Validation of the tracer-balance concept with reference to leucine: 24-h intravenous tracer studies with L-[1-^{13}C]leucine and [^{15}N-^{15}N]urea[1-3]

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ABSTRACT The validity of tracer-derived estimates of whole-body leucine balance was investigated. Seven healthy young adult subjects received an adequate protein diet for 6 d; at 1800 on the last day, L-[1-^{13}C]leucine and [^{15}N-^{15}N]urea were given as primed, continuous intravenous infusions for 24 h. Subjects were in a fasting state for the first 12 h and at 0600 on day 7 they then received hourly 10 equal meals to achieve a fed state. Total leucine intake (diet plus tracer) was 89.4 $mg \cdot kg^{-1} \cdot d^{-1}$. Mean daily leucine oxidation was equivalent to 89.5 ± 3.3 mg leucine/kg. The predicted daily oxidation rate, from measurements made during the last hour of the fast and the fifth hour of the fed period, was 91.2 ± 5.8 mg/kg ($P = 0.25$ from measured). Measured and predicted whole-body leucine balances were 0.76 ± 2.99 and −0.98 ± 5.54 mg/kg, respectively ($P = 0.25$). Urea production exceeded urea excretion by 20%; daily protein oxidation was the same when estimated from leucine oxidation or nitrogen excretion. Thus, the tracer-balance concept is valid, and reliable predictions of total daily leucine oxidation and whole-body leucine balance can be obtained from short-term measurements of leucine oxidation during fasted and fed states. *Am J Clin Nutr* 1994;59:1000–11.

KEY WORDS Indispensable amino acid requirements, bicarbonate recovery, plasma leucine concentration, diurnal rhythm, urea production, urea hydrolysis, leucine balance, leucine oxidation

Introduction

From a series of tracer studies with L-[1-^{13}C]leucine in young adults we determined rates of leucine oxidation during the fasted and fed states (1–5). These determinations were accomplished via tracer protocols lasting ≤ 8 h, with oxidation being measured during the final segment (≈1 h) of a specific metabolic period (fasted and/or fed). From these determinations we predicted the daily rate of oxidation by extrapolating the rates, measured during these brief experimental timeframes, to the entire 12-h fasted and 12-h fed periods. Then, a determination of the daily body leucine balance was made from the difference between daily leucine intake and the predicted daily rate of leucine oxidation [(1-h rate during fast × 12) + (1-h rate during fed state × 12)]. The validity of this approach depends on whether the rate of leucine oxidation fluctuates substantially outside of the measurement periods and, if so, whether the measured rate adequately reflects the mean rate for the entire metabolic phase of interest. Carpenter (6) stated, appropriately, that we do not know in detail how leucine oxidation changes during the night time. Indeed, we showed some years ago a diurnal cycle of body nitrogen loss and balance that was sensitive to dietary protein intake, suggesting significant, diet-dependent changes in the rate of amino acid oxidation throughout the day (7). Whether a major diurnal rhythm, in terms of leucine kinetics, applies to the standard experimental conditions used in our previous studies in which intakes of amino acids were given as small, frequent meals to achieve a relatively constant dietary input throughout the feeding period, must be explored.

Therefore, to evaluate these issues concerning the determination of oxidation and balance over more prolonged periods, we describe herein the results of an experiment involving direct measurement of leucine oxidation throughout a 24-h period in subjects adjusted to a constant, generous leucine intake of 89.4 $mg \cdot kg^{-1} \cdot d^{-1}$. We compared the measured 24-h rate of leucine oxidation with that predicted from extrapolation of rates measured at the end of the fast period and during the fifth hour of the fed state, which mimics the procedure followed in our recent studies (8, 9). In a companion paper (10) we explore these relationships for leucine oxidation, in which leucine intake was designed to equal current, international requirement values (11) or the higher intake that we propose as being the minimum physiological requirement for healthy adults (2, 5). Our working hypothesis was that at a generous intake of leucine, ie, one that substantially exceeds the minimum physiological requirements, healthy adults would be in neutral, whole-body leucine balance. Therefore, the tracer-balance procedure should give an estimate of neutral body leucine balance, or essentially equilibrium, if our techniques are accurate and the tracer-balance concept is valid.

Additionally, we estimated urea production rates and determined urinary nitrogen output to further assess the metabolic and

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TABLE 1
Characteristics of subjects who participated in the bicarbonate recovery and 24-h leucine kinetic studies

Study, subject, and sex	Age	Weight	Height
	y	*kg*	*cm*
Bicarbonate recovery			
1 (M)	20	75.3	186.7
2 (F)	19	74.4	165.1
3 (M)	27	91.7	179.1
4 (M)	20	77.2	190.5
5 (F)	22	61.3	163.8
$\bar{x}$ ± SD	22 ± 3	76.0 ± 10.8	177.0 ± 12.2
24-h Leucine kinetics			
1 (M)	20	86.1	186.1
2 (M)	19	77.0	186.7
3 (M)	19	63.7	168.9
4 (M)	20	70.5	180.3
5 (M)	22	95.7	174.0
6 (F)	19	51.6	155.6
7 (M)	24	71.1	181.6
$\bar{x}$ ± SD	20 ± 2	73.7 ± 14.4	176.2 ± 11.1

nutritional significance of the tracer-derived measurement of leucine-oxidation rates. We have done this, in part, because of the claim (12) that tracer-derived estimates of leucine oxidation overestimate actual rates of dietary leucine oxidation, because of a postulated, significant contribution to total leucine entering the body that arises from microbial synthesis of leucine within the intestinal lumen. If this claim is found to be valid it would have profound implications for the interpretation and nutritional significance of our earlier carbon-13 tracer studies (1–3, 5), as well as for the prevailing and now well-established concept of dietary protein quality in human nutrition.

Subjects and methods

Three studies were conducted. First, a study involving two healthy young adult men who were studied according to the 24-h tracer protocol described below, except that they did not receive any tracer. The purpose was to determine how the background ^{13}C-enrichment of expired carbon dioxide varied over the 24-h period. Second, we determined in five healthy young adults (**Table 1**) the recovery, in expired air, of a 24-h tracer infusion with [^{13}C]labeled sodium bicarbonate (bicarbonate recovery study). The third investigation was the dietary study (24-h leucine kinetics; Table 1); seven adult subjects, six males and one female, aged 19–24 y participated in this phase.

Subjects

All subjects were recruited from within the student population at the Massachusetts Institute of Technology (MIT) and the community of the Boston-Cambridge area. They were studied as outpatients at the Clinical Research Center (CRC) of MIT. All were in good health as determined by medical history, physical examination, analysis for blood cell count, routine blood biochemical profile, and urinalysis. Subjects who smoked cigarettes, consumed five or more alcoholic drinks per week, or drank more than six cups of caffeinated beverages per day were excluded from participation in these studies. Women of childbearing age were encouraged to volunteer. One female agreed to participate and she was studied during the 7–10-d period after onset of menstrual bleeding. Women who were taking contraceptive pills were excluded from this initial series of studies. A negative pregnancy test (plasma human chorionic gonadotropin) was required 2–3 d before the dietary study started.

Daily energy intake was estimated to maintain body weight, and intakes for all subjects were close to 188 $kJ \cdot kg^{-1} \cdot d^{-1}$ (45 $kcal \cdot kg^{-1} \cdot d^{-1}$) for this study sample. Subjects were encouraged to maintain their customary levels of physical activity but were asked to refrain from excessive or competitive exercise.

The purpose of the study and the potential risks involved were explained to each subject. Written consent was obtained in accordance with the protocol approved by the MIT Committee on the Use of Humans as Experimental Subjects and the Advisory Committee of the MIT CRC.

The subjects were admitted as inpatients to the MIT Medical Department for the last phase of the study, which involved a 24-h stable-isotope-tracer protocol. They received financial compensation for their participation in the experiment and all remained healthy throughout the study period.

