ d^{-1})						
Amino acid	3	6	9	13	18	21	24
	mg/g mixture						
L-Tryptophan	15.72	15.73	15.71	15.65	15.65	15.62	15.57
L-Threonine	47.47	47.49	47.43	47.25	47.23	47.17	47.01
L-Isoleucine	63.33	63.37	63.28	63.05	63.02	62.93	62.73
L-Leucine[1]	28.30	28.28	28.24	28.16	28.12	28.08	28.02
L-Lysine HCl	84.31	84.36	84.24	83.93	83.89	83.77	83.51
L-Methionine	2.77	5.55	8.31	11.98	16.57	19.30	22.01
L-Cystine	0	0	0	0	0	0	0
L-Phenylalanine	55.10	55.13	55.05	54.85	54.82	54.75	54.57
L-Tyrosine	41.06	41.08	41.02	40.87	40.85	40.80	40.67
L-Valine	70.82	70.86	70.76	70.50	70.47	70.37	70.15
L-Histidine HCl	30.91	30.93	30.88	30.77	30.76	30.71	30.62
L-Arginine HCl	76.18	76.23	76.12	75.84	75.81	75.70	75.46
L-Alanine	193.00	193.11	192.84	192.12	192.04	191.77	191.16
L-Aspartic acid	12.03	12.03	12.02	11.97	11.97	11.95	11.91
L-Glutamic acid	29.74	29.76	29.72	29.61	29.59	29.55	29.46
Glycine	127.35	124.13	122.58	122.11	117.93	116.40	116.43
L-Proline	40.63	40.65	40.60	40.45	40.43	40.37	40.24
L-Serine	81.26	81.31	81.20	80.89	80.86	80.75	80.49
Total[2]	1000	1000	1000	1000	1000	1000	1000

[1] 9.44 mg leucine $\cdot$ kg^{-1} $\cdot$ d^{-1} was added to each mix every day, except on the infusion day, when this amount of leucine was infused as tracer.
[2] 1.11 g mixture $\cdot$ kg^{-1} $\cdot$ d^{-1} was given to the subjects and provided 160 mg N $\cdot$ kg^{-1} $\cdot$ d^{-1}.

leucine oxidation and balance. On the day of the infusion study, the subjects received 10 isoenergetic, isonitrogenous, small meals at hourly intervals beginning at 0600 on day 7 and lasting until 1500 (together, these meals were equivalent to the 24-h dietary intake for that day). A similar feeding pattern was imposed on the subjects on day 6 as well so that the feeding pattern on the day of the infusion was not suddenly different from that on the previous day.

Analyses of breath samples for $^{13}CO_2$ enrichment by isotope ratio mass spectrometry (Europa Scientific Ltd, Crewe, United Kingdom) and of blood samples for ^{2}H and ^{13}C enrichments of plasma α-ketoisocaproic acid and leucine by gas chromatography–mass spectrometry (Varian, Palo Alto, CA) were performed as previously described (13, 17).

Leucine oxidation and balance calculations

Leucine oxidation ($\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$) was computed for consecutive half-hourly intervals (4, 18) as the ratio of the $^{13}CO_2$ production rate ($\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$) to the plasma [$^{13}C$]α-ketoisocaproic acid enrichment (moles percent excess) at that time. Leucine balance ($mg \cdot kg^{-1} \cdot d^{-1}$) was computed as the difference between the leucine input (dietary leucine + intravenous tracer) and the leucine output (sum of leucine oxidation at half-hourly intervals).

Statistical methods and data evaluation

Data are presented as means ± SDs. The weight change and metabolic variables were analyzed by using mixed-models analysis of variance. The model for weight change over the 6-d experimental diet periods included a factor for diet period. The models for 12-h leucine oxidation and flux included diet period, metabolic phase (fasted compared with fed), methionine intake, and the interaction between methionine intake and metabolic phase. Model contrasts were used to make pairwise comparisons of interest, as appropriate, on the basis of the significance of the interaction and main effects. The model for 24-h IAAB (leucine) included diet period and methionine intake; comparisons versus zero balance were made using the model. A two-sided *P* value of 0.05 indicated significance for all tests of interaction and main effects; *P* values of pairwise comparisons were adjusted by using Tukey's method. The data were analyzed by using SAS version 8.2 (SAS Institute Inc, Cary, NC).

