Whole-Body Nitrogen and Splanchnic Amino Acid Metabolism Differ in Rats Fed Mixed Diets Containing Casein or Its Corresponding Amino Acid Mixture[1]

Maren Daenzer, Klaus Jürgen Petzke, Brian J. Bequette* and Cornelia C. Metges[2]

Deutsches Institut für Ernährungsforschung (DIfE), D-14558 Bergholz-Rehbrücke, Germany and
**Rowett Research Institute, Bucksburn, Aberdeen, AB295SB, Scotland, UK*

ABSTRACT Whole-body and splanchnic metabolism of dietary amino acids derived from casein (CAS) or the corresponding crystalline L-amino acid mixture (AA) were compared. Male adult rats were adapted for 9 d to two isoenergetic, isonitrogenous diets (15 g/100 g protein, 5 g/100 g fat) containing either CAS or AA. On d 10, the rats were fed a single mixed meal (3 g dry mass) containing either intrinsically ^{13}C-labeled goat casein or the amino acid mixture containing [U-$^{13}C_6$] leucine and [α-^{15}N] lysine. Rats were killed before and 1, 3, 5 and 7 h after meal ingestion and samples of plasma, stomach wall and contents, small intestine and liver were collected. ^{13}C and ^{15}N enrichments of free and protein-bound amino acids in plasma and tissues were analyzed by gas chromatography-combustion isotope ratio mass spectrometry. Urinary nitrogen excretion was higher ($P < 0.05$) and weight gain lower ($P < 0.05$) in rats given the AA diet, indicating a lower whole-body net protein synthesis. Free ^{13}C-leucine from the AA diet appeared in the intestinal mucosa free pool more rapidly ($P < 0.05$) than the CAS-^{13}C-leucine, probably due to the faster transit through the stomach of the AA group. However, the incorporation of dietary leucine into plasma and liver proteins was higher in the CAS group 7 h after the meal ($P < 0.05$), whereas lysine incorporation into liver protein was higher in the AA group ($P < 0.05$). We conclude that whole-body protein homeostasis is better supported by dietary casein-bound than crystalline free amino acids, and that protein-bound leucine, but not lysine, is used more efficiently for liver protein synthesis than dietary free leucine. J. Nutr. 131: 1965–1972, 2001.

KEY WORDS: • *^{13}C leucine* • *^{15}N lysine* • *splanchnic tissue* • *stable isotopes* • *mass spectrometry*

Dietary protein quality is usually assessed by amino acid composition and protein digestibility (1). Recent observations that the molecular form of the ingested nitrogen (free amino acids, peptides or proteins) and the type of dietary protein (casein vs. whey) affect amino acid absorption kinetics, the degree of whole-body amino acid oxidation and utilization for protein synthesis (2–6), suggest that these variables may also require consideration when assessing protein quality. To date, however, the influence of these factors has been assessed only by examination of the metabolic utilization of one amino acid, leucine.

Work conducted by Young and colleagues (7) at the Massachusetts Institute of Technology has attempted to better define the amino acid requirement pattern for humans. In large part, these dietary studies have involved the use of defined L-amino acid mixtures and assessment of amino acid balance (requirement) based on intravenous infusion of selected tracer amino acids. In light of the fact that the molecular form of the dietary nitrogen may affect the efficiency of amino acid utilization, it is relevant to reevaluate these amino acid requirement patterns by assessment of dietary protein-bound amino acid use. Furthermore, results from human and pig studies also suggest that there is substantial utilization of dietary amino acids by the splanchnic tissues during the absorptive process (8–12). In humans, first-pass splanchnic uptake (gut and liver) of leucine was higher when ingested as casein-bound leucine compared with free crystalline leucine (2, unpublished data). Consequently, the use of the intravenous tracer balance technique may overestimate amino acid requirements due to the failure to consider first-pass metabolism (splanchnic uptake), and the degree to which this occurs may differ among amino acids and with different forms of protein.

The objectives of this study were as follows: *1*) to compare whole-body nitrogen homeostasis and growth efficiency of rats offered diets based on either casein or a free amino acid mixture equivalent to the amino acid pattern of casein; and *2*) to compare the fate of dietary leucine and lysine in the splanchnic tissues and in plasma when these amino acids are ingested as casein (intrinsically labeled) or as part of a mixture of crystalline free amino acids (extrinsically labeled), simulating the amino acid pattern of casein. We also examined the appearance of a range of other indispensable and dispensable casein-bound amino acids in splanchnic tissues and in plasma.

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[2] To whom correspondence should be addressed.
E-mail: metges@www.dife.de

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TABLE 1

Composition of mixed diets containing either an L-amino acid mixture (AA) or casein (CAS) fed to adult rats during the experimental period (d 1–10).

Component	AA	CAS
	g/kg diet	
Wheat starch[1]	580	580
N-source		
AA mixture	150	—
Casein[2]	—	150
Sucrose[3]	100	100
Sunflower oil[4]	50	50
Cellulose[5]	50	50
Mineral mixture[6]	50	50
Vitamin mixture[6]	20	20

[1] Kröner GmbH & Co.KG, Ibbenbüren/Westfalen, Germany.
[2] Dauermilchwerk Peiting GmbH, Landshut, Germany.
[3] Südzucker, Zeitz, Germany.
[4] Europe GmbH, Hamm, Germany.
[5] J. Rettenmaier und Soehne GmbH, Rosenberg, Germany.
[6] Altromin, Lage, Germany. Vitamin mixture (mg/kg diet): vitamin A (5.16), cholecalciferol (0.013), E (163.95), K-3 (10.0), thiamine (20.04), riboflavin (20.32), B-6 (15.3), B-12 (0.041), niacin (50.0), panthothenate (50.1), folic acid (10.01), biotin (0.20), choline chloride (1011.5), *p*-amionobenzoic acid (100.0), inositol (111.0), vitamin C (20.0), methionine (3.5). Mineral mixture (mg/kg diet): Ca (9500.8), Mg (750.8), P (7500.5), Na (2500.5), K (7042.7), S (2810.6), Fe (180.1), Mn (100.8), Zn (30.4), Cu (12.2), J (0.45), F (4.19), Se (0.314), Co (0.13), Ni (0.002).

