Isotopic Evidence for the Differential Regulation of Arginine and Proline Synthesis in Man

Heiner K. Berthold, Peter J. Reeds, and Peter D. Klein

Arginine and proline derive from the metabolism of Δ^1-pyrolline-5-carboxylate, a product of intestinal glutamic acid metabolism. We studied the extent of glutamate, arginine, and proline synthesis in four adult fed and fasted women. The subjects ingested a single dose of a mixture of uniformly (U)-^{13}C-labeled amino acids and carbohydrate of algal origin. Frequent blood samples were taken for 24 hours. All the mass isotopomers of plasma lysine, glutamate + glutamine (GLX), arginine, and proline were measured using negative chemical ionization, selected-ion monitoring gas chromatography–mass spectrometry. In this isotopic approach, the appearance of U-^{13}C-amino acid in the plasma reflects entry of the dietary amino acids, and the appearance of ^{13}C in lower mass isotopomers demonstrates synthesis of the respective amino acids by the subject. All the mass isotopomers (including [M + 4]) of GLX became enriched with ^{13}C. We suggest that the [M + 4] isotopomer of GLX reflects synthesis of the amino acid from α-ketoglutarate derived from the metabolism of U-^{13}C-carbohydrate by the bacterial flora. Arginine labeling showed two patterns. The [M + 5] isotopomer of plasma arginine was labeled as rapidly as [M + 6] (ie, tracer) arginine, and we propose that the appearance of the [M + 5] isotopomer reflects the synthesis of citrulline from dietary [M + 5]-glutamate in first pass. The [M + 1] to [M + 3] isotopomers of arginine were also labeled for a prolonged period of time, suggesting that systemic glutamate was also a precursor for arginine synthesis. In fed subjects, only the [M + 5] isotopomer of proline was significantly labeled. Fasting was associated with increased labeling of the [M + 1] to [M + 3] isotopomers of both arginine and proline, suggesting an increase in the contribution of de novo synthesis to their plasma flux. We conclude that proline synthesis either is strictly regulated by dietary proline or is substantially compartmentalized.

THE TRADITIONAL CLASSIFICATION of amino acids as either essential or nonessential was originally established using a nutritional approach involving measurement of nitrogen balance during consumption of synthetic diets in which single amino acids were deleted.[1] This showed that some amino acids (eg, glutamate, alanine, and aspartate) are not obligatory components of the diet. It is now known that the synthesis of some "nonessential" amino acids (eg, tyrosine, cysteine, glycine, proline, and arginine) requires the provision of preformed carbon skeletons derived from other amino acids. Under certain metabolic, developmental, or pathophysiologic conditions, one or more of this group of amino acids may limit the conservation and deposition of body protein,[2-4] and as a group, they are now often designated as "conditionally essential."[5,6]

Both nonessential and conditionally essential amino acids are involved in metabolic functions apart from protein synthesis. Arginine appears to have substantial effects on the immune system,[7] perhaps because it is the precursor for nitric oxide synthesis.[8,9] This implies that arginine may play an indirect role in the regulation of blood pressure[10] and in the development of higher cognitive function.[11] Furthermore, the main carbon precursors for net arginine synthesis, glutamate + glutamine (GLX), are involved in physiologic functions (such as neurotransmitter and glutathione synthesis) unrelated to their role in protein metabolism.

In view of the physiologic significance of some nonessential and conditionally essential amino acids, it remains important to understand factors that regulate their biosynthesis, especially in man. To do so, there is a need to develop appropriate stable-isotope tracer techniques. Much of the available quantitative information on nonessential and conditionally essential amino acid synthesis has been based on a method that ascribes differences between the measured entry rate of a given nonessential amino acid and its calculated rate of entry from the diet and from body protein breakdown to biosynthesis.[12-13] An alternative approach is to administer labeled precursors and measure the rate of amino acid synthesis from label incorporation rather than label dilution.[14,15] This has been applied recently to the measurement of arginine synthesis[16] in human beings.

We have also shown that after a mixture of organic nutrients uniformly labeled with ^{13}C are ingested, the rate of appearance and the distribution of ^{13}C label among the low mass isotopomers potentially allows the dissection of some of the complexities of carbon metabolism in vivo.[17] Here, we report the results of a study in human subjects in which U-^{13}C-algal biomass was used as a tracer to examine the biosynthesis of glutamate, arginine, and proline and to study the effect of an overnight fast on these pathways.