Diet and experimental design

For the definitive diet study, each subject received for 6 d a weight-maintaining diet that was based on egg protein (nitrogen

TABLE 2
Composition of the diet used in studies of 24-h leucine kinetics

Component	Amount
Egg-protein formula (g/d)[1]	
Egg-white solids[2]	72
Whole-egg solids[2]	20
Beet sugar	210
Safflower oil[3]	55
Orange sherbet[4]	195
Unsweetened flavored beverages[5]	1245
Protein-free cookies (g/d)[1]	270
Supplements	
Multivitamin-multimineral (capsules)[6]	1
Sodium chloride (g)[7]	6
Calcium (mg)[8]	1000
Potassium (mg)[9]	3912
Choline (mg)[10]	500

[1] For a 70-kg subject, with an energy intake of 13.17 MJ/d (3150 kcal/d).
[2] Henningsen Foods, Inc, White Plains, NY.
[3] PET Inc, St Louis.
[4] National Dairy Products Corp, Philadelphia.
[5] Kool-Aid; Kraft General Foods, Inc, White Plains, NY.
[6] One-a-Day (Miles Inc, Elkhart, IN): vitamin A, 5000 IU; vitamin C, 60 mg; thiamin, 1.5 mg; riboflavin, 1.7 mg; niacin, 20 mg; vitamin D, 400 IU; vitamin E, 30 IU; vitamin B-6, 2 mg; folic acid, 0.4 mg; vitamin B-12, 6 μg; biotin, 30 μg; panthotenic acid, 10 mg; chloride, 34 mg; iron, 18 mg; calcium, 130 mg; phosphorous, 100 mg; iodine, 150 μg; magnesium, 100 mg; copper, 2 mg; zinc, 15 mg; chromium, 10 μg; selenium, 10 μg; molybdenum, 10 μg; manganese, 2.5 mg; and potassium, 37.5 mg.
[7] Eli Lilly & Co, Indianapolis.
[8] TUMS; SKB Corp, Pittsburgh.
[9] K-LYTE; Bristol Lab, 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.

× 6.25) to supply 1 $g \cdot kg^{-1} \cdot d^{-1}$ and was the source of nitrogen and indispensable amino acids (**Table 2**). 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 (8). Nonprotein energy from fat was ≈40% and from carbohydrate was ≈60%. The main sources of carbohydrate were beet sugar and wheat starch, so as to attain a low carbon-13 content in the diet and to achieve a relatively steady, background breath $^{13}CO_2$ enrichment over a 24-h period (*see* below). The daily leucine intake provided by the egg diet during this period was 80 mg/kg. When the tracer [1-^{13}C]leucine was given intravenously during the 24-h infusion period, it supplied additionally ≈9.4 mg/kg for that day (*see* below).

Other nutrients were given in adequate amounts. A choline supplement of 500 mg was given daily. Dietary fiber was provided as 20 g microcrystalline cellulose (Avicel, FMC Co, Philadelphia) daily.

The total daily dietary intake was consumed as three isoenergetic, isonitrogenous meals (at 0800, 1200, and 1800). Every morning, body weight and vital signs were monitored. On average, two of three meals were consumed at the MIT CRC under the supervision of the dietary staff and/or one of the investigators. On the basis of the strong rapport developed between the volunteer subjects and one of the investigators (AE El-K), as well as the CRC dietary staff, it is our judgement that all subjects complied with the dietary requirements of the protocol.

After 6 d of the diet described above, a 24-h stable-isotope-tracer-infusion study was performed in each subject. This began on day 6 at 1800, continuing to day 7 at 1800; constant intravenous infusions of [1-^{13}C]leucine and [^{15}N-^{15}N]urea were given as described below.

24-h tracer-infusion protocol

The primed, continuous intravenous tracer-infusion approach was used in these experiments and a standard design was applied in all three studies. In the diet study subjects were given their last regular meal on day 6 at 1500. The tracer infusions were then begun at 1800, with subjects sleeping from 0000 to 0600 of the following day (day 7). Subjects were then given 10 isoenergetic, isonitrogenous small meals hourly between 0600 and 1500 (which together were equivalent to the 24-h dietary intake), and the 24-h tracer infusion was terminated at 1800 (day 7). Throughout the 24-h study the subjects remained in bed, in a reclined position, except during sleep when they laid supine. This tracer design, therefore, divided the 24-h day into two distinct 12-h metabolic periods (fasted and fed). Moreover, this design was implemented to achieve two additional requirements; first, we wanted to maintain the subjects' usual sleeping patterns and, second, we planned for the feeding phase to occur during the second half of the 24-h study. Our purpose was to minimize potential recycling of tracer. Because whole-body protein breakdown increases with fasting and decreases with feeding (13–17), the final 12-h fed period would lessen the likelihood of tracer recycling into plasma. Also, this is why the tracer-infusion studies began and finished in the evening. To avoid disturbing the subjects for purposes of breath sampling and to permit the possibility of uninterrupted sleep, blood (and not breath) $^{13}CO_2$ enrichments were determined between 0000 and 0600 in the [^{13}C]bicarbonate-recovery and leucine-urea tracer-infusion studies. Note, however, that recycling of leucine tracer would not affect the precision of the estimation of oxidation because this index is based on measurement of the isotopic enrichment of the precursor in plasma, namely α-ketoisocaproic acid (KIC).

Figure 1 depicts the design of the 24-h leucine-urea tracer-infusion studies, including collection of consecutive, complete 3-hourly urine samples; indirect calorimetry at hourly intervals; and half-hourly drawings of blood samples for plasma carbon-13 enrichment of α-KIC and urea nitrogen-15 enrichment. Selected time points were used for determination of plasma leucine concentrations and plasma urea concentrations were measured at every 3-h time point.

Primed, constant intravenous infusions of [1-^{13}C]leucine (99.3 atom %; Tracer Technologies, Inc, Somerville, MA) and [^{15}N-^{15}N]urea (99 atom %; from Cambridge Isotope Laboratories, Cambridge, MA) were given through a 20-gauge, 5-cm catheter placed into an antecubital vein on the nondominant side. The lines were inserted after local anesthesia with 1% lidocaine (Lyphomed; Fujisawa Div, Deerfield, IL). The leucine infusion was given at a known rate of ≈2.8 $\mu mol \cdot kg^{-1} \cdot h^{-1}$; the prime was 4.2 μmol/kg, administered over ≈1 min. The urea infusion rate was 7 $\mu mol \cdot kg^{-1} \cdot h^{-1}$; the prime was 88 μmol/kg. The bicarbonate pool was primed with 0.8 μmol/kg [^{13}C]sodium bicarbonate (99 atom %; Cambridge Isotope Laboratories). Tracers were prepared in physiological 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.

Blood sampling (3.5 mL per sample) 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. The catheter was introduced in an antiflow position to facilitate blood drawing, 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. The patency of the sampling catheter was maintained by slow infusion of normal saline.

Indirect calorimetry

Total carbon dioxide production ($\dot{V}CO_2$) and oxygen consumption ($\dot{V}O_2$) rates were determined with the aid of the indirect calorimeter (Deltatrac; SensorMedics, Anaheim, CA) with a ventilated-hood system. Measurements were performed according to a standardized procedure between hourly breaks throughout the 24-h period. During sleep, 3 h of measurements were sufficient to determine an average sleeping metabolic rate. This approach was found to provide reliable data on $\dot{V}CO_2$ over the 24 h, because $\dot{V}CO_2$ achieved a plateau during sleep and another plateau was obtained at ≈2 h after starting the multiple small-meal feeding. Our gas-analysis system was calibrated, by using a standard gas sample provided by the manufacturer (96% O_2 and 4% CO_2), every 4–6 h. The drift in oxygen and carbon dioxide measurements was < 0.3% and 0.03%, respectively. Each week, flow calibration was verified by performing pure ethanol burns with a kit provided by the manufacturer. After combusting 5 mL pure ethanol, the difference between measured and predicted total $\dot{V}CO_2$ was < 5% and the average respiratory quotient (RQ) was between 0.64 and 0.69. The transparent, plexiglass ventilated hood permitted our subjects to read or watch television during the periods of measurement.