We also estimated a breakpoint for the relation between leucine oxidation or leucine balance and methionine intake. A two-phase linear regression model was fit to the 24-h oxidation data (IAAO) to estimate at what methionine intake ($mg \cdot kg^{-1} \cdot d^{-1}$) the oxidation no longer decreased with increasing dietary methionine. A mixed-models analysis of variance regression model estimated the intercept and slope of one line segment and the intercept of the second line segment, and the slope of the second line segment was restricted to zero. Biologically, the slope of the second line should be zero, but we first tested whether the slope was significantly different from zero before implementing the restriction. The model was constrained so that the 2 line segments intersected at the unknown breakpoint. The breakpoint was estimated as $-1 \times$ the ratio of the difference between intercepts to the difference between slopes (19). The 95% CI for the breakpoint was calculated by using Fieller's theorem. The analysis was repeated by using daily IAAB (leucine) data to determine when the balance no longer increased with increasing dietary methionine. The analysis was implemented by using PROC NLMIXED, which accounted for multiple measurements on each subject.

On execution of the planned analyses, we noted that the breakpoint model did not fit the data well at the lowest intakes and that perhaps a step function or a model with 2 breakpoints would be more appropriate. As a post hoc analysis suggested by the data, a comparison of fit between a series of additional models was performed for 24-h leucine oxidation and balance. The goal was to explore whether the data suggested another model, and if so, what it implied for the estimated mean methionine requirement. The simplest model compared assumed no breakpoint and fit a straight-line relation between leucine oxidation (or balance) and methionine intake. Because we did not think that our data could fit the step function model, we instead fit a sigmoid model of the form

$$Y = \beta_0 + \beta_1 \times F[(\text{intake} - \mu)/\sigma] \quad (1)$$

where $F[\ldots]$ is the cumulative normal distribution function, and σ is restricted as $\sigma = 1$ for estimation; note that this functional form for a sigmoid model was chosen for statistical convenience. The mean requirement was estimated for this model as $\mu + 2.6\sigma$ because ≈99% of the area under a normal curve lies within ± 2.6 SDs of the mean. These 2 models were also compared with the saturated mixed-models analysis of variance model of the primary analysis and with the breakpoint model.

The fits of nested models (ie, when one model is a simplification of another model) were compared by using likelihood ratio tests, which compare the difference in the values of $-2 \times$ the log likelihood between 2 models with a chi-square distribution with df equal to the difference in the number of variables estimated in the 2 models; a decrease in $-2 \times$ the log likelihood of 3.84 with the addition of 1 model variable indicates a significant improvement in model fit at the 0.05 level. The fits of nonnested models were compared by simulating the distribution of the difference in the values of $-2 \times$ the log likelihood and comparing the observed difference with the simulated distribution.

RESULTS

Anthropometry

The subjects' anthropometric measures were comparable to those of the subjects in our previous series of studies (11–13). During the 6-d experimental diet periods, the subjects experienced a small but significant ($P < 0.001$) mean weight loss of 0.35 ± 0.43 kg across the diet periods. There was no significant difference in weight loss between the diet periods ($P = 0.12$).

Leucine oxidation

As shown in **Table 3**, there was no interaction between methionine intake and metabolic phase ($P = 0.42$), indicating that the effects of methionine intake on leucine oxidation are similar across metabolic phases. Across methionine intakes, leucine oxidation was significantly lower in the fasted phase than in the fed phase ($P = 0.009$). Regardless of metabolic phase, there was a significant effect of methionine intake on leucine oxidation ($P < 0.0001$). Total leucine oxidation was significantly lower at the 13-, 18-, and 24-$mg \cdot kg^{-1} \cdot d^{-1}$ intakes than at the 3-, 6-, and 9-$mg \cdot kg^{-1} \cdot d^{-1}$ intakes (each $P < 0.05$). The 3-, 6-, and 9-$mg \cdot kg^{-1} \cdot d^{-1}$ intakes did not differ significantly from one another, and the 13-, 18-, 21-, and 24-$mg \cdot kg^{-1} \cdot d^{-1}$ intakes did not differ significantly from one another.