SUBJECTS AND METHODS

Study Protocol

Subjects. The study protocol was approved by the Baylor Institutional Review Board for Human Research. Informed consent was obtained from the subjects.

Four healthy female volunteers were recruited from the student population on campus and paid for their participation. Before the subjects were enrolled in the study, they underwent a complete physical examination, medical history, and standard laboratory screening. None of the subjects had intercurrent medical condi-

From the US Department of Agriculture/Agricultural Research Service (USDA/ARS) Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX.

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Present address: H.K.B., Department of Clinical Pharmacology, University of Bonn, Sigmund Freud Strasse 25, 53105 Bonn, Germany.

Address reprint requests to Peter J. Reeds, PhD, Children's Nutrition Research Center, Baylor College of Medicine, 1100 Bates St, Houston, TX 77030.

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tions, a history of drug or alcohol abuse, metabolic diseases, obesity, recent weight loss or weight gain, or current use of any medications except oral contraceptive steroids. All subjects were nulliparous, and three used oral contraceptive steroids. They were studied between days 4 and 12 of their respective menstrual cycles. Subjects' characteristics are listed in Table 1.

Study procedure. Each subject was studied twice, once in the continuously fed state and once in the fasted state. The interval between trials was exactly 4 weeks. For the fed phase of the study, subjects entered the Metabolic Research Unit at 7 AM on the prestudy day after an overnight fast. On this day and on the day of study, subjects ingested a commercially available liquid formula (Liquid Ensure, Ross Laboratories, Columbus OH). On the prestudy day, the liquid formula was given in five equal portions at 8 AM, noon, 3 PM, 6 PM, and 9 PM. Subjects had free access to nonalcoholic, caffeine-free, noncaloric fluids until midnight.

On the study day, subjects consumed two meals (each providing 1/30 of their daily intake) at 7 and 7:30 AM. From 8 AM until 9 PM, they consumed food in hourly meals. Each hourly meal provided 1/15 of their daily intake. At 7:50 AM, subjects ingested ^{13}C-labeled *Spirulina platensis* mixed with 100 mL water. This feeding regimen was adopted to maintain a metabolic steady state during the fed phase of the study.

For the fasting study, the schedule for the prestudy day was exactly as described earlier, except subjects fasted from 9 PM on the prestudy day until 8 AM on the poststudy day.

Formula and tracer composition. The liquid diet (Liquid Ensure) supplied 4.43 J/mL with a caloric distribution of 14% protein (sodium and calcium caseinates 88%, soy protein isolate 12%), 31.5% fat (corn oil 100%, linoleic acid 56.6%, linolenic acid 1.3%, nonessential fatty acids 42.1%, cholesterol <5 mg/kJ), and 54.5% carbohydrate (corn syrup 70%, sucrose 30%). Each subject received 1.23 g protein and 146 kJ/kg body weight per day. The hourly intake of lysine, proline, arginine, and glutamine/glutamic acid from the formula is listed in Table 2.

U-^{13}C-labeled *Spirulina platensis* was obtained from Martek (Columbia, MD). We used the lipid-free, water-soluble fraction. This was lyophilized and ground to a fine powder; 23% of the dry matter was protein (N × 6.25) and the remainder was complex carbohydrate. Each subject received 239 mg (55 mg protein) *Spirulina* per kilogram body weight. The amounts of U-^{13}C-lysine, glutamate, proline, and arginine ingested are also listed in Table 2.

Blood collection. A catheter (silicone rubber, 21-gauge) was inserted into a wrist or antecubital vein at 7 AM on the study day and kept patent by occasional flushing with NaCl (154 mmol/L). Blood (5 mL) was drawn into evacuated tubes containing Na_2EDTA and immediately centrifuged at 3,600 × *g* at room temperature; plasma was separated, divided into 0.5-mL aliquots, and stored at −70°C until analyzed. Blood samples were taken at 5, 10, and 20 minutes, at 20-minute intervals until 2 hours, at 2-hour intervals until 8 hours, and at 4-hour intervals until 24-hours.

