Am J Physiol Endocrinol Metab
278: E1000–E1009, 2000.

Kinetics of L-[1-^{13}C]leucine when ingested with free amino acids, unlabeled or intrinsically labeled casein

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Metges, Cornelia C., Antoine E. El-Khoury, Ambalini B. Selvaraj, Rita H. Tsay, Alan Atkinson, Meredith M. Regan, Brian J. Bequette, and Vernon R. Young. Kinetics of L-[1-^{13}C]leucine when ingested with free amino acids, unlabeled or intrinsically labeled casein. *Am J Physiol Endocrinol Metab* 278: E1000–E1009, 2000.—In two groups of five adults, each adapted to two different dietary regimens for 6 days, the metabolic fate of dietary [1-^{13}C]leucine was examined when ingested either together with a mixture of free amino acids simulating casein (extrinsically labeled; *condition A*), along with the intact casein (extrinsically labeled; *condition B*), or bound to casein (intrinsically labeled; *condition C*). Fed state leucine oxidation (Ox), nonoxidative leucine disposal (NOLD), protein breakdown, and splanchnic uptake have been compared using an 8-h oral [1-^{13}C]leucine and intravenous [$^{2}H_3$]leucine tracer protocol while giving eight equal hourly mixed meals. Lower leucine Ox, increased NOLD, and net protein synthesis were found with *condition C* compared with *condition A* (19.3 vs. 24.9; 77 vs. 55.8; 18.9 vs. 12.3 $\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$; $P < 0.05$). Ox and NOLD did not differ between *conditions B* and *C*. Splanchnic leucine uptake calculated from [1-^{13}C]- and [$^{2}H_3$]leucine plasma enrichments was between 24 and 35%. These findings indicate that the form in which leucine is consumed affects its immediate metabolic fate and retention by the body; the implications of these findings for the tracer balance technique and estimation of amino acid requirements are discussed.

leucine oxidation; leucine flux; amino acid mixture; intrinsic label

PREVIOUS STUDIES CONDUCTED in our laboratory on the relationship between amino acid kinetics and amino acid intake have largely involved use of a diet providing nitrogen in the form of a crystalline L-amino acid mixture (e.g., Refs. 1, 10, 11, 24, 29, 32, 44). This gave us the opportunity to vary precisely the dietary intake of each indispensable amino acid separately, while maintaining total nitrogen intake constant, which is a prerequisite for estimating the amino acid requirement using the tracer-balance technique (48).

However, differences in the time course of absorption have been reported when amino acids are ingested either as free amino acids or small peptides or bound to proteins. Peptide amino acids are known to be absorbed more rapidly than free amino acid mixtures (31, 41, 42), with free amino acids appearing in the peripheral plasma more quickly than amino acids arising from intact proteins (22, 23).

Results on growth and nitrogen utilization from studies comparing whole proteins, peptides, and corresponding amino acid mixtures are, however, conflicting. Nitrogen balance in the rat (19) and in human subjects (35) did not differ between whole protein, hydrolyzed protein, and free amino acid mixtures of identical pattern. Also, no difference in feed efficiency was seen when comparing free amino acids and the equivalent peptide mixture of casein and egg white proteins (8). On the other hand, net protein utilization was observed to be greater with small peptides from milk protein than with an equivalent amino acid mixture in normal rats and animals with a resection of the biliopancreatic duct (34).

Studies by Batterham and Bayley (2) in the pig indicated that oxidation of free phenylalanine, as an index of amino acid utilization, is greater when the diet contains free lysine compared with protein-bound lysine.

The possible effects of the rate and pattern of absorption on the fate of dietary amino acids emerging from different molecular forms of nitrogen intake are important to investigate because *1*) our tentative Massachusetts Institute of Technology (MIT) amino acid requirement pattern (48) has been derived, in part, from dietary studies using L-amino acid mixtures, and *2*) under circumstances where a net protein catabolism occurs, such as protein-energy malnutrition, renal disease, sepsis and traumatic injury (30), and sarcopenia of the elderly (36), the qualitative nature of the dietary amino acid supply might affect amino acid losses and retention.

Therefore, we have conducted a study to explore the prandial metabolic fate of ^{13}C-labeled dietary leucine when it is ingested as a component of mixed meals either bound to a protein (casein; intrinsically labeled) or together with a mixture of crystalline free amino acids (extrinsically labeled) simulating the casein amino

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acid pattern. As a control, leucine kinetics were measured also when free labeled leucine was given together with the intact protein (casein). In contrast to many published studies (5–7, 9, 21, 45, 46) concerned with prandial and postprandial aspects of amino acid metabolism and the molecular form of the ingested tracer amino acid that did not involve dietary adjustment or adaptation periods to the experimental diets, the present study included a 6-day adjustment period on each experimental diet before the tracer study. Amino acid absorption appears to be readily adaptable to the prevailing protein/amino acid uptake (28), and so it was important to standardize the dietary background of the study population. Additionally, our investigation included an assessment of the splanchnic first-pass uptake of leucine under all three tracer conditions, using a simultaneous intravenous/oral tracer infusion paradigm.

MATERIALS AND METHODS

Subjects. Fourteen young adult volunteers, recruited from the student population of MIT and from the community of the Boston-Cambridge area, were randomly assigned to three study groups and were studied as outpatients at the MIT Clinical Research Center (MIT-CRC). They were healthy according to medical history, physical examination, analysis of blood cell count, routine blood biochemical profile, and urinalysis. Subjects who smoked, consumed five or more alcoholic beverages per week, or five cups of caffeinated beverages per day were excluded from participation. Women were studied during the 5- to 10-day period after onset of menstrual bleeding. A negative pregnancy test (on the basis of plasma human choriogonadotropin concentrations) 2–3 days before starting the dietary periods was required from each female subject.

One woman and four men were included in each of *groups 1* and *2*, whereas two men and two women were studied in *group 3*. Mean age, weight, and height in *groups 1*, *2*, and *3*, respectively, were as follows: age (yr) 20.6 ± 1.7, 21.6 ± 1.1, 26.8 ± 0.5; weight (kg) 69.2 ± 8.2, 74.6 ± 8.3, 71.8 ± 13.9; height (cm) 173.2 ± 11.4, 175 ± 5.4, 176.5 ± 6.4. Written consent was obtained from each subject after explanation of the risks involved and the purpose of the study, which had been approved by the MIT Committee on the Use of Humans as Experimental Subjects and the MIT-CRC Advisory Committee. The subjects, who were paid for their participation in the studies, were instructed to maintain their usual level of activity and were asked to refrain from excessive or competitive exercise.

