Threonine requirement of young men determined by indicator amino acid oxidation with use of L-[1-^{13}C]phenylalanine[1–4]

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ABSTRACT

Background: Threonine is an indispensable amino acid with a complex degradative pathway. Use of the indicator amino acid oxidation technique should provide an estimate of the threonine requirement that is not affected by its metabolic pathway.

Objective: Our objective was to determine the requirement for threonine in men by using the indicator amino acid oxidation method and to provide statistical estimates of the population mean and 95% CIs of the threonine requirement. We hypothesized that the current World Health Organization estimate of the threonine requirement, 7 $mg \cdot kg^{-1} \cdot d^{-1}$ (based on nitrogen balance studies), is too low.

Design: Six healthy men each received 6 different threonine intakes while consuming an energy-sufficient diet with 1.0 g L-amino acid mixture $\cdot kg^{-1} \cdot d^{-1}$. The effect of graded alterations in dietary threonine intake on phenylalanine flux and oxidation was studied by using L-[1-^{13}C]phenylalanine as the indicator amino acid.

Results: The results of two-phase linear regression crossover analysis showed that the mean threonine requirement, based on indicator oxidation, was 19.0 $mg \cdot kg^{-1} \cdot d^{-1}$ with an upper safe intake of 26.2 $mg \cdot kg^{-1} \cdot d^{-1}$.

Conclusions: This is the first application of the indicator amino acid oxidation technique in humans to study the requirement for an indispensable amino acid with a complex degradative pathway. We found that the upper safe intake for 95% of the population is almost 4-fold higher than the current World Health Organization estimate. *Am J Clin Nutr* 2000;71:757–64.

KEY WORDS Threonine, indicator amino acid oxidation, amino acid requirement, stable isotope, phenylalanine, men

INTRODUCTION

Current dietary recommendations for indispensable amino acids in adults are based on nitrogen balance studies performed 40 y ago (1, 2); these studies involved a small number of subjects who were only tested at a few different amino acid intakes. Many researchers in the field have suggested that these requirement estimates need revision (3–6). Plasma free amino acid concentrations have been used to estimate amino acid requirements (7), and although this method is not sensitive enough for routine use, the results suggested higher requirements than those determined from nitrogen balance studies. Requirements have also been estimated by using direct amino acid oxidation, and these results suggested that the nitrogen balance estimates are low, with actual requirements being 2- to 3-fold higher (3, 8). Most recently, a new experimental method called indicator amino acid oxidation (IAAO) was used to determine amino acid requirements (9–11). Results from these studies support the proposed higher requirements for indispensable amino acids. With the IAAO method, the oxidation of an indicator amino acid is studied during feeding with graded amounts of the test amino acid. The oxidation of the indicator amino acid decreases as intakes of the test amino acid increase. Once the requirement is reached and then exceeded, there is no further decrease in the oxidation of the indicator amino acid.

Threonine is a totally indispensable amino acid because it has no metabolic precursor (12). The upper end of the range for recommended intake, 7 $mg \cdot kg^{-1} \cdot d^{-1}$ (1, 2), is based on nitrogen balance studies performed in only 3 healthy men (13). By using direct amino acid oxidation, Zhao et al (14) estimated the requirement for threonine to be between 10 and 20 $mg \cdot kg^{-1} \cdot d^{-1}$.

For the direct oxidation method to be completely reliable, the tracer amino acid must have only a single degradative pathway, oxidation to carbon dioxide (4). However, threonine has 2 degradative pathways in mammals (15, 16), and therefore this condition is not met. In contrast, threonine requirements in humans could be measured by IAAO because an alternative amino acid is used as the indicator and the method is therefore independent of the partitioning of carbon atoms from threonine degradation among several possible pools (15, 16). We proposed that use of the IAAO method would allow us to improve upon the current estimate of

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TABLE 1
Subject characteristics and energy intakes

Subject	Age	Height	Weight[1]	Energy intake[2]	BMI
	y	*cm*	*kg*	*MJ/d*	*kg/m²*
1	20	177	87.7 ± 0.8	8.45	28.0
2	39	188	80.0 ± 1.8	7.68	22.6
3	27	164	60.0 ± 0.2	6.68	22.3
4	22	174	98.1 ± 1.4	9.12	32.4
5	24	191	83.3 ± 0.6	8.15	22.8
6	27	173	78.4 ± 0.3	7.85	26.2
$\bar{x}$ ± SD	26.5 ± 6.8	177.8 ± 10.1	81.3 ± 12.6	7.99 ± 0.8	25.7 ± 4.0

[1] $\bar{x}$ ± SD values were calculated for all study periods for each subject (*n* = 6); weight did not change significantly for any subject over the course of the 6 studies.

