Total sulfur amino acid requirement in young men as determined by indicator amino acid oxidation with L-[1-^{13}C]phenylalanine[1–4]

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ABSTRACT

Background: Determining the sulfur amino acid (SAA) requirements of humans has remained elusive because of the complex nature of SAA metabolism. Current recommendations are based on nitrogen balance studies.

Objective: The goal of the present study was to determine the methionine requirement of men fed a diet devoid of cysteine (total SAA requirement).

Design: Six men were randomly assigned to receive 6 graded intakes of methionine: 0, 6.5, 13.0, 19.5, 26.0, and 32.0 mg·kg^{-1}·d^{-1}. The total SAA requirement was determined by measuring the oxidation of L-[1-^{13}C]phenylalanine to $^{13}CO_2$ ($F^{13}CO_2$). The mean total SAA requirement was estimated with use of a linear regression crossover analysis, which identified a breakpoint of the $F^{13}CO_2$ response to methionine intake.

Results: On the basis of the mean measures of $F^{13}CO_2$, the mean requirement and population-safe intake (upper limit of the 95% CI) of total SAAs were found to be 12.6 and 21 mg·kg^{-1}·d^{-1}, respectively.

Conclusion: Although the mean SAA requirement is consistent with current guidelines for the total SAA intake, the population-safe intake is substantially higher than the currently recommended total SAA intake. *Am J Clin Nutr* 2001;74:756–60.

KEY WORDS Sulfur amino acids, indicator amino acid oxidation, amino acid requirement, stable isotopes, phenylalanine, methionine, men

See corresponding editorial on page 709.

INTRODUCTION

Methionine is a nutritionally indispensable amino acid required for the normal growth and development of all mammals (1, 2), whereas cysteine is conditionally indispensable (3, 4). In addition to its required role in protein synthesis, methionine supplies the methyl group for numerous methylation reactions and the sulfur atom for cysteine formation (5–8). Through the intermediate *S*-adenosylmethionine, methionine is the source of the methyl groups of choline, creatine, and both DNA and RNA intermediates (1, 5, 6, 8). Cysteine is involved in the protein synthesis and biosynthesis of taurine, sulfate, and glutathione (6).

Total sulfur amino acids (SAAs) are the first limiting amino acids in several foods (9); therefore, knowledge of the mean requirement and population-safe intake of SAAs is important for making recommendations about protein and amino acid intakes in humans. The current population-safe SAA requirement of 13 mg·kg^{-1}·d^{-1} (10) is based largely on the nitrogen balance studies of Rose et al (11). However, because nitrogen balance tends to underestimate nitrogen losses and is influenced by excess energy intake, amino acid requirements are susceptible to underestimation (12–16).

Recent tracer studies of methionine kinetics and balance (17) determined that the upper range of the total SAA requirement in humans exceeds the recommended intake of 13 mg·kg^{-1}·d^{-1} set by the FAO/WHO/UNU in 1985 (10). The complex nature of SAA metabolism has restricted the study of SAAs to intermediary metabolic pathways and to their interrelations with key cofactors and substrates such as vitamin B-12, vitamin B-6, folate, choline, betaine, and creatine (3, 18–22).

However, despite the well-developed models used to investigate the details of SAA metabolism (1, 22), these studies generally cannot provide quantitative physiologic estimates of requirements for either methionine or cysteine. Indicator amino acid oxidation (IAAO) offers a solution by monitoring the oxidation of an independent indispensable indicator amino acid in response to graded intakes of a given indispensable test amino acid (15). IAAO, therefore, is useful in elucidating the requirements of metabolically complex indispensable amino acids such as SAAs. L-[1-^{13}C]Phenylalanine in the presence of an excess of tyrosine was shown previously to be an acceptable tracer to use

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TABLE 1
Subject characteristics and energy intakes[1]

Characteristic	Value
Age (y)	29 ± 6
Weight (kg)	84.7 ± 14.5
BMI (kg/m^2)	26.78 ± 2.47
Energy intake (MJ/d)[2]	13.84 ± 2.34
Lean body mass (kg)[3]	66.5 ± 13.3

[1] $\bar{x}$ ± SD.
[2] Calculated from the resting metabolic rate, as determined by indirect calorimetry, multiplied by an activity factor of 1.7.
[3] Assessed by bioelectrical impedance analysis.

for indicator oxidation in adults (23–25). The objective of the present study was to use IAAO with L-[1-^{13}C]phenylalanine to estimate the total SAA requirement in young men.