Experimental design and diets. The dietary tracer protocol for *groups 1* and *2* consisted of two separate dietary periods of 6 days (adaptation period) with an 8-h tracer ingestion/infusion protocol being conducted on *day 7* during the fed state; *group 3* was studied on *day 7* with tracer-free diets only. The order of the dietary periods was randomized for each subject. Between the two dietary periods, a break of 3 wk occurred when subjects consumed their usual free-choice diets. In *group 1*, [1-^{13}C]leucine was ingested on one occasion as protein bound to casein (intrinsically labeled; [^{13}C]Leu-casein), and in the second study the free [1-^{13}C]leucine tracer was added to unlabeled casein (extrinsically labeled; casein + [^{13}C]Leu) just before meal feeding. *Group 2* subjects were studied after completion of the study in *group 1*. In *group 2*, [1-^{13}C]leucine was given orally as either protein bound to casein ([^{13}C]Leu-casein) or with an L-amino acid mixture patterned as in casein (AA + [^{13}C]Leu). Subjects in *group 3* participated in tracer-free studies only and received on one occasion either an unlabeled crystalline L-amino acid mixture simulating the casein pattern or unlabeled goat's casein. On the second occasion, they received either an L-amino acid mixture or unlabeled cow's casein. One subject was studied with all three diets.

The diets were isocaloric and isonitrogenous (161 mg $N \cdot kg^{-1} \cdot day^{-1}$) and contained the same generous amounts of leucine (115 mg leucine$\cdot kg^{-1} \cdot day^{-1}$; inclusive of leucine tracers). To keep leucine intake equal on all days, small amounts of crystalline leucine were given during the first 6 days to adjust for the leucine tracer intake on *day 7*. Energy intake was close to 45 kcal$\cdot kg^{-1} \cdot day^{-1}$ (188 kJ$\cdot kg^{-1} \cdot day^{-1}$) to maintain body weight and consisted of protein-free wheat starch cookies and a flavored protein-free formula in which the amino acid mixture or the casein was blended, respectively (Table 1). About 40% of nonprotein energy was from fat (safflower oil, butter), and 60% was from carbohydrate (beet sugar, starch). Vitamins and minerals were given to meet or exceed the recommended allowances or safe and adequate intakes (Table 1 and Ref. 37). No other foods or beverages were allowed, except tap water, decaffeinated tea or coffee with or without artificial sweetener, and bouillon. Diets were consumed as three daily equal meals (at 0800, 1200, and 1800) in the dining room of the MIT-CRC. Two out of three meals were consumed under supervision of the CRC dietary staff. Every morning, the subject's body weight and vital signs were recorded. On the day of the tracer protocol (*day 7*), oral

Table 1. *Composition of the diets based on casein or an L-amino acid mixture simulating the casein pattern*

Component	Amount
L-Amino acid mixture, g/day	78.3
Casein, g/day	78.5
Leucine supplement, mg/day	660.8
Phenylalanine supplement, mg/day	525.6
Beet sugar	80.0
Protein-free formula, g/day	
Orange sherbet	230.0
Safflower oil	65.0
Beet sugar	123.0
Unsweetened flavored beverage	883.0
Protein-free cookies, g/day	270.0
Supplements	
Multivitamin-multimineral capsules	1
Sodium chloride, g/day	6
Calcium, mg/day	1,000
Potassium, mg/day	3,912
Choline, mg/day	500
Microcellulose, g/day	20

Data are for a 70-kg subject with an energy intake of 13.17 MJ/day. L-Amino acid, leucine supplement, and phenylalanine supplement were from Ajinomoto. Casein used was either cow's casein (Alanate^{R}; New Zealand Milk Products) or goat's casein (Dr. Brian Bequette; Rowett's Research Institute). Orange sherbert was from National Dairy Products. Safflower oil was from PET. Unsweetened flavored beverage was Kool-Aid from Kraft General Foods. Multivitamin-Multimineral capsules were One-a-Day from Miles and contained the following: 5,000 IU vitamin A, 60 mg vitamin C, 1.5 mg thiamin, 1.7 mg riboflavin, 20 mg niacin, 400 IU vitamin D, 30 IU vitamin E, 2 mg vitamin B6, 0.4 mg folic acid, 6 μg vitamin B12, 30 μg biotin, 10 mg panthotenic acid, 34 mg chloride, 18 mg iron, 130 mg calcium, 100 mg phosphorus, 2 mg copper, 15 mg zinc, 10 μg chromium, 10 μg selenium, 10 μg molybdenum, 2.5 mg manganese, and 37.5 mg potassium. Sodium chloride was from Eli Lilly. Calcium was from TUMS (SKB). Potassium was from K-LYTE (Bristol). Choline was from Lee Nutrition. Microcellulose was from Avicel (FMC).

tracers were given with the eight small hourly meals, the composition of which was the same as the 6 days before the tracer protocol (Table 1). Leucine and energy intake during these 8 h corresponded to two-thirds of the total daily intake.

Casein-bound [1-^{13}C]leucine was derived from goat's milk. For this purpose, a lactating goat received a nonprimed, continuous infusion (0.8 g/h) of [1-^{13}C]leucine [99 atom% excess (APE); MassTrace, Woburn, MA] via a jugular vein catheter. After the start of the isotope infusion, milk was collected by hand, initially for every hour for 12 h and thereafter every 6 h until 24 h after the infusion was stopped. Fresh milk samples were placed immediately on ice and were processed within 1 h to separate total casein from fat, whey proteins, and lactose, all as described previously (3). A maximum ^{13}C enrichment of 29 ^{13}C APE in the leucine moiety of casein was achieved. The batches included in the experimental diets were checked for absence of ^{13}C enrichment in alanine, glycine, serine, glutamic acid/glutamine, proline, and aspartic acid/asparagine by gas chromatography-combustion-isotope ratio mass spectrometry (32a).

For the tracer phase of the experiment, the labeled casein was given at a rate that corresponded to a [1-^{13}C]leucine intake of 2.5 μmol·kg^{-1}·h^{-1}. To achieve this, the labeled goat's casein was diluted with unlabeled casein. Because unlabeled goat's casein was not available in sufficient amounts, we used unlabeled cow's casein (New Zealand Milk Products, Santa Rosa, CA) during diet *days 1–6* instead of unlabeled goat's casein. To make sure that the use of unlabeled cow's casein did not result in a different metabolic response, we compared the amino acid pattern (Table 2) and checked the background $^{13}CO_2$ breath enrichment (Fig. 1), which were both similar.

