Leucine Kinetics in Reference to the Effect of the Feeding Mode as Three Discrete Meals

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In a recent study, we observed that the 24-hour leucine oxidation measured when three equal meals providing a generous intake of leucine (~90 mg · kg^{-1} · d^{-1}) are eaten during the day is 16% lower ($P < .01$) than that for the same diet given as 10 hourly, equal meals. We hypothesized that the pattern of meal intake at a lower level of dietary leucine would affect the 24-hour rate of leucine oxidation and possibly improve the retention of dietary leucine. A total of 11 healthy adults participated in this investigation. The daily leucine intake was 182 μmol · kg^{-1} · d^{-1} (38 mg · kg^{-1} · d^{-1}) given with an L-amino acid diet. All subjects received three discrete meals daily for 6 days prior to a 24-hour intravenous (IV) tracer infusion of L-[1-^{13}C]-leucine on day 7 (study 1). Four of these subjects participated in two additional studies of similar design. Study 2 involved giving [1-^{13}C]-leucine as a constant IV infusion together with tracer added to the amino acid mixture at each meal time. In study 3, subjects received the three meals with added [1-^{13}C]-leucine tracer while [^{2}H$_3$]-leucine was given as a constant IV infusion. Total leucine oxidation in studies 1 and 2 was 238 ± 66 and 231 ± 85 μmol · kg^{-1} · d^{-1}, respectively. Leucine balance was positive, amounting to 18% of the total (diet + tracer) intake. The estimated mean nitrogen balance was +8 mg · kg^{-1} · d^{-1}. Leucine oxidation was higher ($P < .01$) for breakfast than for the lunch meal. This difference was associated with lower insulin and higher plasma leucine concentrations at breakfast versus lunch periods. The results from study 3 suggest that the higher rate of leucine oxidation observed at breakfast as compared with lunch is not due to a difference in the immediate splanchnic fate of absorbed leucine from each meal. In comparison to our previous small frequent-meal studies, the pattern of meal feeding influences overall leucine utilization at both generous and limiting leucine intakes. Hence, it is possible that the pattern of meal feeding may affect estimations of amino acid requirements using the tracer-balance approach. Longer-term dietary studies will be needed to establish whether and the extent to which this is so.

THE EFFECT of the meal feeding pattern on whole-body leucine metabolism has been evaluated recently in our laboratory.[1] Using a 24-hour [^{13}C]-leucine tracer protocol, we found that for a generous leucine intake, daily leucine oxidation is about 16% lower when the intake is provided as three isocaloric meals, rather than 10 hourly small meals, with the first of these given after a 15-hour overnight fast.[2] This observation has further support and a possible explanation from data demonstrating that after a large single meal there is an apparent marked increase in whole-body protein synthesis,[3] which was not observed previously with a continuous feeding mode.[4-6] In addition, other whole-body leucine kinetic parameters, such as the rate of leucine appearance or protein breakdown,[3,7] might be affected differently by large versus small meals. These various findings led us to consider the possibility that a more physiologic feeding pattern (discrete large meals) rather than continuous feeding or frequent small meals might affect leucine metabolism, particularly oxidation, when dietary leucine is provided at a more limiting, or "minimum requirement," level.

We have defined the physiologic minimum requirement for leucine as the minimum dietary intake needed to maintain a balance between intake and irreversible oxidation.[8-10] This was found to occur at a dietary leucine intake of about 40 mg · kg^{-1} · d^{-1} using a 24-hour tracer protocol (12-hour fast/12-hour fed) with food given as hourly small meals.[8] The primary aim of this study was therefore to define the 24-hour leucine balance at the Massachusetts Institute of Technology (MIT)-proposed minimum requirement intake level of leucine (~40 mg · kg^{-1} · d^{-1}) with subjects on a "physiologic" three-meal feeding pattern.

From an initial pilot study involving the three-discrete-meals protocol, we observed two interesting and unexpected phenomena. The first was a significant decrease in 24-hour leucine oxidation, which appeared larger than we may have predicted from our previous study.[1] Second, we observed a different oxidation rate in response to two identical meals, with the rate for the breakfast meal being higher than the rate for the lunch meal. This difference was not observed in our previous 24-hour ^{13}C-leucine infusion study,[1] wherein subjects were given a generous intake of leucine.

We suspected that these preliminary findings for leucine oxidation might be explained, in part, by the uptake of dietary leucine by splanchnic tissues during the first pass and that some leucine was oxidized in situ, before equilibration with the intravenously administered tracer. Consequently, there would be an underestimation of leucine oxidation when the three large meals were given. We also wanted to assess whether the lower leucine oxidation rate observed at lunch compared with breakfast was due to a suppression of the total whole-body leucine oxidation rate or was related to the immediate fate of dietary leucine, during its first pass within the splanchnic region and its distribution into pathways of protein synthesis and oxidation in situ.

Therefore, the aims of this study were also to determine whether (1) at a limiting leucine intake the meal feeding pattern has a critical effect on the immediate fate of dietary leucine and (2) the attenuated leucine oxidation rate following the lunch meal is a consequence of a different fate of dietary leucine or is

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due to a reduced availability of endogenous leucine for oxidation.

SUBJECTS AND METHODS

Subjects

Eleven healthy subjects (weight, 73.5 ± 8.8 kg; height, 178.0 ± 5.0 cm; age, 24.1 ± 4.5 years, mean ± SD) participated in the study. Subjects were evaluated by medical history and physical examination. In addition, blood and urine samples were collected for a routine clinical screening in the Clinical Research Center (CRC) laboratories. The study and the consent form were approved by the MIT Committee on the Use of Humans as Experimental Subjects and the Advisory Committee of the MIT CRC. Informed consent was obtained from each subject, and they were paid for participation in the study.

