The 24-h kinetics of leucine oxidation in healthy adults receiving a generous leucine intake via three discrete meals

Antoine E El-Khoury, Melchor Sánchez, Naomi K Fukagawa, Ray E Gleason, Rita H Tsay, and Vernon R Young

ABSTRACT  The significance of meal size and frequency for the 24-h leucine tracer-balance technique was examined. Continuous measurements of leucine oxidation throughout a 24-h d were performed in six healthy, young adults who were given a weight-maintaining diet (188 kJ·kg⁻¹·d⁻¹; 1 g protein·kg⁻¹·d⁻¹) for 6 d followed by primed, continuous intravenous infusions of L-[1-¹³C]leucine and [¹⁵N-¹⁵N]urea. The 24-h study was started at 1800 on day 6 and three equal discrete meals were given at 2000, 0600, and 1200. Leucine oxidation was assessed from plasma [¹³C]α-ketoisocaproate enrichment and ¹³CO₂ excretion. The mean (± SD) leucine oxidation after each meal (over 6 h) was not significantly different (P > 0.5) among the three discrete meals: 20.0 ± 3.9, 20.2 ± 1.9, and 20.3 ± 2.4 mg·kg⁻¹·d⁻¹ for the meals given at 2000, 0600, and 1200, respectively. Twenty-four-hour leucine oxidation was 75.0 ± 7.8 mg·kg⁻¹·d⁻¹ for a leucine dietary intake of 80 mg·kg⁻¹·d⁻¹ for the meals given at 2000, 0600, and 1200. Leucine oxidation was paralleled by plasma leucine concentrations. Further, leucine oxidation and urea excretion predicted relatively similar values for 24-h protein oxidation. These data are compared with results from our similar previous studies using a multiple-small-meal feeding protocol. Am J Clin Nutr 1995;62:579–90.

KEY WORDS  Tracer-balance concept, feeding mode, humans, bicarbonate recovery, plasma leucine concentration, diurnal pattern, urea kinetics, leucine balance, protein oxidation, indispensable amino acid requirements

INTRODUCTION  We recently described the 24-h pattern and rate of leucine oxidation at generous (1), low, and inadequate (2) dietary intakes of leucine in healthy adults who received their intake via small frequent meals during the 12-h fed phase of a continuous 24-h L-[1-¹³C]leucine tracer kinetic study. The pattern of leucine oxidation within the 12-h fed period was dependent on the intake of leucine. At the generous intake, leucine oxidation was sustained at a high amount, whereas at low intakes leucine oxidation declined, more profoundly the lower the leucine intake. These previous studies were conducted to validate the [¹³C]leucine tracer-balance concept (1) and to provide estimates of whole-body leucine balance at different leucine intakes (1, 2) compared with those we made previously (3) from extrapolation of isotopic data obtained from tracer protocols lasting ≤ 8 h. Because these various studies involved a continuous feeding mode (10 hourly small meals given from 0600 to 1500), it is important to know whether the daily total leucine oxidative loss is affected by the pattern of dietary leucine intake. Therefore, we conducted a series of 24-h L-[1-¹³C]leucine intravenous-tracer protocols, essentially as carried out previously, and with the same subjects who received a generous leucine intake as hourly small meals (1), except that the dietary leucine was provided in the form of three discrete meals. We report here the results from this new series and draw comparisons with those based on frequent small meals (1).

In addition, we previously showed, with the hourly small meals, that there is a good match between 24-h protein oxidation as derived from leucine oxidation data and urea excretion data; furthermore, these estimates, in turn, were consistent with the dietary protein intake data (1). These relations are now examined for a different feeding mode: three discrete meals. Thus, urea kinetics (production, excretion, and hydrolysis) after a primed, constant, intravenous infusion of [¹⁵N-¹⁵N]urea are compared with leucine kinetics over 24 h and over shorter intervals (6 h) after each of the three discrete meals. In particular, it was relevant to measure urea production to compare urea hydrolysis (production minus excretion) among the different meal-eating patterns.

SUBJECTS AND METHODS  Three separate but related studies were performed (Table 1). The first involved four healthy young adults who were studied according to the 24-h tracer protocol described below, except that they did not receive any tracer (tracer-free studies). The purpose was to determine how the background [¹³C] enrichment of expired carbon dioxide varied over the 24-h period. The

1 From the Laboratory of Human Nutrition, School of Science and Clinical Research Center, Massachusetts Institute of Technology, Cambridge, MA.
2 Supported by NIH grants RR88, DK 15856, and 42101.
3 Address reprint requests to AE El-Khoury, Massachusetts Institute of Technology, Clinical Research Center, 40 Ames Street, Room E17–445, Cambridge, MA 02142.
Received August 16, 1994.
Accepted May 22, 1995.
second study involved the determination, in a second group of three healthy young adults, of the recovery in expired carbon dioxide of infused $^{13}$C-sodium bicarbonate (bicarbonate studies), under the experimental conditions of the 24-h protocol described below. The third investigation was the complete diet and experimental design

For the third investigation (leucine-urea studies), each subject received for 6 d a weight-maintaining diet that was based on egg protein (N X 6.25) to supply 1.0 g egg protein · kg$^{-1}$ · d$^{-1}$ as the source of total nitrogen and indispensable amino acids (Table 2) (1). In addition to the egg-protein-based drink, part of the energy requirement was supplied in the form of protein-free, wheat-starch cookies, as previously described (4). About 40% of nonprotein energy was from fat and was 60% from carbohydrate. The main sources of carbohydrate were beet sugar and wheat starch, to attain a low $^{13}$C content in the diet and to minimize changes in background breath $^{13}$CO$_2$ enrichment over a 24-h period (see below). The daily leucine intake provided by the egg protein during this period was 80 mg/kg. The intravenous tracer [1-$^{13}$C]leucine, given during the 24-h infusion period, supplied an additional 9.7 mg/kg for that day (see below). Other nutrients were given at adequate amounts and dietary fiber was supplied as 20 g microcrystalline cellulose/d (Avicel; FMC Co, Philadelphia).