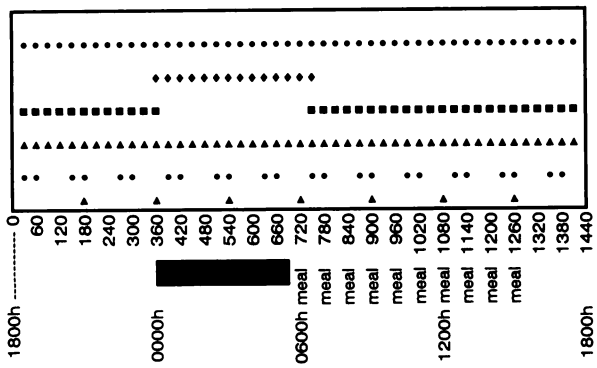

FIG 1. Schematic outline of the 24-h L[1-^{13}C]leucine infusion study beginning at 1800 on day 6 of the experimental diet period. The numbers on the axis are as follows: 1, blood samples; 2, blood samples drawn for $^{13}CO_2$; 3, expired air samples for $^{13}CO_2$ enrichment; 4, continuous 24-h infusion of [^{15}N-^{15}N]urea and of [1-^{13}C]leucine; 5, alternate, 1-h periods used for measurement of indirect calorimetry; and 6, end of 3-h complete periods of urine collection. Minutes (0 to 1440) refer to experimental time; time of day is indicated by hours.

Breath $^{13}CO_2$ background enrichment

The design depicted in Figure 1 was used to study two young adult male subjects in this phase. Our aim was to determine whether the experimental diet was "carbon-13 neutral" and did not markedly alter breath $^{13}CO_2$ baseline enrichment throughout the 24-h infusion study. No tracers were given but hourly breath samples were collected, as described above. The results are shown in **Figure 2,** with data plotted separately for each subject. From these findings a correction was made for the change in $^{13}CO_2$ background enrichment during the 24-h [1-^{13}C]tracer studies, as follows; data for these two subjects were averaged for each time point and this value was applied to the carbon-13 enrichment determined for each half-hourly period during the 24-h tracer study.

[^{13}C]Bicarbonate recovery

Because $^{13}CO_2$ production from the in vivo oxidation of [1-^{13}C]leucine has to be corrected for the recovery of [^{13}C]bicarbonate in breath, it was necessary to determine the appropriate correction factor for the present experimental conditions. In previous, 8-h tracer studies in our laboratories, mean recovery of intravenously infused sodium [^{13}C]bicarbonate was 0.70 of the dose in the fasting state and 0.82 in the fed condition (18). To determine whether these recovery factors could be used in the present tracer studies, five young adults were given the experimental diet for 4 d before a 24-h infusion of [^{13}C]sodium bicarbonate (99 atom %; Cambridge Isotope Laboratories). The tracer was given intravenously in an antecubital vein as described above for leucine-urea infusions. The infusion rate was 3.9 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (n = 4) and an additional subject was infused at a lower rate of 1 $\mu mol \cdot kg^{-1} \cdot h^{-1}$.

Isotope safety testing and preparation were similar to that described above for leucine and urea, with additional precautions taken to decrease the exchange of the bicarbonate solution with room air carbon dioxide. The tracer was dissolved in physiological saline and the final solution was then sampled for immediate analysis. Samples were also analyzed after the infusion had been completed. Analysis of carbon dioxide content was performed with a carbon dioxide analyzer (Total CO_2 analyzer no 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 5.73 ± 1.66% ($\bar{x}$ ± SD). Measurements at the termination of the infusion confirmed that no loss of carbon dioxide had occurred during the 24-h period.

Collection and analysis of samples

Breath samples were collected half-hourly, after collection of three baseline samples at −30, −15, and −5 min. Breath gas was collected in disposable rubber bags by a mechanism that permitted removal of dead-space air. The air sample was then transferred into three, 15-mL nonsilicon-coated glass tubes (Venoject, Terumo Medical Corp, Elkton, MD). Transfer was accomplished by means of a 35-mL disposable syringe and thin needle (0.5 × 16 mm). The total carbon dioxide content of each tube was ≈20 μmol. The samples were stored at room temperature until analyzed by isotope ratio mass spectrometry (MAT Delta E; Finnigan, Bremen, Germany) (18).

Blood samples were collected at 30-min intervals between 0000 and 0600. Three baseline samples at −30, −15, and −5 min were taken before administration of the leucine tracer. 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). These tubes were stored at room temperature for a maximum of 12 h. To liberate the carbon dioxide from the blood bicarbonate, 2 mL of 85% (vol:vol) phosphoric acid was injected into the evacuated tube and the contents vortexed. The evacuated tube was then backfilled with nitrogen to bring it to atmospheric pressure, then left to stand overnight. The liberated carbon dioxide was transferred to a plain nonsilicon-coated 15-mL Venoject tube, which was subsequently backfilled with nitrogen to bring it to atmospheric pressure. The $^{13}CO_2$ enrichment was then measured as for the breath samples.

To validate the method for preparation and measurement of blood $^{13}CO_2$ enrichment, a volunteer subject was given a bolus dose of [^{13}C]sodium bicarbonate to provide a range of blood and

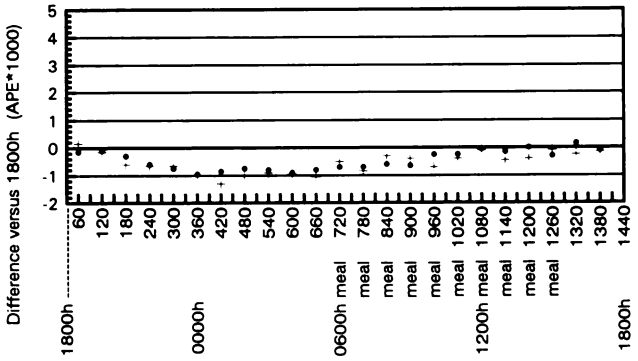

FIG 2. Change in the background ^{13}C-enrichment of expired carbon dioxide in two subjects (● and +), who were studied for 24 h but without receiving the leucine and bicarbonate tracers. Results are expressed as the difference in enrichment [atoms percent excess (APE) × 1000] from the initial 1800-h value. Minutes (60 to 1440) refer to experimental time; time of day is indicated by hours.

breath gas samples that were drawn simultaneously. Both sets of samples were processed and measured as indicated above. The $^{13}CO_2$ enrichment of all samples was expressed as atoms percent excess (APE) × 1000 above baseline and a graphic comparison of results made with breath $^{13}CO_2$ enrichment assigned to the x axis, and blood $^{13}CO_2$ enrichment on the y axis. The enrichment range tested was from 2.5 to 25 APE × 1000 above background. The y intercept and slope were −0.15% and 0.989 respectively; the correlation coefficient was 0.997 (n = 11). This indicated excellent agreement between blood and breath $^{13}CO_2$-enrichment values. Furthermore, this good agreement was confirmed in each study because samples taken at 0000 and at 0600 were measured for both breath and blood $^{13}CO_2$ enrichments. The interassay precision of the $^{13}CO_2$ quality-control samples, run seven times over a period of months with study samples, was 0.014%.

At 30-min intervals throughout the 24-h study period and at −30, −15, and −5 min before the 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.