SUBJECTS AND METHODS

Subjects

Six healthy men participated in the study on an outpatient basis at the Clinical Investigation Unit at The Hospital for Sick Children, Toronto. Subject characteristics are described in **Table 1**. None of the subjects had a history of recent weight loss, unusual dietary practices, or endocrine disorders, and none were using medication at the time of entry into the study. The design and aims of the study and the potential risks involved were fully explained to each subject and informed, written consent was obtained. All procedures were approved by the Ethics Review Board of The Hospital for Sick Children. Subjects received financial compensation for their participation.

Experimental design

The study design was based on the noninvasive IAAO model of Bross et al (26). Each subject randomly received each of 6 dietary methionine intakes: 0, 6.5, 13.0, 19.5, 26.0, and 32.0 mg·kg^{-1}·d^{-1}. Each study consisted of a 2-d adaptation period to a prescribed diet. The diet provided 1.0 g protein·kg^{-1}·d^{-1} and was followed by a single study day on which phenylalanine kinetics were measured with the use of L-[1-^{13}C]phenylalanine at 1 of the 6 dietary methionine intakes and a crystalline amino acid intake of 1.0 g·kg^{-1}·d^{-1}. The dietary study periods were separated by ≥1 wk; all subjects completed all 6 studies within 3 mo.

Dietary and energy intakes

Dietary intakes during the 2-d adaptation period were provided in the form of milk shakes (Scandishake; Scandipharm, Birmingham, AL), which were weighed in daily portions for each subject and supplemented with additional protein (Promod; Ross Laboratories, Columbus, OH) and energy (Caloreen; Nestlé Clinical Nutrition, North York, Canada), depending on each subject's requirement. Subjects were instructed to add a predetermined volume of homogenized milk containing 3.25% fat to their daily portion and to drink the milk shake at regular meal times throughout the day. Energy intakes were based on each subject's resting metabolic rate, as determined by indirect calorimetry (Vmax 29n; Sensormedics, Yorba Linda, CA), multiplied by an activity factor of 1.7 (Table 1). Subjects also consumed a daily multivitamin supplement (Centrum; Whitehall-Robins Inc, Mississauga, Canada) containing 0.4 mg folic acid, 3 mg vitamin B-6, and 9 μg vitamin B-12 for the entire duration of the 6 studies. No other food or beverages, including diet products containing artificial sweeteners, were consumed during the adaptation period.

On each of the 6 study days, the diet was provided as an experimental formula developed for amino acid kinetic studies (27). Briefly, a liquid formula (protein-free powder, product 80056: Mead Johnson, Evansville, IN) flavored with orange and fruit crystals (Tang and Kool-Aid, respectively; Kraft Foods Canada, Toronto) and protein-free cookies supplied the main source of energy in the diet. A crystalline amino acid mixture (1.0 g·kg^{-1}·d^{-1}), based on the amino acid composition of egg protein, provided the only source of amino nitrogen in the diet. Energy intakes were prescribed as above. The macronutrient composition of the experimental diet, expressed as a percentage of dietary energy, was 55% carbohydrate, 35% fat, and 10% protein. The diets were prepared and weighed in the metabolic kitchen and were portioned into isoenergetic, isonitrogenous meals. The diet was consumed as hourly meals; each meal represented one-twelfth of the subjects' total daily protein and energy requirements. Subjects had free access to water throughout the study day. The amount of L-[1-^{13}C]phenylalanine given during the study day was subtracted from the dietary provision of phenylalanine such that the total phenylalanine intake was 14 mg·kg^{-1}·d^{-1}; 40 mg tyrosine·kg^{-1}·d^{-1} was supplied to ensure an excess.