Tracer protocol and sample collection. On the morning of *day 7*, the subjects reported to the outpatient unit of the MIT-CRC at 0630 after an overnight fast. After recording body weight and vital signs, a 20-gauge 5-cm catheter was placed, under sterile conditions, into an antecubital vein of the nondominant arm for infusion. A second 20-gauge 3.2-cm catheter was inserted in a dorsal hand vein for blood sampling. Between samplings of blood, the intravenous lines were kept open with a slow drip of sterile physiological saline. Throughout the 8-h tracer protocol, the subjects remained in bed in a reclined position. The tracer protocol began at about 0815 with oral prime doses of [^{13}C]sodium bicarbonate (0.6 μmol/kg), L-[1-^{13}C]leucine (3.75 μmol/kg), and L-[$^{2}H_2$]-phenylalanine (2.5 μmol/kg) mixed into 24 ml distilled water. Simultaneously, prime doses of L-[$^{2}H_3$]leucine (3.75 μmol/kg) and L-[*ring*-$^{2}H_5$]phenylalanine (2.5 μmol/kg) were administered by vein in 10 ml sterile physiological NaCl solution over a 3-min period. Immediately after the priming doses were given, the first small hourly meal was ingested (*time 0*); the meal consisted of a small portion of cookies and either the intrinsically [1-^{13}C]leucine-labeled casein (corresponding to 2.5 μmol·kg^{-1}·h^{-1} [1-^{13}C]leucine), the unlabeled amino acid mixture plus free [1-^{13}C]leucine (2.5 μmol·kg^{-1}·h^{-1}), or unlabeled casein plus free [1-^{13}C]leucine (2.5 μmol·kg^{-1}·h^{-1}). These tracers were mixed into the protein-free formula (~150 ml/meal). In addition, [$^{2}H_2$]phenylalanine (2.5 μmol·kg^{-1}·h^{-1}) was given as oral tracer in distilled water (8 ml/h). Simultaneously, an intravenous continuous infusion of L-[$^{2}H_3$]leucine (2.5 μmol·kg^{-1}·h^{-1}) and L-[*ring*-$^{2}H_5$]phenylalanine (2.5 μmol·kg^{-1}·h^{-1}) was started. These tracers were continuously infused for 8 h by means of a screw-driven syringe pump (Harvard Apparatus, Millis, MA). When [1-^{13}C]leucine bound to casein was given, a small amount of unlabeled leucine was added to keep leucine intake equal with all treatments. Leucine intake with each meal was 9.9 mg·kg^{-1}·h^{-1}. Meals were isoenergetic (15.6 kJ·kg^{-1}·h^{-1}) and isonitrogenous (13.3 mg N·kg^{-1}·h^{-1}). Each meal contained ~11 g of fat, 40 g of carbohydrates, and 6 g of casein or amino acid mixture. Drinking water was allowed ad libitum. In the tracer-free studies, the same protocol was performed, but the leucine and phenylalanine tracers were replaced by the corresponding unlabeled amino acid, and the amounts of amino acid tracers usually given by intravenous infusion were given orally. The results of the labeled phenylalanine studies will be published separately.

Tracers were prepared from sterile powders of high chemical purity (>99%), high optical purity, and high isotopic enrichment under sterile conditions in either physiological NaCl (intravenous tracers) or distilled water (oral tracers). Tests for sterility and pyrogenicity were performed by an independent laboratory (Micro Test Laboratories, Agawam, MA). The L-[1-^{13}C]leucine and [$^{2}H_3$]leucine [all 99 atom% (AP)] were obtained from MassTrace, as were the L-[$^{2}H_2$]-

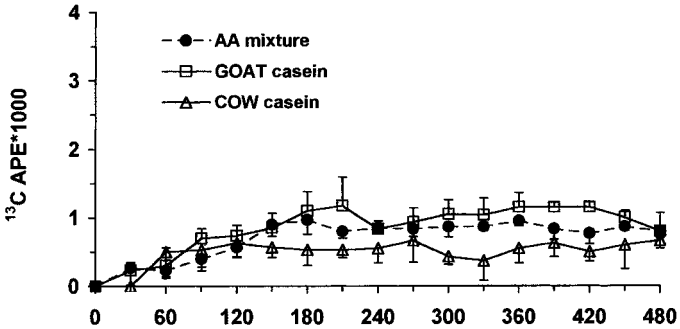

Fig. 1. Tracer free studies: comparative changes in the background ^{13}C enrichment of breath CO_2 when subjects were studied according to the tracer protocol when receiving as the N source either an L-amino acid (AA) mixture (●) or casein (cow and goat; △ and □) but without the leucine, phenylalanine, and bicarbonate tracers. Values are expressed as difference from the prefeeding values [atom percent excess (APE) × 1,000].

Table 2. *Amino acid pattern of goat's and cow's casein and composition of L-amino acid mixture*

	Goat's Casein	Cow's Casein	AA Mixture
Alanine	29.28	35.96	31.92
Glycine	14.56	22.08	19.96
Leucine	105.28	106.68	105.07
Isoleucine	52.96	57.60	52.85
Valine	75.20	65.44	75.05
Serine	55.04	58.96	54.92
Threonine	56.80	48.48	56.67
Proline	76.16	63.20	75.99
Aspartic acid	—	—	68.73
Asparagine + aspartic acid	70.24	81.88	—
Glutamine	—	—	221.47
Glutamine + glutamic acid	190.24	188.48	—
Cystine	4.48	4.16	4.47
Methionine	29.68	26.60	30.81
Phenylalanine	52.96	54.04	52.84
Tyrosine	44.00	58.12	43.90
Lysine	76.48	79.36	95.04
Histidine	26.24	25.92	35.27
Arginine	29.92	36.56	36.00
Tryptophan	12.00	11.48	11.97

Units are mg amino acid (AA)/160 mg N. Lysine contained ·HCl in amino acid mixture. Histidine contained·HCl ·H_2O in amino acid mixture. Arginine contained ·HCl in amino acid mixture.

phenylalanine and the L-[*ring*-$^{2}H_5$]phenylalanine (99 AP). The [^{13}C]sodium bicarbonate (99 AP; Cambridge Isotope Laboratories, Andover, MA) was prepared as a solution of 25 mg sodium bicarbonate/ml distilled water.

Before withdrawal of each of the blood samples, the hand was placed in a custom-made warming box at 65°C for 10 min, to achieve arterialization of venous blood. The samples were collected in prechilled heparinized tubes before the start of the tracer administration (baseline samples) and at 30-min intervals starting at the zero time point for 480 min. Blood samples were promptly centrifuged (1,500 *g* for 10 min at 4°C), and the plasma was stored at −20°C until used for analysis. In *group 2*, at each time point, an additional 1-ml blood sample was drawn into prechilled EDTA tubes containing Trasylol (100 μl/ml blood; Bayer, Kankakee, IL) for insulin and glucagon determination. These plasma samples were stored at −80°C. In *group 3* (tracer-free studies) no blood was taken. Breath samples were collected into rubber bags every 30 min, immediately transferred to 15-ml evacuated glass tubes (Monoject; Cardinal Health), and stored at room temperature until analyzed. Total carbon dioxide production and oxygen consumption rates were determined with the aid of the indirect calorimeter (Deltatrac or Vmax; Sensormedics, Anaheim, CA) by using a ventilated hood system. Measurements were performed for 20 min during each hour.