For isotopic analysis of [^{13}C]KIC, the procedure, briefly, was as follows: 300 μL plasma was deproteinized with methanol. After centrifugation, the supernate was evaporated to dryness under nitrogen and redissolved in 200 μL distilled water. The quinoxalinol derivative was prepared according to Rocchiccioli et al (19) by addition of a 2% solution of 1,2-phenylenediamine in hydrochloric acid with heating. The solution containing the partially derivatized KIC was extracted twice with ethyl acetate and evaporated to dryness for 1 h at 90 °C. The residue was taken up in 50 μL *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA; Pierce, Rockford, IL) and 50 μL pyridine. The quinoxalinol-*t*-BDMS derivative was formed by leaving it to stand overnight at room temperature. One microliter was sufficient for gas chromatography/mass spectrometry analyses by selective ion monitoring (SIM).

Measurements of plasma [^{13}C]KIC isotope enrichment were made on a Hewlett-Packard 5890 Series II gas chromatograph coupled to an HP 5971 quadrapole mass spectrometer and an HP RTE-A data system (Hewlett Packard, Palo Alto, CA). Electron-impact ionization was conducted at 70 eV. The analysis of KIC was carried out on a 30 m × 0.25 mm DB 1701 fused-silica column (J and W Scientific, Folsom, CA), temperature programmed from 140 to 300 °C at 10 °C/min. KIC eluted at 7.2 min and was monitored at its base peak m/z 259[M-57]$^+$ and carbon-13 labeled species at m/z 260. Plasma [^{13}C]KIC enrichments were measured against calibration graphs prepared from standard mixtures of labeled [1-^{13}C]KIC, ranging from 0% to 10% mole fraction. The calibration graphs used to convert the measured ion ratios to mole fractions in this study were determined six times over a period of 6 mo; the y intercept and slope were 22.2% and 1.06, respectively. Their coefficients of variation were 0.6% and 3.2%, respectively. For the present analysis the theoretical y intercept is 21.7%.

The intraassay precision of the sample preparation procedure was assessed by separately processing five samples from the same labeled plasma through the complete procedure. The CV for a sample with a [^{13}C]KIC enrichment of 3% mole fraction was 1.9%. The interassay precision was assessed by taking four unknown samples through the complete procedure over a period of 3 mo. It was found to be 4.4%.

Plasma [di-^{15}N]urea enrichment was determined on a 50-μL plasma sample to which 2 mL methanol was added. The methanolic extract was evaporated to dryness and a *t*-butyldimethylsilyl derivative prepared. Measurement of isotope dilution was carried out as described above, except that an HP 5988A quadrapole was used. A DB 1301 fused-silica column was used and temperature programmed from 110 to 280 °C at 30 °C/min. Urea eluted at 6.5 min, and was monitored at its base peak m/z 231 [M-57]$^+$ and [^{15}N]urea at m/z 233. The plasma enrichments of the [^{15}N-^{15}N]urea were measured against calibration graphs prepared by using standard mixtures ranging from 0% to 10% mole fraction. The calibration graphs used to convert the measured ion ratios in the plasma samples to mole fractions, determined six times over a period of 3 mo, had a y intercept of 8.5% and a slope of 1.19. The CVs of the mean y intercept and slope were 0.48% and 0.45%, respectively. The theoretical y intercept was 8.9%.

The intraassay precision of the measurement of a sample with a [^{15}N-^{15}N]urea enrichment of ≈5% mole fraction was 0.2% (n = 5). The interassay precision of the complete procedure, based on five samples measured over a period of 1 mo, was 2.1%.

The concentrations of leucine in plasma and infusates were determined as previously described by using an HPLC procedure (8). 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 (20), with an auto analyzer. Urinary urea excretion was corrected for the changes in body urea pool (*see* below). Total urinary nitrogen concentration was determined by microKjeldahl analysis.

Evaluation of primary data

Leucine oxidation. Leucine oxidation was computed for consecutive half-hourly intervals to improve the accuracy of the 24-h leucine-oxidation value, because there was a variable rate of leucine oxidation throughout the 24-h period. Moreover, before the plateau in plasma [^{13}C]KIC enrichment had been reached during the first 60–120 min of the 24-h infusion, leucine oxidation during each of these initial half-hourly intervals was computed as being equal to the leucine oxidation during the first half-hourly interval of the initial plateau period, which began at 120 min. Therefore, leucine oxidation was directly measured for 44 of the total 48 half-hourly intervals. For each half-hourly interval, leucine oxidation was computed as follows:

$$\text{leucine oxidation } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\ \text{min}^{-1}) = {}^{13}CO_2 \text{ production } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\ \text{min}^{-1}) / [{}^{13}\text{C}]\text{KIC enrichment (APE} \times 10^{-2})$$

where

$$^{13}CO_2 \text{ production } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\ \text{min}^{-1}) = \dot{V}CO_2\ (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\ \text{min}^{-1}) \times {}^{13}CO_2 \text{ enrichment}^{\#} \times (\text{APE} \times 1000) \times \frac{1}{10^5} \times \frac{1}{R^{\#\#}}$$

where # is the average of the two enrichments at the two time points determining the half-hour interval (this average was corrected for $^{13}CO_2$ background, as described earlier); ## is R_{fast} or R_{fed}, the mean [^{13}C]bicarbonate fraction recovered during fasting

(0.766) and feeding (0.851), respectively (*see* Results); and [^{13}C]KIC enrichment is the average of the two enrichments determining the half-hour interval.

In addition, within each metabolic state, $\dot{V}CO_2$ over the time interval when it was not directly measured was derived as the arithmetic average of $\dot{V}CO_2$ 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 also computed for half-hourly intervals as follows:

$$\text{Recovery} = [^{13}CO_2 \text{ expired (A)}]/[^{13}CO_2 \text{ infused (B)}]$$

where

$$\text{A } (\mu\text{mol}) = \dot{V}CO_2\ (\mu\text{mol}) \times {}^{13}CO_2 \text{ enrichment (APE} \times 1000) \times \frac{1}{10^5}$$

$$\text{B } (\mu\text{mol}) = \text{measured } CO_2\ (\mu\text{mol/mL}) \times \text{volume infused (mL)} \times \text{tracer enrichment} \left(\text{atom \%} \times \frac{1}{10^2}\right)$$

Leucine balance. Predicted 24-h leucine balance (input − predicted output) was computed as follows:

Input (μmol/kg) = dietary leucine + intravenous tracer leucine

Predicted Output (μmol/kg)

= (oxidation during the selected fasting hour × 12)

+ (oxidation during the selected feeding hour × 12)

Measured 24-h leucine balance (input − measured output) was computed as follows:

Input (μmol/kg) = dietary leucine + intravenous tracer

Output (μmol/kg) = sum of the determined oxidation for the 48 half-hourly intervals

Urea metabolism and protein oxidation. The 24-h urea excretion rate was computed as the sum of the eight 3-hourly urea excretions, each corrected for changes in body urea pool as follows:

Urea N excretion (corrected) = urea N excretion

− [(plasma urea N at start − plasma urea N at end) × (estimated total body water/0.92)]

as described by Fern et al (22), with total body water estimated from equations by Watson et al (23). The 24-h urea production rate was computed as the sum of the 48 half-hourly urea production rates, by using the same tracer-based approach as described above for leucine oxidation. Half-hourly urea production was computed as follows:

$$UR_{prod}\ (\text{mg urea N} \cdot \text{kg}^{-1} \cdot 30\ \text{min}^{-1}) = [^{15}\text{N}]\text{urea infusion rate/plasma } ^{15}\text{N urea enrichment}$$

where

$$\text{Infusion rate (mg urea } ^{15}\text{N} \cdot \text{kg}^{-1} \cdot 30\ \text{min}^{-1}) = \text{measured (mg urea N/mL)} \times \text{volume infused } (\text{mL} \cdot \text{kg}^{-1} \cdot 30\ \text{min}^{-1}) \times \text{tracer enrichment} \frac{1}{10^2}\ (\text{atom \%})$$

Plasma [^{15}N-^{15}N]urea enrichment (APE × 10^{-2}) is the average of the two enrichments measured at the two time points determining the particular half-hourly interval. Twenty-four–hour protein oxidation ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), as derived from nitrogen excretion, was computed as follows:

(Urinary urea N excreted + urinary nonurea N excreted + 8) × 6.25

Here we assume that 8 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ is sufficient to account for unmeasured fecal nitrogen loss under these conditions. Twenty-four–hour protein oxidation ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), derived from urea production, was computed as follows:

(Urea N production + urinary nonurea N excreted + 8) × 6.25

Twenty-four–hour protein oxidation ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), derived from leucine oxidation, was computed as follows:

24-h Leucine oxidation ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) × (100/8) × [I/(I + i)]

where 100/8 assumes an average of 8% leucine (by wt) in whole-body, mixed proteins, and (I/I + i) is a correction factor for the additional oxidation of leucine due to the nonmassless tracer (i), because, at generous intakes of leucine, any additional leucine given in this way will be disposed of by irreversible oxidation; without this correction there would be a spurious overestimation of protein oxidation, providing that neutrality in leucine balance is present. I is dietary leucine intake.