MATERIALS AND METHODS

Animals, diets and experimental design

The experiment was performed in accordance with the guidelines of the ethics committee of the Ministry of Agriculture, Nutrition and Forestry (State Brandenburg, Germany, Permission No. 48–3560-0/3). Two groups (n = 22/group) of male adult rats (Shoe Wistar, Tierzucht Schoenwalde, Germany) weighing ~170 g were housed in individual cages under controlled conditions of light (12-h light:dark cycle) and temperature (22°C). The rats were adapted for 9 d to two adequate isoenergetic and isonitrogenous diets (**Table 1**) containing either casein (CAS)[3] or a free crystalline L-amino acid mixture simulating the pattern of casein (AA) as the sole dietary nitrogen sources (**Table 2**). The diets were offered as two meals per day (2 × 8 g dry mass), and the rats were trained to consume the meal within 30 min. To prevent spillage, food was moistened (food powder/water, 1:2). Rats consumed water ad libitum.

In the morning of d 10 after overnight food deprivation, rats were fed a meal identical to the respective diet (3 g dry mass corresponding to 0.45 g protein) containing either ^{13}C-labeled goat casein or the amino acid mixture labeled with [U-$^{13}C_6$] leucine and α-^{15}N lysine (Tables 1 and 2). At 1, 3, 5 and 7 h after the complete uptake of their meals, groups of 4 or 5 randomly selected rats per dietary treatment were killed by decapitation after injection of barbiturate (Ketamin/Rompun, Albrecht/Bayer, Aulendorf/Leverkusen, Germany). Four rats in each diet group were not fed and were used to provide baseline values for background ^{13}C and ^{15}N enrichments. Plasma, stomach and small intestinal contents, gastric tissue, mucosa of the proximal part of the small intestine and liver were collected immediately. Additionally, as a proxy for amino acid oxidation, urinary nitrogen excretion and body weight gain were measured during the experimental period (d 1–10).

[3] Abbreviations used: AA, L-amino acid mixture; AP, atom %; APE atom % excess; CAS, casein; EA-IRMS, elemental analyzer isotope ratio mass spectrometry; FA, fractional appearance; GC-C-IRMS, gas chromatography-combustion isotope ratio mass spectrometry.

^{13}C-labeled casein was derived from goat's milk. A lactating goat received nonprimed, continuous intravenous infusion of a hydrolysate procured from 7 g of uniformly ^{13}C-labeled algae protein (Celtone-C; Martek Biosciences, Columbia, MD). The milk was collected by hand in several batches over 24 h after the start of infusion. Casein was separated as described previously (13) and the isolated casein powder was washed several times with deionized water. The casein batch with the highest enrichment of leucine and lysine (Table 2) was used for the feeding experiment (Table 1). In the amino acid mixture [U-$^{13}C_6$] leucine [99 atom % (AP); Mass Trace, Woburn, MA] and [α-^{15} N] lysine dihydrochloride (95 AP; VEB Berlin-Chemie, Berlin, Germany) were mixed with unlabeled leucine and lysine, respectively (Table 2).

Sample preparation and analytical methods

Urinary nitrogen excretion, tissue nitrogen contents and amino acid concentration. On d 9, the rats were placed in metabolic cages. Urine was collected over 24 h into HCl, and urinary nitrogen excretion was calculated on the basis of a micro-Kjeldahl analysis (14). Nitrogen content of tissue and gastric contents was determined by means of an elemental analyzer (Vario EL N, Elementar Analysensysteme GmbH, Hanau, Germany). The proximal and distal part of small intestine (upper and lower third) were removed and contents and empty weights were weighed for each rat. Plasma free amino acid concentrations were analyzed after acid precipitation (deproteinization with 5-sulfosalicylic acid) by ion exchange chromatography.

TABLE 2

Amino acid composition of casein (CAS) and the L-amino acid mixture (AA) fed to adult rats during the experimental period (d 1–10), and ^{13}C or ^{15}N amino acid enrichments [atom % excess (APE)] in labeled casein and the L-amino acid mixture fed as a component of a mixed diet on experimental d 10

	CAS		AA	
Amino acids[1]	*mg/160 mg N*	APE	*mg/160 mg N*	APE
Histidine[2]	27.5	0.391	30.1	NA[3]
Isoleucine	46.9	0.332	47.1	NA
Leucine	90.8	0.467	91.1	0.892[4]
Lysine[2]	74.9	0.683	79.0	0.446[5]
Methionine	19.3	0.319	19.5	NA
Phenylalanine	49.7	0.422	49.9	NA
Threonine	48.5	0.299	48.7	NA
Tryptophan	14.7	ND[6]	14.8	NA
Tyrosine	42.6	ND	42.8	NA
Valine	73.8	0.421	74.1	NA
Alanine	22.1	0.319	22.2	NA
Arginine[2]	29.1	ND	31.9	NA
Asparagine[7] + aspartic acid	62.7	0.096	62.9	NA
Glutamine[7] + glutamic acid	218.0	0.139	216.4	NA
Glycine	11.4	0.412	11.5	NA
Proline	116.0	0.422	117.0	NA
Serine	50.4	0.174	52.7	NA