The same diet as described above (Table 2) was used previously (1), except on the infusion day when the 24-h intake was divided into 10 small hourly meals. In the present inves-

### TABLE 1

Characteristics of subjects who participated in 24-h studies with the three discrete meal-feeding mode

<table>
<thead>
<tr>
<th>Study, subject, and sex</th>
<th>Age (y)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracer-free studies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (M)</td>
<td>24</td>
<td>70.4</td>
<td>176.5</td>
</tr>
<tr>
<td>2 (M)</td>
<td>22</td>
<td>81.8</td>
<td>180.3</td>
</tr>
<tr>
<td>3 (M)</td>
<td>22</td>
<td>74.0</td>
<td>171.4</td>
</tr>
<tr>
<td>4 (M)</td>
<td>23</td>
<td>71.1</td>
<td>172.7</td>
</tr>
<tr>
<td>x ± SD</td>
<td>22.7 ± 1.0</td>
<td>74.3 ± 5.2</td>
<td>175.2 ± 4.0</td>
</tr>
<tr>
<td>Bicarbonate studies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (M)</td>
<td>20</td>
<td>74.5</td>
<td>186.7</td>
</tr>
<tr>
<td>2 (M)</td>
<td>20</td>
<td>75.8</td>
<td>190.5</td>
</tr>
<tr>
<td>3 (F)</td>
<td>22</td>
<td>62.4</td>
<td>163.8</td>
</tr>
<tr>
<td>x ± SD</td>
<td>20.7 ± 1.2</td>
<td>70.9 ± 7.4</td>
<td>180.3 ± 14.4</td>
</tr>
<tr>
<td>Leucine-urea studies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (M)</td>
<td>20</td>
<td>86.6</td>
<td>186.1</td>
</tr>
<tr>
<td>2 (M)</td>
<td>19</td>
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<td>168.9</td>
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<tr>
<td>3 (M)</td>
<td>20</td>
<td>70.6</td>
<td>180.3</td>
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<tr>
<td>4 (M)</td>
<td>22</td>
<td>95.6</td>
<td>174.0</td>
</tr>
<tr>
<td>5 (F)</td>
<td>19</td>
<td>50.7</td>
<td>155.6</td>
</tr>
<tr>
<td>6 (M)</td>
<td>24</td>
<td>70.9</td>
<td>181.6</td>
</tr>
<tr>
<td>x ± SD</td>
<td>20.7 ± 2.0</td>
<td>73.3 ± 15.8</td>
<td>174.4 ± 11.0</td>
</tr>
</tbody>
</table>

1 Weight on the 24-h study day.

### TABLE 2

Composition of the diet used in the tracer-free, bicarbonate, and leucine-urea studies

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg-protein formula (g/d)$^1$</td>
<td>72</td>
</tr>
<tr>
<td>Egg-white solids$^2$</td>
<td>20</td>
</tr>
<tr>
<td>Whole-egg solids$^2$</td>
<td>210</td>
</tr>
<tr>
<td>Beet sugar</td>
<td>55</td>
</tr>
<tr>
<td>Safflower oil$^4$</td>
<td>195</td>
</tr>
<tr>
<td>Orange sherbet$^1$</td>
<td>1245</td>
</tr>
<tr>
<td>Unsweetened flavored beverages$^4$</td>
<td>270</td>
</tr>
<tr>
<td>Protein-free cookies (g/d)</td>
<td></td>
</tr>
<tr>
<td>Supplements</td>
<td></td>
</tr>
<tr>
<td>Multivitamin-multimineral (capsules)$^6$</td>
<td>1</td>
</tr>
<tr>
<td>Sodium chloride (g)$^7$</td>
<td>6</td>
</tr>
<tr>
<td>Calcium (mg)$^9$</td>
<td>1000</td>
</tr>
<tr>
<td>Potassium (mg)$^9$</td>
<td>3912</td>
</tr>
<tr>
<td>Choline (mg)$^{10}$</td>
<td>500</td>
</tr>
</tbody>
</table>

$^1$ For a 70-kg subject, with an energy intake of 13.17 MJ (3150 kcal/d).
$^3$ PET Inc, St Louis.
$^4$ National Dairy Products Corp, Philadelphia.
$^6$ One-A-Day; Miles Inc, Elkhart, IN.
$^7$ Eli Lilly & Co, Indianapolis.
$^8$ TUMS; SKB Corp, Pittsburgh.
$^9$ K-LYTE; Bristol Laboratory, Evansville, IN: orange-flavored tablets, to dissolve in water, four tablets per day, each containing 978 mg K (25 mEq K).
$^{10}$ Lee Nutrition Inc, Cambridge, MA.
tigation we maintained a similar feeding pattern (three discrete, isoenergetic, isonitrogenous meals) both before and during the 24-h tracer-infusion study. Before the tracer phase, for 6 d, the total daily dietary intake was consumed as three isoenergetic, isonitrogenous meals (at 0800, 1200, and 1800). Usually two of these three meals were consumed at the MIT CRC under the supervision of the dietary staff and/or one of the investigators.

After the initial 6 d, a 24-h stable-isotope-tracer-infusion study was performed in each subject, beginning on day 6 at 1800 and continuing to day 7 at 1800. Constant intravenous infusions of [1-13C]leucine and [15N-15N]urea were given as described below. The above diet and feeding details were the same for the tracer-free and [13C]bicarbonate studies, except that a 4-d dietary period preceded the 24-h [13C]bicarbonate infusion.

24-h tracer-infusion protocol

We used a primed, continuous, intravenous infusion of tracer. Subjects received their second regular meal on day 6 at 1200. The tracer infusions were then begun at 1800 with subjects sleeping from 0000 to 0600 of the following day (day 7) (Figure 1). After the infusion started at 1800 on day 6, the third meal of day 6 was given at 2000. On day 7, the first meal was given at 0600 and the second meal at 1200 (Figure 1). The third meal of day 7 was given after the 24-h infusion ended at 1800.

Throughout this 24-h phase, the subjects remained in bed in a reclined position, except during sleep when they lay in the supine position. As discussed previously (1, 2), recycling of tracer does not affect the estimation of leucine oxidation because this index is based on the ratio of 13CO2 production to 13C enrichment of the precursor in plasma, α-ketoisocaproic acid (KIC). Nevertheless, the design of the 24-h infusions attempted to minimize problems of isotope recycling because it was preferable to incorporate the overnight fasting period into the first half of the 24-h study. As suggested previously (5), recycling of tracer is less significant during feeding (compared with fasting). To avoid disturbing the subjects and to permit the possibility of uninterrupted sleep, blood (and not breath) 13CO2 enrichments were determined between 0000 and 0600 in the bicarbonate and leucine-urea studies.

The design of the 24-h leucine-urea tracer-infusion studies is depicted in Figure 1, including collection of consecutive, complete, three-hourly urine samples; indirect calorimetry over 1-h periods; and half-hourly drawing of blood samples to determine plasma 13C enrichment of α-KIC and urea 15N enrichment. Selected time points were used for determination of plasma leucine concentrations (more frequent measurements during the first 2 h after each of the three discrete meals; see Results). Plasma urea concentrations were measured at every 3-h time point.