Table 1. Characteristics of Female Subjects

Subject No.	Age (yr)	Weight (kg)	Height (cm)	Body Mass Index (kg · m^{-2})	Body Fat (%)
1	28.1	55.0	166	19.9	26.5
2	22.8	56.9	170	19.7	27.0
3	23.8	64.3	174	21.6	22.4
4	24.2	51.5	156	21.1	30.5
Mean ± SD	24.7 ± 2.3	56.9 ± 5.4	166.5 ± 7.6	20.6 ± 0.9	26.6 ± 3.3

Table 2. Amino Acid Contribution From Liquid Ensure and Uniformly Labeled *Spirulina platensis*

	Ensure Amino Acid (mg/kg/h)	U-^{13}C-Amino Acid (mg/kg)
Lysine	5.30	2.34
GLX	17.6	6.12
Arginine	2.84	2.73
Proline	7.52	3.31

Analytical Methods

Isolation of plasma free amino acids. Plasma (0.5 mL) was acidified with 0.5 mL acetic acid (1 mol/L). Disposable columns were prepared with 1 mL AG50 (200 mesh, 8% cross-linked, H^+ form) cation-exchange resin. After two washes with deionized water (2 mL), amino acids were eluted with 2 mL 3-mol/L NH_4OH, followed by 1 mL water. The NH_4OH fraction was then dried under nitrogen.

Derivatizaiton of amino acids. GLX, lysine, and proline were analyzed as their *N*-propyl ester *N*-heptafluorobutyramide derivatives, synthesized in a two-step procedure. The dried amino acid extracts were esterified with a solution (1 mL) of *n*-propanol and acetyl chloride (5:1 vol:vol) at 100°C for 1 hour, and then evaporated to complete dryness under nitrogen. The dried residue was reacted with 100 μL heptafluorobutyric anhydride at 60°C for 20 minutes. Arginine was analyzed as the guanido-N,N^{δ}-ditrifluoroacetyl, 2-perfluoroalkyl-3-oxazoline-5-on-arginine derivative. This was prepared by heating the dried amino acid extract in 100 μL of a mixture of 1,2 dichloroethane and trifluoroacetic anhydride (4:1 vol:vol) at 100°C for 120 minutes. Excess reagents were removed by evaporation at room temperature in a gentle stream of nitrogen. The samples were then redissolved in ethyl acetate (~0.5 mL). An aliquot of *Spirulina* was hydrolyzed in 5.4 mol/L HCl for 24 hours at 110°C and the amino acids were isolated and derivatized in a manner similar to that used for the plasma amino acids.

Gas chromatography–mass spectrometry. The amino acids were analyzed on a Hewlett Packard (Atlanta, GA) 5988A gas chromatography–mass spectrometry system using negative-ion chemical ionization with methane as the reagent gas. The samples (1 μL) were injected splitless onto a 30-m, 0.32-mm diameter, 1.0-μm film thickness, DB-5 capillary column (J & W Scientific, Rancho Cordova, CA) with helium as the carrier gas. Chromatography was effected with a linear temperature gradient (80°C to 250°C at 10°C/min). Selected-ion monitoring was performed on the respective [M-HF] fragment ions of the amino acids and all their associated mass isotopomer ions. Enrichment measurements were performed on baseline and enriched samples, as well as on the tracer material. [M-HF + X]/[M-HF] isotope ratios were calculated, and these data were used to solve a set of simultaneous linear equations to obtain the abundance of each isotopomer expressed as a percentage of all isotopomers, including the unlabeled [M + 0] amino acid molecules, ie, the molar enrichments.

Calculations

Isotopomer enrichment pattern correction. The growth of an algal culture in an atmosphere of $^{13}CO_2$ results in the biosynthesis of a large proportion of uniformly labeled amino acids. These are detected as mass isotopomers of a mass that is X mass units higher ([M + X]) than the unlabeled molecule, where X is the number of carbon atoms. However, a small ($<4\%$) proportion of molecules in the labeled biomass contain both ^{12}C and ^{13}C atoms, in random distribution. These are detected and quantified as isotopomers with masses of [M + 1] . . . [M + (X − 1)].

In contrast to stable-isotope studies in which only a single

labeled molecule is given as tracer and a simple distribution pattern of labeled molecules is seen, administration of uniformly ^{13}C-labeled molecules leads to the formation of a complex pattern of, potentially, X + 1 isotopomers containing 1 ... X additional atoms of ^{13}C. Thus, any given metabolite can contain either no ^{13}C or 1 to X ^{13}C atoms. Calculation of the excess molar enrichment of each isotopomer (ie, its contribution to the total number of metabolite molecules) requires the solution of (X + 1) simultaneous linear equations of the general form $I = AijX_0 + AijX_1 + \ldots + AijX_x$, in which the ion intensity (I) at a specific mass is the sum of (X + 1) terms consisting of the product of the abundance coefficient (Aij) and an unknown mole fraction (X_n). To obtain the solution for the unknown mole fractions, matrix algebraic transformations are performed.