[1] Sigma-Aldrich Chemie GmbH, Steinheim; Serva Finebiochemica, Heidelberg, Germany.
[2] Amino acid in HCl-form in the L-amino acid mixture.
[3] NA, Natural ^{13}C(~1.11 AP) and ^{15}N (~0.37 AP) abundances, respectively.
[4] ^{13}C; [U-$^{13}C_6$]leucine (Mass Trace, Woburn, MA).
[5] ^{15}N; [α-^{15}N]lysine (VEB Berlin-Chemie, Berlin, Germany).
[6] ND, not determined.
[7] L-Amino acid mixture contained glutamine and asparagine, but not glutamic and aspartic acids.

Amino acids were detected by postcolumn ninhydrin detection (TRI-ONE ninhydrin reagent, Pickering Laboratories, Mountain View, CA) using HPLC units (Beckman Instruments GmbH, Munich, Germany) and a step-change elution method with increasing temperature, lithium-concentration and pH (several buffers from Laborservice Onken, Gründau, Germany) (14). For calculation of the amino acid concentration, an external standard solution was used. Measurement of amino acid concentration of dietary casein and tissue proteins was performed as previously described (15).

Amino acid isolation and determination of ^{13}C and ^{15}N enrichments. After killing, organ tissues were quickly removed, washed with ice-cold NaCl solution (0.15 mol/L), weighed and frozen immediately in liquid nitrogen. Mucosa samples were scraped from the proximal and distal 15 cm of the small intestine using a plastic spatula. Frozen organ and mucosa tissues were lyophilized, homogenized with mortar and pestle and stored in an exsiccator until analyzed.

Free amino acids were extracted from 20–30 mg lyophilized tissues after homogenization using 3 × 2 mL of 0.1 mol/L HCl. The acidic extracts were centrifuged at 800 × *g* for 10 min and the amino acids in the supernatant were purified by cation exchange chromatography (Dowex AG 50W-X8; Na-form; 200 mesh, Fluka Chemie AG, Steinheim, Germany) (16). Plasma free amino acids were isolated by ion exchange chromatography as described earlier (17). Protein-bound amino acids in tissues and plasma were isolated from freeze-dried, finely ground tissues (2–5 mg) or from 100 μL plasma by acid precipitation with 3.5 mL of 0.6 mol/L trichloroacetic acid. The protein precipitates were washed with ethanol and ether and underwent a 24-h acid hydrolysis (6 mol/L HCl, 110°C). The amino acids from hydrolyzed proteins were purified by cation exchange before derivatization (16).

The ^{13}C and ^{15}N enrichments of the crystalline leucine and lysine to be mixed into the amino acid mixture were determined separately using an elemental analyzer (EA 1108, Fisons Instruments, Italy) coupled to an isotope ratio mass spectrometer (IRMS; Delta C-IRMS, Finnigan MAT, Bremen, Germany). Enrichments in free and protein-bound amino acids of tissues, plasma and labeled goat's casein were determined after derivatization to their *N*-pivaloyl-*i*-propylesters and were measured by an IRMS (Delta S-IRMS) coupled on-line to a gas chromatograph (GC; HP 5890, Hewlett-Packard, Waldbronn, Germany) via a combustion interface (18,19). Briefly, for derivatization the amino acid isolates were dried and dissolved in thionylchloride/*i*-propanol solution and heated at 100°C. The propylated product was dried and dissolved in pyridine. After addition of pivaloylchloride, the amino acids were acylated and methylene chloride was added after cooling. The mixture was then passed over a silica gel column and the filtrate was dried in a gentle nitrogen stream and redissolved in ethylacetate for injection. The chemicals used were all of analytical grade and purchased from several suppliers (Fluka Chemie, AG, Buchs, Switzerland; Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

The ^{15}N and ^{13}C abundances, determined by EA-IRMS or gas chromatography-combustion (GC-C)-IRMS, were measured against a laboratory standard, which had been calibrated against international standards (air and PDB Belemnite carbonate, respectively). ^{15}N or ^{13}C enrichments were expressed as AP and atom % excess (APE) above baseline as described recently (19). ^{13}C enrichments measured by GC-C-IRMS were corrected for the contribution of extra carbon introduced during the derivatization process. On the basis of stoichiometric mass balance and isotopic fractionation, an empirical ^{13}C correction equation was derived for individual amino acids (19).

Calculations and statistical analysis. To compare the incorporation of dietary amino acids from either casein or the amino acid mixture, data were normalized for differences in the amino acid enrichment in the diet. This was accomplished by calculating the fractional appearance (FA) from enrichments of lysine and leucine in tissues (stomach, intestinal mucosa, liver) or plasma, and diet as follows:

$$FA = E_{\text{tissue or plasma}}/E_{\text{diet}}$$

where *E* is the enrichment of individual amino acids in tissue or plasma and the diet in APE.

For calculation of FA, we assumed that the amino acid enrichment in meals represented the enrichment at the site of absorption. A direct comparison of FA from ^{15}N and ^{13}C lysine was possible because rates of carbon and nitrogen losses during lysine degradation are closely related (20).