Tracer protocol

NaH^{13}CO$_3$ (99 atom%) and L-[1-^{13}C]phenylalanine (99 atom%) were used as tracers (Mass Trace, Woburn, MA). Isotope solutions were prepared in deionized water and stored at −20°C. Oral priming doses of NaH^{13}CO$_3$ (0.176 mg/kg) and L-[1-^{13}C]phenylalanine (0.664 mg/kg) were given with the fifth hourly meal. An hourly oral dosing protocol of L-[1-^{13}C]phenylalanine (1.2 mg·kg^{-1}·d^{-1}) was commenced simultaneously and continued throughout the remaining 6 h of the study.

Sample collection and analysis

We previously showed that plasma amino acid enrichments can be determined from urine (26, 28, 29). Baseline samples of breath carbon dioxide and urine were collected 60, 45, and 30 min before the isotope protocol began. A background isotopic steady state was achieved in all subjects within 4 h of the commencement of feeding. Breath carbon dioxide and urine samples were also collected at 30-min intervals during isotopic steady state, 120–320 min after isotope administration began, and were stored at −20°C. Breath samples were collected in disposable Haldane-Priestley tubes (Venoject; Terumo Medical Corp, Elkton, MD) with use of a collection mechanism that permits the removal of dead-air space (30). Samples were stored at room temperature until analyzed. Carbon dioxide production was measured during each study day for 30 min with a variable-flow indirect calorimeter (Vmax 29n; Sensormedics).

The enrichment of ^{13}C in breath carbon dioxide was measured on a continuous-flow isotope ratio mass spectrometer (PDZ Europa Ltd, Cheshire, United Kingdom). Breath ^{13}CO$_2$ enrichments were expressed as atom% over a reference standard of compressed carbon dioxide gas. Urinary L-[1-^{13}C]phenylalanine enrichment was measured by gas chromatography (selected ion monitoring negative chemical ionization)–mass spectrome-

TABLE 2
Effect of methionine intake on phenylalanine flux and oxidation as measured by the rate of $^{13}CO_2$ release[1]

Methionine intake ($mg \cdot kg^{-1} \cdot d^{-1}$)	Phenylalanine flux	Phenylalanine oxidation
	$\mu mol \cdot kg^{-1} \cdot h^{-1}$	
0 (n = 6)	53.1 ± 5.9[a]	4.7 ± 0.3[a]
6.5 (n = 5)	37.0 ± 10.4[b]	2.6 ± 1.3[b]
13 (n = 5)	48.8 ± 7.6[a]	2.9 ± 1.4[b]
19.5 (n = 5)	45.6 ± 6.8[a]	2.6 ± 0.4[b]
26.0 (n = 5)	48.6 ± 8.5[a]	2.6 ± 0.9[b]
32.5 (n = 6)	56.5 ± 10.7[a]	3.4 ± 1.1[b]

[1] $\bar{x}$ ± SD. Methionine had a significant effect on both phenylalanine flux and oxidation, P = 0.01. Flux and oxidation rates were also significantly different among individuals, P = 0.01. Means in a column with different superscript letters are significantly different, P = 0.05.

try (model 5890 gas chromatograph and model 5988A mass spectrometer; Hewlett-Packard, Mississauga, Canada). Urine samples (500 μL) were deproteinized and acidified with 250 μL of 400 g trichloroacetic acid/L and centrifuged at 7000 × g at room temperature for 5 min. Amino acids were separated from the supernatant fluid with a cation-exchange resin (Dowex 50W-X8, 100–200 mesh H^+; Bio-Rad Laboratories, Richmond, CA) and were derivatized according to the method of Patterson et al (31) to their N-heptafluorobutyramide n-propyl esters. Selected-ion chromatograms were obtained by monitoring mass-to-charge ratios of 383 and 384 for L-[1-^{13}C]phenylalanine corresponding to the unenriched (m) and enriched (m+1) peaks, respectively. The areas under the peaks were integrated with use of Hewlett-Packard G1034C MS-CHEM software (α Omega Technologies, Brielle, NJ). Isotopic enrichment was expressed as molecule% excess.