Sample analysis. Carbon dioxide from breath was cryogenically trapped, and ^{13}C enrichment was analyzed by isotope ratio-mass spectrometry (Delta E; Finnigan MAT, Bremen, Germany). Plasma concentrations of leucine, isoleucine, valine, phenylalanine, and tyrosine were measured by ion exchange chromatography, postcolumn ninhydrin reaction, with detection at 570 nm for primary amino acids using an HPLC system (Beckman System Gold; Beckman Instruments, San Ramon, CA). Aliquots of plasma samples were deproteinized with 5% sulfosalicylic acid containing a known concentration of norleucine as internal standard. Supernatant (50 μl) was injected on a lithium spherogel column with a buffer flow rate of 0.8 ml/min at a temperature of 40°C. The amino acid pattern of goat's and cow's casein was determined after hydrolysis by a method that we have described recently (40).

The enrichments of plasma [1-^{13}C]- and [$^{2}H_3$]leucine, as well as the corresponding enrichments of the transamination product α-ketoisocaproic acid (α-[1-^{13}C]KIC and α-[$^{2}H_3$]KIC), were measured by electron-impact gas chromatography-mass spectrometry (70 eV) using an HP 5890 gas chromatograph coupled to an HP 5988 quadrupole mass spectrometer (Hewlett Packard, Palo Alto, CA). Plasma amino acids were isolated by cation exchange resin (Bio-Rad AG 50W-X8, 100–200 mesh, H+ form; Bio-Rad, Melville, NY) with 1 ml of 3 M NH_4OH followed by 1 ml double deionized H_2O. After the sample was dried under a stream of N_2, 50 μl acetonitrile and 50 μl *N*-methyl-*N*-tert-butydimethylsilyl trifluoroacetamide (Pierce, Rockford, IL) were added, and the tightly capped vial was heated for 1 h at 60°C to form tert-butyldimethylsilyl (*t*-BDMS) derivatives. One microliter was injected in splitless mode (purge on time 0.5 min) on a DB-1301 fused silica column (30 m × 0.25 mm ID; 0.25 μm; J and W Scientific, Folsom, CA). The temperature was programmed from 160 to 280°C at 15°C/min and then from 280 to 300°C at 20°C/min. Leucine eluted at ~5 min and was monitored for mass-to-charge ratio (*m*/*z*) 302–305. Unlabeled leucine was measured at its base peak *m*/*z* 302 [*M*-57]$^+$, [1-^{13}C]leucine at *m*/*z* 303, and [$^{2}H_3$]leucine at *m*/*z* 305.

α-KIC enrichments were measured in quinoxalinol-*t*-BDMS derivatives, which were prepared as described previously (12), except that acetonitrile was used instead of pyridine. Separation of α-KIC derivatives was performed on a temperature-programmed DB 1301 column (30 m × 0.25 mm ID; 0.25 μm; J and W Scientific; 100–280°C at a rate of 30°C/min). α-KIC eluted at ~6.9 min, and selected ion monitoring was carried out for *m*/*z* 259 [*M*-57]$^+$, *m*/*z* 260, and *m*/*z* 262 for natural, α-[1-^{13}C]KIC, and α-[$^{2}H_3$]KIC, respectively.

For purposes of calibration, a training data set was created by compiling the mass spectral response of graded mixtures of [1-^{13}C]- and [$^{2}H_3$]leucine and α-[1-^{13}C]- and α-[$^{2}H_3$]KIC together with unlabeled leucine and unlabeled α-KIC, respectively, over a 0–10 mole fractional range for each tracer. Multilinear regression was then used to generate a prediction equation correlating the ion pair area ratios ($m+1/m+0$, $m+3/m+0$) obtained spectrometrically on standards (100% tracee and tracers) as the explanatory variable for tracer-to-tracee mole ratios. To account for a potential cross-contribution of one tracer into the target ion of the other tracer (e.g., [1-^{13}C]leucine contribution to $m+3$), ion pair area ratios $m+1/m+0$ and $m+3/m+0$ for both tracers were considered in the prediction equation. The equation was then applied in determining ion pair mole ratios from the corresponding spectral information obtained with the plasma samples, and the resulting mole ratios were converted algebraically into tracer mole percent excess (MPE) for each tracer, after subtraction of the corresponding baseline plasma values for each set of samples. Analysis of replicate standards ($n = 5$) under these conditions showed a coefficient of variation ranging from 2 to 7%, the latter values being those for the detection of 0.5 MPE of either leucine or α-KIC isotopolog in the presence of a 5 MPE of the other. Also, by way of validation, the accuracy of estimates for this methodological approach was found to fall within 5% of expected values on average, based on analysis of sham mixtures prepared gravimetrically and with known tracer(s)-to-tracee composition (2 MPE).

Hormone concentrations were measured for *group 2* only (AA + [^{13}C]Leu vs. [^{13}C]Leu-casein). Plasma insulin concentrations were determined by an enzyme immunoassay (Mercodia Insulin ELISA; ALPCO American Laboratory Products, Windham, NH). An RIA was used to measure plasma glucagon concentrations (Euro-Diagnostica, Malmö, Sweden).

Background breath $^{13}CO_2$ enrichments. Under conditions identical to those followed in the main experiment (*day 7*; 8 small hourly meals), four additional subjects (*group 3*) were studied without administration of any tracer. Breath samples were taken at 30-min intervals. The aim was to determine the breath $^{13}CO_2$ background enrichment throughout the 8-h study and to use the values to correct for the ^{13}C due to diet alone (Fig. 1).

Data evaluation. Leucine oxidation (Leu Ox) was computed for each half hourly interval during the 8-h tracer protocol. Leucine oxidation during the first hour of the study was taken to be equal to that measured at the end of the 2-h period to avoid any possible effect of the bicarbonate prime

$$\text{Leu Ox } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\ \text{min}^{-1}) = \frac{^{13}CO_2 \text{ production } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\ \text{min}^{-1})}{^{13}\text{C plasma } \alpha\text{-KIC enrichment (MPE)}} \times 100$$

where

$$^{13}CO_2 \text{ production } (\mu\text{mol}\cdot\text{kg}^{-1}\cdot 30\ \text{min}^{-1}) = \dot{V}_{CO_2}\ (\text{mmol}\cdot\text{kg}^{-1}\cdot 30\ \text{min}^{-1}) \times {}^{13}CO_2 \text{ breath enrichment} \times 1/R$$

where $^{13}CO_2$ breath enrichment is calculated as APE × 1,000 (corrected for background shift due to diet only), and R is recovery of $^{13}CO_2$ [as reported for fed state: 79% (27)].

After determining that isotopic steady state existed for the last 4 h of the tracer protocol (by testing for absence of a significant slope when analyzed with linear regression), mean plateau enrichment values were used to calculate leucine rate of appearance (R_a) as noted below. Leucine turnover (R_a) was calculated as follows

$$\text{Leu } R_a = \text{ir} \times (E_i/E_p - 1)$$

where ir is the tracer infusion rate or rate of oral administration ($\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$), E_i is the enrichment in APE of the administered isotope ([1-^{13}C]Leu, [$^2H_3$]Leu), and E_p is the enrichment of the respective leucine isotope or of its transamination product α-KIC in plasma at each isotopic steady state.