Sequestration (*S*) of dietary leucine and lysine into liver protein 7 h after meal ingestion was derived from the following equation:

$$S\ (\text{mg}) = \text{liver protein (g N)} \times \text{amino acid concentration (mg/g N)} \times FA$$

All results are expressed as means ± (SD). A nonparametric Mann-Whitney test (Wilcoxon test) was employed to evaluate the differences between diet groups (21). A value of $P < 0.05$ was considered to be significant.

RESULTS

Growth performance, organ weights and urinary nitrogen excretion. Body weight gains of rats fed the CAS diet were higher than for rats fed the AA diet (20.6 ± 20.0 vs. 12.7 ± 7.4 g/9 d; $P < 0.05$). Final body weights on d 10 did not differ between the CAS group (191.2 ± 19.8 g) and the AA group (187.0 ± 26.4 g) due to slight differences in initial body weights. Total organ weights (AA vs. CAS; stomach, 1.41 ± 0.15 vs. 1.34 ± 0.27; liver, 7.17 ± 1.10 vs. 7.53 ± 1.08; small intestine, 4.15 ± 0.39 vs. 3.93 + 0.34 g) and total nitrogen content of empty stomach, liver and small intestine were not different between groups and did not change during the 7 h of observation (data not shown). The 24-h urinary nitrogen excretion on d 9 was higher for rats fed the AA diet compared with those fed CAS (204.5 ± 49.5 vs. 154.1 ± 55.0 mg N; $P < 0.05$). Because dietary nitrogen intake was identical, this indicates higher nitrogen retention in rats fed the diet containing the intact protein.

Amino acid concentrations in plasma and tissue proteins and gastric nitrogen content. Concentrations of plasma isoleucine, tyrosine, valine, alanine and glutamine measured after overnight food deprivation were higher in the CAS group, but threonine and serine were lower compared with the AA diet group (**Table 3**). At 1 and 3 h after consumption of the CAS and AA meals, plasma concentrations of almost all indispensable amino acids increased relative to the baseline values, with the notable exceptions of phenylalanine, threonine and tyrosine, which did not change after CAS consumption. For indispensable amino acids, no differences were seen between diet groups at 1 and 3 h after the meal, although concentrations tended to be higher after AA consumption (isoleucine, threonine, tyrosine, valine: $0.05 < P < 0.2$). Variations in plasma leucine and lysine concentrations during the 7 h after meal consumption were of similar magnitude with both diets (ranges AA vs. CAS; leucine, 92–182 vs. 125–180 μmol/L; lysine, 547–723 vs. 574–725 μmol/L). Most notable among the dispensable amino acids was the significantly greater increase from baseline for alanine, aspartic acid, glutamine and glycine at 1 h after consumption of the AA diet compared with the CAS diet. By contrast, the increase from baseline for asparagine was greater in rats fed CAS than in those fed the AA diet. Plasma and tissue protein concentrations of leucine and lysine were not affected by diet (data not shown). At 3 and 5 h after meal consumption, rats fed the CAS diet had more nitrogen in their stomach contents (CAS vs. AA; 3 h, 233 ± 111 vs. 71 ± 38 mg N; 5 h, 44 ± 25 vs. 19 ± 6 mg N; $P < 0.05$). However, there were no differences in the dry matter weight and nitrogen level in the intestinal contents between rats fed the two diets at 7 h postmeal (data not shown).

TABLE 3

Postabsorptive baseline and changes in plasma free amino acid concentrations of adult rats from baseline at 1 (Δ 0–1 h) and 3 (Δ 0–3 h) h after administration of single mixed meals containing casein (CAS) or an L-amino acid mixture (AA) on experimental d 10[1]

	Postabsorptive baseline		Δ 0–1 h		Δ 0–3 h	
Amino acids	AA	CAS	AA	CAS	AA	CAS
			μmol/L			
Isoleucine	54.2 ± 7.4	75.4 ± 14.7[2]	29.5 ± 15.9[4]	17.8 ± 10.5[4]	43.8 ± 35.4	19.8 ± 9.5[5]
Leucine	91.8 ± 13.2	124.7 ± 23.9	78.8 ± 31.9[5]	42.6 ± 34.5[4]	90.7 ± 66.3[4]	52.3 ± 27.8[5]
Lysine	547.4 ± 50.4	574.1 ± 87.1	200.9 ± 109.0[4]	114.2 ± 64.5[4]	108.9 ± 63.2[4]	153.0 ± 93.8[4]
Methionine	47.4 ± 7.5	46.9 ± 11.0	98.3 ± 36.4[5]	92.9 ± 37.4[5]	89.4 ± 20.1[5]	112.1 ± 49.1[5]
Phenylalanine	43.1 ± 8.5	56.0 ± 14.3	27.1 ± 15.4[4]	5.6 ± 14.8	25.7 ± 23.7	3.1 ± 13.1
Threonine	473.7 ± 106.2	312.0 ± 7.4[2]	294.0 ± 161.2[4]	110.0 ± 113.5	286.7 ± 238.9	112.4 ± 92.8
Tyrosine	53.7 ± 11.0	75.9 ± 7.2[2]	73.0 ± 30.5[5]	22.2 ± 40.6	18.4 ± 22.7	26.0 ± 34.0
Valine	111.9 ± 16.2	159.6 ± 23.6[2]	204.5 ± 71.2[5]	124.4 ± 56.8[5]	229.5 ± 78.7[5]	138.3 ± 54.1[5]
Alanine	458.5 ± 72.7	596.6 ± 25.7[2]	388.4 ± 114.2[5]	131.1 ± 85.0[2,4]	155.0 ± 103.0[4]	175.1 ± 112.0[4]
Arginine	143.0 ± 18.1	145.9 ± 27.6	20.2 ± 21.9	9.8 ± 20.4	19.0 ± 41.5	12.4 ± 17.4
Asparagine	61.4 ± 14.3	76.6 ± 5.8	−9.5 ± 1.3[5]	16.9 ± 8.6[3,4]	−17.2 ± 8.0[5]	22.2 ± 12.7[3,4]
Aspartic acid	17.1 ± 5.2	28.3 ± 8.5	2.8 ± 5.4	−6.9 ± 2.6[2]	12.3 ± 19.9	−6.9 ± 2.1
Glutamic acid	89.8 ± 52.3	150.3 ± 14.8	5.4 ± 17.0	−21.3 ± 20.6	12.1 ± 13.1	−27.5 ± 20.8[2,4]
Glutamine	612.2 ± 61.7	797.2 ± 95.4[2]	294.1 ± 89.0[5]	−11.8 ± 53.6[3]	304.3 ± 130.7[5]	12.9 ± 66.1[3]
Glycine	400.6 ± 15.5	363.0 ± 47.7	5.6 ± 32.0	−58.9 ± 10.1[2,5]	−34.4 ± 2.1[5]	−47.4 ± 24.3[5]
Serine	339.6 ± 12.0	280.0 ± 17.7[3]	146.0 ± 74.5[5]	30.9 ± 21.6[4]	93.2 ± 77.6	37.0 ± 21.4[4]