[2] Energy intake was calculated by using the 1985 World Health Organization energy report equations for age (1).

the threonine requirement in men. Our objectives were to determine the requirement for threonine in men by using the IAAO method and to provide statistical estimates of the population mean and 95% CIs for the threonine requirement.

SUBJECTS AND METHODS

Subjects

Six healthy adult male volunteers with a mean (±SD) age of 26.0 ± 7.2 y (range: 19–39 y) were recruited for participation in this study; their characteristics are shown in **Table 1**. None of the subjects had a history of recent weight loss, unusual dietary practices, chronic disease, endocrine disorder, or atypical sleeping or exercise schedules. Studies were performed on an outpatient basis in the Clinical Investigation Unit at The Hospital for Sick Children in Toronto.

The purpose of the study and the potential risks involved were explained fully to each subject and written consent was obtained. The subjects received financial compensation for the loss of earnings that resulted from participation in the study. Approvals from the University of Toronto Human Experimentation Committee and the Human Subjects Review Committee of The Hospital for Sick Children in Toronto were obtained.

Experimental design

We used a previously described experimental approach of IAAO studies (9–11). Briefly, the total experimental period for each subject was 18 d, divided into two 9-d periods during which the subjects were free-living, except when the oxidation studies were being performed. These 2 periods were separated by ≥2 wk but <1 mo to allow for a break from the experimental diet. On days 3, 6, and 9 of each dietary period, each subject was assigned (in random order) an amount of the test amino acid. During each dietary period, subjects consumed a diet containing 14 mg phenylalanine $\cdot$ kg^{-1} $\cdot$ d^{-1} to ensure adequate dietary phenylalanine. This amount meets the requirement of 95% of adult males, as previously determined by amino acid oxidation when tyrosine was present in relative excess (8). It has been shown that the indicator amino acid is most sensitive to the intake of the test amino acid when the former is consumed at its required amount (17). The amino acid composition of the diet was based on egg protein and contained generous amounts of both tyrosine and threonine (40 and 47 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}, respectively) (18).

The estimate of threonine requirement is 7 mg $\cdot$ kg^{-1} $\cdot$ d^{-1} by nitrogen balance (1) and is between 10 and 20 mg $\cdot$ kg^{-1} $\cdot$ d^{-1} by direct oxidation (14). We predicted an estimated requirement close to 20 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}, on the basis of the assumption that the estimate from direct oxidation was low as a result of undetected partition of some oxidized threonine into glycine (16). On days 3, 6, and 9 of each dietary period, the subjects received a test intake of dietary threonine at 1 of 7 amounts (5, 10, 15, 20, 25, 30, or 35 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}), assigned in random order, and then measurements of phenylalanine flux and oxidation were performed. Each subject was therefore studied at 6 different amounts (36 studies in all); thus, we had sufficient test threonine intakes for estimation of the population mean and 95% CIs. The distribution of the number of studies at each test amount is shown in **Table 2.** The requirement breakpoint estimate depends particularly on the slope of the line for the lowest intake amount. To better define the slope of this line, we assigned 6 studies each to the 2 lowest amounts, which also meant it was only possible to assign 4 studies to each of the 2 highest amounts.

Energy requirements

The estimated energy requirement of each subject was calculated by using the equations for prediction of resting metabolic rate contained in the 1985 FAO/WHO/UNU Expert Consultation (1); these equations use the individual's age, sex, weight, and height. Resting metabolic rate was also measured before each study (after a 12-h overnight fast) by continuous computerized open-circuit indirect calorimetry (Sensormedics 2900; Sensormedics, Anaheim, CA) with a ventilated hood for 30–40 min once the subject had adapted to the equipment. The system uses a paramagnetic oxygen analyzer and an infrared carbon dioxide analyzer. Air flow is adjusted with a mass flow meter and carbon dioxide concentration is maintained in the range of 0.5–0.8% by adjustment of flow rate. The system is calibrated against nitrogen and span gases, 20% oxygen and 4% carbon dioxide (Linde Mixed Medical Gas; Union Carbide, Toronto). Resting metabolic rate values were multiplied by an activity coefficient of 1.7 to obtain an appropriate energy intake (19). Subjects were encouraged to maintain their normal levels of physical activity and were provided with preprinted forms on which to record all activities throughout the study.