Protocol

During the first 6 days, all 11 subjects received a "minimum requirement" intake of leucine, 38 mg · kg^{-1} · d^{-1},[8] and adequate but not excessive total nitrogen.[11] At the end of this experimental diet period, a 24-hour tracer protocol was performed in which ^{13}C-leucine was infused intravenously (IV) (study 1). Three equal meals were consumed at 8:00 PM (dinner), 6:00 AM (breakfast), and 12:00 noon (lunch).

Four of the subjects also received the same diet as in study 1 for two additional 7-day diet periods. All diet periods were separated by an interval of 6 to 30 days, during which time the subjects consumed their free-choice diets. On day 7 of each experimental period, a 24-hour tracer protocol was performed. In study 2, ^{13}C-leucine was infused IV at the same rate as in study 1, and at each meal time, a known quantity of about one third of the dietary leucine was replaced with ^{13}C-leucine. The goal of study 2 was to further label the expired CO_2 with ^{13}C arising from dietary leucine that was oxidized before possible equilibration of absorbed leucine with the IV tracer. Hence, with this protocol, we anticipated a higher $^{13}CO_2$ enrichment than in study 1. It seemed possible that some of the dietary leucine (unlabeled and labeled) would be compartmentalized and metabolized during the first pass in the splanchnic region, and thus there might be a relatively smaller increase for plasma ^{13}C-ketoisocaproate (^{13}C-KIC) than for the $^{13}CO_2$ enrichment in breath. Thus, the ratio of $^{13}CO_2$ production to plasma ^{13}C-KIC (used to calculate leucine oxidation), would then be higher than for study 1. Thus, the difference between the two estimates of 24-hour leucine oxidation (studies 1 and 2) would be interpreted as being due to a first-pass splanchnic oxidation of leucine. Study 3 was then performed in the same four subjects, but in this case, they received a continuous IV infusion of $^{2}H_3$-leucine at the same rate as the ^{13}C-leucine tracer in study 1, and at each meal time, about one third of the dietary leucine was replaced by ^{13}C-leucine (as in study 2). The aim of study 3 was to investigate the fate of dietary leucine, particularly in reference to the difference in the rate of total leucine oxidation between breakfast and lunch observed in study 1 and study 2 (shown later).

We have chosen to analyze only the differences in leucine kinetics between breakfast and lunch for several reasons. From our preliminary data, major and persistent differences in leucine oxidation appeared between these two meals. Moreover, with the composition of the three meals being identical, they differed only in the period of postabsorption or fasting that preceded them, with breakfast served after a 10-hour fast and lunch consumed only 6 hours after breakfast. It was thought that this comparison would best reveal any effect of the fasting period on leucine metabolism prior to meal intake.

For study 3, it was considered possible from the ratio of $^{13}CO_2$ excretion and dietary leucine enrichment to estimate dietary leucine oxidation during the meals. Moreover, using non–steady-state equations, we also attempted to estimate the rate of appearance of dietary leucine in the sampled plasma compartment, as well as leucine release from endogenous sources. We are aware that non–steady-state equations, although used here and by other investigators,[3,12] have not been validated fully for leucine. On the other hand, by comparing data for the two meals, it was believed that possible limitations in the estimation of the metabolic parameters, due to non–steady-state conditions, would be similar. Hence, even if the interpretation of our data may not be accurate in a quantitative sense, the qualitative aspects of the comparison between meals would still seem valid.

Diet

Daily energy intake was constant for each subject, with a range of 41 to 45 kcal/kg (172 to 188 kJ/kg). No significant or persistent body weight changes occurred during the experimental periods. Nonprotein energy was provided as 40% from fat (safflower oil and butter) and 60% from carbohydrate (beet sugar and wheat starch). Nitrogen (160 mg · kg^{-1} · d^{-1}) was supplied as an L-amino acid mixture (Table 1). Vitamins, minerals, choline, and fiber were supplied as daily supplements. Additional details have been reported previously.[8]

Tracer Study

On the afternoon of day 6 of the experimental diet period, subjects were admitted to the infusion room of the MIT Medical Department. Two indwelling catheters were inserted, one in a forearm vein to infuse the tracer and the other in a retrograde direction in a dorsal vein of the hand, from which to draw blood samples. The hand was heated in a heating box (air temperature 68°C) to obtain arterialized venous blood. After collecting blood and breath samples to measure baseline isotopic enrichment, in studies 1 and 2, IV priming boluses of [^{13}C]-sodium bicarbonate 0.8 μmol · kg^{-1} (99 atom %; Cambridge Isotope Laboratories, Cambridge, MA) and L-[1-^{13}C]-leucine 4.2 μmol · kg^{-1} (99 atom %; MassTrace, Somerville, MA) were administered. Then, L-[1-^{13}C]-leucine was infused continuously (2.8 μmol · kg^{-1} · h^{-1}) throughout each 24-hour study. In study 3, L-[5,5,5-$^{2}H_3$]-leucine (99 atom %; MassTrace) was administered as an IV primed-continuous infusion (2.8 μmol · kg^{-1} · h^{-1}). In studies 2 and 3, L-[1-^{13}C]-leucine 19.7 μmol · kg^{-1}

Table 1. Composition of L-Amino Acid Mixtures Used to Supply Intake of Leucine 38 mg · kg^{-1} · d^{-1}

Amino Acid	g/kg Mixture
L-Tryptophan	15.97
L-Threonine	48.19
L-Isoleucine	64.30
L-Leucine*	26.04
L-Lysine HCl	77.47
L-Methionine	30.37
L-Cystine	22.52
L-Phenylalanine	55.94
L-Tyrosine	41.69
L-Valine	71.90
L-Histidine HCl-H_2O	31.38
L-Arginine HCl	77.35
L-Alanine	196.0
L-Aspartic acid	12.21
L-Glutamic acid	30.20
Glycine	74.74
L-Proline	41.25
L-Serine	82.51

*Leucine 9.4 mg · kg^{-1} BW · d^{-1} was added every day to the daily diet except on the tracer infusion day, when this amount was supplied by the tracer infusion, to maintain a constant total leucine intake throughout the study.

was substituted for unlabeled leucine and mixed with each meal. Thus, about one third of dietary leucine was labeled in these two studies.