[1] Values are means ± SD, $n = 4$ or 5.
[2,3] Significantly different between CAS and AA: [2] $P < 0.05$; [3] $P < 0.01$.
[4,5] Significantly different from baseline; [4] $P < 0.05$; [5] $P < 0.01$.

Fractional appearance of dietary lysine and leucine in stomach, intestinal mucosa, liver and plasma. Labeled lysine and leucine from both diets were already incorporated into stomach tissue by 1 h after meal ingestion, with incorporation continuing to increase in a curvilinear manner up to 7 h postmeal (**Fig. 1***A*, *B*). The fractional appearance of dietary leucine into stomach proteins tended to be higher ($P = 0.1$) than for lysine, independent of diet, presumably reflecting differences in leucine and lysine contents of the diet (Table 2) and of the stomach proteins (ranges; leucine, 66–72 vs. lysine, 50–57; mg/g). The appearance of leucine and lysine from the AA diet into the free pool of the proximal small intestinal mucosa was more rapid (peaking at 1 h) than for the CAS diet, whereas appearance of these amino acids from the CAS diet into the mucosa free pool was much slower (peaking at 3 h) (**Figs. 2***A* and **3***A*). By 7 h after consumption of the diets, the differences in free pools of proximal intestinal mucosa between diet groups had disappeared. The incorporation of dietary leucine and lysine into protein of proximal mucosa was higher 3 h after AA ingestion ($P < 0.05$) (Figs. 2*A* and 3*A*). In contrast, by 7 h after meal consumption, ~8–10% of the leucine label, and 4–8% of the lysine label from the CAS diet were recovered in the distal small intestinal mucosa as free and protein-bound amino acids, which was significantly higher compared with the AA diet (data not shown).

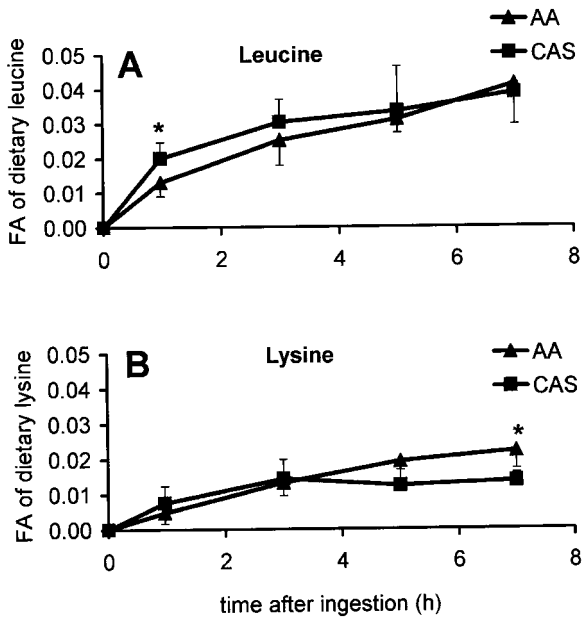

FIGURE 1 Time course of fractional appearance (FA) of dietary leucine (*panel A*) and lysine (*panel B*) administered as free (AA; triangles) or casein-bound (CAS; squares) amino acids in protein-bound amino acids of stomach tissue of adult rats. Values are means ± SD, $n = 4$ or 5. *Significant differences between diet groups ($P < 0.05$).