Experimental diet

The experimental diet was specifically developed for use in studies of amino acid metabolism (18) and has been used in such

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

	Threonine intake ($mg \cdot kg^{-1} \cdot d^{-1}$)						
	5 (*n* = 6)	10 (*n* = 6)	15 (*n* = 5)	20 (*n* = 6)	25 (*n* = 5)	30 (*n* = 4)	35 (*n* = 4)
	$\mu mol \cdot kg^{-1} \cdot h^{-1}$						
Phenylalanine flux	25.1± 7.0	21.9 ± 4.8	24.9 ± 3.5	22.2 ± 2.8	22.4 ± 5.1	23.3 ± 6.5	19.3 ± 4.3
Phenylalanine oxidation	1.8 ± 0.6[a]	1.3 ± 0.2[b,c]	1.4 ± 0.4[a,b]	1.3 ± 0.4[c]	1.5 ± 0.4[b,c]	1.3 ± 0.4[c]	0.7 ± 0.2[c]

[1] Threonine intake did not significantly affect phenylalanine flux but did have a significant effect on the rate of $^{13}CO_2$ release from oxidation of L-[1-^{13}C]phenylalanine, $P = 0.002$. The oxidation rate also differed significantly among individuals, $P = 0.002$. Means with different superscript letters are significantly different, $P = 0.05$.

studies previously (8–11). All amino nitrogen was supplied as a crystalline amino acid mixture that simulated the composition of intact egg protein and was consumed in the amount of 1 g protein$\cdot kg^{-1} \cdot d^{-1}$. This mixture was needed to maintain a phenylalanine intake equal to the requirement, thereby ensuring maximum sensitivity of the method (20), and to ensure that other amino acids in the diet were kept constant. This mixture was also needed to control threonine intake so that dietary amounts ranging from deficient to excessive, based on recommendations (1) and direct oxidation studies (14), could be studied. The remainder of the diet was provided as nonprotein energy sources and it met the energy, vitamin, and mineral requirements of the subjects. The main source of energy in the diet was a flavored liquid-formula diet (Protein Free Powder, product no. 80056; Mead Johnson, Evansville, IN), with the remainder of the energy supplied by 2 types of protein-free cookies prepared in the research kitchen of The Hospital for Sick Children (18). Supplemental foods were not permitted during the study, except for clear tea, decaffeinated coffee, and water. The diet was formulated to provide 10% of energy as protein, 53% as carbohydrate, and 37% as fat. Energy intake was calculated as described above and was constant for each individual during the study period.

Diets were prepared and weighed (model PE2000 Mettler scale; Mettler, Nanikon, Switzerland) in the research kitchen and were then portioned into 4 isoenergetic, isonitrogenous meals that were consumed daily at 0800, 1200, 1600, and 2000. Each meal consisted of a bottle of flavored, protein-free formula, into which the amino acid mixture had been added, and one of each type of protein-free cookie.

Anthropometry

Standing heights were measured to the nearest 0.1 cm by using a wall-mounted stadiometer (Holtain Ltd, Croswell, Crymych, United Kingdom). After voiding their urine, subjects dressed in only light clothing were weighed on a balance scale (model 2020 Toledo scale; Toledo, Windsor, Canada) to the nearest 0.1 kg in the morning before each dietary period and on all experimental study days. Body mass index (BMI) was calculated as weight in kg divided by height in m^2.

Isotope infusion studies

The stable isotopes used in these studies were $NaH^{13}CO_3$ (Cambridge Isotope Laboratories, Woburn, MA) with a 90% enrichment and L-[1-^{13}C]phenylalanine (Tracer Technologies Inc, Somerville, MA) with a 99% enrichment at the carboxyl carbon. Quality-control tests performed by Tracer Technologies on the tracer [^{13}C]phenylalanine showed a single spot by thin-layer chromatography, 99% enrichment at the specified carboxyl carbon position by nuclear magnetic resonance and gas chromatography with mass spectrometry, and absence of the D-isomer confirmed by gas chromatography with a chiral column. Stock solutions of $NaH^{13}CO_3$ (3.30 g/L), priming L-[1-^{13}C]phenylalanine (6.65 g/L), and constant-infusion L-[1-^{13}C]phenylalanine (3.00 g/L) were prepared in normal saline solution. The solutions were prepared with sterile water by passage through a 0.22-μm filter (Millipore, Bedford, MA) under a laminar flow hood and were then dispensed into single-dose vials in the Department of Pharmacy of The Hospital for Sick Children. Each batch of the infusates was shown to be sterile and free of bacterial growth over 7 d in culture and to be pyrogen-free by the limulus amebocyte lysate test (21).