The problem is as follows. Because of the natural occurrence of stable isotopes of all the elements (C, N, H, O, and F) in the derivatives used for gas chromatography–mass spectrometry analysis, any given isotopomer contributes to the intensity of the signal associated with any other isotopomer, especially those of 1 and 2 higher m/e excess ratios. In principle, measurements of these contributions should be made empirically by measuring the mass spectra of isotopically pure samples of all ^{13}C isotopomers of the specific derivative. However, in practice, only the mass spectra of the derivatives of baseline and the administered tracer material are available. However, the contributions of the spectrum from any isotopomer to the spectrum of any other are a predictable function of the natural abundance of each isotope of the constituent elements and the numbers of atoms of each element in the derivative. In the present study, therefore, the contributions of the signal from each isotopomer to those of the other isotopomers were calculated using a statistical algorithm that uses the known values for the natural abundances of the stable isotopes of the constituent elements and presumes that 1 to (X-1) of each one of these atoms in a given molecule are replaced by these isotopes. The computer software that allows these calculations is available free of charge from Dr D.L. Hachey at the Children's Nutrition Research Center. The results presented herein are expressed as percent molar enrichments, ie, 100 × the contribution of each isotopomer to the total quantity of metabolite (the sum of all isotopomers, including molecules containing only ^{12}C).

Calculation of amino acid synthesis. For this calculation, we used the previously published approach.[12-14] The initial step is the calculation of the rate of body protein breakdown using the kinetics of a labeled essential amino acid—in this report, U-^{13}C-([M + 6])-lysine.

The area under the isotopic enrichment × time curve (AUC_8) of [M + 6]-lysine was calculated over the first 8 hours of each study by the trapezoidal method. The lysine entry rate (milligrams per kilogram per 8 hours) was then calculated as

$$\frac{[\text{dose given}]}{[AUC_8 \times 0.125]} - \text{dose given}, \qquad \text{Eq 1}$$

where dose is expressed as milligrams U-^{13}C-lysine per kilogram body weight and AUC_8 is moles [M + 6]-lysine per mole total lysine per hour. The denominator 0.125 is included to account for the 8-hour calculation period. The choice of 8 hours as the period over which to calculate the AUC is a compromise between the theoretical requirement for extrapolation to infinity and the increasing likelihood that as the time over which the AUC is calculated increases, the likelihood of label recycling affecting the result also increases. In the present study, we chose 8 hours because by this time the U-^{13}C-isotopomers of the amino acids had largely cleared from the plasma. For example, by 8 hours the molar enrichment of [M + 6]-lysine was approximately 0.20 mol/100 mol. This represented approximately 7% of the peak enrichment and was also the level below which, in our hands, analytical errors become a significant factor. As a point of reference for lysine, the AUC calculated over 8 hours was 92% of the value calculated at 24 hours (data not shown).

In the fasted state, lysine only enters the plasma from body protein degradation; in the fed state, the lysine entry rate is converted to entry from body protein by subtracting the lysine intake. Data on lysine were then converted to protein equivalents on the assumption that body protein contains 72 g lysine/kg protein.[18]

The total entry rates of glutamate, proline, and arginine were then calculated likewise from the areas under the isotopic enrichment time curves of their uniformly labeled isotopomers. Entry from body protein (calculated from the lysine data) was then calculated by assuming that body protein contains 110, 72, and 68 g/kg protein of GLX, arginine, and proline, respectively.[18]

Differences between means within an amino acid associated with feeding state were assessed by Student's *t* test for paired samples. *P* less than .05 (two-tailed) was considered as statistically significant.

RESULTS

The molar enrichments of all mass isotopomers of glutamate (A,B), arginine (C,D), and proline (E,F) over the first 8 hours after ingestion of labeled algal biomass are shown in Fig 1 (A,C, and E for mass isotopomers [M + X] and [M + (X − 1)], and B, D, and F for mass isotopomers [M + 1] to [M + (X − 2)]). Only the [M + 6] isotopomer of lysine was detected (data not shown). With the exception of proline in the fed state, statistically significant ^{13}C enrichment was found in all isotopomers, although the magnitude of isotopic enrichments differed between the various isotopomers.