Leucine balance

With respect to leucine balance (Table 3), the results were essentially the same whether expressed as an absolute balance or

TABLE 3
Leucine oxidation and flux at 7 daily methionine intakes in well nourished Indian men[1]

	Methionine intake (mg·kg^{-1}·d^{-1})						
Leucine index	3	6	9	13	18	21	24
Oxidation (mg leucine·kg^{-1}·d^{-1})[2]							
12-h Fasted	24.6 ± 5.0	22.9 ± 3.7	23.5 ± 2.7	19.1 ± 1.9	20.7 ± 2.3	21.8 ± 2.7	20.4 ± 2.5
12-h Fed	26.1 ± 3.7	24.7 ± 3.7	26.1 ± 3.9	22.6 ± 2.5	20.8 ± 4.4	22.1 ± 3.8	21.3 ± 2.0
Total (24 h)	50.8 ± 7.4	47.6 ± 4.8	49.6 ± 3.6	41.8 ± 3.5[3]	41.4 ± 6.3[3]	43.9 ± 3.6	41.7 ± 1.8[3]
Total intake (mg leucine·kg^{-1}·d^{-1})	39.7 ± 0.6	39.6 ± 0.8	39.3 ± 0.4	39.1 ± 0.5	39.3 ± 0.8	39.6 ± 0.9	39.7 ± 0.8
24-h Balance[4]							
Intake − oxidation (mg leucine·kg^{-1}·d^{-1})	−11.0 ± 7.4[5]	−8.0 ± 4.9[5]	−10.3 ± 3.6[5]	−2.7 ± 3.7[6]	−2.1 ± 6.5[6]	−4.3 ± 3.6[5,6]	−2.0 ± 2.3[3]
Percentage of intake (%)	−27.8 ± 18.6	−20.2 ± 12.5	−26.2 ± 9.4	−6.9 ± 9.6	−5.4 ± 16.9	−11.0 ± 9.0	−5.2 ± 5.8
Flux (μmol·kg^{-1}·30 min^{-1})[7]							
12-h Fasted	49.8 ± 4.9	48.1 ± 6.1	47.7 ± 4.5	44.2 ± 4.3	49.2 ± 5.0	48.0 ± 6.4	48.2 ± 6.3
12-h Fed	48.8 ± 4.5	50.2 ± 4.5	47.7 ± 3.3	46.0 ± 3.6	45.2 ± 5.3	47.8 ± 5.6	48.2 ± 6.5
Total (24 h)	49.3 ± 3.8	49.2 ± 4.8	47.7 ± 3.4	45.1 ± 3.7	47.2 ± 5.0	47.9 ± 5.5	48.2 ± 5.9

[1] $\bar{x}$ ± SD; n = 9 per intake.
[2] Mixed-models ANOVA indicated significant effects of methionine intake ($P < 0.0001$) and of metabolic phase (fasted compared with fed; $P = 0.009$) but no significant interaction between intake and phase.
[3] Significantly different from 3-, 6-, and 9-mg·kg^{-1}·d^{-1} intakes, $P < 0.05$ (mixed-models ANOVA and Tukey's method).
[4] There was a significant effect of methionine intake, $P < 0.0001$ (mixed-models ANOVA).
[5] Significantly different from zero, $P < 0.001$ (mixed-models ANOVA).
[6] Significantly different from 3- and 9-mg·kg^{-1}·d^{-1} intakes, $P < 0.05$ (mixed-models ANOVA and Tukey's method).
[7] There was a significant interaction between methionine intake and metabolic phase, $P = 0.02$ (mixed-models ANOVA).