Blood and breath samples were collected every 30 minutes for determination of $^{13}CO_2$ enrichment, plasma [^{13}C]-KIC and [^{13}C]- and [$^2H_3$]-leucine enrichment, and plasma leucine concentration, all as previously described.[2] Blood was collected in chilled heparinized tubes and immediately centrifuged, and the plasma was stored at −20°C before analysis. As in previous 24-hour studies, to allow the subjects to sleep between midnight and 6:00 AM, blood, but not breath, samples were drawn for determination of ^{13}C enrichment in CO_2 during this time interval.[2] Breath and blood samples for $^{13}CO_2$ enrichment were collected as previously described[2] and stored at room temperature until analysis by isotope ratio mass spectrometry (MAT Delta E; Finnigan, Bremen, Germany). Total CO_2 production was measured by indirect calorimetry (Deltatrak; SensorMedics, Yorba Linda, CA) for about 25 to 30 minutes every hour.

Complete 24-hour urine was collected during the 6-day experimental period. During the tracer study, consecutive 3-hour urine collections were obtained as previously described.[2] Plasma and urinary urea nitrogen concentration was determined by a modified version of the procedure of Marsh et al[13] using an autoanalyzer. Urinary urea excretion was corrected for changes in the body urea pool (see calculations). Total urinary nitrogen was determined by micro-Kjeldahl analysis.

Serum insulin concentrations were measured before each meal and hourly for 3 hours after the beginning of each meal by a double-antibody radioimmunoassay (Incstar, Stillwater, MN).

Data Analysis

Leucine oxidation rates were computed for consecutive half-hour intervals as described previously[2] in study 1 and study 2. Because the present experimental conditions were essentially the same as those used previously[1] (except for the amount of dietary leucine), we used the [^{13}C]-bicarbonate recovery factors determined earlier[1] to correct for $^{13}CO_2$ retained in the whole body. We corrected $^{13}CO_2$ enrichment for changes in $^{13}CO_2$ background enrichment also as described previously.[1]

Leucine Oxidation

Total leucine oxidation ([Tot Leu Ox] micromoles per kilogram per 30 minutes) was calculated as Tot Leu Ox = $^{13}CO_2$ production/$E_{^{13}C\text{-}KIC}$, where $^{13}CO_2$ production ($\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$) is VCO_2 ($\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$) × $^{13}CO_2$ enrichment (APE × 1,000) × 1/R, where R is the correction factor for bicarbonate recovery.[1] As noted already, this recovery factor was determined previously in three subjects studied under essentially identical experimental conditions[1] except for the level of dietary leucine and the source of dietary "protein." The $^{13}CO_2$ recovery factor was specific for each half-hour interval of leucine oxidation. $E_{^{13}C\text{-}KIC}$ is the plasma enrichment of [^{13}C]-KIC in studies 1 and 2, expressed as mole percent excess (MPE). Twenty-four-hour Tot Leu Ox is the sum of the 48 consecutive Tot Leu Ox measurements. Twenty-four-hour leucine balance is the difference between 24-hour Leu intake and 24-hour Tot Leu Ox.

Dietary leucine oxidation (Diet Leu OX) was calculated in study 3 for breakfast and lunch, covering a 6-hour period in each case as follows: Diet Leu OX = $^{13}CO_2$ production/E_{diet}, where $^{13}CO_2$ production is expressed as the area under the curve (AUC) over a 6-hour period and E_{diet} (MPE) is ^{13}C-leucine enrichment of the total dietary leucine.

Total nitrogen (Tot N) excretion was calculated as Tot N = urea N excretion (corrected) + nonurea N excretion + 8, where urea N excretion (corrected) is urea N excretion − (Δplasma $urea_{0\text{-}1440}$ × estimated TBW/0.92) as described by Fern et al,[14] with TBW (total body water) estimated from the equation used by Watson et al.[15] We also added 8 mg $N \cdot kg^{-1} \cdot d^{-1}$ to the N excretion to approximate unmeasured N losses of catabolic origin, including those via feces and sweat, as described previously.[2]

Leucine Kinetics

The whole-body rates of leucine appearance (Ra) and disappearance (Rd) were calculated for non–steady-state conditions after breakfast and lunch using Steele's equations[16] as modified by Proietto et al.[17] The applicability of these equations to describe leucine kinetics and the rationale for the use of a correction factor for the endogenous leucine pool size and a volume of distribution of $0.5\ L \cdot kg^{-1}$ body weight (BW) have been discussed elsewhere.[3] Thus, Tot Leu Ra = $[I_{IV} - [pV \times C(t) \times dE_{IV\text{-}leu}/dt]]/E_{IV\text{-}leu}(t)$, where I_{IV} is the IV infusion rate ($\mu mol \cdot kg^{-1} \cdot min^{-1}$) of $^2H_3$-leucine corrected for isotopic purity and p equals 0.25 and represents a correction factor applied to the volume of distribution ($V = 0.5\ L \cdot kg^{-1}$ BW), since leucine does not mix instantaneously with the total endogenous pool. Based on the literature,[3,12] we have used an effective volume of distribution (pV) of $0.125\ L \cdot kg^{-1}$ BW. C(t) is the mean plasma leucine concentration between two consecutive time points (μmol/L), $dE_{IV\text{-}leu}/dt$ (MPE) is the derivative of plasma $^2H_3$-leucine enrichment, and $E_{IV\text{-}leu}(t)$ is the mean plasma $^2H_3$-leucine enrichment (MPE) between two consecutive time points. Tot Leu Rd = Tot Leu Ra − (pV × dC/dt), where dC/dt is the time derivative of the plasma leucine concentration.