For rats fed both diets, the appearance of leucine in the plasma free pool peaked at 1 h after meal consumption and then gradually decreased (Fig. 2*B*). The appearance of lysine in the plasma free pool reached a quasi plateau between 1 and 5 h after consumption of both meals, and then decreased at 7 h (Fig. 3*B*). In contrast to the mucosa free pool kinetics, the FA of dietary lysine and leucine in the liver and plasma free pools did not differ between the diet groups, although both amino acids reached a peak at 1 h postmeal (Figs. 2, 3). A significantly higher incorporation of leucine in plasma and liver protein was observed 7 h after CAS ingestion (Fig. 2*B* and 2*C*), whereas dietary lysine incorporation into liver protein was significantly higher 3 and 7 h after AA consumption (Fig. 3*C*). Consequently, total sequestration of leucine into liver proteins at 7 h after consumption of the CAS diet was greater than in rats fed the AA diet ($P < 0.05$), whereas sequestration of lysine into liver proteins did not differ between groups (AA

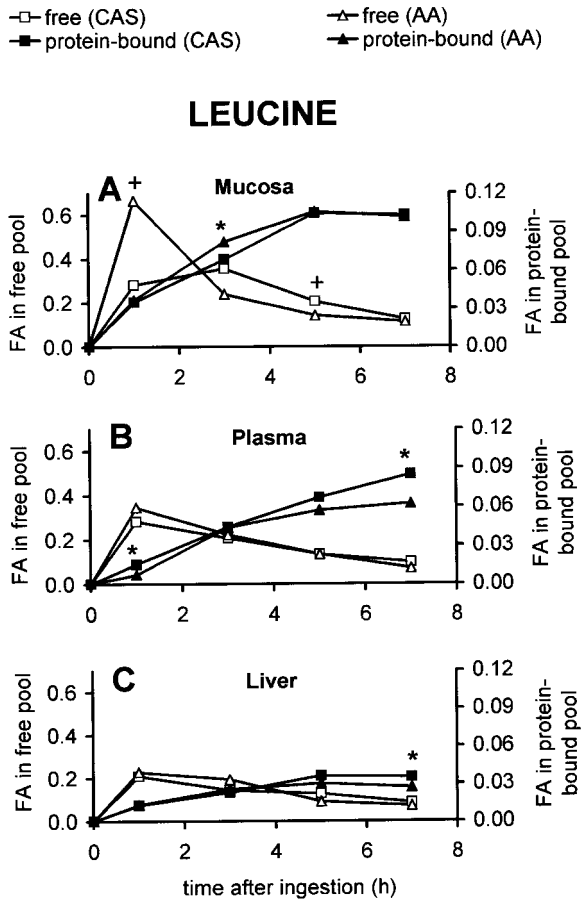

FIGURE 2 Time course of fractional appearance (FA) of dietary leucine administered with a single mixed meal as free (AA; triangles) or casein-bound (CAS; squares) amino acids in the free (open symbols) and protein-bound (closed symbols) amino acid pools of proximal small intestinal mucosa (*panel A*), plasma (*panel B*), and liver (*panel C*) of adult rats. Values are means, $n = 4$ or 5. For clarity SD bars have been omitted. *Significant differences between diet groups in the protein-bound pools ($P < 0.05$). $^{+}$Significant differences between diet groups in the free pools ($P < 0.05$).

vs. CAS; leucine, 2.66 ± 0.22 vs. 3.83 ± 0.46 mg ($P < 0.01$); lysine, 1.62 ± 0.11 vs. 1.31 ± 0.61 mg).

Appearance of other dietary indispensable amino acids into small intestinal mucosa and plasma free pools after the casein (CAS) meal. Except for leucine and lysine, the FA of individual amino acids into the mucosa and plasma free pools did not reflect the compositional pattern of these amino acids in the CAS diet (compare Table 2 and **Fig. 4**). Thus, valine, which is similar in concentration to lysine in casein, appeared in relatively higher amounts in the small intestinal mucosa and plasma free pools compared with lysine. Although methionine and alanine have similar concentrations in casein, the FA of alanine was much lower (Fig. 4). The glycine content of casein is lower than that of alanine, but the FA of glycine in the intestinal mucosa free pool was higher than that of alanine (Fig. 4). An exception to these kinetic patterns is proline, which is the second highest in concentration in casein and also had the highest FA in small intestinal mucosa and plasma free pools (Fig. 4). Notably, the FA of phenylalanine, glycine, aspartic acid, and serine in the mucosa free pool were significantly greater compared with their FA in the plasma free pool ($P < 0.05$).

DISCUSSION

The objectives of this study were to compare whole-body nitrogen homeostasis and the appearance of dietary leucine and lysine in splanchnic tissues and plasma when rats consumed mixed diets containing either intact protein or the corresponding free amino acid equivalent.

Urinary nitrogen excretion, a proxy for whole-body amino acid oxidation, was higher in rats fed the AA diet. Lower growth rates and decreased nitrogen retention have been also observed when pigs and rats are fed diets containing free amino acids compared with those fed whole proteins (22–26). Why free amino acid–based diets are used less efficiently than diets containing intact proteins is unclear, but evidence to date suggests that differences in the kinetics of amino acid absorption and consequently postabsorptive utilization may be important. The amount of nitrogen in the stomach contents of rats fed the CAS diet was higher at 3 and 5 h postmeal, suggesting a slower gastric emptying time after ingestion of the casein meal. This may be due to the clotting of casein in the stomach and possibly the release of opioid peptides, leading to a slower gastric emptying (4,5,27).