Statistical methods

Directly derived values obtained via measurement vs those obtained by the indirect (predicted) approach (ie, those from extrapolation) first were evaluated by using the graphical method of Bland and Altman (24), as well as by comparisons based on the paired *t* test. One-way analysis of variance (ANOVA), followed by pair-wise comparisons among means by using the Newman-Keuls test, was used to compare protein-oxidation data obtained via the different calculation procedures described above. Paired *t* tests were used to compare protein oxidation with protein intake and to compare leucine-oxidation rates (measured or extrapolated) during fasting and feeding. One-sample *t* test was used to determine whether measured and predicted leucine balances differed from zero.

To compare plasma leucine concentrations with leucine-oxidation data, the following procedure was used: For each time point at which the plasma leucine concentration was measured, it was matched with the corresponding half-hourly value (starting at that particular time point) for the rate of leucine oxidation. A subject × condition (fasted vs fed) × time × leucine (concentration vs oxidation) repeated-measures ANOVA was used to analyze the data. In addition, correlations were calculated, within each condition, to determine the relationship between plasma leucine concentration and the rate of leucine oxidation. Data analyses were done by using *SAS* software, version 6 (SAS Institute Inc, Cary, NC).

Results

For precise determination of leucine oxidation from the continuous 24-h [1-^{13}C]leucine tracer infusion, it was necessary to establish, first, the factors to be used for the retention of $^{13}CO_2$

under these conditions. As summarized in **Figure 3,** the retention of $^{13}CO_2$ was essentially steady for each metabolic state. A mean value of 76.6 ± 2.0% was found for the liberation of the infused bicarbonate during the fasted state and 85.1 ± 2.5% during the fed state. These mean values were used to correct the $^{13}CO_2$-output data after the 24-h infusions with the [1-^{13}C]leucine tracer.

The patterns of total carbon dioxide output, $^{13}CO_2$ enrichment, $^{13}CO_2$ excretion, and plasma [^{13}C]KIC enrichment throughout the 24-h tracer period for subjects receiving the generous intake of leucine (89.4 mg·kg^{-1}·d^{-1}) are shown in **Figure 4, a–d.** From these data the pattern and rate of whole-body leucine oxidation emerges: these results are depicted in **Figure 5.** It can be seen that under these feeding conditions, leucine oxidation increased when the first small meal was consumed and remained relatively constant until ≈1 h after the last small meal taken at 1500. Note that the last meal was given at this time because it was our purpose to capture, via measurement, all of the irreversible oxidative loss of leucine for the entire daily intake of 89.4 mg·kg^{-1}·d^{-1} (including the tracer); this required terminating feeding sometime before the end of the tracer-infusion period. The pattern of leucine oxidation closely paralleled the change in plasma leucine concentration (**Fig 6**), confirming and extending the close association between the rate of leucine oxidation and plasma leucine concentrations that we observed in earlier studies with young adults receiving different intakes of dietary leucine (25). However, a repeated-measures ANOVA showed a difference in the pattern of leucine oxidation vs that for plasma leucine concentration ($P < 0.01$), which indicates that the rate of leucine oxidation is not an exact derivative of the plasma leucine concentration. In addition, a negative correlation ($r = -0.63$, $P < 0.01$) was observed between leucine-oxidation rates at equivalent times measured during fasting and feeding, suggesting some relationship between oxidation status in fasting vs feeding, which may play a role in determining the 24-h pattern in the rate of leucine oxidation.

The measured rates of leucine oxidation during the periods from 1800 to 0600 (fasted state) and 0600 to 1800 (fed state) and during the last hour of the fast (a) and fifth hour of the fed (b) periods are summarized in **Table 3.** From these results total daily leucine-oxidation rate, obtained by summation of the actual, or measured, rates, was equivalent to 89.5 ± 3.3 mg leucine·kg^{-1}·d^{-1}. Essentially 61% of this oxidative loss occurred during the 12-h fed period.

The predicted rates were obtained from extrapolation of the rates measured during the last hour of fast (a) and fifth hour of the fed (b) periods (Table 3). This predicted daily oxidation rate was 91.2 ± 5.8 mg leucine·kg^{-1}·d^{-1}, or only 1.9% higher (P

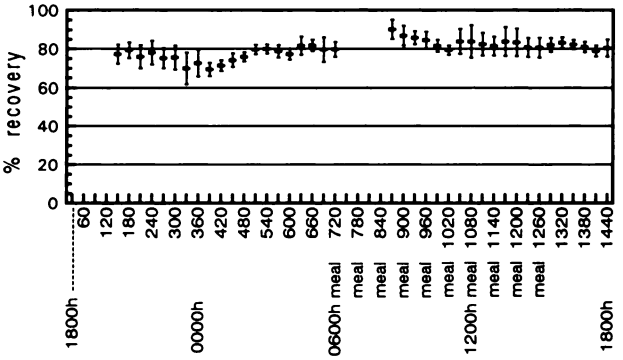

FIG 3. The recovery (expressed as % of infused label) of sodium [^{13}C]bicarbonate when given as a continuous intravenous tracer infusion throughout a 24-h period. The experimental 24-h tracer protocol was comparable with that described in Figure 1. Mean data for five subjects. Bars at each time point represent + or − 1 SD. Minutes (60 to 1440) refer to experimental time; time of day is indicated by hours.