Different isotopomers also showed different kinetics. The [M + X] isotopomer appeared rapidly in the plasma and, irrespective of the amino acid, disappeared from plasma with essentially the same rate constant (data not shown). The [M + (X − 1)] isotopomers of GLX and arginine also appeared rapidly, and the kinetics of [M + 5]-arginine were essentially the same as those of [M + 6]-arginine. [M + 4]-glutamate remained labeled for a prolonged period of time. Label appearance in isotopomers [M + 1] to [M + (X − 2)] for both amino acids was slower in onset and more prolonged.

Proline showed a distinctive pattern of enrichment. In the fed state (Table 3), the only isotopomer to be enriched significantly above zero was [M + 5], ie, the isotopomer that the subjects ingested. In the fasted state, there was a low but statistically significant ^{13}C enrichment in all but the [M + 3] isotopomer.

The areas under the isotopic enrichment × time curves over the first 8 hours of each study are listed in Table 3. These results show in a more formal manner that in the fed state there was no statistically significant ^{13}C incorporation into any isotopomer of proline, other than the uniformly labeled form. The data also illustrate the large proportional increase in the labeling of low mass isotopomers of proline that was associated with the fasted state.

In Table 4, entry rates calculated from the kinetics of the U-^{13}C-tracer are listed. Fasting was associated with a significant change in entry rate only for proline. The