Plasma amino acid concentrations reflect the net difference

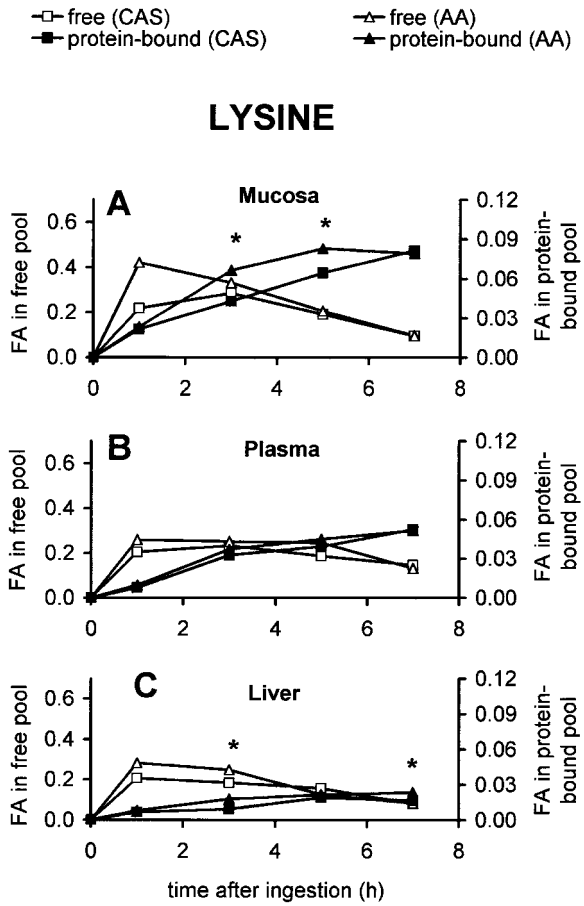

FIGURE 3 Time course of fractional appearance (FA) of dietary lysine administered with a single mixed meal as free (AA; triangles) or casein-bound (CAS; squares) amino acids in the free (open symbols) and protein-bound (closed symbols) amino acid pools of proximal small intestinal mucosa (*panel A*), plasma (*panel B*), and liver (*panel C*) of adult rats. Values are means, $n = 4$ or 5. For clarity SD bars have been omitted. *Significant differences between diet groups in the protein-bound pools ($P < 0.05$). $^{+}$Significant differences between diet groups in the free pools ($P < 0.05$).

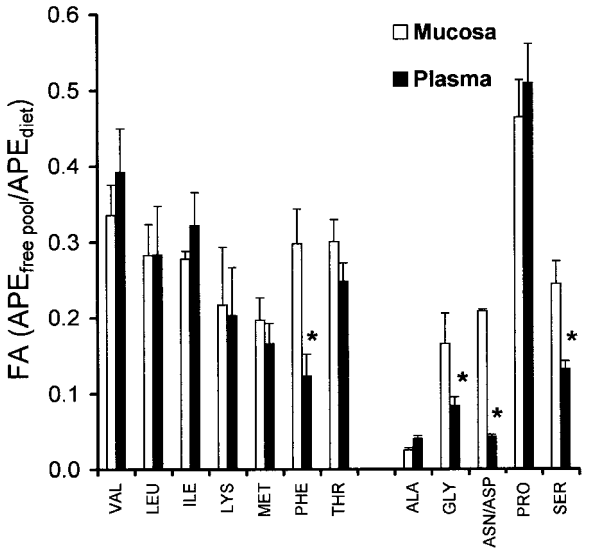

FIGURE 4 Fractional appearance (FA) of casein-derived amino acids in the free amino acid pools of proximal small intestinal mucosa and plasma 1 h after the ingestion of the intrinsically labeled casein meal in adult rats. Values are means ± SD, $n = 4$ or 5. *Significant differences between mucosa and plasma ($P < 0.05$).

between the release of amino acids by the splanchnic bed and their uptake (and release) by the peripheral tissues. The generally smaller increase of plasma amino acids after the CAS meal may be indicative of a higher first-pass splanchnic uptake of casein-derived amino acids compared with the free amino acid mixture. This interpretation would be consistent with the finding that splanchnic uptake of casein-derived leucine was higher than that of crystalline leucine in humans (2, unpublished data), resulting in lower plasma concentration of leucine, as well as isoleucine and valine (2). Similar differences in the kinetics of plasma concentrations of isoleucine, leucine and lysine were observed when subjects consumed a meal of cottage cheese compared with one containing an L-amino acid mixture simulating cottage cheese (28).

Another important aspect in interpreting plasma free amino acid levels after a meal is the meal composition. Large differences in plasma amino acid concentrations among dietary proteins were found when only pure (protein) nitrogen sources were ingested (6,3,29). Our data do not show a largely different variation of plasma leucine or lysine concentrations, respectively, between diets. This is most likely due to the fact that in this study, as well as in other studies (2, unpublished data), mixed meals that also contained carbohydrates and fat were given. It was shown earlier that the degree of plasma amino acid concentration enhancement depends on the composition of the meal (29,30).

Because almost all of the enterally delivered glutamic acid and most of the glutamine is taken up during the first pass by gut and liver (11), the difference seen in postmeal plasma glutamine concentration (Table 3) points to either a decreased splanchnic uptake or an increased endogenous glutamine production when glutamine is ingested as a free amino acid. However, it cannot be excluded that the high plasma glutamine concentration was due to the fact that no glutamic acid was included in the L-amino acid mixture (Table 2), which may affect splanchnic amino acid transporter activities and the glutamate-glutamine metabolic pathway (11).

There were no differences in organ weights and nitrogen (protein) content, nor were leucine and lysine concentrations in tissue proteins different between rats fed the AA or CAS meals. Thus, FA could be compared between rats fed the two diets without the confounding effect of changes in amino acid pool size. The appearance of dietary-derived labeled lysine and leucine in stomach protein within 1 h after meal consumption suggests that these dietary amino acids are rapidly taken up by the stomach mucosa and either incorporated directly into gastric proteins (first-pass use) or incorporated into gastric proteins (second-pass use) subsequent to intestinal absorption into the blood (31–33). However, it cannot be excluded that in spite of careful rinsing of the splanchnic organs, some food particles continued to adhere to the mucosa, carrying with them labeled amino acids. The lower incorporation of dietary lysine into stomach tissue protein in comparison to leucine, independent of the nitrogen source, is likely to be due to a lower lysine content in stomach tissue protein.