Leucine splanchnic uptake (Leu Spl Upt) was computed from plasma enrichments (E) of orally ($E_{[1\text{-}^{13}C]Leu}$) and intravenously administered leucine ($E_{[^2H_3]Leu}$), at isotopic steady state, normalized for infusion or administration rate (ir) of tracers, as follows

$$\text{Leu Spl Upt} = \{1 - [(E_{[1-^{13}C]Leu}/ir_{[1-^{13}C]Leu})/(E_{[^2H_3]Leu}/ir_{[^2H_3]Leu})]\} \times 100$$

This value is the fraction of leucine taken up by the gut and the liver during its first pass.

The relationship between leucine rate of appearance and individual components of whole body protein turnover is represented by

$$R_a = D + I + i = NOLD + Ox$$

where D is protein degradation, I is leucine dietary intake, i is leucine tracer intake, NOLD is the nonoxidative leucine disposal via protein synthesis, and Ox is leucine oxidation. Hence

$$NOLD = R_a - Ox$$

whereas

$$D = R_a - (I + i)$$

The fraction of turnover that was oxidized (FR_{Ox}) during the experiment at isotopic steady state was calculated as follows

$$FR_{Ox}(\%) = (\text{Leu Ox}/\text{Leu } R_a) \times 10^2$$

The foregoing estimates used steady-state equations because we have found in our previous studies with small frequent meals that results obtained using an approach for non-steady-state conditions gave essentially identical estimates (16). Furthermore, the present approach used for estimation of leucine oxidation gives values that agree well with predicted rates of leucine oxidation based on considerations of nitrogen excretion and balance (12).

Statistical summary. Values are expressed as means ± SD. All analyses were conducted separately for the two groups of subjects. For outcomes that were measured with two leucine tracers (e.g., rate of appearance: ig [1-^{13}C]Leu or iv [$^2H_3$]Leu) the effect of the form of the leucine tracer was determined using a paired *t*-test. For all outcomes, paired *t*-tests were used to compare tracer forms within each group. Means were considered to be significantly different at $P < 0.05$.

RESULTS

Plasma α-KIC and leucine enrichments. Plasma α-[$^2H_3$]KIC enrichments were significantly higher than α-[1-^{13}C]KIC enrichments with the exception of AA + [^{13}C]Leu. Plasma [^{13}C]leucine and [$^2H_3$]leucine enrichments were significantly different with all tracer forms (Table 3).

These enrichment data were used to determine the kinetic parameters of leucine metabolism, including rates of leucine appearance and oxidation.

Leucine oxidation. The course of total CO_2 production did not differ between the diets in both groups. The pattern of $^{13}CO_2$ excretion throughout the 8-h tracer study for *group 1* receiving diets supplying intrinsically labeled casein or [1-^{13}C]leucine added to unlabeled casein (extrinsically labeled) differed only marginally. In contrast, different patterns of $^{13}CO_2$ excretion emerged when subjects (*group 2*) ingested diets containing [1-^{13}C]leucine added extrinsically to an L-amino acid mixture simulating the casein pattern (AA + [^{13}C]Leu) compared with the intrinsically labeled casein ([^{13}C]Leu-casein; Fig. 2). Figure 3 shows the course of the whole body leucine oxidation derived from the

Table 3. *Mean plasma α-KIC and leucine enrichments (^{13}C, $^2H_3$; 240–480 min), leucine rates of appearance based on α-KIC enrichments, and prandial leucine oxidation with different tracer forms*

	[^{13}C]leucine	[$^2H_3$]leucine
Plasma KIC enrichment, MPE		
Group 1		
Casein + [^{13}C]Leu	1.54 ± 0.29	2.01 ± 0.04*
[^{13}C]Leu-casein	1.53 ± 0.22	1.85 ± 0.12*
Group 2		
AA + [^{13}C]Leu	1.60 ± 0.14†	1.72 ± 0.16
[^{13}C]Leu-casein	1.41 ± 0.15	1.74 ± 0.10*
Plasma leucine enrichment, MPE		
Group 1		
Casein + [^{13}C]Leu	1.66 ± 0.13	2.21 ± 0.10*
[^{13}C]Leu-casein	1.62 ± 0.10	2.10 ± 0.19*
Group 2		
AA + [^{13}C]Leu	1.50 ± 0.12	2.09 ± 0.11*
[^{13}C]Leu-casein	1.35 ± 0.27	2.00 ± 0.09*
Rate of appearance, $\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$		
Group 1		
Casein + [^{13}C]Leu	87.49 ± 22.12	63.30 ± 3.15*
[^{13}C]Leu-casein	88.08 ± 17.79	67.94 ± 4.13*
Group 2		
AA + [^{13}C]Leu	80.65 ± 8.46‡	72.46 ± 6.34
[^{13}C]Leu-casein	96.25 ± 8.31	71.58 ± 4.17*
Leucine oxidation, $\mu mol \cdot kg^{-1} \cdot 30\ min^{-1}$		
Group 1		
Casein + [^{13}C]Leu	21.40 ± 2.91	
[^{13}C]Leu-casein	18.17 ± 1.87	
Group 2		
AA + [^{13}C]Leu	24.88 ± 2.12‡	
[^{13}C]Leu-casein	19.29 ± 1.94	

Values are means ± SD. KIC, ketoisocaproic acid; MPE, mole percent excess; Leu, leucine. *Significantly different between [^{13}C]leucine (ig) and [$^2H_3$]leucine (iv) tracer. †Significantly different from intrinsically labeled casein, $P < 0.01$. ‡Significantly different from intrinsically labeled casein, $P < 0.05$.

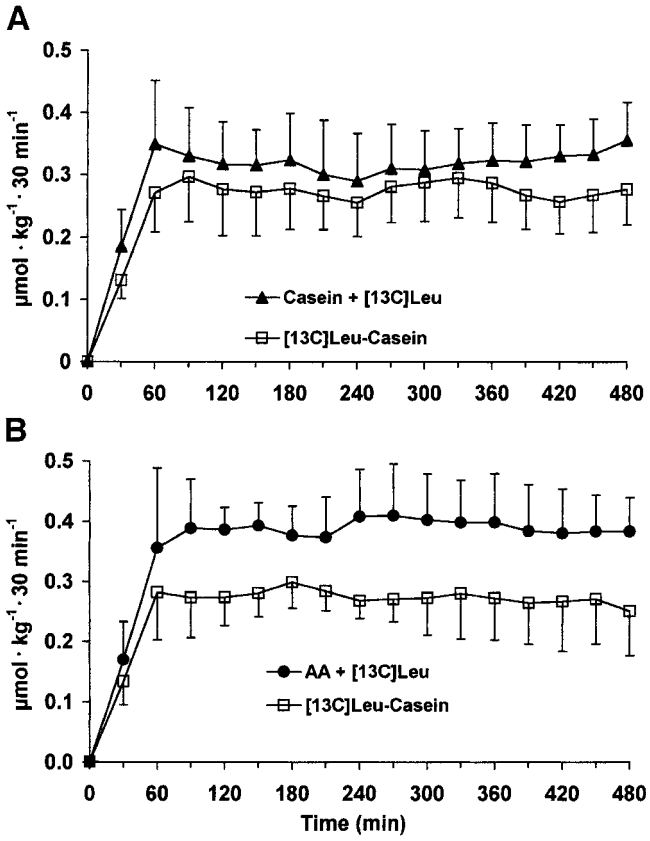

Fig. 2. $^{13}CO_2$ production (µmol·kg^{-1}·30 min^{-1}) expressed as mean ± SD per 30-min interval. *A*: *group 1* (n = 5) receiving, as a tracer, either intrinsically [1-^{13}C]leucine (Leu)-labeled casein (□) or [1-^{13}C]leucine added to unlabeled casein (▲). *B*: *group 2* (n = 5) receiving, as a tracer, either intrinsically [1-^{13}C]leucine-labeled casein (□) or [1-^{13}C]leucine added to the free L-amino acid mixture (●). Small meals were ingested at 60-min intervals starting at *time 0*.