as a percentage of leucine intake. Daily leucine balance was significantly affected by methionine intake ($P < 0.0001$) and was significantly different from zero balance at the 3-, 6-, 9-, and 21-mg·kg^{-1}·d^{-1} intakes ($P < 0.001$). Leucine balance was significantly lower at the 3- and 9-mg·kg^{-1}·d^{-1} intakes than at the 13-, 18-, 21-, and 24-mg·kg^{-1}·d^{-1} intakes (each $P < 0.05$), and the 6-mg·kg^{-1}·d^{-1} intake tended to be lower than the 13-, 18-, 21-, and 24-mg·kg^{-1}·d^{-1} intakes but was significantly lower than the 24-mg·kg^{-1}·d^{-1} intake only ($P < 0.05$). The 3-, 6-, and 9-mg·kg^{-1}·d^{-1} intakes did not differ significantly from one another, and the 13-, 18-, 21-, and 24-mg·kg^{-1}·d^{-1} intakes did not differ significantly from one another.

Leucine flux

For leucine flux (Table 3), there was a significant interaction between intake and metabolic phase ($P = 0.02$), indicating that the effects of methionine intake on leucine flux differed between metabolic phases. In the fasted state, there was no effect of methionine intake on leucine flux ($P = 0.24$), whereas in the fed state, there was a trend toward an effect of methionine intake on leucine flux ($P = 0.09$).

Breakpoint analysis

The results of fitting a two-phase linear regression model to the data are summarized in **Table 4**. The 24-h leucine oxidation data estimated a breakpoint at a mean methionine intake of 14 mg·kg^{-1}·d^{-1} (95% CI: 11, 23 mg·kg^{-1}·d^{-1}), and the 24-h leucine balance data estimated a breakpoint at a mean methionine intake of 15 mg·kg^{-1}·d^{-1}. These analyses indicate that the mean methionine requirement of these subjects is 15 mg·kg^{-1}·d^{-1}. The mean daily rate of leucine oxidation at methionine intakes at or above the breakpoint was 42 mg·kg^{-1}·d^{-1}, or essentially the daily intake of leucine.

Statistical model comparisons

The results of the statistical analysis of the relation between 24-h leucine balance or 24-h leucine oxidation and methionine intake are summarized in **Table 5**. With respect to leucine balance, the likelihood ratio comparisons indicated that a model that assumed a simple linear relation between 24-h leucine balance and methionine intake was overly simplified in comparison with the other 3 models (saturated, $P = 0.06$; breakpoint, $P = 0.05$; sigmoid, $P < 0.01$). In contrast, the saturated model was not a significant improvement in fit over the breakpoint and sigmoid models, which suggests that the relation between daily leucine balance and methionine intake may be adequately described by either the breakpoint or the sigmoid model. Direct comparison of these 2 models suggests that the sigmoid model is a slightly better fit ($P = 0.07$) to the data. As shown in **Figure 1**, the models differed in their fit at the lower intakes, which was the region of concern, whereas their fits at the higher intakes were essentially identical, each estimating a mean leucine balance of −3 mg·kg^{-1}·d^{-1} at methionine intakes ≥18 mg·kg^{-1}·d^{-1}. Importantly, the 2 models estimated similar mean methionine requirements; the breakpoint model estimated a mean requirement of 15 mg·kg^{-1}·d^{-1}, and the sigmoid model similarly suggested a mean requirement of 14 mg·kg^{-1}·d^{-1}.

With respect to 24-h leucine oxidation, the results concur with those for 24-h leucine balance (Table 5 and **Figure 2**), suggesting that the relation between 24-h leucine oxidation and methionine intake may be adequately described by either the breakpoint or the sigmoid model. However, the sigmoid model is a slightly better fit to the data, especially at the lower intakes. Both of the 2 models estimate a mean methionine requirement of 14 mg·kg^{-1}·d^{-1}.