Primed, constant, intravenous infusions of L-[1-13C]leucine (99.3 atom %; Tracer Technologies, Inc, Somerville, MA) and [15N-15N]urea (99 atom %; Cambridge Isotope Laboratories, Andover, MA) were given through a 20-gauge, 5-cm catheter placed into an antecubital vein on the nondominant side, as previously described (1). The leucine infusion was given at a known rate of ≈2.8 μmol · kg⁻¹ · h⁻¹; the prime was 4.2 μmol/kg, administered over ≈1 min. The urea infusion rate was ≈7 μmol · kg⁻¹ · h⁻¹; the prime was 88 μmol/kg. The bicarbonate pool was primed with 0.8 μmol/kg [13C]sodium bicarbonate (99 atom %; Cambridge Isotope Laboratories). Tracers were prepared in physiologic saline under sterile conditions and tested for sterility and nonpyrogenicity by an independent laboratory (Findley Research Inc, Fall River, MA) before use. The tracers were infused with the aid of a screw-driven pump (model 919; Harvard Apparatus, Millis, MA), in a total volume of ≈8 mL/h, as previously described (1). Blood sampling was performed through a 20-gauge, 3.2-cm catheter placed into a superficial vein of the dorsal hand or the wrist on the nondominant side, while the hand was placed into a custom-made warming box maintained at 68 °C for 15 min before withdrawal of each sample, to achieve arterIALIZATION of venous blood, as previously described (1).

Indirect calorimetry

Total carbon dioxide production (VCO2) and oxygen consumption (VO2) rates were determined with the aid of the indirect calorimeter (DeltaTrac; SensorMedics, Anaheim, CA) by using a ventilated-hood system. Measurements were performed according to a standardized procedure between hourly breaks throughout the 24-h period, as previously described (1).

Studies of breath 13CO2 background enrichment

Under conditions identical to those followed in the main experiment, four additional young adult male subjects were studied in this phase. Our aim was to determine the evolution of breath 13CO2 background (baseline) enrichment throughout the 24-h infusion study. No tracers were given but hourly breath samples were collected. The results are shown in Figure 2. From these findings, a correction was made for the change in 13CO2 background enrichment during the 24-h bicarbonate and leucine-urea studies, as previously described (1).
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expired carbon dioxide in four subjects who were studied for 24 h but did not receive any tracer. Results are expressed as the difference in enrichment (atoms percent excess (APE) X 1000) from the initial value preceding 2000. Minutes (0 to 1440) refer to experimental time; time of day is indicated by "M".

Recovery of $^{13}$C bicarbonate

Three other young adults were given the experimental diet for 4 d before a 24-h infusion with $[^{13}$C]sodium bicarbonate (99 atom %; Cambridge Isotope Laboratories). The tracer was intravenously infused into an antecubital vein, as described previously (1). Analysis of carbon dioxide content was performed with a carbon dioxide analyzer (analyzer # 446; Instrumentation Laboratory, Lexington, MA), which was calibrated by using both the manufacturer’s and prepared standards (from 10 to 80 mmol CO$_2$/L) to cover the full range of possible carbon dioxide concentrations in the samples. The difference between measured and expected infusate carbon dioxide concentrations was -6.21 ± 0.03% (x ± SD). At the end of the 24-h infusion, the measurements confirmed that no additional loss of carbon dioxide had occurred during the 24-h period.

As discussed previously (1), because $^{13}$CO$_2$ production from the in vivo oxidation of [1-13C]leucine has to be corrected for the recovery (%) of $^{13}$C bicarbonate in breath, it was necessary to determine the appropriate correction factors for the present experimental conditions. Because of the rapid changes in $^{13}$C recovery induced by each of the three discrete meals, we used a specific mean correction factor for each of the consecutive half-hourly intervals.

Collection and analysis of samples

Breath samples were collected half-hourly, after three baseline samples taken at -30, -15, and -5 min before the infusion of tracers began. Breath gas was collected as previously described (1). The samples were stored at room temperature until analyzed by isotope ratio mass spectrometry (MAT Delta E; Finnigan, Bremen, Germany) (1).

Blood samples were collected at 30-min intervals between 0000 and 0600. Three baseline samples at -30, -15, and -5 min were taken before the leucine tracer was given. Two milliliters of blood was used for $^{13}$CO$_2$ analysis. After the blood sample was drawn it was injected immediately via a thin needle (0.5 × 16 mm) into a sodium-heparin–coated, 15-mL capped evacuated tube (Venoject; Terumo Medical Corp, Elkton, MD). Sample handling, processing, and analysis were described previously (1).

At 30-min intervals throughout the 24-h study period and at -30, -15, and -5 min before tracer was given, 3.5-mL blood samples were drawn into heparinized tubes and centrifuged for 15 min at 1200 × g in a refrigerated centrifuge (4 °C). Plasma was stored at -20 °C until analyzed. The procedures for isotopic analysis of $[^{13}$C]KIC and [di-$^{15}$N]urea were previously described in detail (1). The concentrations of leucine in plasma and infusates were determined as previously described by using an HPLC procedure (1). Infusates of the tracers were analyzed in duplicate.

Plasma and urinary urea nitrogen concentrations were determined by means of a modified version of the procedure of Marsh et al (6), using an auto analyzer. Urinary urea excretion was corrected for the changes in body urea pool (see below). Total urinary nitrogen concentrations were determined by micro-Kjeldahl analysis.

Evaluation of primary data and statistical methods

Leucine oxidation

Leucine oxidation was computed for consecutive half-hourly intervals. Moreover, before the plateau in plasma $[^{13}$C]KIC enrichment had been reached during the first 60–90 min of the 24-h infusion, leucine oxidation during each of these initial half-hourly intervals was reasonably assumed to be equal to the leucine oxidation rate during the first half-hourly interval of the initial plateau period, which began at 90 min. Therefore, leucine oxidation was directly measured for 45 of the total 48 half-hourly intervals. For each half-hourly interval, leucine oxidation was computed as previously described (1), except that the $^{13}$CO$_2$ recovery factor was specific for each half-hourly interval (see below). In addition, within each metabolic state, VCO$_2$ over the time interval when it was not directly measured was derived as the arithmetic average of VCO$_2$ measured just before and just after this interval.