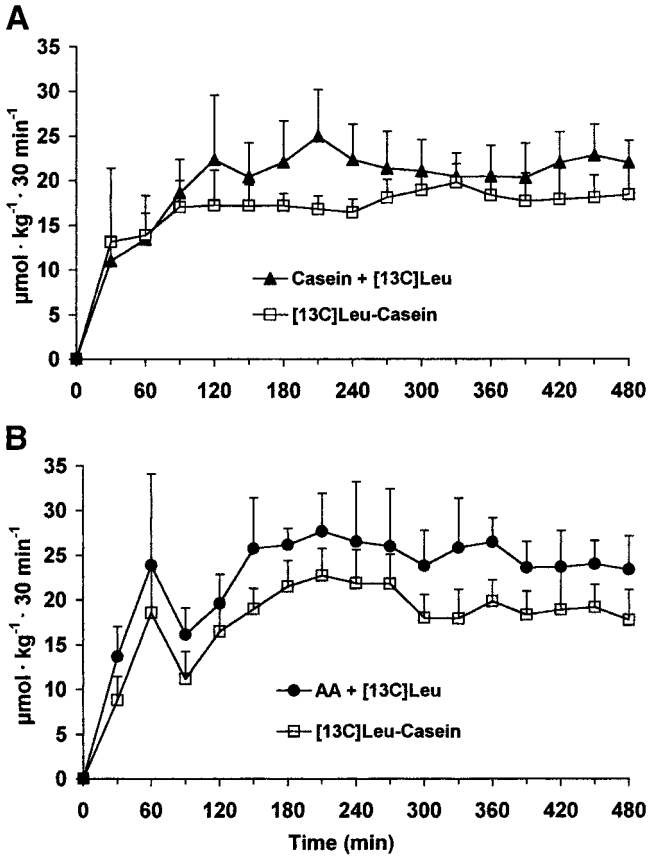

Fig. 3. Leucine oxidation (µmol·kg^{-1}·30 min^{-1}) for each 30 min-interval throughout the 8-h tracer study. The leucine tracer was supplied as either free [1-^{13}C]leucine together with unlabeled casein (▲) or as intrinsically labeled casein (□) in *A* or as free [1-^{13}C]leucine together with a free L-amino acid mixture (●) or an intrinsically labeled casein (□) in *B*. Eight small mixed meals providing 9.9 mg/kg leucine each were fed at 60-min intervals starting at *time 0*.

$^{13}CO_2$ output and plasma α-[^{13}C]KIC enrichments for *groups 1* and *2*.

The rates of leucine oxidation during the last 4 h of the 8-h feeding period (240–480 min) and leucine rates of appearance after oral and intravenous tracer administration derived from plasma α-[^{13}C]KIC and α-[$^{2}H_3$]KIC enrichments are summarized in Table 3 for *groups 1* and *2*. Leucine oxidation was significantly higher when free leucine was given together with the crystalline amino acid mixtures compared with the diet with the intrinsically labeled casein. For tracer AA + [^{13}C]Leu, total leucine intake was 37.14, and for intrinsically labeled casein it was 36.69 µmol·kg^{-1}·30 min^{-1}. Hence, in comparison with the leucine oxidation data (Table 3), this suggests a substantial leucine retention during this 8-h period by both experimental groups.

Rate of appearance, nonoxidative leucine disposal, protein degradation, and splanchnic uptake. The rate of appearance was lower for the intravenously administered [$^{2}H_3$]leucine tracer (Table 3) with the exception of the AA + [^{13}C]Leu tracer. The rate of appearance measured after the intake of amino acid mixture plus free [^{13}C]leucine was significantly lower than after the intrinsically labeled casein (Table 3; $P < 0.05$).

With the use of plasma α-[^{13}C]KIC enrichment, nonoxidative leucine disposal and also the difference between nonoxidative leucine disposal and protein degradation (net protein synthesis) was significantly higher, whereas the fraction of the turnover oxidized was significantly lower when the intact intrinsically labeled casein was ingested (*group 2*; Table 4).

Splanchnic uptake calculated from the plasma leucine enrichment ratio of orally (1-^{13}C) and intrave-

Table 4. *NOLD, D, NOLD-D, and FR_{Ox} calculated from α-[^{13}C]KIC plasma enrichment data*

	Group 1		*Group 2*	
	Casein + [^{13}C]Leu	[^{13}C]Leu-casein	AA + [^{13}C]Leu	[^{13}C]Leu-casein
NOLD	66.09 ± 17.36	70.02 ± 14.81	55.77 ± 10.38	76.97 ± 9.11*
D	49.78 ± 18.16	50.89 ± 14.19	43.52 ± 8.46	58.11 ± 8.35
NOLD-D	16.31 ± 3.04	19.13 ± 1.83	12.25 ± 2.36	18.86 ± 4.45†
FR_{Ox}, %	25.24 ± 5.05	21.15 ± 3.96	31.45 ± 5.87	20.22 ± 2.89†

Values are means ± SD. Units are µmol·kg^{-1}·30 min^{-1}. NOLD, nonoxidative leucine disposal; D, protein degradation; NOLD-D, net protein synthesis; FR_{Ox}, fraction of flux oxidized. *Significantly different from AA + [^{13}C]Leu, $P < 0.05$. †Significantly different from AA + [^{13}C]Leu, $P < 0.01$.

nously ($^2H_3$) administered leucine tracers was not different between tracer forms AA + [^{13}C]Leu and intrinsically labeled casein (*group 2*: 0.28 ± 0.08 vs. 0.35 ± 0.11) and casein + [^{13}C]Leu and intrinsically labeled casein (*group 1*: 0.26 ± 0.02 vs. 0.24 ± 0.09), respectively.

Hormones and leucine plasma concentrations. Plasma insulin showed a meal-driven pattern, whereas the amplitude appeared to be smaller with the casein diet compared with the free amino acid mixture. The mean overall plasma insulin level did not differ significantly between the two diets (Fig. 4*A*). No systematic differences appeared to occur for plasma glucagon concentration (Fig. 4*B*), and the insulin-to-glucagon ratio (data not shown) was not significantly different between the intact protein and amino acid diets.