DISCUSSION

The findings in the present study add to the earlier tracer-derived balance data that we generated with the use of the diet-adapted, 24-h direct or IAAO and IAAB paradigm to quantify adult amino acid requirements (11–13, 17, 18, 20–22) in South Asian (Indian) and American subjects. This pattern of amino acid

TABLE 4
Two-phase regression analysis of the relations between 24-h leucine oxidation or 24-h leucine balance and methionine intake

Variable	Breakpoint estimate[1]	Equation for variable	
		Below breakpoint	Above breakpoint[2]
	mg methionine · kg^{-1} · d^{-1}		
24-h Leucine oxidation (mg · kg^{-1} · d^{-1})	14 (11, 23)	Oxidation = 54 − 0.8 × intake	Oxidation = 42 ± 0.9
24-h Leucine balance (mg · kg^{-1} · d^{-1})	15 (11, 27)	Balance = −14 + 0.7 × intake	Balance = −3 ± 0.9

[1] 95% CI, which was calculated by using Fieller's theorem, in parentheses.
[2] Estimate ± SE. The 2 line segments intersect at the breakpoint.

requirements (for leucine, lysine, and threonine) is similar to the amino acid pattern recommended for adults by a recently convened WHO/FAO/UNU Expert Consultation on Protein and Amino Acid Requirements (23). The finding in the present study of a mean methionine requirement of 15 mg · kg^{-1} · d^{-1} in the absence of a source of dietary cystine also generally confirms and extends the findings from short-term tracer DAAB (3–6) and IAAO (7) studies.

In the tracer model used in the short-term DAAB experiments (3), methionine "oxidation" (transsulfuration) was measured with the use of a dual-stable-isotope labeled methionine (L-[^{13}C,^{2}H$_3$]methionine) tracer protocol, in which 80% of plasma methionine enrichment was assumed to represent intracellular precursor pool enrichment. Recent evidence suggests that intracellular precursor pool enrichments may in fact be even lower (10) and that plasma homocysteine or cystathionine enrichments may be more representative of intracellular precursor enrichment, at between 50% and 60% of the value of plasma methionine enrichment. The calculated methionine oxidation rates from the short-term DAAB studies would increase, therefore, by 40% if corrected for this gradient between intracellular and extracellular methionine enrichments (3, 8), leading to a mean negative methionine balance at a methionine intake of 13 mg · kg^{-1} · d^{-1}. The present study, which used a 24-h IAAB approach and which was independent of these precursor pool considerations because the indicator amino acid tracer used was [^{13}C]leucine, essentially confirmed the conclusions from the findings of earlier short-term IAAO (7) and DAAB studies (3–6); in the present study, we found that the mean methionine requirement was ≈15 mg · kg^{-1} · d^{-1}. Thus, the question arises why there was reasonable concordance between the 24-h IAAO and IAAB and short-term IAAO estimates of the methionine requirement if the estimate of methionine oxidation was in error. It seems likely that the ratio of plasma methionine enrichment to intracellular methionine enrichment suggested by the findings of MacCoss et al (10) may not be strictly applicable to the present experiment. There is a need for studies that perform simultaneous measurements of methionine, homocysteine, and cystathionine pool enrichments under the dietary conditions used in the present study.

The short-term DAAB estimates of the methionine requirement were also based on intravenous administration of the tracer and therefore did not take into account the possibility of a significant first-pass catabolism of methionine in the splanchnic area. In this case, the intravenous tracer studies may also have underestimated the methionine requirement, particularly in the fed state. At high methionine intakes, labeled methionine appears to be oxidized more extensively when given orally than when administered intravenously (24). However, more recent studies (25) showed that when the first-pass effect of splanchnic area, which in the fed state is low and close to zero, is included in the experiment, the net methionine balance at a requirement level of methionine intake is not significantly different from zero. The first-pass removal of cysteine is apparently of more importance to considerations of methionine-cysteine relations than to considerations of methionine requirements alone (25). The present study, which used the 24-h IAAO and IAAB method, has the advantage of not being confounded by considerations of the splanchnic fate of the tracer.