Bicarbonate recovery

The fraction of $^{13}$C bicarbonate recovered during the 24-h $[^{13}$C]bicarbonate infusions was computed for each half-hourly interval, as follows:

\[
\text{Recovery} = \frac{^{13}\text{CO}_2 \text{ expired (A)}}{^{13}\text{CO}_2 \text{ infused (B)}}
\]

where

\[
A (\mu\text{mol}) = V\text{CO}_2 (\mu\text{mol}) \times ^{13}\text{CO}_2 \text{ enrichment} (\text{APE} \times 1000) \times 1/10^5
\]

where APE is atom percent excess.

\[
B (\mu\text{mol}) = \text{measured } CO_2 (\mu\text{mol/mL}) \times \text{tracer enrichment} (\text{atom } % \times 1/10^2).
\]
Measured 24-h leucine balance

Balance was computed as (input − output):

\[
\text{Input (µmol/kg)} = \text{dietary leucine + intravenous tracer}
\]

\[
\text{Output (µmol/kg)} = \text{sum of the determined oxidation for the 48 half-hourly intervals}
\]

Urea metabolism and protein oxidation

The urea excretion rate was computed as previously described (1) and corrected for changes in body urea pool as follows:

\[
\text{Urea N excretion (corrected)} = \text{urea N excretion} - [(\text{plasma urea N at start} - \text{plasma urea N at end}) \times (\text{estimated total body water/0.92})]
\]

as described by Fern et al (7), with total body water estimated from equations by Watson et al (8). The urea production rate was computed in two ways:

1) Method B: as (urinary urea N excreted + urinary nonurea N excreted + 8) × 6.25. We assume that 8 mg · kg\(^{-1} \cdot \text{d}^{-1}\) is sufficient to account for both unmeasured fecal nitrogen loss that derives from the catabolism of amino acids and the nitrogen lost via sweat (mainly as urea) under these conditions. Here, we assume that 4 mg N · kg\(^{-1} \cdot \text{d}^{-1}\) (out of 9 mg N · kg\(^{-1} \cdot \text{d}^{-1}\) obligatory fecal nitrogen loss; 9) is derived from amino acid catabolism. We also assume a urea nitrogen sweat loss of ∼4 mg N · kg\(^{-1} \cdot \text{d}^{-1}\) (10, 11). Therefore, when these calculations are performed for a 6-h period, the assumed value for these routes of nitrogen loss is 2 mg · kg\(^{-1} \cdot \text{d}^{-1}\). This method attempts to account for the nitrogen loss that is related to amino acid catabolism, because it is the latter that is traced by the [1,13C]leucine technique.

2) Method C: it accounts for all nitrogen lost via feces (∼9 mg N · kg\(^{-1} \cdot \text{d}^{-1}\); see 9) and all nitrogen lost via miscellaneous routes (∼8 mg N · kg\(^{-1} \cdot \text{d}^{-1}\); 12), without consideration for any specific metabolic source of the nitrogen lost. It adds 17 mg N · kg\(^{-1} \cdot \text{d}^{-1}\) (instead of 8 mg) to the urinary nitrogen excretion described above. Therefore, for a 6-h period, it adds 4.25 mg N · kg\(^{-1} \cdot \text{d}^{-1}\).

As will be shown below (see Results), the overall conclusions are not affected by the choice of method (method C compared with B).

Protein oxidation (mg · kg\(^{-1} \cdot \text{d}^{-1}\)), derived from urea production, is computed as shown above for urea excretion (methods B and C) except that urea production is used instead of urea excretion. Protein oxidation (mg · kg\(^{-1} \cdot \text{d}^{-1}\)), derived from leucine oxidation, was computed as follows:

\[
\text{Protein oxidation} = \left[\text{24-h leucine oxidation (mg · kg}^{-1} \cdot \text{d}^{-1}\right] - \text{leucine tracer (mg · kg}^{-1} \cdot \text{d}^{-1}\right] \times 100/8
\]

where 100/8 assumes an average of 8% leucine (by wt) in whole-body, mixed proteins (13); and leucine tracer is a correction factor for the additional oxidation of leucine due to the nonmass tracer. (At generous intakes of leucine, any additional leucine given in this way would be disposed of by irreversible oxidation.) A similar calculation of protein oxidation (mg · kg\(^{-1} \cdot \text{h}^{-1}\)) was performed over 6-h periods after each of the three discrete meals.

Statistical methods

Analysis of variance (ANOVA), followed by pair-wise comparisons among means by using the Student-Newman-Keuls test, was used to compare leucine oxidation data over the 6-h period after the meal among the three discrete meals of the 24-h day. A paired \(t\) test was used to compare data derived from the same subjects. Linear-regression analysis was used to determine the slope for bicarbonate recovery data over time, as well as for leucine oxidation data over time. Data analyses were done with SAS Software, version 6 (SAS Institute, Inc, Cary, NC). Data in text are means ± SDs.

RESULTS

The temporal pattern of \(^{13}\text{CO}_2\) recoveries in breath is depicted in Figure 3. Recovery (% of infused \(^{13}\text{C}\) bicarbonate increased immediately after each of the three discrete meals was given, and progressively declined thereafter over a 2.5-h interval after the beginning of each meal. Although comparison of recovery over the initial 4th half-hourly interval (72.3 ± 6.9%) did not differ \((P = 0.868)\) from that for the last (48th) half-hourly interval (73.2 ± 3.4%), linear-regression analysis on the entire data set indicated a small but significant \((P < 0.01)\) increase in recovery with time. However, the

\[
\begin{align*}
\text{FIGURE 3. Mean (± SD) recovery (expressed as % of infused label) of sodium [\(^{13}\text{C}\)]bicarbonate when given as a primed, continuous, intravenous tracer infusion throughout a 24-h period. The experimental protocol was comparable with that described in Figure 1. n = 3. Minutes (0 to 1440) refer to experimental time; time of day is indicated by hours. Discrete meals are indicated by "M".}
\end{align*}
\]
estimate of leucine oxidation, discussed below, took this change into account as described in Methods.

\[ \text{VCO}_2 \text{(mmol} \cdot \text{kg}^{-1} \cdot \text{30 min}^{-1}) \] and \[ \text{\textsuperscript{13}CO}_2 \] enrichments over baseline (APE \( \times 1000 \)) derived from leucine-urea studies are plotted in Figure 4. Total \[ \text{VCO}_2 \] (Figure 4) and the \[ \text{\textsuperscript{13}CO}_2 \] recoveries derived from the other subjects (Figure 3) showed qualitatively similar temporal patterns, as we had expected from the earlier relations found between \[ \text{VCO}_2 \] and \[ \text{\textsuperscript{13}CO}_2 \] recoveries (14–16). In addition, Figure 4 shows the expected decline in \[ \text{\textsuperscript{13}CO}_2 \] enrichments that coincided with the increase in \[ \text{VCO}_2 \].