Dietary leucine Ra (Exo Ra) was calculated according to the method of Proietto et al[17] as follows: Exo Ra = [Tot Leu Ra × $E_{PO\text{-}leu}(t)$ + [pV × C(t) × $dE_{PO\text{-}leu}/dt$]]/E_{diet}, where Tot Leu Ra is calculated as before from the IV continuous infusion with $^2H_3$-leucine, $E_{PO\text{-}leu}(t)$ is the mean plasma ^{13}C-leucine enrichment in study 3 between two consecutive time points (oral tracer), and $dE_{PO\text{-}leu}/dt$ is its time derivative. The Rd of dietary leucine (Exo Rd) is Exo Rd = Exo Ra − [pV × C(t) × $dE_{PO\text{-}leu}/dt$]/E_{diet}. Endogenous leucine Ra (Endo Ra), taken as representative of whole-body protein breakdown, is calculated as Endo Ra = Tot Leu Ra − Exo Ra − I_{IV}.

The difference between Tot Leu Rd and Tot Leu Ox was taken to be nonoxidative leucine disposal (NOLD). Assuming that all dietary leucine is absorbed, then it is leucine used for whole-body protein synthesis. NOLD = Tot Leu Rd − Tot Leu Ox. Finally, the contribution of dietary leucine to protein synthesis (Exo NOLD) was calculated as Exo NOLD = Exo Rd − Diet Leu OX.

Tracer Infusion Time

Because we used a 24-hour constant tracer infusion protocol for comparison to the rates of leucine oxidation measured under similar experimental conditions except for a higher leucine intake,[1] the question arises as to whether there is sufficient recycling of tracer during the study to confound the interpretation of the data. In a prior study, Schwenk et al[18] reported a 30% recycling of leucine tracer over a 24-hour period in subjects who received the tracer at 12:00 noon or one day that continued through to 12:00 noon on the next day following an overnight fast. This finding has not been supported in the literature, nor has this been our experience. Thus, two studies[19,20] showed very little or no recycling over 10 hours and about 3% recycling per day over 5 days of ^{13}C-leucine infusion. Simulation experiments supported the experimental finding.[19] Further, in our previous 24-hour tracer studies,[2] we found little evidence of a significant degree of tracer recycling as judged from the differences in ^{13}C-KIC enrichment during the early versus later phase of the prolonged tracer study. In any event, recycling would be essentially no consequence when comparing leucine kinetics between breakfast and lunch, in view of the even shorter time frame involved (6 hours). As stated earlier, estimates of leucine oxidation are not at all influenced by recycling, even if this were significant, since the use of plasma KIC enrichment would correct for this.

Statistical Methods

Results are expressed as the mean ± SD unless otherwise stated. Two-way (mean × time) ANOVA for repeated measures was used to compare plasma leucine and insulin concentration data between break-

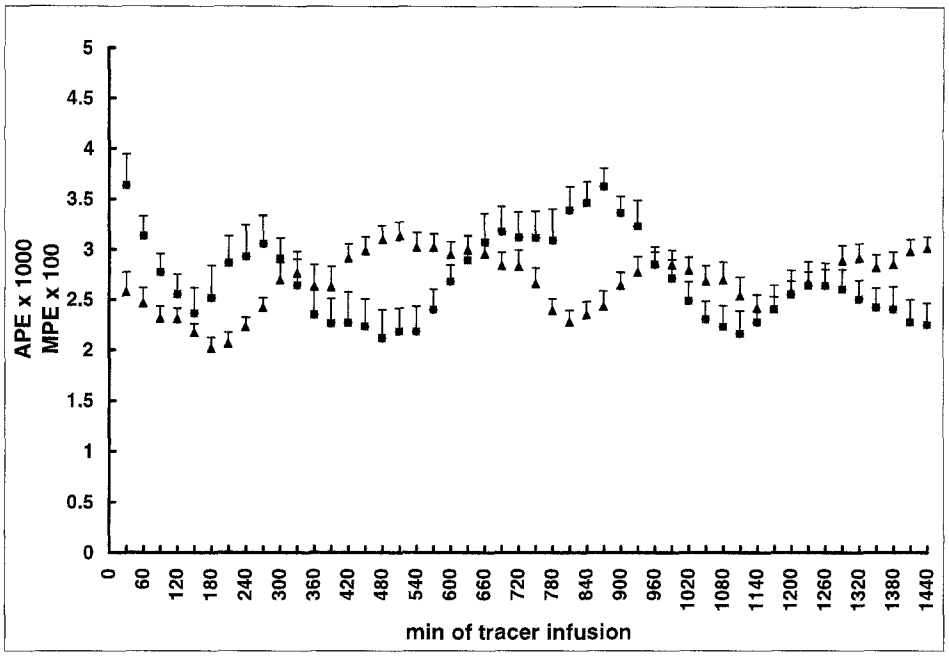

Fig 1. Level and pattern of breath $^{13}CO_2$ enrichment (■, APE × 1,000) and of plasma [^{13}C]-KIC enrichment (▲) throughout the 24-hour period. Meals: 120 minutes, dinner; 720 minutes, breakfast; 1,080 minutes, lunch. Data are the mean ± SE.

fast and lunch. A paired *t* test was used to compare mean values between studies 1 and 2 and between breakfast and lunch in studies 1 and 3. A one-sample paired *t* test was used for comparison of leucine and nitrogen balance data to zero.

RESULTS

The temporal patterns of breath $^{13}CO_2$ and plasma [^{13}C]-KIC enrichment are shown in Fig 1 (study 1), and Fig 2 shows the 24-hour pattern of the plasma leucine concentration (study 1). Plasma leucine peaked about 30 minutes after beginning each meal. The postbreakfast (B) AUC for the plasma leucine concentration (AUC_B) was 581 ± 48 μmol · L^{-1} · 6 h^{-1}, significantly higher than for the postlunch (L) AUC_L, which was 504 ± 53 μmol · L^{-1} · 6 h^{-1} ($P < .001$). The peak increment above the premeal concentration was not different (B 35.1 ± 26.4 *v* L 37.1 ± 25.1 μmol · L^{-1}, P = NS). Plasma insulin concentrations were higher (ANOVA, $P < .05$) during and after lunch versus during and after breakfast (Fig 3).