Isotope kinetics

Phenylalanine kinetics were calculated according to the stochastic model of Matthews et al (32), previously used by Zello et al (23). Isotopic steady state in the metabolic pool was represented by plateau in free L-[1-^{13}C]phenylalanine in urine and $^{13}CO_2$ in breath. The difference between mean breath $^{13}CO_2$ enrichments of the 3 baseline and the 7 plateau samples was used to determine atoms percent excess above baseline at isotopic steady state.

Phenylalanine flux ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) was measured during isotopic steady state from the dilution of the L-[1-^{13}C]phenylalanine infused into the metabolic pool with urinary enrichments of L-[1-^{13}C]phenylalanine (23, 32). The rate of $^{13}CO_2$ release from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$, in $\mu mol\ ^{13}CO_2 \cdot kg^{-1} \cdot h^{-1}$) was calculated according to the method of Matthews et al (32) from $^{13}CO_2$ expiration by using a factor of 0.82 to account for the $^{13}CO_2$ retained in the body, in the fed state, as a result of bicarbonate fixation (33). The rate of L-[1-^{13}C]phenylalanine oxidation ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) was calculated from $F^{13}CO_2$ and urinary free phenylalanine enrichment (23, 32).

Statistical analysis

Repeated-measures analysis of variance was performed on primary and derived variables to assess the effects of methionine intake, of the order in which the test intakes were administered, of subject, and of interactions (SAS, version 6.6; SAS Institute Inc, Cary, NC). The significance of differences between intakes was examined by using Tukey's post hoc test. Results are expressed as means ± SDs. In all cases, results were considered to be statistically significant at $P < 0.05$.

Estimates of the mean and population-safe intakes of total SAAs for men were derived by breakpoint analysis with a 2-phase linear regression crossover model similar to that described previously (23). This model minimizes the residual SE in a stepwise partitioning of data points between 2 regression lines. The 95% CI for the mean total SAA requirement was calculated by using Fieller's theorem; the upper limit of the 95% CI was used to represent the population-safe intake of total SAAs. The requirement for individual subjects was determined by visual inspection. To identify the possible sources of variation in individual $F^{13}CO_2$ responses, an analysis of covariance was carried out between $F^{13}CO_2$ (dependent variable) and methionine intake and lean body mass (independent variables). Lean body mass was chosen because of the strong relation between SAA metabolism and creatine biosynthesis (34) and because of our previous observation of a relation between lean body mass and the lysine requirement of men (35).

RESULTS

Mean (±SD) baseline L-[1-^{13}C]phenylalanine enrichments were not significantly different between study days, illustrating no significant carryover of isotope between studies. Mean L-[1-^{13}C]phenylalanine flux and oxidation at the 6 dietary methionine intakes are shown in **Table 2**. The effect of dietary methionine intake on $F^{13}CO_2$ is shown in **Figure 1**. Individual data points are shown in **Figure 2**.

Linear regression crossover analysis resulted in the identification of a mean total SAA requirement of 12.6 $mg \cdot kg^{-1} \cdot d^{-1}$ and of a population-safe intake of total SAAs of 21 $mg \cdot kg^{-1} \cdot d^{-1}$. The inclusion of lean body mass in the analysis of covariance model relating methionine intake to $F^{13}CO_2$ was not significant. Results indicated that 19% of the intersubject variation in $F^{13}CO_2$ was explained by differences in body composition.