Figure 5 depicts the relation between the pattern in \[ \text{\textsuperscript{13}CO}_2 \] production (numerator in the equation to derive leucine oxidation; see Methods) and the \[ \text{\textsuperscript{13}C} \] abundance over baseline in plasma \( \alpha \)-KIC (denominator in the above mentioned equation; see Methods). The relative changes induced by feeding (increase in \[ \text{\textsuperscript{13}CO}_2 \] production and decrease in the \[ \text{\textsuperscript{13}C} \] abundance over baseline in plasma \( \alpha \)-KIC) are similar in size, suggesting that under our experimental conditions both components of the equation were equally affected by feeding. This observation further reinforces the appropriateness of the use of plasma \( \text{\textsuperscript{13}C} \)-\( \alpha \)-KIC as the enrichment of the precursor pool for whole-body leucine oxidation, and its immediate response to a change in leucine oxidation, as discussed previously by us (1) and others (17, 18).

The 24-h pattern and rate of leucine oxidation is shown in Figure 6. In view of the generous dietary intake of leucine, leucine oxidation increased with feeding over the 2–3 h after each meal and declined progressively thereafter to reach its baseline oxidation rate \( \approx 5–6 \) h after beginning each meal. In addition, leucine oxidation over the first (4th) half-hourly interval (11.56 \( \pm \) 1.88 mmol \( \cdot \) kg\(^{-1} \cdot \) 30 min\(^{-1} \)) was not different (\( P = 0.313 \)) from the leucine oxidation over the last (48th) half-hourly interval (10.43 \( \pm \) 0.79 mmol \( \cdot \) kg\(^{-1} \cdot \) 30 min\(^{-1} \)). Further, when linear-regression analysis was performed on the entire data set, it indicated that the slope relating leucine oxidation to length of tracer infusion was not different from zero (\( P = 0.34 \)), suggesting that tracer-infusion duration, per se, had no effect on the precision of our estimate of leucine oxidation.

Plasma leucine concentrations (Figure 7) showed a 24-h pattern that was qualitatively similar to the pattern found for leucine oxidation (compare with Figure 6). This relation extends our earlier and similar findings with multiple small meals (1, 2).
Table 3 shows individual data on 24-h leucine input (diet and tracer), leucine output (oxidation), and 24-h leucine balance. The latter was positive (+14.81 ± 7.67 mg leucine · kg⁻¹ · d⁻¹) and slightly higher than the neutrality that we had expected for these experimental conditions of generous dietary leucine intake; this is discussed further below.

Leucine oxidation data over the 6-h intervals after each of the three equal discrete meals were compared and these results are shown in Table 4. Leucine oxidation was not different (P > 0.05) among the three equal meals, being ~20 mg/kg for each of the three separate 6-h intervals. The time of the meal, therefore, did not appear to affect the extent of leucine oxidation under these experimental conditions involving generous leucine and nitrogen intakes. In addition, when values were expressed as a percentage of total 24-h leucine oxidation, the time of the meal also had no effect and there was little variation in the values among subjects, suggesting that one could predict the 24-h leucine oxidation value by extrapolating from data over 6 h after a meal providing one-third of the total daily intake.

Table 5 summarizes the urinary nitrogen data (urea nitrogen and total nitrogen) where the urea component has been corrected for the changes in body urea pool over the time intervals considered (see Methods). The mean contribution of 24-h urinary nonurea nitrogen to 24-h total urinary nitrogen output was ~13.7%. The lowest urinary urea and total nitrogen values were observed between 0300 and 0600, on the fasting interval following hours 8, 9, and 10 after the last meal (given at 2000) (Figure 8).

From these various data (Tables 3, 4, and 5) estimates of protein oxidation can be derived (see Methods) by using either the rate of leucine oxidation or that for nitrogen excretion. As summarized in Table 6, when method B was used there was close agreement (P = 0.504) between the two methods (A and B) for deriving daily (24 h) protein oxidation. When method C was used, in comparison with method A, the difference was significant (P = 0.022); the mean difference in this case was ~73 mg N · kg⁻¹ · d⁻¹, or a discrepancy of ~8.2%. When the time frames of urine collections matched the 6-h intervals immediately following meals, such as for the meals given at 0600 and 1200, agreement between method B (compared with method A) and method C (compared with A) was also found to be very good. Consequently, protein oxidation rates were similar for the three equal discrete meals, whether based on leucine oxidation or on nitrogen excretion (Table 6).

Urea nitrogen production is plotted in Figure 9. It declined over the time interval from 0000 to 0600 (3.8 ± 0.6 compared with 3.1 ± 0.5 mg urea N · kg⁻¹ · 30 min⁻¹; P = 0.002) as was to be expected for the fasting phase. Over the 6-h periods after each meal, urea production was either relatively constant or increased slightly after an apparent delay (Figure 9).

Similar computations of protein oxidation were performed (see Methods) by using urea nitrogen production (instead of excretion). For all subjects, 24-h protein oxidation from urea nitrogen production exceeded the protein oxidation values obtained from nitrogen excretion (methods B or C) or leucine oxidation (Figure 10).

Comparisons may also be performed over 3-h intervals and the conclusions are similar to those above. A summary of the data on protein oxidation (with the four methods outlined above) over 3-h intervals is plotted in Figure 11. About a twofold difference between values derived from urea nitrogen production compared with other methods was found for the period between 0300 and 0600. This is the 3-h interval corresponding to the maximum period of fasting, under the conditions of this study (~7–10 h after the meal given at 2000). These findings imply that a maximum overestimation of protein oxidation occurs during fasting when urea nitrogen production is used as the basis for computation.

With reference to our earlier studies with multiple small meals (1), Table 7 compares nitrogen and leucine kinetics within each and between the two eating patterns because daily dietary intakes were identical and the same subjects were studied in both the present and earlier investigation (1). Furthermore, subjects were studied in random order for the effects of eating pattern on leucine kinetics. In contrast with the multiple-small-meal eating pattern in which dietary protein intake matched protein oxidation, as derived from leucine oxidation or urea excretion, the latter two variables were lower than the dietary intake for the present three discrete meal-eating pattern (Table 7). Protein oxidation as derived from urea production was higher than for protein intake with both feeding modes, but somewhat less significantly so with the three discrete meals (P = 0.04 and P = 0.056, respectively). When the two eating patterns were compared, all variables were significantly different (P < 0.05), except urea production (and consequently protein oxidation derived from urea production).