Leucine Kinetics

Total leucine oxidation was 237.8 ± 66.4 μmol · kg^{-1} · d^{-1} (study 1). Leucine balance was positive in eight of 11 subjects, with a group mean of +55.0 ± 65.5 μmol · kg^{-1} · d^{-1} (*v* neutral balance, $P < .05$).

Figure 4 depicts the estimate of 24-hour leucine oxidation in

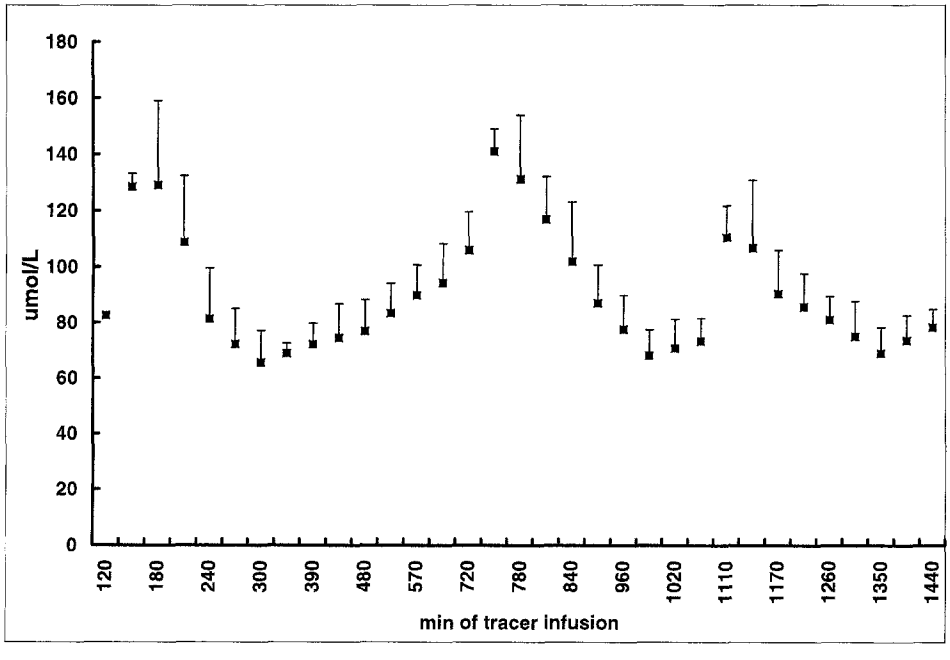

Fig 2. Level and pattern of plasma leucine concentration throughout the 24-hour period. Data are the mean ± SD. For other details, see Fig 1.

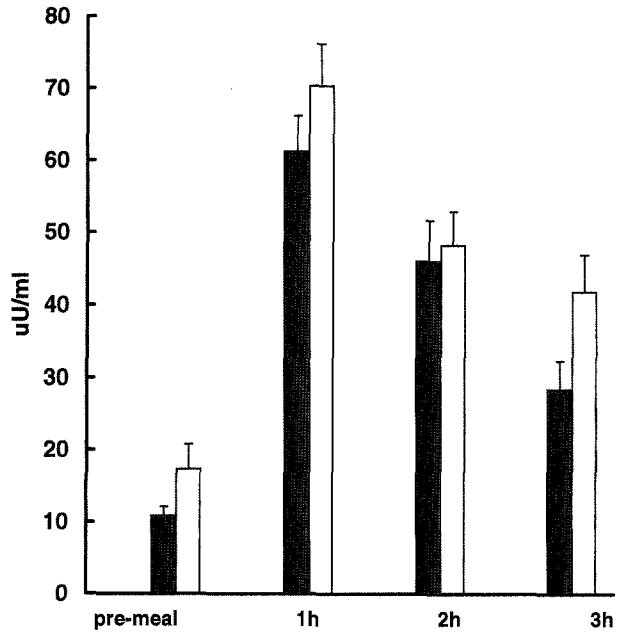

Fig 3. Plasma insulin concentrations for study 1 before and 1, 2, and 3 hours after breakfast (■) and lunch (□). Data are the mean ± SE.

study 1 (IV tracer only, 11 subjects) and study 2 (IV and oral tracer, four subjects). Total leucine oxidation in study 2 was 231.0 ± 84.7 μmol · kg^{-1} · d^{-1} (*v* study 1, *P* = NS).

Table 2 lists the leucine kinetic parameters for the breakfast and lunch periods for four subjects who participated in both study 1 and study 3. The AUC for total Ra tended to be larger for the 6-hour period postbreakfast versus postlunch (*P* = .06). This appears to be due to a higher rate of leucine release via body protein breakdown (Endo Ra) at breakfast (*v* lunch, *P* < .05); the dietary leucine Ra (Exo Ra) was not different between these two meals. The latter also accounts for about

Table 2. Comparison of Kinetic Parameters of Leucine Metabolism (μmol · kg^{-1} · 6 h^{-1}) at Breakfast Versus Lunch

Parameter	Breakfast	Lunch	*P*
Total leucine oxidation	81.4 ± 15.5	59.0 ± 19.7	<.01
Total leucine Ra	498.1 ± 59.4	475.8 ± 44.8	.06
Total leucine Rd	506.9 ± 56.4	476.2 ± 45.1	<.05
NOLD	425.6 ± 54.8	417.2 ± 47.6	.2
Endogenous leucine Ra	413.7 ± 53.6	389.4 ± 44.5	<.05
Exogenous leucine Ra	67.5 ± 6.1	69.5 ± 4.1	.4
Exogenous leucine Rd	65.4 ± 5.9	68.6 ± 4.2	.2
Exogenous leucine oxidation	8.0 ± 2.5	8.6 ± 3.8	.3
Exogenous NOLD	57.3 ± 8.3	60.0 ± 7.6	.2

NOTE. Data are the mean ± SD for 4 subjects.