DISCUSSION

The first published estimates of total SAA requirements were based on the nitrogen balances of 6 men (11). The current

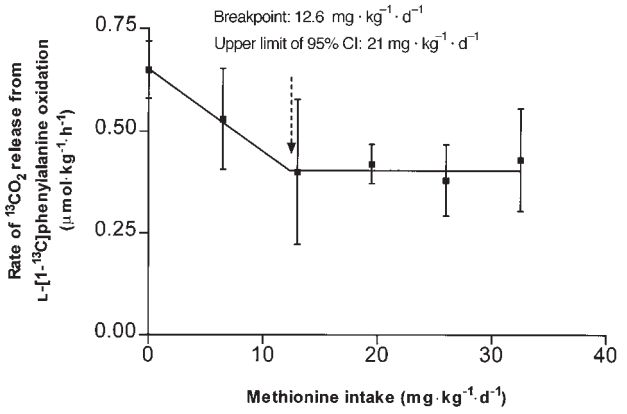

FIGURE 1. Mean (±SD) rate of $^{13}CO_2$ release from L-[1-^{13}C]phenylalanine oxidation at methionine intakes of 0, 6.5, 13.0, 19.5, 26.0, and 32.5 $mg \cdot kg^{-1} \cdot d^{-1}$ in 6 men (n = 36 observations). The dashed arrow indicates the mean total sulfur amino acid requirement of 12.6 $mg \cdot kg^{-1} \cdot d^{-1}$.

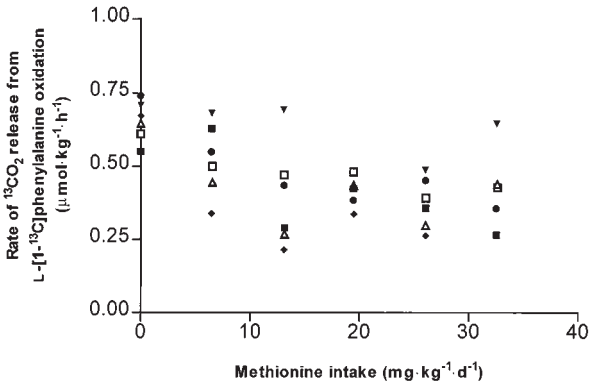

FIGURE 2. Relation between the rate of $^{13}CO_2$ release and methionine intake in 6 men (n = 36 observations).

population-safe intake of SAAs for adults recommended by the FAO/WHO/UNU is 13 mg·kg^{-1}·d^{-1} (10), which is based on the highest estimated individual requirement to achieve positive nitrogen balance in studies carried out by Rose et al (11) in men and by Reynolds et al (36) in women. Human nutrient requirements, except for energy, are set according to a statistical model that uses the mean requirement plus 2 SDs to determine a population-safe intake for a given nutrient (37). In nitrogen balance studies used to estimate total SAA requirements, one can easily calculate a mean requirement and an SD because individual data are provided for each of the 6 subjects in the original paper by Rose et al (11). When we recalculated this nitrogen balance data, we found a mean requirement of 13.2 mg·kg^{-1}·d^{-1}. To this new mean, we added 2 times the SD to arrive at an estimated population-safe total SAA intake of 18 mg·kg^{-1}·d^{-1}. This value is similar to the population-safe intake found in the present study (21 mg·kg^{-1}·d^{-1}) and is consistent with the 24-h balance estimates discussed below (17, 38).

Given the complexity of SAA metabolism, estimating total SAA requirements by direct oxidation tracer methods is extremely difficult. This is because the carboxyl carbon of methionine is not directly lost to the bicarbonate pool, nor is it irreversibly oxidized to carbon dioxide during degradation; a condition that must be met for the principles of direct oxidation to apply (15). However, Young et al (17) suggested that the current FAO/WHO/UNU population-safe SAA intake of 13 mg·kg^{-1}·d^{-1} is too low on the basis of 24-h balance studies using L-[methyl-$^{2}H_3$,1-^{13}C]methionine as a tracer. In that study, 5 men were fed 13 mg SAAs·kg^{-1}·d^{-1} and tracer oxidation was monitored over 24 h. Although all of the subjects did not achieve balance at that intake, some subjects were close enough to zero balance for the authors to conclude that the true mean total SAA requirement was not much different from the FAO/WHO/UNU population-safe intake (17). The authors also suggested that for all subjects to achieve methionine balance, the population-safe intake should be set at ≈25 mg·kg^{-1}·d^{-1}. These results were confirmed in a later study (38).