Isotope infusion studies were carried out on days 3, 6, and 9 of the study period in a temperature-controlled metabolic facility at The Hospital for Sick Children. The meals at 0800 and 1200 were divided into 6 equal parts, which the subjects consumed hourly beginning 2 h before infusion of the isotope (18); this ensured a metabolic steady state. The amount of dietary phenylalanine in the last 4 meals was reduced by a quantity equivalent to the amount of L-[1-^{13}C]phenylalanine tracer infused so that the total phenylalanine intake remained unchanged. We used L-alanine to keep the total amino acid content constant, decreasing its amount as threonine intake increased. The test threonine intake and the nonlabeled phenylalanine were provided separately on study days so that their exact intakes would be known. Three baseline breath and blood samples were obtained before infusion of the isotope, which was infused via a 21-gauge peripheral catheter inserted aseptically into the antecubital fossa vein of the left arm. Each subject received a priming dose of $NaH^{13}CO_3$ (0.176 mg/kg) and a priming dose of L-[1-^{13}C]phenylalanine (0.665 mg/kg). Immediately thereafter, a constant infusion of L-[1-^{13}C]phenylalanine (1.2 $mg \cdot kg^{-1} \cdot h^{-1}$) was administered for 4 h via a calibrated syringe pump (IVAC 710; Eli Lilly Inc, San Diego).

Once the isotope infusion started, blood and breath samples were taken every 30 min during the 120–240 min after priming, when steady state was achieved. Arterialized venous blood (3 mL) was sampled from a 21-gauge peripheral catheter inserted into a superficial vein on the dorsum of the right hand; the catheter was kept patent by infusing the line with heparin (10 000 US Pharmacopoeia U/L) between sampling. To arterialize the venous blood, the hand was heated inside a heating device at 60 °C for ≥15 min before blood sampling (22). Blood samples were drawn into heparin-containing syringes and stored on ice until centrifugation at 1500 × *g* for 10 min at 4 °C. Plasma was

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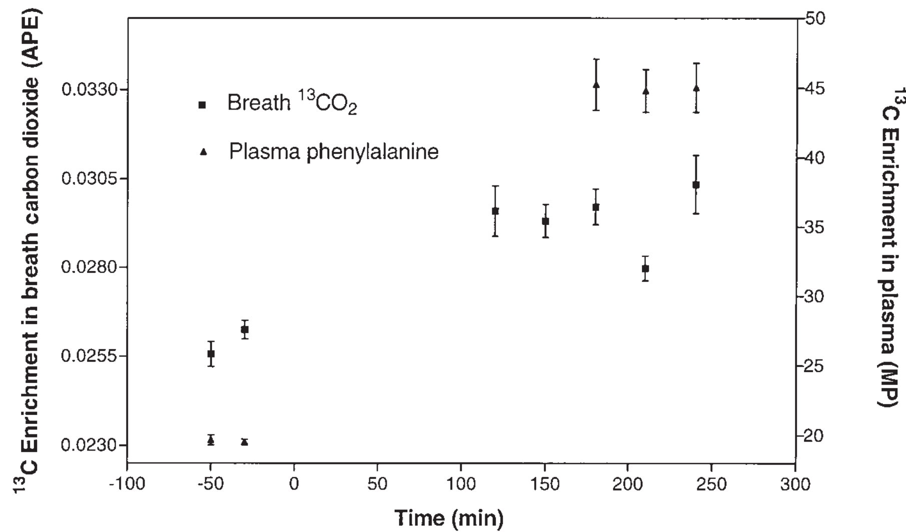

FIGURE 1. Mean (±SEM) breath $^{13}CO_2$ enrichments and plasma L-[1-^{13}C]phenylalanine enrichments at baseline and plateau (n = 6). APE, atoms percent excess; MP, molar ratio as a percentage.

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stored at −20°C until it was analyzed for [^{13}C]phenylalanine enrichment.

Three 7-min baseline breath samples were collected 1 h before the infusions started. Thereafter, 7-min breath samples were collected every 30 min during the 4-h infusion period. The collection of breath samples and the procedure for trapping expired carbon dioxide were reported in detail previously (23). In addition to the usual breath-sample collection, indirect calorimetry (Sensormedics 2900; Sensormedics) was carried out to measure the total carbon dioxide production rate simultaneously with breath sampling both before and every 30 min throughout the isotope infusion. A ventilated face mask (Scott 802167–30; Sensormedics) was used.

Analytic procedures

The percentage enrichment of the expired $^{13}CO_2$ was measured on a dual-inlet magnetic sector isotope ratio mass spectrometer (VG Micromass 602D; VG Isogas Ltd, Cheshire, England) by using techniques described previously (24). Breath samples were analyzed in duplicate. Breath enrichments from baseline samples and from those taken during the isotope infusion were expressed as atoms percent excess (APE) $^{13}CO_2$ over a reference standard of compressed carbon dioxide gas.