90% of the leucine ingested and for about 14% of the leucine entering the circulation. The rate of total leucine disposal (Tot Leu Rd) was also higher after breakfast than after lunch (*P* < .05), but the amount of leucine used for protein synthesis (NOLD) was not different between the two meal periods. Total leucine oxidation during the 6-hour period following dinner, breakfast, and lunch was respectively 61.1 ± 21.4, 71.0 ± 18.2, and 52.8 ± 18.6 μmol · kg^{-1} (n = 11); the difference was statistically significant between breakfast and lunch (*P* < .01).

Only about 10% of dietary leucine was oxidized over the 6-hour period covered by each of the meals, and the contribution of dietary leucine (Exo Ox) to total leucine oxidation was not different between meals, most of the leucine provided with the meals was retained apparently for protein synthesis. Further, if this approximation of the oxidative fate of dietary leucine is reasonable, it would mean that the efficiency of retention of dietary leucine is about 70% under these experimental conditions.

Total N excretion after correcting for changes in the body urea pool was 152.2 ± 12.9 mg · kg^{-1} · d^{-1}. The difference between total nitrogen intake and excretion was +7.8 ± 12.9 mg · kg^{-1} · d^{-1} (*v* neutral balance, *P* = .08). Total urinary

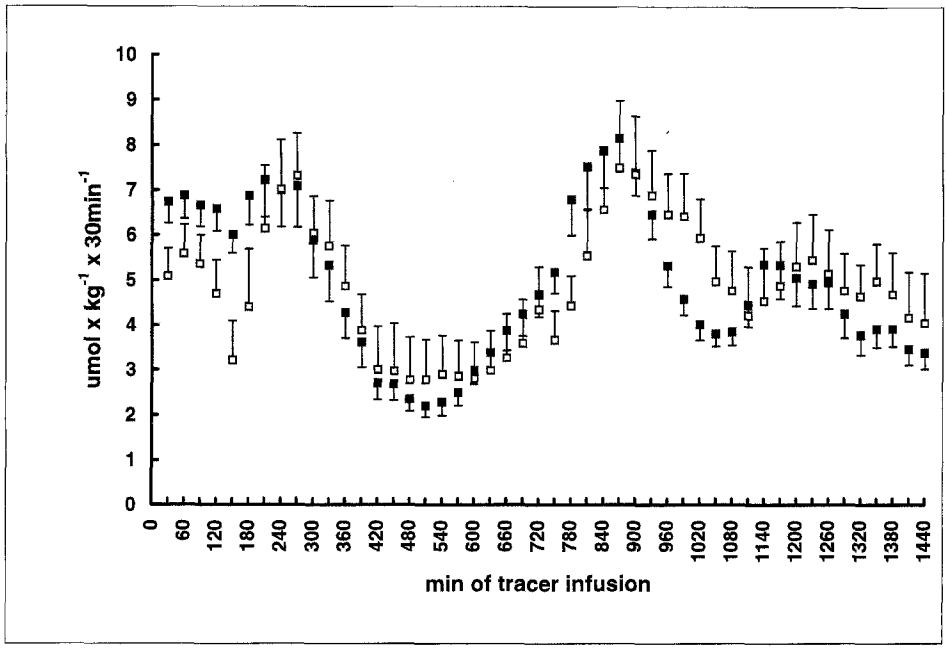

Fig 4. Rate and pattern of leucine oxidation throughout the 24-hour period in study 1 (■) and study 2 (□). Data are the mean ± SE. For other details, see Fig 1.

nitrogen excretion was not different during the 6-hour period after breakfast versus after lunch (51 ± 3 *v* 54 ± 2 mg · kg^{-1} · 6 h^{-1}, P = NS).

DISCUSSION

The main goal of this study was to explore whether the pattern of meal feeding can affect the rate of leucine oxidation at a limiting but not grossly inadequate intake. If so, we wanted to assess whether this might affect our estimates of the leucine requirement obtained with the ^{13}C-tracer-balance approach.[8]

Based on tracer-balance methodology, the minimum leucine requirement has been defined as the lowest intake at which a neutral balance is achieved between intake and whole-body oxidation.[21] From a series of previous studies,[8,22] we concluded that about 38 to 40 mg · kg^{-1} · d^{-1} is the lowest intake of leucine at which the difference between intake and oxidation is not different from zero. In these studies, we used either a 3-hour fast/5-hour fed or 12-hour fast/12-hour fed tracer-protocol approach, with either continuous intragastric administration of the L-amino acid formula or consumption of the diet as hourly small meals.

Recently, we found that at a generous leucine intake (89 mg · kg^{-1} · d^{-1}) divided into three bulk meals[1] instead of frequent small meals[2] and given during the 24-hour tracer study, the daily oxidation rate of leucine was reduced by 15 mg · kg^{-1} · d^{-1}. This bulk meal pattern apparently spared leucine from oxidative loss, to the extent of about 16% of the intake. In the present study with leucine at our proposed minimum requirement level of intake, the sparing effect of the bulk feeding mode on leucine oxidation appeared similar, or about 18% of the intake. The nitrogen balance was in accordance with the positive leucine balance: the difference between N intake and excretion as estimated here was about +7.8 mg N · kg^{-1} · d^{-1}. When N balance is expressed as protein balance, about 50 mg protein · kg^{-1} body weight was retained during the infusion day. If we assume that leucine is about 8% of whole-body proteins,[2] this N retention corresponds to a predicted positive leucine balance of approximately 4 mg · kg^{-1} · d^{-1}. This compares reasonably well with the leucine balance, measured directly by the tracer technique, of 7.2 mg · kg^{-1} · d^{-1}. Hence, both estimates indicate a positive state of body leucine and protein balance under the present experimental conditions. Since a positive balance is unlikely to be sustained over a prolonged period, the precise significance of these findings with respect to estimates of the minimum physiological requirement for leucine remains unclear. It is possible that the subjects had not fully adjusted to the apparent anabolic state created by the experimental design and that, with time, the positive retention of N and leucine would have eroded.