IAAO is an independent method of estimating indispensable amino acid requirements in humans. Since its first applications in humans (23), IAAO has evolved into a relatively noninvasive and efficient means of elucidating the indispensable amino acid needs of children and adults (26, 28). The technique monitors the oxidation of an independent, indispensable indicator amino acid in response to graded intakes of an indispensable test amino acid. As the intake of the test amino acid approaches its requirement, the oxidation of the indicator decreases such that further increments in the test amino acid will have no effect on the oxidation of the indicator amino acid (25). Our minimally invasive IAAO model (26) was modified to include a 2-d adaptation to the protein intake used in the oxidation studies (39). The lack of dietary restriction on test amino acid intake allows for a wider range of intakes to be studied, resulting in a stronger and more reliable estimated requirement of the test amino acid.

One of the strengths of using IAAO in the present study was that it determines the amount of methionine required for both protein synthesis and cysteine biosynthesis. This implies that enough cysteine is synthesized from methionine to permit optimal protein synthesis. In the event that methionine requirements for cysteine biosynthesis were not reached, cysteine would then become the first limiting amino acid for protein synthesis because we did not provide an exogenous source of cysteine in the present study. If this were the case, we could not have observed a plateau in the $F^{13}CO_2$ response at methionine intakes beyond the breakpoint. Furthermore, the obligatory oxidation levels observed in the present study are comparable with previous IAAO studies with lysine, threonine, and tryptophan, suggesting that there are no additional amino acid needs for protein synthesis beyond the breakpoint for methionine. Therefore, the total SAA requirements found in the present study represent the amount of dietary methionine needed to fulfill all the functions of methionine in vivo. However, it cannot be concluded from the present study whether the amount of cysteine required for the synthesis of glutathione, taurine, or sulfate was achieved with methionine intakes at the breakpoint for protein synthesis. This is an important consideration for deciding on appropriate dietary reference intakes for SAAs; additional research is required on this issue.

Ensuring adequate B-vitamin nutriture when attempting to study SAA metabolism is of the utmost importance. We provided a multivitamin supplement that provided more than adequate quantities of vitamin B-6, vitamin B-12, and folic acid. Vitamin B-6 is required as the cofactor for pyridoxal-*P* for the proper conversion of homocysteine to cysteine (7, 8). Both cystathionine β-synthase and γ-cystathionase are dependent on pyridoxal-*P* for catalysis of their respective reactions (7, 8). Vitamin B-6 is also necessary for the methylation of tetrahydrofolate to 5,10-methylenetetrahydrofolate (7, 8). Vitamin B-6 is also a cofactor in reactions involved in cysteine metabolism, catalyzed by cysteine lyase, aspartate 4-decarboxylase, and cystathionine β-lyase (7, 8). Cobalamin is required as a cofactor in the remethylation of homocysteine to methionine by 5-methyltetrahydrofolate–homocysteine *S*-methyltransferase (7, 8). The remethylation of homocysteine to methionine is also highly dependent on folic acid nutriture (7, 8).

In the present study, we found a mean total SAA requirement of 12.6 mg·kg^{-1}·d^{-1}, with a population-safe intake of 21.0 mg·kg^{-1}·d^{-1}. This latter amount is 60% greater than the current recommended total SAA requirement of 13.0 mg·kg^{-1}·d^{-1} (10). Both the mean and safe IAAO-determined values agree with values predicted from 24-h balance data (17, 38) and values recalculated from early nitrogen balance data (11). We conclude that 12.6 mg·kg^{-1}·d^{-1} is a reasonable estimate of the average SAA requirement (37, 40). Setting a dietary reference intake for total SAAs will depend on the analysis of more individual data, which may modify our current estimated population requirement of 21 mg·kg^{-1}·d^{-1}.

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