Plasma samples (200 μL) were deproteinized and acidified with an equal volume of 20% trichloroacetic acid (wt:vol) and were then centrifuged at 9000 × g for 5 min at room temperature. Amino acids were separated from the supernate by a cation exchange resin (Dowex 50W-X8, 100–200 mesh H^+ form; Bio-Rad Laboratories, Richmond, CA) and were derivatized to their *N*-heptafluorobutyryl *n*-propyl esters by using the method described by Patterson et al (25). Amino acid enrichment was measured on a gas chromatograph (Hewlett-Packard model 5890, series II; Hewlett-Packard, Mississauga, Canada) attached to a quadrupole mass spectrometer (VG Trio-2; VG Isogas Ltd). Separation of the amino acid derivatives was performed with helium as the carrier gas on a 30-m × 0.32-mm (inside diameter) × 1.0-μm (film thickness) fused silica capillary column (HP5; Hewlett-Packard) coupled directly to the ion source, which was operated under conditions of negative chemical ionization with ammonia as the reactant gas. Each amino acid was analyzed by splitless injection on an automatic sampler (HP 7673 injector; Hewlett-Packard). Selected ion chromatographs were obtained by monitoring the mass-to-charge ratios of 383 and 384 for [^{13}C]phenylalanine, corresponding to the unenriched (m) and enriched (m+1) peaks, respectively. The areas under the peaks were integrated by a Digital DECp 450D_2LP computer (Digital Instruments, Santa Barbara, CA) with the LAB-BASE program (VG Isogas Ltd).

Data analysis

The model used here to study phenylalanine metabolism was described previously by researchers who used a constant infusion approach to study amino acid oxidation (26). The overall model was as follows:

$$Q = S + O = B + I \quad (1)$$

where Q is the rate of phenylalanine flux, S is the rate of phenylalanine incorporation into protein synthesis, O is the rate of phenylalanine oxidation, B is the rate of breakdown of phenylalanine from tissue protein, and I is the rate of exogenous phenylalanine intake. Rates are expressed as μmol phenylalanine·kg^{-1} body wt·h^{-1}. Isotopic steady state in the metabolic pool was represented by the plateau in $^{13}CO_2$ enrichments in breath and [^{13}C]phenylalanine enrichments in plasma, which was observed in the period 120–240 min after the start of isotope administration (**Figure 1**). For calculations of kinetic indexes, we used mean breath and plasma isotope enrichment values of 5 samples at plateau, corrected for enrichment at baseline (mean

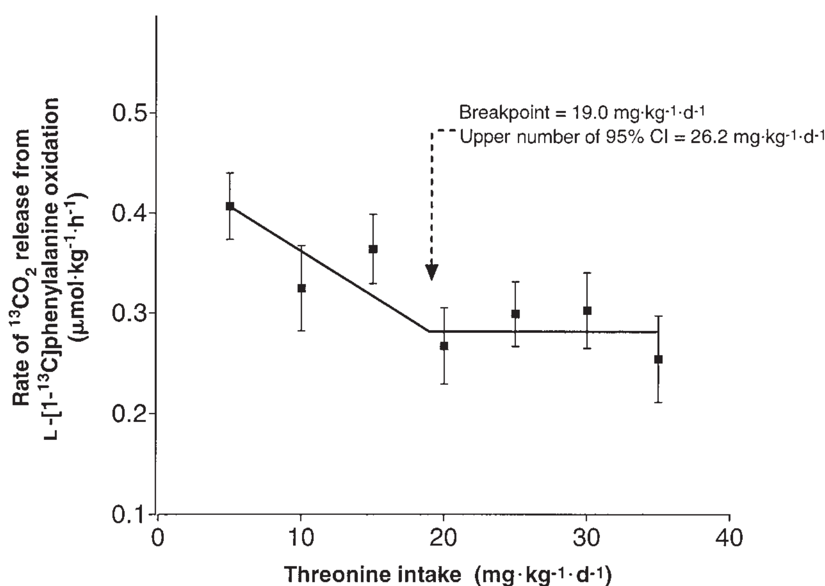

FIGURE 2. Effect of threonine intake on oxidation of L-[^{13}C]phenylalanine determined from the rate of release of $^{13}CO_2$ ($F^{13}CO_2$): mean (±SEM) oxidation rate (■) at each of the 7 threonine intakes for all 6 subjects (*n* = 36 observations). The threonine intake at the breakpoint between the 2 regression lines represents the mean threonine requirement of 19 $mg \cdot kg^{-1} \cdot d^{-1}$. The linear regression equation for the estimated mean threonine requirement is $y = 0.280077 - 0.160506C - 0.008426Cx$, where $C = 1$ for the first line and $C = 0$ for the second line.

of 2 samples) before infusion. The isotopic enrichment was calculated by using the following equation:

$$\text{Enrichment (APE)} = [R_s - R_b/(1 + R_s) - R_b] \times 100 \qquad (2)$$

where R_s and R_b were the ratios of (m+1) to (m) for the enriched sample and the sample at natural abundance, respectively (26), where *m* is the molecular ion.