We considered the possibility that we were significantly underestimating the whole-body rate of leucine oxidation, especially during absorption of leucine from the meals. Thus, it was possible that there was an underestimation of 24-hour leucine oxidation as a result of the tracer administration route. By administering ^{13}C-leucine by the IV route only, we may have failed to fully label the leucine pool(s) in the splanchnic region and a portion of dietary leucine may have been oxidized in the splanchnic area during its first pass, before mixing and equilibrating with the labeled leucine infused peripherally. We then performed study 2 as an extension of study 1.

The rationale for study 2 was that if a significant first-pass leucine oxidation occurred, it would result in higher $^{13}CO_2$ production without a parallel increase in plasma ^{13}C-KIC relative to study 1. However, we failed to detect any significant difference in the 24-hour leucine oxidation rate between the two studies, suggesting that the oxidation of dietary leucine within the splanchnic region is small and unlikely to complicate our estimate of 24-hour leucine carbon balance. This finding is also consistent with our previous observation obtained with a small-meal feeding pattern and showing no difference in the rate of oxidation of ^{13}C-leucine[23] whether given IV or orally in subjects at different levels of dietary protein intake.

The data for dietary leucine oxidation obtained from study 3 further support the view that there is a relatively low level of leucine oxidation within the splanchnic region during the absorptive phase of metabolism. Our computations indicate that about 10% of dietary leucine is oxidized during the prandial phase. Even if all of this occurred in the splanchnic area during its first pass, it would amount to no more than about 7 to 8 μmol · kg^{-1} · meal^{-1}. In comparison, Boirie et al[3] determined that about 30% of dietary leucine from a large meal is oxidized. Although it is still a relatively small percent of the total dietary leucine intake, the difference between our estimates and those of Boirie et al[3] probably are related to the higher amount of leucine given in their test meal, about 360 μmol · kg^{-1}, in comparison to that supplied in the present study, 75 μmol · kg^{-1}.

Leucine oxidation during the night was also lower compared with the rate observed in our previous small-meal study.[8] This is probably casually related to differences in the length of the fasting period. In the small-meal protocol,[8] subjects fasted for 15 hours before the beginning of the fed period at 6:00 AM the next day. During the progression of this fast, an increasing oxidation rate was observed together with an increasing plasma leucine concentration.[8] In the present study, we also observed a trend for an increased leucine oxidation rate and plasma leucine concentration after midnight and prior to breakfast, but this was preceded earlier by a decrease in the leucine concentration and in the oxidation rate for a few hours after dinner. Consequently, both the leucine oxidation rate (Fig 5A) and plasma leucine concentration (Fig 5B) before breakfast reached a lower peak than in the previous study.[8]

It seems possible that the protein-conserving influence of the pattern of food intake is mediated by insulin. Insulin is a potent suppressor of tissue and whole-body protein breakdown.[24,25] It has been shown that infusion of insulin decreases plasma amino acid concentrations[24-26] and probably tissue levels, so less leucine is presumably available for oxidation. Although plasma insulin concentrations were not measured in the small-meal study,[8] it is reasonable to assume that they were lower as compared with the present study, since no food was given between 3:00 PM and 6:00 AM, whereas in the present study a meal was given at 8:00 PM. Another metabolic process of importance during fasting is gluconeogenesis,[27] the rate of which appears to increase linearly over the first 22 hours of a fast.[28,29] The need for gluconeogenic precursors such as alanine and glutamine, during the night might therefore determine an increasing rate of amino acid release from muscle, at the same