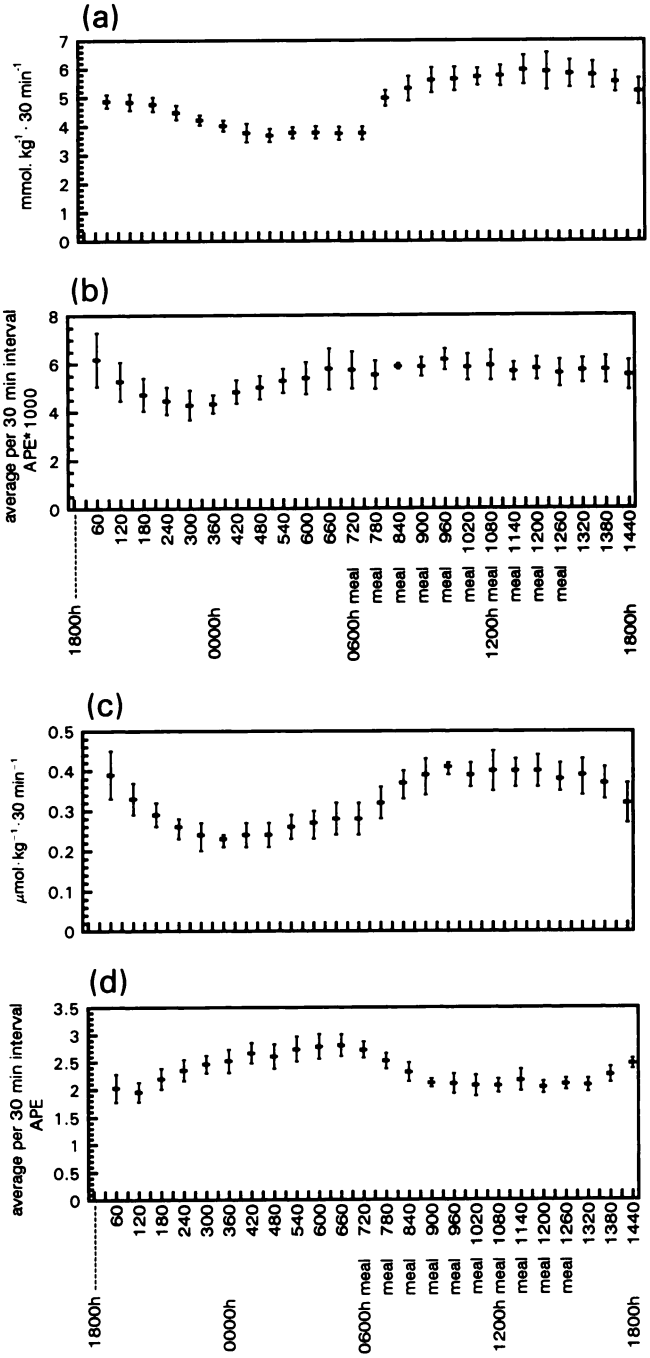

FIG 4. The pattern of change throughout a 24-h continuous intravenous infusion with [1-^{13}C]leucine for a, total carbon dioxide production; b, ^{13}C-enrichment of expired carbon dioxide; c, μmol $^{13}CO_2$ expired; d, carbon-13 enrichment [atoms % excess (APE)] of plasma ketoisocaproate. From these four sets of data total leucine oxidation can be calculated (*see* Fig 5). Minutes (60 to 1440) refer to experimental time; time of day is indicated by hours.

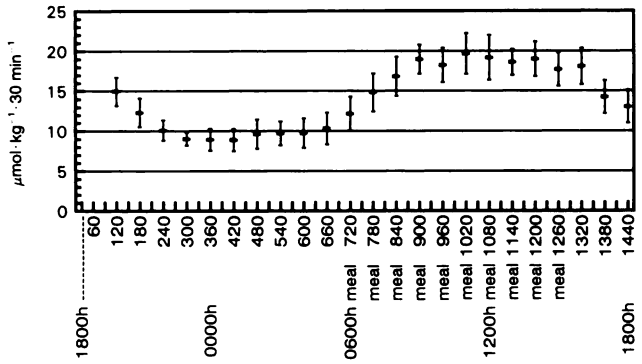

FIG 5. Leucine oxidation for each 30-min interval throughout a 24-h tracer infusion with [1-^{13}C]leucine. $\bar{x}$ ± SD; n = 7. Feeding began with small meals at 0600 to provide 80 mg dietary leucine·kg^{-1}·d^{-1} during the fed periods. Minutes (60 to 1440) refer to experimental time; time of day is indicated by hours.

= 0.25) than the measured value. The precise difference between measured and predicted daily leucine oxidation varied according to the combination of specific fast and fed hours chosen for predicting the 24-h oxidation loss. When all reasonable combinations of measurements for fast (11, 12, 13, 14, or 15 h) with fed (3, 4, or 5 h) were used the range of differences between measured and predicted was from −6.1% to +4.1%; the overall mean difference was −0.8% ($P > 0.1$). However, from subject to subject the direction of the difference was variable (+ or −), as found by the graphical method (24).

Summarized in **Table 4** are the estimates of daily leucine balance for the seven subjects, based on the measured daily rate of leucine oxidation. These are compared with predicted balances derived from the extrapolation of leucine-oxidation rates measured during the last hour of the fast and during the fifth hour of the fed period. The means for the measured and predicted balances were not significantly different (P = 0.25). Of possibly greater interest was the observation that both the measured (t = 0.73) and predicted (t = 0.50) balances did not differ from a neutral, or zero whole-body leucine balance. Hence, under our experimental conditions, the generous leucine intake was matched precisely by a quantitatively equivalent rate of daily leucine oxidation. On the assumption that our tracer model was appropriate and the analytical precision good, this finding was to be expected because our subjects were young adults whose body protein mass (and, therefore, leucine content) was presumably neither increasing nor decreasing at least at a daily rate that could

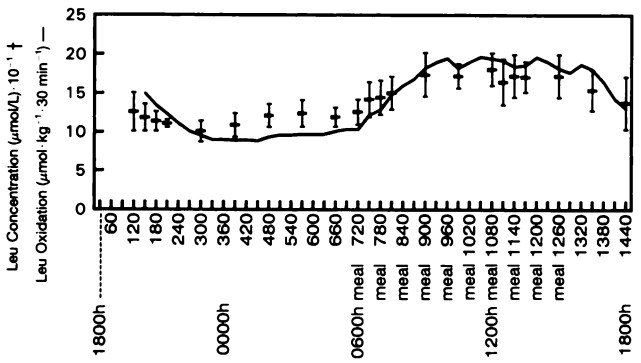

FIG 6. Relationship between the concentration of plasma free leucine and the oxidation of leucine (solid line) at various times during the 24-h tracer-infusion protocol. $\bar{x}$ ± SD; n = 7. Minutes (60 to 1440) refer to experimental time; time of day is indicated by hours.

TABLE 3
Leucine oxidation measured at various times throughout the 24-h study: comparison of measured and predicted daily leucine-oxidation rates[1]

Period of leucine oxidation	Value[1]	Percent difference from measured value[2]
	mg leucine/kg	*%*
Measured for		
12-h fast (1800 to 0600)[3,4]	34.7 ± 4.0	—
12-h fed state (0600 to 1800)[5]	54.8 ± 4.2	—
Last hour of fast (a)	2.7 ± 0.5	—
5th h of fed state (b)	4.9 ± 0.6	—
24 h day (1800 to 1800)[6]	89.5 ± 3.3	—
Predicted for		−6.7 ± 8.6
12-h fast, from (a)[7]	32.5 ± 5.8	(−2.2 ± 2.8)
		+7.0 ± 6.9
12-h fed state, from (b)	58.7 ± 6.6	(3.9 ± 3.7)
		+1.9 ± 3.9
24 h day (12a + 12b)	91.2 ± 5.8	(1.7 ± 3.1)

[1] $\bar{x}$ ± SD.
[2] $\bar{x}$ ± SD (CI of the absolute difference: $\bar{x}$ ± 2.447 SEM, where $t_{0.05(2),6}$ = 2.447).
[3] Significantly different from 12-h fed state (0600 to 1800), $P < 0.001$ (paired t test).
[4] Not significantly different from 12-h fast, from (a), P = 0.13 (paired t test).
[5] Not significantly different from 12-h fed state from (b), P = 0.055 (paired t test).
[6] Not significantly different from 24-h (12a + 12b), P = 0.25 (paired t test).
[7] Significantly different from 12-h fed state from (b), P = 0.001 (paired t test).

TABLE 4
Computed daily leucine balance from measured and predicted daily leucine-oxidation rates

Subject	Measured balance	Predicted balance
	mg leucine · kg^{-1} · d^{-1}	
1	1.76	0.83[1]
2	−1.37	−3.39
3	−3.58	−4.97
4	−0.57	−4.56
5	5.83	9.95
6	2.02	−5.78
7	1.21	1.04
$\bar{x}$ ± SD	0.76 ± 2.99[2]	−0.98 ± 5.54[2,3]

[1] Extrapolated daily oxidative loss based on (last hour fast × 12) + (5th h of fed × 12).
[2] Predicted vs measured: P = 0.25 (NS) (paired t test). Measured vs zero: t = 0.73 (NS) (one sample t test). Predicted vs zero: t = 0.50 (NS) (one sample t test).
[3] CI of the absolute difference with measured value: $\bar{x}$ ± 2.447 SEM, where $t_{0.05(2),6}$ = 2.447; (−1.74 ± 3.12).