Comparison of the FA of amino acids in free and protein-bound pools allowed an assessment of the efficiency of transfer and use of amino acids derived from the two diets. During the early postprandial period (0–1 h) dietary labeled amino acids appeared more rapidly and to a greater extent in plasma and tissue free pools (15 times higher than in protein-bound pools), whereas in the later postprandial period (7 h), these dietary amino acids had been incorporated mainly into proteins. Compared with rats fed the AA diet, the FA of leucine derived from CAS were higher in plasma and liver proteins. Total sequestration of leucine into liver constitutive proteins was also higher in rats fed the CAS meal. Stoll et al. (8) reported that in contrast to the small intestinal mucosa, in which the majority of the metabolized amino acids were catabolized (i.e., oxidized), the liver utilizes a nutritionally important amount of dietary amino acids for protein synthesis. Our results indicate that the availability of dietary leucine and lysine for protein synthesis in the liver (constitutive and plasma export proteins), but not in the small intestinal mucosa, depends on the molecular form of the ingested nitrogen source. Presumably, the slower gastric emptying after consumption of the CAS diet, and thus a slower rate of amino acid absorption, prevents the rapid increase in intracellular free leucine concentration in splanchnic tissues, resulting in less induction of catabolism. Consistent with this view are the observations that leucine oxidation is lower, splanchnic (gut plus liver) uptake of dietary leucine is higher and nonoxidative leucine disposal (protein synthesis) is greater in humans consuming a diet containing casein vs. one containing crystalline amino acids (2, unpublished data).

The differences in absorption rate of amino acids derived from dietary protein-bound vs. free amino acids is analogous to the "slow" and "fast" protein concept of Boirie et al. (6). These authors observed that leucine derived from dietary casein was used for tissue protein synthesis and supported higher postprandial protein gain in humans than leucine derived from whey proteins. The authors attributed these results to the slower rate of digestion and absorption of the casein than the whey proteins. Our study offers the additional opportunity to examine the metabolic fate of two amino acids, lysine and leucine, at the same time. Dietary lysine and leucine displayed differential incorporation kinetics into liver and plasma proteins, and furthermore, these differences were dependent upon whether the CAS or AA diet was fed (Figs. 2, 3). These results not only suggest differences in the sites of catabolism of these amino acids but also that the form of dietary nitrogen influences the efficiency of use of these amino acids. This result is not surprising given the known differences in the major sites of catabolism of leucine (muscle) and lysine (liver) in the body. Our results also support the long-held view that it is not

possible, and certainly misleading, to extrapolate metabolic results from one amino acid to another. Future studies will be necessary to examine a larger range of amino acids and whether the form and composition of protein-amino acids in the diet have similar or different effects on general amino acid utilization. Such results have important implications for the establishment of nutritional requirements for amino acids and the system by which protein sources are scored for quality.

This study also provided the opportunity to trace the metabolic fates of other dietary protein (casein)-bound indispensable amino acids in plasma and splanchnic tissues. Differences in leucine and lysine concentrations in dietary casein were largely reflected in their FA in the intestinal mucosa, possibly indicating similar pool sizes and/or similar turnover rates of these amino acids in the intestinal mucosa. By contrast, although valine has a concentration similar to that of lysine in casein, the transfer of dietary valine into the small intestinal mucosa occurred at a higher rate than for lysine. Again, these differences may reflect a larger free lysine pool and/or a higher turnover rate for lysine than for valine in the small intestine. Interestingly, almost 50% of the proline enrichment in the dietary casein was recovered in the mucosa, which probably indicates that de novo synthesis from glutamate and proline oxidative breakdown during h 1 after meal ingestion were very low. The rather low FA for alanine, relative to its composition in casein, may reflect a substantial net production of alanine by intestinal tissue (9). With the exceptions of alanine and proline, however, the FA of other dispensable amino acids monitored was lower into plasma compared with the mucosa (Fig. 4). One explanation for this may be a greater splanchnic uptake of glycine, aspartic and glutamic acids, and serine in comparison to alanine, proline and the other indispensable amino acids. Consequently, a greater splanchnic removal of these amino acids is compatible with the unchanged or lower plasma concentrations of glycine, aspartic and glutamic acids, and serine after the CAS meal. Furthermore, phenylalanine showed a 100% greater FA into the mucosa than into plasma, which is supported by observations of Stoll et al. (34,35) in which 76% of enteral phenylalanine in pigs was extracted by splanchnic tissues and 59% of dietary phenylalanine was used for mucosal protein synthesis.

In summary, we have shown that dietary protein-bound amino acids (casein), as opposed to crystalline amino acids, support higher rates of weight gain and nitrogen retention in rats. In part, this appears to be related to a slower gastric emptying rate and slower rates of appearance of free amino acids in the blood circulation when rats are fed casein, all of which result in a greater efficiency of transfer of amino acids into tissue and plasma proteins. Further, differences were noted in the FA of amino acids derived from casein into tissues, with leucine transfers into liver and plasma proteins occurring at a higher efficiency than for lysine. We conclude that the molecular form in which dietary amino acids are consumed influences their oxidation as well as their peripheral and splanchnic availability for protein synthesis, and that differences in the metabolic utilization of dietary derived amino acids makes it unjustifiable to extrapolate results from one amino acid to another in assessing overall protein efficiency and quality.

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