The mean plasma leucine concentration during the intake of AA + [^{13}C]Leu was significantly higher ($P < 0.01$) than after the casein diet (Fig. 5). There was no difference in plasma leucine concentrations between the two casein-based diets (data not shown). The concentrations of isoleucine and valine in plasma were also higher when the free amino acid mixture was consumed compared with the casein diet. Plasma phenylalanine and tyrosine concentrations were apparently not different among the diets (data not shown).

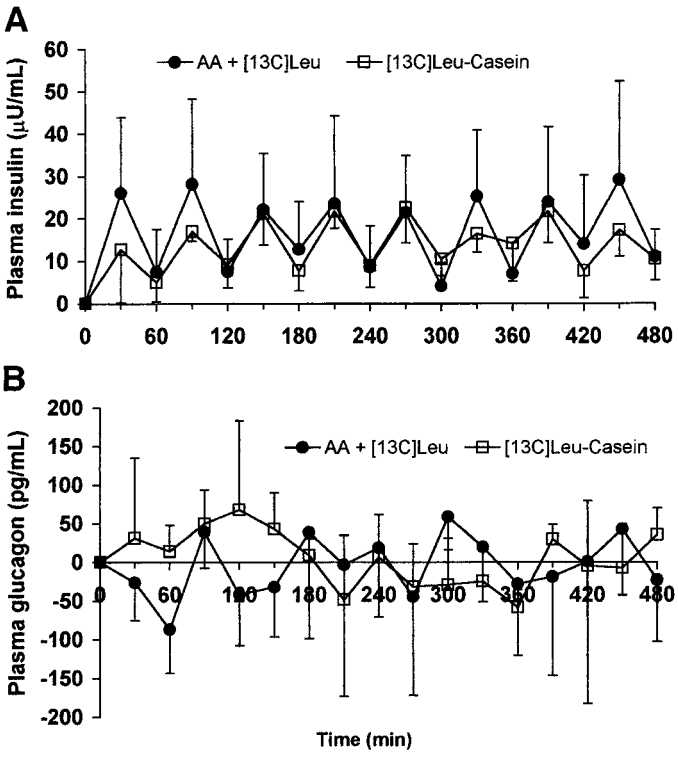

Fig. 4. Temporal evolution of plasma insulin (μU/ml; *A*) and plasma glucagon (pg/ml; *B*) concentration (as difference vs. 0 baseline) during the 8-h tracer study. Eight small mixed meals providing 9.9 mg/kg leucine each were given at 60-min intervals starting at *time 0*. Values are shown for *group 2* receiving as tracer either free [1-^{13}C]leucine together with a free L-amino acid mixture (●) or intrinsically labeled casein (□).

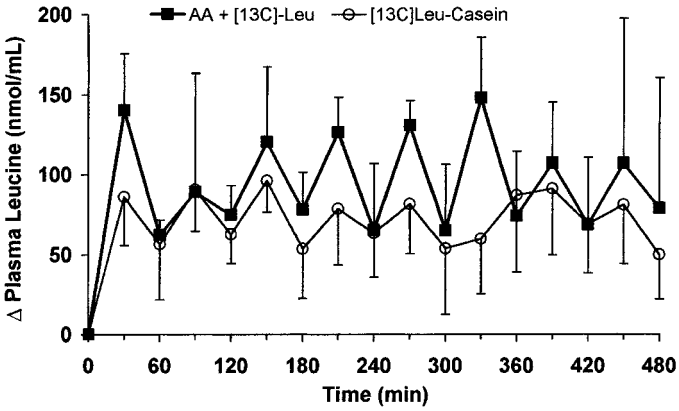

Fig. 5. Temporal evolution of free plasma leucine concentration (nmol/ml; as difference vs. 0 baseline) in *group 2* during the 8-h tracer study. Eight small mixed meals providing 9.9 mg/kg leucine each were given at 60-min intervals starting at *time 0*. The tracer forms were either free leucine together with a free L-amino acid mixtures (■) or intrinsically labeled casein (○).

DISCUSSION

This study has revealed that, during the fed state, leucine oxidation is higher when a [^{13}C]leucine tracer is ingested as free [^{13}C]leucine together with a free amino acid mixture compared with an intrinsically [^{13}C]leucine-labeled casein (Table 3). Together with the finding of a higher NOLD with the intrinsically labeled casein (Table 4), this suggests that a higher proportion of dietary leucine derived from intact casein is utilized during the absorptive phase for whole body protein synthesis in comparison with that from an equivalent intake of free L-amino acids.

These observations have a number of important implications, as follows.

The first implication is the impact of the present results on leucine requirements as estimated by the tracer-balance technique. Previously, we conducted 24-h tracer studies in which the minimal physiological requirement for leucine and other indispensable amino acids was estimated by the tracer-balance technique (e.g., see Refs. 1, 11, 12, and 29). The requirement values derived from these studies are two to three times higher than the current recommendations by the Food and Agricultural Organization (FAO)/World Health Organization (WHO)/United Nations (UNU) (15), which were based on results from nitrogen balance experiments. Although a more recent UN expert group has acknowledged that these latter values are no longer nutritionally relevant (14), the UN has not yet adopted a new set of tentative new values for adult human requirements for indispensable amino acids such as those proposed by the MIT group (48). Detailed discussions of this ongoing debate have been published (20, 33, 47, 49).

As noted in the RESULTS, all of the subjects were in a marked positive leucine balance during the 8-h tracer period, although balance was lower when the L-amino acid mixture was consumed (Table 3). However, because the subjects were receiving a generous intake of leucine, about eight times the required intake accord-

ing to the FAO/WHO/UNU (15) or three times that which we have proposed as the leucine requirement (11, 12, 48), it would be reasonable to predict that a daily body leucine equilibrium would prevail for all of the diets in essentially all of these healthy adults. On this basis, the rate of leucine oxidation during the postabsorptive period would probably compensate for fed-state differences, and the rate at this time would be predicted to be lower when [1-^{13}C]leucine plus the L-amino acid mixture was given compared with the casein-based diets. The investigations by Pacy et al. (38) on the diurnal rhythm of leucine and nitrogen metabolism in subjects consuming different intakes of protein would appear to support this prediction. To test this hypothesis, it will be necessary to conduct continuous 24-h tracer studies, as previously carried out in our laboratory. This too has implications for the design of studies concerned with establishing the whole body mechanisms responsible for determining body protein balance at different energy and protein intakes (5–7, 21, 38).