**DISCUSSION**

A previous series (1, 2) of 24-h leucine kinetic studies were conducted to validate the tracer-balance concept (1) and to further confirm our earlier conclusions that the minimum physiologic requirement for leucine was substantially higher than the current international recommendation of 14 mg · kg⁻¹ · d⁻¹ (2). We have proposed a tentative new leucine requirement value of 39 mg · kg⁻¹ · d⁻¹ (2, 3). These recent 24-h tracer studies involved giving small, equal, frequent (hourly) meals throughout the 12-h fed phase of the 24-h tracer protocol. Hence, the question arises as to whether the daily rate of leucine oxidation might be affected by the size and frequency of meals. Changes in meal frequency affect rat growth and lipid metabolism (19), for example. There is less suitable information available from studies, especially in humans, to indicate the nature and extent of the effect of meal composition, size,
and frequency on dietary protein and amino acid utilization (20). Hence, the present experiment represents an initial phase in our investigation of this issue and so we used here a generous leucine intake to compare the present data with the results reported for comparable, published findings (1) but where a multiple-small-meal feeding mode was used during the 24-h tracer protocol.

Clearly, the pattern of leucine oxidation throughout the 24-h period differed between the present three-discrete-meal format and that found for the multiple-small-meal design (compare Figure 6 in this study with Figure 5 in reference 1). We had expected, based on previous studies, that the leucine-containing meals would give rise to an increased rate of leucine oxidation followed by a decline: these changes might depend on the amount of leucine given. However, under the present experimental conditions, the total amount and, therefore, the rate of leucine oxidation was the same for all three meals (Table 4). This might not be true if there is a limiting or deficient intake of leucine, at least when we consider the temporal pattern of leucine oxidation that we have seen in healthy subjects consuming daily intakes ≥39 mg leucine · kg⁻¹· d⁻¹ (2), where leucine oxidation had reached a low rate by the end of the 10-h feeding phase. Hence, in this context it is important to know whether the morning, lunch, and evening meals are equivalent and frequency on dietary protein and amino acid utilization.

It was an unanticipated outcome of this experiment to find a consistent, moderately positive daily leucine balance (≈17% of the diet and tracer intake) with the three-equal-meal design. We had earlier found a neutral daily leucine balance when the multiple-small-meal design was used (1). Because we determined directly the ¹³C0₂ recovery values for the three-meal condition and showed previously that the collection and analytical techniques used here are precise and accurate, physiologic and/or metabolic reasons for the positive balance should be considered.

First, it is possible that unmeasured leucine in fecal and miscellaneous losses might explain, at least in part, the moderate positivity in 24-h leucine balance. Because we found previously (1) that there was body leucine equilibrium and good agreement between protein oxidation derived from leucine oxidation data and protein intake, leucine fecal and miscellaneous losses were probably quite small. Further, the colon has been shown to be capable of absorbing amino acids.

**TABLE 5**

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Urinary urea nitrogen²</th>
<th>Urinary total nitrogen³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1800–2100</td>
<td>15.6 ± 3.6</td>
<td>17.1 ± 3.5</td>
</tr>
<tr>
<td>2100–0000</td>
<td>12.7 ± 4.7</td>
<td>14.5 ± 4.1</td>
</tr>
<tr>
<td>0000–0300</td>
<td>12.5 ± 4.2</td>
<td>15.0 ± 5.3</td>
</tr>
<tr>
<td>0300–0600</td>
<td>7.6 ± 3.6</td>
<td>9.7 ± 5.0</td>
</tr>
<tr>
<td>0600–0900</td>
<td>15.8 ± 7.0</td>
<td>18.1 ± 7.8</td>
</tr>
<tr>
<td>0900–1200</td>
<td>12.9 ± 11.1</td>
<td>14.8 ± 11.2</td>
</tr>
<tr>
<td>1200–1500</td>
<td>13.2 ± 4.8</td>
<td>14.5 ± 6.0</td>
</tr>
<tr>
<td>1500–1800</td>
<td>17.6 ± 5.4</td>
<td>21.4 ± 7.6</td>
</tr>
<tr>
<td>24-h period</td>
<td>108.0 ± 12.0</td>
<td>125.1 ± 11.5</td>
</tr>
</tbody>
</table>

¹ x ± SD; n = 6.
² Corrected for changes in body urea pool over each interval (see Methods).
³ Urinary urea nitrogen (corrected) + urinary nonurea nitrogen.

---

**FIGURE 8.** Urinary urea nitrogen excretion (corrected for the changes in body urea pool size) over each 3-h interval. x ± SD; n = 6. Minutes (0 to 1440) refer to experimental time; time of day is indicated by hours. Discrete meals are indicated by “M”.

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**TABLE 4**

Comparison of leucine oxidation rate for 6 h after each of three equal discrete meals, by meal period

<table>
<thead>
<tr>
<th>Subject</th>
<th>2000–0200</th>
<th>0600–1200</th>
<th>1200–1800</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg leucine · kg⁻¹· 6 h⁻¹</td>
<td>%</td>
<td>mg leucine · kg⁻¹· 6 h⁻¹</td>
</tr>
<tr>
<td>1</td>
<td>14.02</td>
<td>22.06</td>
<td>18.26</td>
</tr>
<tr>
<td>2</td>
<td>21.64</td>
<td>27.37</td>
<td>21.82</td>
</tr>
<tr>
<td>3</td>
<td>25.11</td>
<td>28.93</td>
<td>22.70</td>
</tr>
<tr>
<td>4</td>
<td>18.02</td>
<td>25.49</td>
<td>19.71</td>
</tr>
<tr>
<td>5</td>
<td>18.97</td>
<td>25.48</td>
<td>20.88</td>
</tr>
<tr>
<td>6</td>
<td>22.55</td>
<td>29.94</td>
<td>17.86</td>
</tr>
</tbody>
</table>

x ± SD²
20.05 ± 3.90
26.54 ± 2.84
20.21 ± 1.94
27.02 ± 1.83
20.33 ± 2.39
27.14 ± 1.72

¹ Percent of 24-h leucine oxidation.
² Analysis of variance (ANOVA), using the Student-Newman-Keuls test, showed that the three equal discrete meals resulted in leucine oxidations (over 6 h) that were not significantly different.
0600-1200 (6 h) 224 ± 24 218 ± 43 232 ± 43

Table 6:
Protein oxidation derived from leucine oxidation compared with protein oxidation derived from urinary nitrogen excretion

<table>
<thead>
<tr>
<th>Time interval</th>
<th>From leucine oxidation (A)</th>
<th>From nitrogen excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000-0200 (6 h)</td>
<td>222 ± 49</td>
<td>B</td>
</tr>
<tr>
<td>0600-1200 (6 h)</td>
<td>224 ± 24</td>
<td>C</td>
</tr>
<tr>
<td>1200-1800 (6 h)</td>
<td>226 ± 30</td>
<td>237 ± 18</td>
</tr>
<tr>
<td>1800-1800 (24 h)</td>
<td>815 ± 98</td>
<td>832 ± 72</td>
</tr>
</tbody>
</table>

1 x ± SD.
2 (Corrected for leucine tracer) × 100/8 (see Methods).
3 Compared with B, P = 0.771; compared with C, P = 0.841; compared with meals given at 2000-0200 and 1200-1800 (A), NS (ANOVA).
4 Compared with B, P = 0.532; compared with C, P = 0.186.
5 Compared with other meal (0600-1200), P = 0.481.
6 Compared with B, P = 0.504; compared with C, P = 0.022.