Flux (*Q*) was determined from the dilution of the infused tracer L-[1-^{13}C]phenylalanine in the metabolic plasma pool at isotopic steady state by using standard equations (26). Similarly, the rate of release of $^{13}CO_2$ was calculated from carbon dioxide production rates and breath $^{13}CO_2$ enrichment at the isotopic steady state, as described previously (26), and included a correction for retention of label in the bicarbonate pool in the fed state (27). Phenylalanine oxidation was determined from the rate of release of $^{13}CO_2$ and plasma L-[1-^{13}C]phenylalanine enrichment at the isotopic steady state by using standard equations (25).

Statistical analysis

A two-factor (threonine intake and subject) general linear model (28) was used to assess the relations among the rate of release of $^{13}CO_2$, phenylalanine flux, and phenylalanine oxidation. Nonsignificant interactions ($P \geq 0.1$), such as the order in which the test intakes were given, were dropped from the model to produce the final reduced model. The significance of specific differences between groups was examined by using the least-significant-differences multiple range test.

The mean requirement for threonine was determined by breakpoint analysis with a two-phase linear regression crossover model (29) similar to that described previously (17, 20). This model minimizes the residual SE in a stepwise partitioning of data points between 2 regression lines. The 95% CI for the mean threonine requirement was calculated by Fieller's theorem (28) and the upper number of the 95% CI was considered the safe amount for threonine intake. In all cases, results were considered to be significant at $P \leq 0.05$.

RESULTS

Subject characteristics and energy intakes are shown in Table 1. Weight did not change significantly over the two 9-d study periods, which provided evidence that the subjects were in energy balance. Mean values of [1-^{13}C]phenylalanine flux and oxidation for the 6 individual subjects at the 7 different test threonine intakes are shown in Table 2. Phenylalanine flux was not significantly affected by threonine intake (Table 2) or the order in which the intakes were given but differed significantly among subjects ($P = 0.047$). In contrast, the rate of phenylalanine oxidation was significantly affected by threonine intake, although no breakpoint could be identified visually, and again, the order of test intakes had no significant effect. At any given threonine intake, the rate of phenylalanine oxidation differed significantly among subjects ($P = 0.002$).

Two-factor general linear model results revealed that both subject ($P = 0.002$) and threonine intake ($P = 0.012$) had significant effects on the rate of release of $^{13}CO_2$ ($F^{13}CO_2$) from oxidation of tracer [1-^{13}C]phenylalanine. Although the subjects differed significantly in their absolute $F^{13}CO_2$, they showed a similar pattern in which $F^{13}CO_2$ decreased with increasing threonine intake to a point that was close to the requirement, after which it remained relatively stable. The mean (±SEM) values for $F^{13}CO_2$ ($n = 6$) at threonine intakes ranging from 5 to 35 $mg \cdot kg^{-1} \cdot d^{-1}$ are shown in **Figure 2**. Regression analysis showed that phenylalanine catabolism, as measured by $F^{13}CO_2$, decreased as threonine intake increased from 5 to 20 $mg \cdot kg^{-1} \cdot d^{-1}$. By using a two-phase linear regression crossover model, a breakpoint in the $F^{13}CO_2$ response curve was