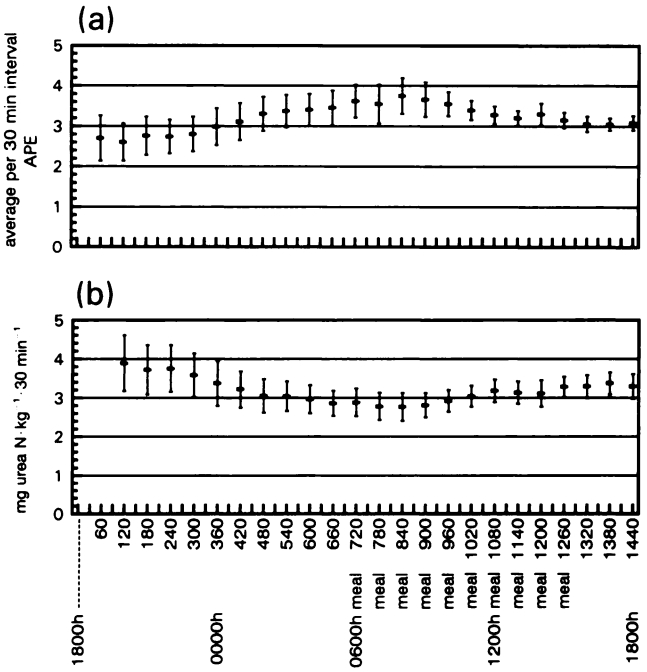

FIG 7. Plasma enrichment of [^{15}N-^{15}N]urea (a) and plasma urea nitrogen appearance (production; b) with a continuous infusion of labeled urea. Details of the 24-h protocol are given in Figure 1. $\bar{x} \pm$ SD; $n = 7$. Minutes (60 to 1440) refer to experimental time; time of day is indicated by hours.

be detected reliably by any currently available method. The determination of neutral body leucine balance from the tracer data at this generous leucine intake validates the tracer-balance concept.

The plasma ^{15}N-enrichment of the dilabeled urea is shown for the 24-h period in **Figure 7,**a. It is evident that the concentration of nitrogen-15 in plasma urea tended to rise over the 12-h fast period, indicating a fall in the rate of urea production as fasting progressed (Fig 7, b) and then increasing when subjects received small protein-containing, isonitrogenous meals at hourly intervals beginning at 0600 (Fig 7, b). These plasma-enrichment values were used to determine the daily rate of urea nitrogen production, as described in Methods; **Table 5** summarizes the values obtained. Under these conditions, mean urea nitrogen production was 155 ± 18 mg·kg^{-1}·d^{-1} compared with a urea excretion rate of 124 ± 14 mg·kg^{-1}·d^{-1} (**Fig 8**). The difference between urea production and excretion is due, presumably, to the hydrolysis of urea within the intestinal lumen; for these conditions it was 31 mg N·kg^{-1}·d^{-1}, or 20 ± 9% of total urea production.

The leucine-oxidation data can be evaluated further in reference to these urea kinetic and excretion data. This assessment is also summarized in Table 5. The basis of this evaluation and the comparisons made are described in Methods. Our results show that when leucine oxidation is used to estimate daily protein (nitrogen × 6.25) oxidation, the value is 998 ± 38 mg·kg^{-1}·d^{-1}. This is not significantly different from the estimated rate of body protein oxidation derived from measurement of total urinary nitrogen output (urea nitrogen + nonurea nitrogen), together with an assumed loss of metabolic fecal nitrogen of 8 mg·kg^{-1}·d^{-1}. Furthermore, the predicted rate of whole-body protein oxidation from the leucine-oxidation data does not differ from the known

TABLE 5
Urea nitrogen kinetics and estimates of protein oxidation based on these data and on daily leucine oxidation[1]

Index	Value
Urea nitrogen production (mg N · kg^{-1} · d^{-1})	155 ± 18
Urea nitrogen excretion (mg N · kg^{-1} · d^{-1})	124 ± 14
Urea hydrolysis (% of production)	20 ± 9
Protein intake (mg · kg^{-1} · d^{-1})	1006 ± 8
Protein oxidation from [mg protein (N × 6.25) · kg^{-1} · d^{-1}]	
Urea nitrogen excretion + nonurea nitrogen + fecal nitrogen	959 ± 111[2]
Urea nitrogen production + nonurea nitrogen + fecal nitrogen	1157 ± 129[3,4]
Leucine oxidation	998 ± 38[5]

[1] $\bar{x} \pm$ SD.
[2,5] Not significantly different from protein intake (paired t test): [2] $P = 0.352$, [5] $P = 0.531$.
[3] Significantly different from the other two methods, $P < 0.05$ (ANOVA and Student Newman-Keul's test).
[4] Significantly different from protein intake, $P = 0.029$ (paired t test).

total protein intake ($P = 0.531$), which would be expected for adults in body protein equilibrium. However, when the urea production rate rather than the measured urea excretion output was used to estimate protein oxidation, the value significantly exceeded that based on the 24-h measured rate of leucine oxidation, or on protein intake. Thus, under our experimental circumstances, use of urea production data leads to an overestimate of both protein and of leucine oxidation. Finally, these comparisons indicate to us that the determinations of 24-h leucine-oxidation rates were precise and reliable because they matched, quantitatively, the daily leucine intake and also were consistent with the nitrogen-excretion data.

Discussion

To estimate accurately whole-body leucine oxidation from a tracer study with [1-^{13}C]leucine it is necessary to know the fraction of the liberated $^{13}CO_2$ that is retained, or fails to appear in the expired air. From the present study, in five subjects who received a 24-h infusion with [^{13}C]sodium bicarbonate under fasted and fed conditions used to explore the daily (24-h) oxidation of

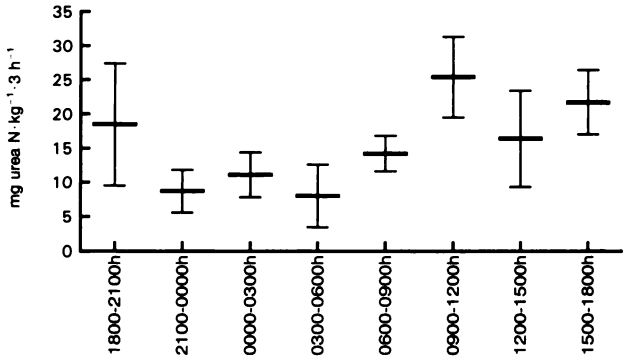

FIG 8. Urea nitrogen excretion over consecutive, 3-h intervals through the 24-h tracer-infusion protocol. $\bar{x} \pm$ SD; $n = 7$.

leucine, the retention was similar to that observed in both short-term and longer-term continuous intravenous tracer-infusion protocols (15, 18, 26–33). Recovery fractions range from 0.51 to 0.95, with the higher recoveries generally being associated with more prolonged tracer infusions and/or where metabolic rates are higher (*see* 29). Thus, recovery factors of 77% and 85% for the fasted and fed states, respectively, were applied in the present 24-h tracer experiments. These are similar, but not identical to the values that we obtained (18) and have applied in our earlier shorter-term tracer studies.