(21), which suggests that free leucine fecal losses would be small and lower than leucine ileal losses.

Second, it can be estimated that ~12 g protein and ~30 μmol free leucine were removed as a result of blood sampling (~200 mL) over the 24-h tracer-infusion period. This surgical withdrawal was not taken into account in our calculations of leucine balance because we did not have evidence that whole-body leucine kinetics and oxidation immediately responded to this experimental manipulation. Nevertheless, it is possible that the withdrawal of 12 g blood protein from the peripheral circulation may have had some impact on the synthesis of export proteins, especially albumin, and so contributed to the observed positive leucine (and apparent nitrogen) balance. Thus, it has been shown that albumin synthesis is stimulated with feeding in human subjects, for example, and that the increase might amount to ~20% or even a 100% change (22, 23). Daily albumin synthesis approximates 12 g in healthy adults and so we cannot rule out an effect of blood withdrawal on leucine kinetics and balance at this time. On the other hand, the amount of blood withdrawn in the present 24-h tracer study was approximately the same as that taken in our earlier, multiple-small-meal, 24-h tracer protocol (1). In that case, the subjects were found to be near body leucine and nitrogen balance equilibrium, suggesting that factors other than loss of blood per se accounted for the more positive nitrogen (and leucine) balance with our present discrete-meal experiment, compared with the previous frequent-small-meal protocol.

Third, it is relevant to focus some attention on the splanchnic uptake and fate of meal-derived leucine and the extent to which this unlabeled leucine is oxidized before becoming fully equilibrated with the body [13C]leucine and KIC pools, which were sampled via the withdrawal of blood from the peripheral venous circulation. Furthermore, the liver accounts for a greater proportion of whole-body leucine oxidation, relative to the fasting state, when dogs receive an l-amino acid mixture either enterally or parenterally (24). Under conditions of fasting in human subjects, Matthews et al (25) concluded that leucine from the intestinal lumen disappears within the splanchnic region but largely escapes oxidation, whereas phenylalanine undergoes an extensive first-pass oxidation. We do not know whether the first-pass uptake of dietary leucine increases, and/or whether a greater proportion of it is immediately
Although the extent to which this first-pass leucine uptake is oxidized, when a generous daily intake is divided across three relatively large meals, compared with values found for lower intakes when divided among smaller, more frequent meals (26). Earlier, we found that when the dietary intake of leucine was ≤ 40 mg · kg⁻¹ · d⁻¹ and given via continuous intragastric infusion there was little difference in the percentage uptake of absorbed leucine by the splanchnic region at various leucine intakes (26). Therefore, assuming, for the present case, a 25% sequestration of absorbed leucine by the splanchnic region (26) after each of the three meals (each supplying 27 mg leucine/kg), it appears that ≈ 20 mg dietary leucine · kg⁻¹ · d⁻¹ disappears during its initial passage within the splanchnic region. Although the extent to which this first-pass leucine uptake is oxidized is not known, it seems reasonable to assume, as we did recently (27), that it is in a proportion similar to the ratio of whole-body leucine oxidation to leucine flux during the fed state (24). Therefore, for the present case, this estimate turns out to be ≈ 4 mg/kg for the three meals. This is a possible amount of dietary leucine oxidized that the intravenous tracer protocol may not have permitted us to estimate. This potential underestimate of leucine output may account for ≈ 30% of the positive leucine balance.

It was noted above that the 24-h urinary excretion of total nitrogen (urea and nonurea nitrogen) was significantly lower for the discrete-meal feeding mode compared with the multiple-small-meal mode (1; Table 7). The mean difference was ≈ 15% and in both studies the 24-h leucine oxidation predicted the 24-h output of nitrogen, more closely when method B was used (as urinary urea and nonurea, plus 8 mg N · kg⁻¹ · d⁻¹ to account for unmeasured fecal and other nitrogen losses). These predictions of nitrogen output by the [¹³C]leucine balance technique were also consistent within each of the subjects studied here, for both the present and previous 24-h studies (1). Additionally, with the two feeding modes, 24-h nitrogen and leucine balances were similar: neutrality with the multiple small meals and positivity with the three-discrete-meal feeding mode (Table 7). Therefore, it appears that with the three discrete meals, our subjects may have been in a positive nitrogen (urea and nonurea) state (24). Therefore, for the present case, this estimate turns out to be 24% lower in both studies, compared with c+b+8, P = 0.02; compared with c+b+17, P = 0.001; compared with a+b+8, P = 0.005; compared with a+b+17, P = 0.056 (nearly significant); compared with a+b+17, P = 0.02; compared with c+b+8, P = 0.001; compared with c+b+17, P = 0.008.

---

**Table 7**

Comparison of nitrogen and leucine kinetics under different eating patterns, for identical daily dietary intakes