An important and as yet unanswered question is whether similar results would have been obtained at the test leucine intake level of 40 mg·kg^{-1}·day^{-1} and/or when evaluated using a 24-h protocol. This would be difficult to test with the present paradigm, because, for a diet containing an intrinsically labeled protein to provide peptide-bound leucine at a level of 40 mg leucine·kg^{-1}·day^{-1} or less, this would require supplementation with a leucine free amino acid mixture so that an adequate total nitrogen intake is given. Thus, although giving the tracer in the form of an intrinsically labeled protein would appear to be the closest simulation of a normal dietary amino acid intake, there are clearly some limitations to this model. Furthermore, the marked differences between the MIT estimates of requirements using the tracer-balance concept and those of Rose (43), which have served as an important data base for the proposed UN requirement values (15), would not be explained by differences in the molecular form of the amino acid ingested, because all of these studies essentially involved use of free amino acid-based diets.

An additional issue with respect to the present and our previous studies on the leucine requirements of healthy adults (12) is that the [^{13}C]leucine tracer was given via the intravenous route in contrast to the oral route in the present study. However, we do not think that this limits the significance of the present findings and their possible implications for the evaluation of amino acid requirements, because we have not found any important differences between estimates of leucine oxidation caused by the route of administration of the [^{13}C]leucine tracer (25).

The second implication is the influence of the nature of intrinsically labeled protein on protein metabolism. We observed a significantly lower leucine oxidation and higher NOLD when the intrinsically labeled casein was given in comparison with the L-amino acid diet (*group 2*; Table 4). First-pass splanchnic extraction of leucine was between 24 and 35%, confirming earlier results (4, 10, 25, 26). Leucine rate of appearance calculated from α-[$^{2}H_3$]KIC and α-[1-^{13}C]KIC enrichments were comparable with results of an earlier study where a 1.5 g protein·kg^{-1}·day^{-1} diet was provided (26).

Although we did not find an effect of the forms of dietary nitrogen or of the leucine tracer administered on estimates of splanchnic extraction, it would be of interest to examine the effect of the molecular form of nitrogen intake on the possible site of leucine oxidation and of NOLD (splanchnic or peripheral). In this context, therefore, the increase of the mean total plasma amino acid concentration in humans was significantly higher after consuming a meal containing a free amino acid mixture simulating cottage cheese protein in comparison with the intact protein control (22). Here, the isoleucine, leucine, and lysine plasma concentrations peaked earlier (30 min after the meal) and higher and declined more rapidly than after the cottage cheese meal was ingested (60 min after the meal; see Ref. 23). We found in the present study a higher mean plasma leucine, isoleucine, and valine concentration during the ingestion of the free amino acid diet that may be causally related to the observed higher rate of leucine oxidation.

The extent to which the difference in leucine oxidation between the L-amino acid mixture and the intrinsically labeled casein is due to a difference in the rate of amino acid absorption per se is not clear. In the pig, peak absorption of amino acids from a milk enzyme hydrolyzate occurred earlier and at a higher level than with an amino acid mixture of identical pattern (41), although the differences disappeared ~1 h after feeding (42). In general, it appears that amino acid absorption from peptides is more rapid than from amino acid mixtures (31), and a recent report showed that feeding of oligopeptides (casein hydrolyzate) induced a higher oxidation of leucine but also a higher rate of protein synthesis and a lesser inhibition of protein breakdown compared with intact casein (9). Furthermore, Beaufrere and co-workers [Boirie et al. (5)] recently proposed the concept of "slow and fast dietary proteins." Their investigation revealed that two major milk proteins (casein and whey) have different metabolic fates related to the apparent rate of amino acid absorption when a single protein meal, without addition of energy substrate, was given. From their results, the slowly absorbed dietary protein promoted postprandial protein deposition by an inhibition of protein breakdown, whereas the "fast" dietary protein stimulated protein synthesis, as well as oxidation. However, the significance of these findings for our understanding of the metabolic basis of the requirements for indispensable amino acids is unclear for a number of reasons, including the fact that a single meal of protein alone was given. Also, from the leucine oxidation data, it appeared that, in their experiment, there was essentially no prandial retention of the dietary leucine. This is not consistent with the apparently high nutritional quality of whey protein, based on nitrogen balance measurements (17, 18). Minimally, studies involving more complete meals would be desirable. Furthermore, intesti-

nal amino acid absorption may adaptively respond to alterations in amino acid/protein intake (28), so a period of adjustment to the test dietary interventions compared would constitute a further improvement in the experimental approach. In summary, the available literature does not permit a clear determination as to whether differences in the rates of amino acid and/or tracer absorption, per se, are responsible for the present findings on the differences in rate of leucine oxidation among the diet/tracer.

Another consideration, with reference to a full interpretation of the nutritional significance of the present findings, is that of the meal pattern. Here we used the frequent, small, and equal meal paradigm that has been applied in most of our amino acid kinetic/requirement studies (11, 29) as well as by other authors (21, 39). However, meal patterns can affect the fate of absorbed amino acids, because we showed that urinary excretion of total nitrogen and leucine oxidation were lower when an isonitrogenous isocaloric intake of three meals per day was given compared with multiple small meals (13). For comparison, in the pig, phenylalanine oxidation was found to be higher when the mixed diet containing free lysine plus grain was given once daily compared with the administration of six equal small meals (2). It is interesting to speculate that the amino acid supply during parenteral nutrition, with the prompt rise in plasma and tissue amino acid concentration, mimics the situation of "fast" protein absorption and possibly limits the efficiency of amino acid retention caused by promotion of amino acid oxidation. Furthermore, to finally assess the nutritional significance of tracer studies of the kind used here and by others, it seems likely that the paradigm might usefully include relatively long-term tracer infusions lasting for at least one complete day.

Also, the question arises as to whether the nature of the specific labeled protein chosen for our study may have influenced the results. That is, would a different intrinsically labeled protein, such as a highly digestible vegetable protein, have demonstrated a lower rate of leucine oxidation and a higher nonoxidative leucine disposal compared with the respective amino acid mixture? Thus a recent study (39) in elderly women showed that when dietary protein intake was increased through addition of vegetable protein, postabsorptive protein breakdown was not inhibited to the same extent as that occurring when animal protein was given. The study also showed that net protein synthesis during the fed period of the day was less with feeding high vegetable protein vs. a high animal protein diet. These kinetic or leucine turnover differences were observed despite the fact that both high-protein diets supplied a generous total nitrogen intake (201–209 mg $N \cdot kg^{-1} \cdot day^{-1}$), and the subjects were in daily body nitrogen balance. Again, we make these points not only to underscore the possibility that the present findings with casein may not necessarily predict those with other good-quality protein sources but also to emphasize caution when drawing nutritional interpretations from leucine kinetics that apply to relatively brief windows of time during the day.

In conclusion, the present and earlier findings reveal that the immediate metabolic fate of absorbed amino acids is determined by a complex interaction of factors, including the molecular form of the amino acid ingested, the amino acid profile, the composition of the meal, the level of intake, and the pattern of meal ingestion.

This research was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-42101 and P-30-DK-40561 RR8 and by a grant of the Deutsche Forschungsgemeinschaft, Bonn, Germany.

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Received 13 May 1999; accepted in final form 29 November 1999.

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