The 24-h pattern of leucine oxidation (Fig 5) showed a prompt, sustained rise in the rate when the small hourly meals were given, beginning at ≈0600. This pattern is similar to that observed by Glugston and Garlick (34) and Garlick et al (35) in their 24-h tracer studies with [1-^{13}C]leucine in which the fed state was achieved by giving a 12-h infusion of a liquid diet, beginning at ≈0800. In that study and the present investigation the dietary intake of leucine exceeded considerably the minimum physiological requirements, either as proposed by the FAO/WHO/UNU (11) or by us (2, 4, 5). Whether this 24-h pattern of leucine oxidation would differ distinctly for leucine intakes at and below requirement amounts remains to be established. In our previous short-term tracer studies, low leucine intakes during the fed state were associated with rates of leucine oxidation either similar to or below those for the fasted state. Hence, it might be anticipated that at lower and inadequate intakes of leucine the 24-h pattern of oxidation would differ substantially from that presented here (Fig 5) for a generous leucine intake. We will explore this issue in detail in our companion paper (10).

We had two main objectives in making continuous measurements of leucine oxidation throughout the 24-h day. The first was to determine whether our previous estimates of daily leucine oxidation, and of daily leucine balance, which we made via extrapolations of leucine-oxidation rates determined over short (1-h) periods during the fasted and fed states, gave reasonably accurate determinations of the actual rates of leucine oxidation throughout both 12-h fast and fed periods as well as for the entire 24-h period. The second aim was to determine whether a [^{13}C]leucine tracer–based estimate of whole-body leucine balance was accurate and, if so, whether the tracer-balance concept could be validated for the generous leucine intake given in this study.

With respect to this latter aim, daily leucine balance for our subjects was 0.76 ± 2.99 mg·kg^{-1}·d^{-1}. This was not different from a zero balance, indicating that a neutral body leucine balance was obtained and confirming that we can accurately determine leucine balance by measuring whole-body leucine oxidation, based on measurement of plasma [^{13}C]KIC as precursor and $^{13}CO_2$ in expired air as product. Losses of leucine per se via urine and of any endogenous leucine via the feces would be expected to be negligible and should not affect the foregoing conclusion. Studies by others (36, 37) have supported use of plasma KIC isotope enrichment to calculate whole-body protein turnover and protein synthesis in the splanchnic region (38). However, whether plasma KIC enrichment is the appropriate measure for estimation of whole-body leucine oxidation still can be questioned but it is important to point out that the measured 24-h rate of leucine oxidation was essentially identical to intake, under conditions of body-protein maintenance in healthy adults. In our opinion this finding offers strong support for the proposed use of plasma α-KIC enrichment as an index of the amount of labeling of leucine in the whole-body tissue pools where it is undergoing oxidation (39).

The comparisons shown in Table 3 between measured and predicted daily oxidation rates of leucine and of measured and predicted daily leucine balances (Table 4), indicate that the various estimates agree very closely with each other. Thus, although predicted values for leucine oxidation and balance were not statistically different from measured values, the mean data indicate a small trend toward a possible overestimation of leucine oxidation. Hence, in previous studies we may have slightly overestimated the minimum physiological requirement for leucine. However, these errors are neither large nor could they explain the marked discrepancies that we have identified between the FAO/WHO/UNU (11) minimum leucine requirement of 14 mg·kg^{-1}·d^{-1}, as derived from nitrogen-balance studies, and that of ≈40 mg·kg^{-1}·d^{-1}, which we set as a tentative minimum requirement in healthy adults (3, 5), based on our carbon-13 tracer investigations.

The daily mean urea production rate for our healthy subjects was 155 mg urea N·kg^{-1}·d^{-1}, which compares well with isotope-tracer-derived published values (37–40) obtained from subjects who were consuming dietary protein at intakes comparable with those used in the present study. Urea excretion was less than urea production, confirming the presence of the well-established process of urea hydrolysis within the intestinal tract (12). Again, our mean value of 20% for urea hydrolysis, expressed as a percentage of urea production, agrees well with other reports for subjects receiving generous intakes of dietary protein (40–45).

From the measured rates of urea production, and estimates of total nitrogen losses via urine and feces, it was possible to estimate the daily rate of protein oxidation and to compare these estimates with those derived from the leucine-oxidation data. The latter data predict that protein oxidation amounted to 998 mg·kg^{-1}·d^{-1}, an estimate that agrees closely with that derived from both measured nitrogen-excretion values and from the subject's actual protein intake. This comparison provides additional support for the accuracy of the measured 24-h leucine-oxidation rate in these experiments. There are no other "gold standards" or reference values that we might use or could depend on to assess whether we had made the best determination of the actual in vivo rate of whole-body leucine oxidation.

When the urea production rate was used to estimate daily protein oxidation, the derived value was significantly higher ($P < 0.05$) than that obtained from either total nitrogen excretion, protein intake, or leucine-oxidation data. This indicates to us that a quantitatively major path followed by the nitrogen (ammonia) released during intestinal urea hydrolysis is its direct reincorporation into urea; there is reasonably good evidence in support of this being the major fate of the urea nitrogen that is released via hydrolysis (46), particularly when intakes of protein are adequate, as in the present case.

A relationship between leucine oxidation, protein oxidation, and urea production and excretion was also evident from their 24-h patterns of change. The lowered rate of urea production during the fast (Fig 7, b) and the increased rate when feeding began was reflected by a lowered rate of leucine oxidation during the fast and a prompt and sustained rise that occurred with ingestion of the hourly meals. From Jungas et al (47), these patterns of association between amino acid oxidation and nitrogen output are presumably reflective of the gluconeogenesis that occurs, apparently, as a normal prandial process.

Finally, these comparisons between leucine kinetics and urea production provide an opportunity to assess an extravagant claim by Jackson (12) that there is a significant synthesis of indispensable amino acids (such as leucine) by the microflora within the intestinal lumen, which are then made available to meet in a quantitatively important way the physiological needs of the host. If this hypothesis was correct, at least for our experimental conditions, a carbon-13–tracer-derived, neutral body leucine balance would not have been obtained and the estimated rates of daily protein oxidation derived from the leucine-oxidation rates would not have agreed as closely as they did when compared with the estimate of protein oxidation based on total nitrogen excretion or on protein intake. We conclude that for the present experimental conditions, which were similar to those used previously in carbon-13 tracer-oxidation studies of human amino acid requirements (1, 2), it is highly unlikely that the tracer-derived estimate of 24-h leucine oxidation significantly overestimated the oxidation of dietary leucine because of a postulated intestinal source of newly formed leucine. Thus, our data fail to support the postulate made by Jackson (12) and we conclude that his hypothesis should be discarded, at least in reference to the present and our previous (1–5) experimental conditions. Furthermore, we are unaware of data from other laboratories that strongly support the Jackson hypothesis for any metabolic and/or nutritional conditions.

In conclusion, we conducted continuous 24-h measurements of [1-^{13}C]leucine oxidation in healthy adults consuming a generous intake of dietary leucine. We observed that they were in neutral whole-body leucine balance; this finding validates the tracer-balance concept, as used previously to estimate the minimum physiological requirements for leucine, and possibly for other indispensable amino acids. Predictions of total daily leucine oxidation can be made with reasonable accuracy (within $\approx$2 $\pm$ 4% of the measured rate) from measurements made during brief (1-h) periods toward the end of an overnight fast and during a fed state after $\approx$4–5 h of receiving equal meals at hourly intervals. Therefore, our previous studies (1–5) involving short tracer-infusion protocols have provided reasonable estimates of the daily leucine-oxidation rate and, thus, of minimum dietary intakes required to balance the irreversible loss of leucine. Confirmation and extension of this assessment, which is based on 24-h leucine kinetic studies for subjects receiving a generous leucine intake, would be desirable by conducting comparable investigations at maintenance and deficient dietary leucine intakes. This will be the topic of our companion paper (10).

We thank the MIT CRC nursing and laboratory staff for their help in the conduct of these studies. The technical assistance of John LoRusso, Mike Kenneway, and Alan Atkinson is appreciated and the willingness and dedication of the subjects who volunteered for these studies is gratefully acknowledged.

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