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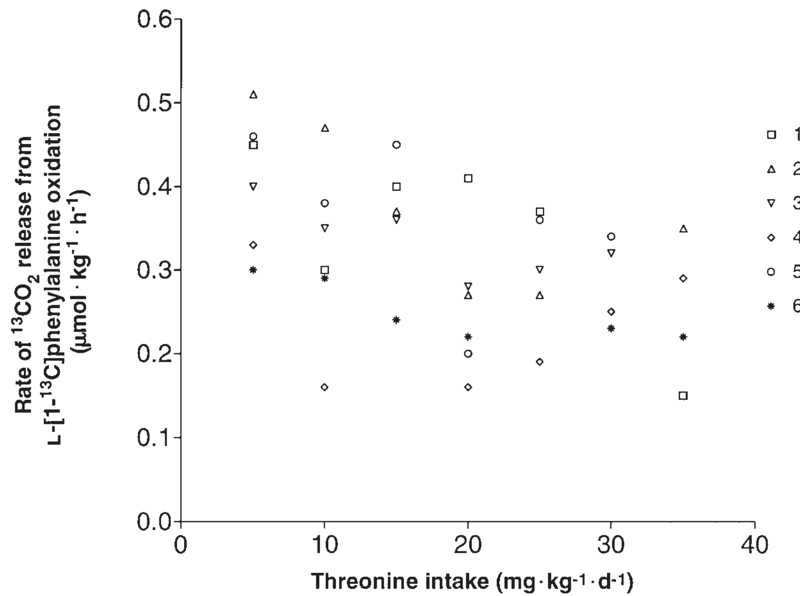

FIGURE 3. Relation between the rate of release of $^{13}CO_2$ (breath $F^{13}CO_2$) and threonine intake for the 6 individual subjects (*n* = 36 observations).

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identified at a dietary threonine intake of 19.0 mg·kg^{-1}·d^{-1}; the upper number of the 95% CI was 26.2 mg·kg^{-1}·d^{-1}. The threonine requirements of the individual subjects were estimated by visual inspection and were found to be 35, 20, 20, 10, 20, and 20 mg·kg^{-1}·d^{-1} (**Figure 3**).

DISCUSSION

The first estimate of threonine requirements in adult humans, published by Rose et al in 1955 (13), was obtained by using the nitrogen balance method in 3 healthy young men. A lower value for the requirement of women was obtained by Leverton et al (30), who also used the nitrogen balance method. These results have been incorporated into the FAO/WHO/UNU upper requirement amount, 7 mg·kg^{-1}·d^{-1} (1). However, the nitrogen balance approach involves several methodologic problems; for details, *see* the excellent review by Fuller and Garlick (5). More recently, Millward (6) recalculated the FAO/WHO/UNU requirements to allow for miscellaneous losses of nitrogen and reported a threonine requirement of 15.7 mg·kg^{-1}·d^{-1}. This value is within the range reported on the basis of direct oxidation (14), which is discussed below.

Attempts to determine total threonine oxidation by using the direct amino acid oxidation method were first made in mammals; researchers measured the production of labeled carbon dioxide after infusion of threonine with either radioisotope or stable isotope labeling of the carboxyl group (31–34). However, these studies failed to account for a major confounder, the sequestration of carbons 1 and 2 of threonine into glycine via the threonine dehydrogenase (TDG) pathway (16). Ballevre et al (16) developed a multitracer method to quantify both major pathways of threonine oxidation in pigs and found a partition in which 80% of oxidation occurred via the TDG pathway and 20% occurred via the threonine dehydratase (TDH) pathway. Furthermore, any increase in oxidation occurred almost exclusively via the TDG pathway (16). This effect of diet in pigs was confirmed by Le Floch et al (15), who also found evidence of extrahepatic oxidation of threonine associated with the decrease in total oxidation when the dietary supply of threonine was reduced. The same group later showed that in pigs, the pancreas is a major site of oxidation of threonine to glycine (35). The relative contributions of the 2 pathways to oxidation of threonine in humans have only been determined in one physiologic state. Darling et al (36) showed conversion of threonine to glycine, presumably via the TDG pathway, in low-birth-weight infants. They estimated that the rate of conversion of threonine to glycine in formula-fed preterm infants amounted to 44% of total threonine degradation, whereas degradation via the TDH pathway accounted for 56% of threonine disposal (36).

The only direct amino acid oxidation study that estimated the threonine requirement in humans was performed by Zhao et al (14). Threonine oxidation rates fell with reduced threonine intake in 8 healthy young men, reaching a relatively constant rate between 10 and 20 mg·kg^{-1}·d^{-1}. This figure is 2–3-fold higher than the current recommendation (1). However, Zhao et al (14) found that excess threonine was not as efficiently oxidized as lysine (37) or leucine (38). They concluded that this could not be the result of alternative oxidative pathways because they could not measure any [^{13}C]glycine. However, research in human infants (36) and in pigs (16, 39) showed partition of threonine metabolism to glycine via TDG. The analytic method used by Zhao et al (14) to measure [^{13}C]glycine (gas chromatography–quadrupole mass spectrometry) was not sensitive enough to detect labeling, as compared with the recently developed method known as gas chromatography–combustion isotope ratio mass spectrometry (36).