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7 ± SD</th>
<th>% Difference compared with small meals</th>
<th>P^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea production (a)</td>
<td>148.9</td>
<td>140.3</td>
<td>193.3</td>
<td>152.0</td>
<td>144.2</td>
<td>182.7</td>
<td>160.2 ± 22.1</td>
<td>2.1 ± 5.6</td>
<td>0.364 (NS)</td>
</tr>
<tr>
<td>Urea excretion (c)</td>
<td>91.3</td>
<td>115.6</td>
<td>115.1</td>
<td>100.9</td>
<td>123.6</td>
<td>101.3</td>
<td>108.0 ± 12.0</td>
<td>-15.2 ± 11.8</td>
<td>0.028</td>
</tr>
<tr>
<td>Nonurea excretion (b)</td>
<td>17.8</td>
<td>15.5</td>
<td>21.8</td>
<td>15.0</td>
<td>12.7</td>
<td>20.0</td>
<td>17.1 ± 3.4</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>Urea hydrolysis</td>
<td>57.6</td>
<td>24.7</td>
<td>78.2</td>
<td>51.1</td>
<td>20.6</td>
<td>81.4</td>
<td>52.3 ± 25.7</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>Leucine oxidation</td>
<td>107.6</td>
<td>139.0</td>
<td>154.1</td>
<td>121.6</td>
<td>129.4</td>
<td>131.7</td>
<td>130.6 ± 15.7</td>
<td>-17.8 ± 8.3</td>
<td>0.003</td>
</tr>
<tr>
<td>Dietary intake</td>
<td>158.3</td>
<td>158.0</td>
<td>168.9</td>
<td>160.0</td>
<td>163.4</td>
<td>166.7</td>
<td>160.2 ± 2.0</td>
<td>0.037 (NS)</td>
<td></td>
</tr>
<tr>
<td>a+b+8</td>
<td>174.7</td>
<td>163.8</td>
<td>223.1</td>
<td>175.0</td>
<td>164.9</td>
<td>210.7</td>
<td>185.4 ± 25.2</td>
<td>0.779 (NS)</td>
<td></td>
</tr>
<tr>
<td>c+b+8</td>
<td>117.1</td>
<td>139.1</td>
<td>144.9</td>
<td>123.9</td>
<td>144.3</td>
<td>129.3</td>
<td>133.1 ± 11.5</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>a+b+17</td>
<td>183.7</td>
<td>172.8</td>
<td>232.1</td>
<td>184.0</td>
<td>173.9</td>
<td>219.7</td>
<td>194.4 ± 25.2</td>
<td>0.779 (NS)</td>
<td></td>
</tr>
<tr>
<td>c+b+17</td>
<td>126.1</td>
<td>148.1</td>
<td>153.9</td>
<td>132.9</td>
<td>153.3</td>
<td>138.3</td>
<td>142.1 ± 11.5</td>
<td>0.011</td>
<td></td>
</tr>
</tbody>
</table>

^1 Data for multiple small meals extracted from a previous study (1) in which these detailed results were not reported.

^2 Comparison with small meals.

^3 Protein oxidation as derived from leucine oxidation (see Methods), divided by 6.25.

^4 With the same eating pattern: compared with leucine oxidation, P = 0.422 (NS); compared with a+b+8, P = 0.040; compared with a+b+17, P = 0.014; compared with c+b+8, P = 0.701 (NS); compared with c+b+17, P = 0.397 (NS).
The possible impact of feeding mode on the utilization of other indispensable amino acids, and of leucine at lower dietary intakes in particular, deserves detailed investigation.

We thank the MIT CRC nursing and laboratory staff for their help in conducting these studies. The technical assistance of Alan Atkinson, Ambalini Selvaraj, and Shihong Wang under the supervision of Thomas E Chapman is appreciated. The willingness and dedication of the subjects who volunteered for these studies are gratefully acknowledged.

REFERENCES


In conclusion

1) It is possible to make meaningful determinations of leucine oxidation in the nonsteady state, and the 24-h pattern of whole-body leucine oxidation for the present feeding mode, involving three discrete meals, differed from that observed previously (1).

2) Under the conditions of three equal meals providing generous leucine and nitrogen intakes, it is possible to extrapolate leucine oxidation data from a 6-h measurement (after one meal) to the 24-h day; there were no apparent differences in the extent of leucine oxidation among the three large meals.

3) In comparison with the multiple-small-meal paradigm (1), whole-body leucine balance was positive. This positive leucine balance correlated well with the nitrogen balance estimate and so it appeared to be a reliable index of leucine utilization and not the result of significant errors of overestimating intake and/or underestimating the oxidative loss of leucine. Hence, the tracer-balance technique also appears to be valid under these discrete-meal-feeding conditions, which agrees with the conclusions drawn earlier for the multiple-small-meal eating pattern (1).

4) The possible impact of feeding mode on the utilization of other indispensable amino acids, and of leucine at lower dietary intakes in particular, deserves detailed investigation.

The urea kinetic data deserve some comment, particularly because the urea production (appearance) rate was remarkably reproducible within each subject across the multiple-small-meal (1) and three-discrete-meal protocols. However, urea excretion was lower for the latter protocol, leading to the finding that urea hydrolysis (production − excretion) was greater when three meals were given (32%) of production) than when multiple small meals were given (18%). A possible interpretation of these data is that there is a greater salvage of urea nitrogen when three discrete meals rather than multiple small meals are given. If this was indeed the case, the retention of urea nitrogen was likely to be in the form of dispensable amino acid nitrogen because we found, as discussed above, that leucine oxidation was an excellent predictor of daily protein oxidation and, thus, of indispensable amino acid retention under these conditions. Alternatively, most, if not all, of the hydrolyzed urea nitrogen might be returned via a futile cycle to the urea pool. This interpretation is based on the following reasoning: 1) leucine oxidation predicted total nitrogen excretion (method B), with the latter being 133 mg N·kg⁻¹·d⁻¹ (Table 7); 2) the amount of protein (amino acid) nitrogen available for urea production would be 133 minus the nonurea nitrogen output, or 133 − 17 = 116 mg N·kg⁻¹·d⁻¹; 3) mean urea nitrogen production (appearance) was 160 mg N·kg⁻¹·d⁻¹, and excretion was 108 mg N·kg⁻¹·d⁻¹; 4) because the total amount of protein nitrogen available for net urea formation was 116 mg N·kg⁻¹·d⁻¹ and urea excretion was 108 mg N·kg⁻¹·d⁻¹, a net equivalent of 8 mg N urea·kg⁻¹·d⁻¹ may have been retained. With urea production (appearance) being 160 mg N·kg⁻¹·d⁻¹ the conclusion may be that of the 52 mg N·kg⁻¹·d⁻¹ urea hydrolyzed, 44 mg was returned to the urea pool. Hence, a futile cycling of urea nitrogen may dominate the fate of the nitrogen liberated via the hydrolysis of urea nitrogen within the intestinal tract. This is also consistent with our previous conclusion that the urea hydrolyzed under feeding conditions of multiple small meals (1) is largely, if not entirely, returned to the urea pool via passage through the amino nitrogen pool and, in part, in the form of aspartic acid (34).

findings are as follows. All of the subjects were in a 24-h positive energy balance because their physical activity was reduced on the day of the tracer study, whereas total food intake remained at the amount received on the previous days. Whereas dietary fat balance (as a percentage of fat intake) was not different among the two feeding modes (+49% in each case), carbohydrate balance was +14% with the multiple small meals and +24% with the three discrete meals. These findings suggest that there may be a causal link between the protein and carbohydrate balances, and this deserves further investigation. It is possible that a higher carbohydrate balance reflected a more potent insulinogenic state and that this, in turn, could have helped promote a higher leucine (and nitrogen) balance (28, 29). Animal studies of macronutrient metabolism with different feeding modes have shown conflicting results, some showing an effect of feeding mode (19, 30) and some no effect (31, 32). Extrapolation of these data to humans remains hazardous (33) and, clearly, more research on humans is needed.