Our observation, based on a post hoc analysis of the data, that the relation between methionine intake and leucine oxidation or balance was described slightly better by a sigmoid function than

TABLE 5
Comparison of models fit to describe the relations between 24-h leucine balance or 24-h leucine oxidation and methionine intake[1]

	24-h Leucine balance				24-h Leucine oxidation			
		Alternative model[3]				Alternative model[3]		
Model[2]	−2LL	Breakpoint	Sigmoid	Saturated	−2LL	Breakpoint	Sigmoid	Saturated
Linear (2)[4]	374.2	3.2	6.9	10.5	372.9	4.6	8.2	12.3
P		0.05	< 0.01	0.06		0.03	< 0.01	0.03
Breakpoint (3)	371.0	—	3.7	7.3	368.3	—	3.6	7.7
P			0.07	0.12			0.06	0.13
Sigmoid (3)	367.3	—	—	3.6	364.7	—	—	4.1
P				0.55				0.44
Saturated (7)[4]	363.7	—	—	—	360.6	—	—	—

[1] −2LL, −2 × the log likelihood.
[2] Number of variables in parentheses.
[3] Values are the difference in −2LL between the model and the alternative model. The *P* values are for the comparison of fit between the 2 models; $P < 0.05$ indicates that the model should be rejected in favor of the alternative model.
[4] Nested models (ie, the linear model is nested in the saturated mixed-models ANOVA model) can be compared directly by comparing the difference in −2LL between the models with a chi-square distribution with df equal to the difference in the number of variables, eg, χ^2 (df = 5, α = 0.05) = 11.1.

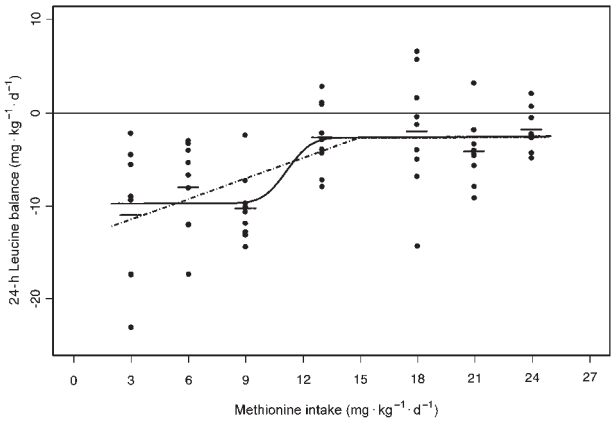

FIGURE 1. Relation between 24-h leucine balance and methionine intake. The observed (●) and mean (—) values are plotted, and the following fitted models are overlaid: the breakpoint model summarized in Table 4 and the sigmoid model, in which balance = $-9.8 + 7.0 \times F(\text{intake} - 11.2)$, and $F(\ldots)$ is the cumulative normal distribution function.

by the two-phase regression breakpoint analysis deserves brief comment from a biological standpoint. Although the breakpoint regression model has been applied appropriately and conveniently by us (12–14, 22) and others (7) in the evaluation of these and comparable test amino acid intake–IAAO data, the actual physiologic response relation is likely to be more complex (26). A substrate saturation kinetics model, based on the concept of enzyme kinetics and rate-limiting steps in metabolic pathways, has been used to characterize nutrient-response relations, including responses to graded intakes of specific indispensable amino acids in growing rats (27, 28); the change in the ratio of plasma lysine to dietary lysine has also been shown to be sigmoid (29). Furthermore, methionine is thought to be the rate-limiting endogenous amino acid for protein synthesis (30), and there are supporting data from studies in rats (31–33) and pigs (34) but

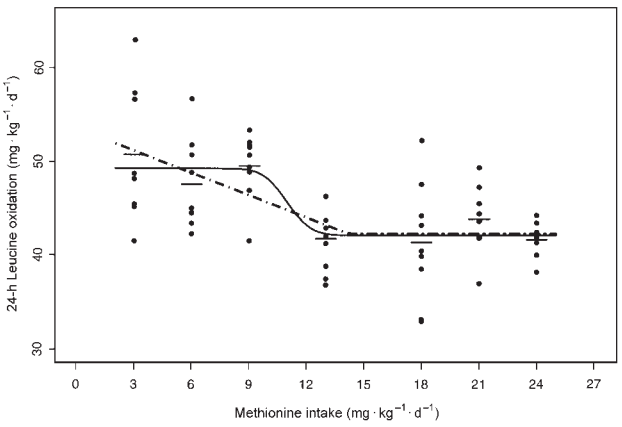

FIGURE 2. Relation between 24-h leucine oxidation and methionine intake. The observed (●) and mean (—) values are plotted, and the following fitted models are overlaid: the breakpoint model summarized in Table 4 and the sigmoid model, in which oxidation = $49.3 - 7.2 \times F(\text{intake} - 11.0)$, and $F(\ldots)$ is the cumulative normal distribution function.