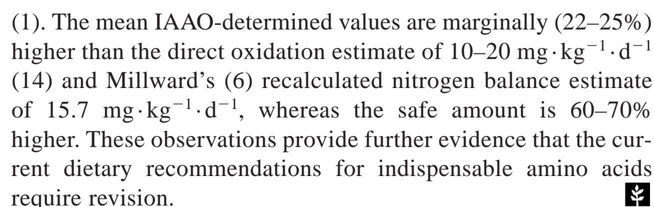
IAAO is a new, independent, and relatively simple method of estimating amino acid requirements in humans. It has been validated extensively in animals (17, 20, 40). This technique is based on the hypothesis that the partition of any indispensable amino acid between oxidation and protein synthesis is sensitive to the amount of the most limiting amino acid in the diet. When an indispensable amino acid is limiting for protein synthesis, all other amino acids are in relative excess and therefore must be

oxidized. This means that increasing the dietary amount of the limiting amino acid in graded quantities will increase the uptake of all dietary amino acids for protein synthesis, which in turn reduces their oxidation, until the requirement point is reached. Once the requirement is reached, further increments in the test amino acid will have no effect on the uptake of other indispensable amino acids for protein synthesis or oxidation (17).

We showed previously that phenylalanine oxidation is not altered by adaptation, over 3, 6, or 9 d, to phenylalanine intakes of either 4.2 or 14.0 $mg \cdot kg^{-1} \cdot d^{-1}$. Because the duration of adaptation for most amino acid requirement studies was 7–10 d, we concluded that the need for prior adaptation is minimal (4, 8, 9). These minimal requirements for adaptation allow for repeated measurements with each subject, and therefore data can be obtained on each subject across the full study range (4, 9–11). Nevertheless, it still remains to be determined whether subjects would show differences if they had adapted to lower intakes of threonine for a period of several weeks.

The precursor pool from which phenylalanine is oxidized is a concern. This is the second study (*see* reference 11) in which we have not been able to obtain a breakpoint estimate from phenylalanine oxidation. Our estimate of phenylalanine oxidation was calculated on the basis of plasma phenylalanine enrichment, which may not represent enrichment at the site of oxidation within the hepatocyte. The actual precursor is [^{13}C]tyrosine, and because its enrichment is likely to be lower than our measured value for plasma phenylalanine, our estimate of phenylalanine oxidation is lower than the true value. However, because we were able to achieve an isotopic steady state in breath $^{13}CO_2$ enrichments, we reasoned that the rate of $^{13}CO_2$ production varies in proportion to the rate of conversion of [^{13}C]phenylalanine to [^{13}C]tyrosine, and subsequent [^{13}C]tyrosine oxidation. This in turn means that changes observed in the rate of $^{13}CO_2$ production are a valid reflection of the use of phenylalanine for protein synthesis and hence permits a valid estimate of the requirement for the test amino acid (in this case, threonine). There was more individual variation in $F^{13}CO_2$ in this study (Figure 3) than we observed in an earlier indicator oxidation study that was performed to determine tryptophan requirements (10). The reasons are not clear, except that the previously mentioned complex degradative pathways for threonine (15, 16) may result in more individual variation. The effect of graded amounts of threonine on the rates of threonine disposal via the TDG and TDH pathways has yet to be studied in humans.

Nevertheless, requirements for indispensable amino acids with more complex metabolism can be measured by IAAO. There is no dietary restriction on test amino acid intake that can be examined, because the requirement for the test amino acid is determined by the oxidation of a different amino acid. All possible dietary quantities of an amino acid can be studied, unlike the direct oxidation method, in which very low intakes are impossible because the amount of isotope infused has to be included as part of the dietary intake. The statistical estimates of breakpoint are also stronger when there is a wider range of intakes of the test amino acid, both below and above the inflection point. We published previously a review of studies that used IAAO to evaluate amino acid requirements in humans (41). In the current experiment, we found a mean threonine requirement of 19.0 $mg \cdot kg^{-1} \cdot d^{-1}$, with an upper safe amount of 26.2 $mg \cdot kg^{-1} \cdot d^{-1}$. This latter amount is almost 4-fold higher than the current recommended threonine requirement of 7 $mg \cdot kg^{-1} \cdot d^{-1}$ (1). The mean IAAO-determined values are marginally (22–25%) higher than the direct oxidation estimate of 10–20 $mg \cdot kg^{-1} \cdot d^{-1}$ (14) and Millward's (6) recalculated nitrogen balance estimate of 15.7 $mg \cdot kg^{-1} \cdot d^{-1}$, whereas the safe amount is 60–70% higher. These observations provide further evidence that the current dietary recommendations for indispensable amino acids require revision.



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