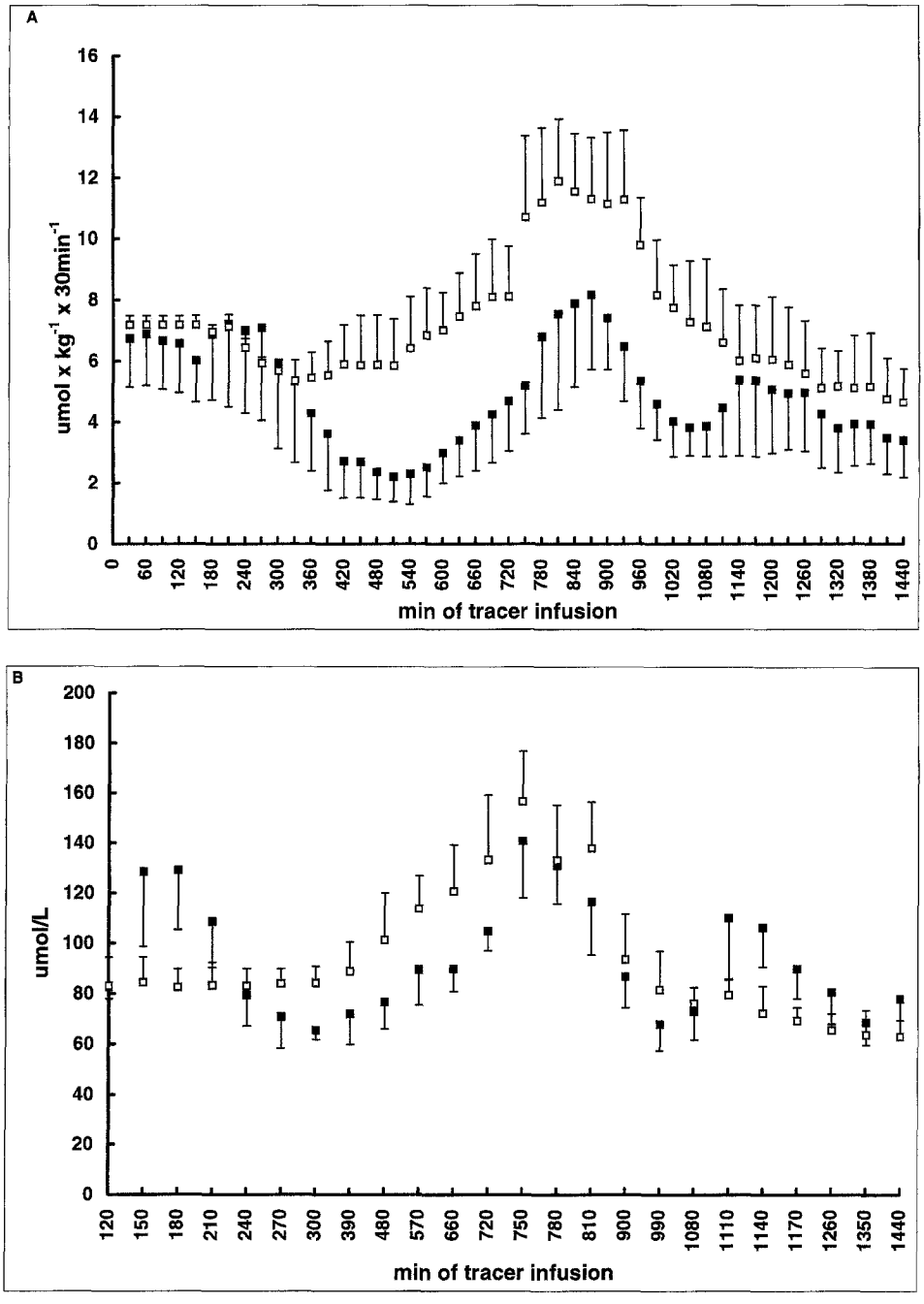

Fig 5. Comparison of leucine oxidation (A) and plasma leucine concentration (B) obtained from study 1 (■) and from a previous study[8] with small frequent meals given between 720 and 1,260 minutes (□). Data are the mean ± SD.

time mobilizing nongluconeogenic amino acids such as leucine that are disposed of via oxidation.

In contrast to our previous bulk-meal study[1] involving a generous intake of leucine and in which leucine oxidation was found to be similar during the 6-hour period following each of the three equal meals, we found differences here in the leucine oxidation rate among meals, especially between breakfast and lunch. Thus, whole-body leucine oxidation was about 71 $\mu mol \cdot kg^{-1}$ during the 6 hours following breakfast, significantly higher than the rate of 50 $\mu mol \cdot kg^{-1}$ after lunch. Also, there was a higher plasma leucine concentration after breakfast than after lunch. By combining simultaneous oral and IV $^{2}H_3$- and ^{13}C-leucine tracers (study 3), we were able to determine whether these differences were due to the handling of dietary leucine. We conclude that dietary leucine appeared in the peripheral blood circulation in equal amounts following each of the two meals and was oxidized in the same proportion and therefore incorporated into protein without differences between meals.

The higher rate of leucine oxidation at breakfast versus lunch then appears to be best explained in relation to the higher plasma leucine concentrations. This may be a consequence of

the higher rate of whole-body protein breakdown (Endo Ra) that occurred before breakfast versus before lunch. Food ingestion suppressed endogenous leucine release to the same absolute extent at both meals, but because of the higher premeal Ra at breakfast, postbreakfast values remained higher than postlunch values. Additionally, insulin concentrations for the 3 hours following the two meals were higher immediately before and after lunch as compared with the breakfast meal period.

The Rd of plasma leucine also was higher at breakfast, but nonoxidative leucine utilization (NOLD) was similar between the two postmeal periods. Thus, at breakfast, the "excess" leucine available peripherally as revealed from the plasma concentrations was not incorporated into proteins but was oxidized. While it is difficult to establish from the present data the metabolic/physiologic basis for these meal-dependent differences, it is again relevant to consider the plasma insulin and leucine concentrations between the two meals. Thus, we have compared the ratio between the AUC of insulin and the AUC of leucine during the first 3 hours after the meals: at lunch (L), the significantly higher Ins/Leu ratio (L 0.51 $\pm$ 0.1 *v* B 0.38 $\pm$ 0.1, $P < .01$) was associated with a lower rate of leucine oxidation. Although the quantitative effect of insulin per se on leucine utilization is still not entirely clear,[30,31] it is possible that at lunch the higher circulating insulin concentrations and the longer prior exposure of tissue to insulin could have increased glucose disposal and inhibited leucine oxidation, possibly as a consequence of insulin-mediated attenuation of the processes of cell and organ protein breakdown.[32]

We appreciate that other counter regulatory hormones, including glucagon, glucocorticoids, and epinephrine, all have effects on leucine kinetics and oxidation[32-34] and that glucagon and corticosteroids, for example, interact with insulin in the metabolic response to feeding and fasting. The diurnal variations in both glucagon and glucocorticoids[35,36] also must be recognized. Hence, to ascribe the present findings to an insulin-mediated mechanism is clearly an oversimplification of a probably more complex pattern of involvement of hormonal factors in the disposal of dietary amino acids.

In conclusion, our data suggest that (1) by feeding three discrete meals instead of multiple small meals, leucine and nitrogen balance might be improved at a "minimum requirement" intake of leucine, at least over a brief period, in agreement with our previous findings in subjects with a generous leucine intake[1]; (2) leucine oxidation during the hepatic first pass of the amino acid appears to be very low; and (3) the pattern of meal administration plays an important role in the regulation of endogenous leucine metabolism. Further investigations are desirable to identify the physiological mechanisms underlying the response of leucine metabolism to the meal feeding pattern, the extent to which this is common to other indispensable amino acids, and its relevance to the quantitative definition of minimum amino acid requirements.

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