not, to our knowledge, from studies in adult humans. However, this remains a possibility, and several additional facts are also relevant: *1*) methionine appears to be the most toxic of the indispensable amino acids (35), *2*) its metabolic intermediates (36) serve as intermediates in various metabolic pathways and also as regulators of key enzymes in the transmethylation and transsulfuration pathways [such a duality of function accounts for the sigmoid behavior of regulatory enzymes (37)], and *3*) when the response between an enzyme and its regulator is sigmoid, the change in enzyme activity (ie, fractional saturation) requires a much lower change in regulator concentration than that necessary when the interaction between enzyme and regulator molecule is described by a hyperbolic function (37). Given these various facts, perhaps the integrated response of whole-body protein metabolism or of amino acid utilization for whole-body protein synthesis to graded, submaintenance-to-maintenance intakes of methionine differs from that for other indispensable amino acids. Hence, the response might be described best by a sigmoid curve, which thus far has not been the case in our comparable studies using lysine (12, 13), threonine (14), and leucine (17) as test amino acids. The estimates of the minimum methionine intake needed to achieve a constant, maximum utilization of the indicator amino acid (leucine) were essentially indistinguishable when judged from the breakpoint and sigmoid response curves. Nevertheless, this post hoc analysis of our data suggests the desirability of a more in-depth investigation of the dose responses of different facets of methionine metabolism to graded intakes of methionine and SAAs (methionine plus cysteine) in healthy adults.

The small average weight loss experienced by the subjects in the present study was similar to that documented in our earlier studies of similar subjects (12–14) and appeared to be due to the negative carbohydrate balance observed in the subjects on day 7 of the feeding period, which was associated with a concomitant water loss (38) related to glycogen breakdown. We have also shown that experiments with normal, habitual diets do not lead to a significant change in weight, and the subjects were, on average, in near-zero carbohydrate balance (39). This difference in substrate oxidation appears to be linked to the high habitual carbohydrate intake (≈70%) of these subjects and the relatively low carbohydrate intake (≈50%) during the experimental diet because of constraints on how much wheat starch and beet sugar could reasonably be included in the experimental diet.

The application of amino acid requirements derived from studies of well-nourished adults in developed countries to populations on a global basis has been questioned because it is thought that there may be adaptive reductions in the requirement for amino acids when habitual diets that are low in protein or amino acids are eaten. On the other hand, there may be increased requirements because of other factors, such as the chronic but subclinical immunostimulation that appears to be present in subjects living in urban slums (40). Cysteine sulfur is derived from methionine through the transsulfuration pathway, and cysteine is important for the synthesis of glutathione (41). Glutathione is specifically important in the immune response to infections (42, 43), and glutathione concentrations in normal subjects (44), subjects with protein-energy malnutrition (45), and subjects with HIV infection (46) have been shown to be dependent on cysteine availability, which in turn would affect the total SAA requirement. Therefore, although it is of primary importance to study amino acid requirements in well-nourished, healthy persons from these populations,

it is now necessary to further define the requirement outside the framework of healthy, affluent, well-nourished Americans or Indians to include persons in developing countries who are habitually exposed to unsanitary and polluted environments. In summary, the present investigation of 24-h [^{13}C]leucine tracer indicator kinetics in well-nourished Indian subjects studied with 7 test intakes of methionine, including the 1985 FAO/WHO/UNU (1) SAA requirement of 13 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}, indicates that the international mean requirement for total SAAs (specifically, methionine in the absence of a dietary cystine source) should be close to 15 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}.

AVK was involved in the study design, data collection, sample and data analysis, and the writing of the manuscript. SV, JV, and TR were involved in data collection and analysis. JG was involved in data collection and sample analysis. VRY and MMR were involved in the study design, data analysis, and the writing of the manuscript. The authors had no conflicts of interest.

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