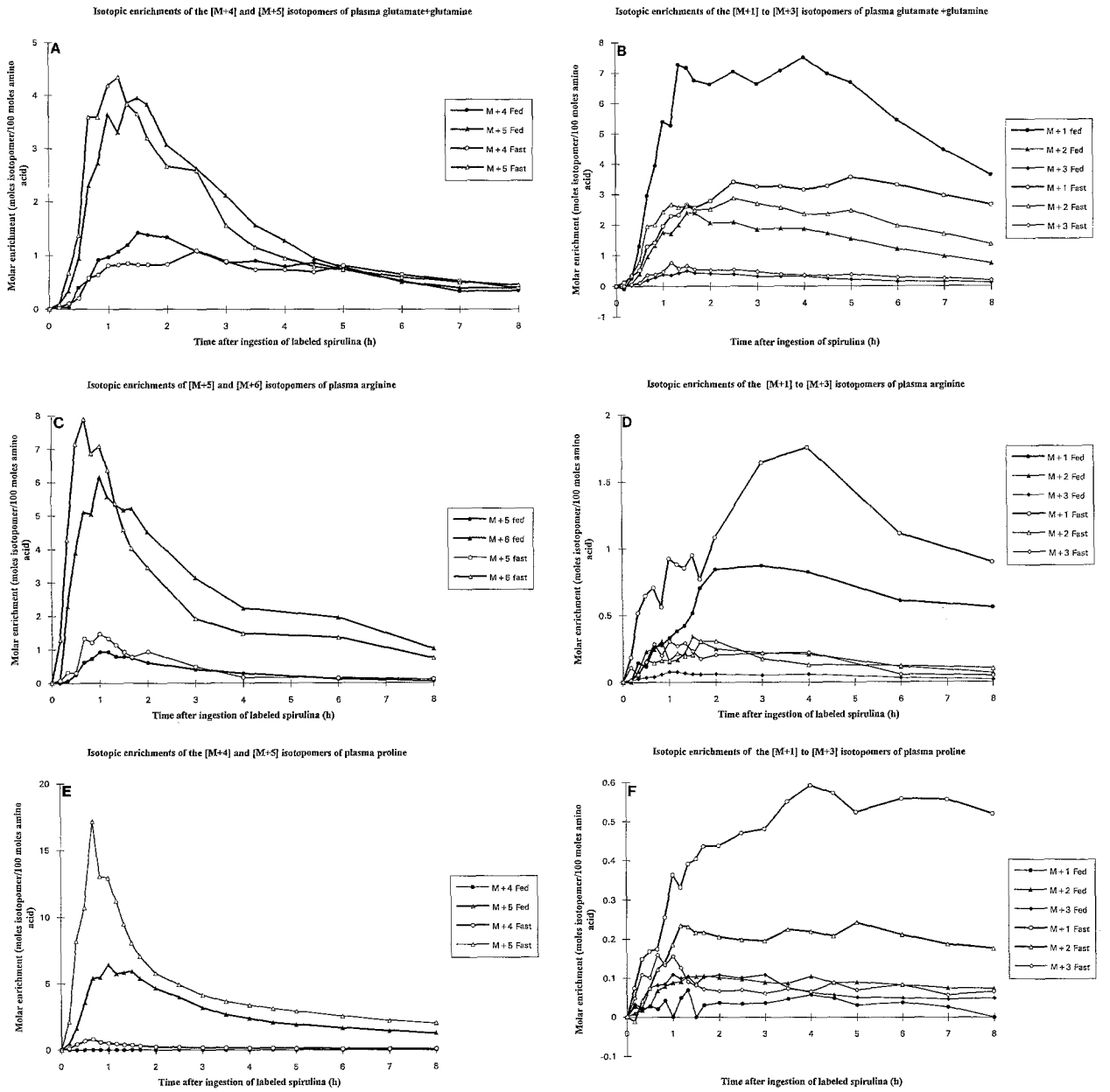

Fig 1. Time course of the mean excess molar enrichment (moles isotopomer per 100 mol amino acid) of the isotopomers of GLX (A,B), arginine (C,D), and proline (E,F) in 4 women in the fed and fasted states over an 8-hour period immediately following the ingestion of U-^{13}C-labeled dry *Spirulina platensis*.

apparent rate of body protein degradation, calculated from the U-^{13}C-lysine data, was 220 ± 35 (mean ± SD) in the fed state and 278 ± 14 mg protein/kg · h^{-1} ($P < .05$) in the fasted state.

These values for protein degradation calculated from the data for lysine were used to calculate the apparent rate of de novo synthesis for each of the "nonessential" amino acids[12-13] (Table 5). Significant GLX synthesis was demonstrated, and this apparently increased from 20% to 50% of flux ($P < .001$) on fasting. However, this method of calculation suggested that there was no endogenous synthesis of either proline or arginine. Although data for the labeling of mass isotopomers less than M + X are not amenable to formal analysis, the appearance of significant ^{13}C in arginine (in the fed and fasted states) and in proline (in the fasted state) demonstrates that under these circumstances the amino acids were being synthesized.

DISCUSSION

Glutamate and glutamine occupy key positions in mammalian nitrogen metabolism. Given the fact that recent data show that a high proportion of dietary glutamate and glutamine are used in first pass by tissues of the splanchnic bed,[15] it is to be expected that de novo synthesis contributes substantially to the flux of both amino acids. In the present study, both were analyzed as glutamate because the deriva-

Table 3. Area Under the Plasma Molar Isotopic Enrichment × Time Curve of Isotopomer [M + 6] for Lys and All Isotopomers for GLX, Prol, and Arg in Four Women in the Fed and Fasted States

	[M + 1]	[M + 2]	[M + 3]	[M + 4]	[M + 5]	[M + 6]
GLX fed	44.2 ± 5.4	11.6 ± 2.4	1.96 ± 0.21	5.70 ± 1.55	11.6 ± 2.8	
Percent total ^{13}C	28.7 ± 1.8	15.1 ± 2.4	3.81 ± 0.70	14.8 ± 3.2	37.7 ± 4.8	
GLX fasted	20.5 ± 3.4‡	15.7 ± 0.7‡	2.75 ± 0.48†	5.01 ± 1.31	10.9 ± 2.2	
Percent total ^{13}C	15.3 ± 3.2	23.4 ± 7.1	6.16 ± 1.7	15.0 ± 4.23	40.6 ± 7.2	
Prol fed	0.25 ± 0.32*	0.36 ± 0.26*	0.34 ± 0.37*	0.07 ± 0.16	22.1 ± 4.4	
Percent total ^{13}C	0.22 ± 0.29	0.63 ± 0.70	0.90 ± 0.81	0.20 ± 0.6	97.8 ± 2.1	
Prol fasted	3.77 ± 0.61§	1.52 ± 0.30§	0.61 ± 0.20	1.63 ± 0.35§	37.1 ± 7.9†	
Percent total ^{13}C	1.89 ± 0.19	1.53 ± 0.18	0.92 ± 0.36	3.27 ± 4.62	93.2 ± 4.1	
Arg fed	4.36 ± 0.75	1.46 ± 0.39	0.38 ± 0.31*	0.21 ± 0.19*	2.47 ± 0.93	21.8 ± 3.2
Percent total ^{13}C	2.86 ± 0.31	1.92 ± 0.9	0.75 ± 0.60	0.55 ± 0.90	8.12 ± 1.98	86.1 ± 4.6
Arg fasted	9.39 ± 1.75‡	1.48 ± 0.41	1.22 ± 0.30†	0.40 ± 0.31*	3.88 ± 1.06	19.0 ± 5.0
Percent total ^{13}C	6.21 ± 1.7	1.96 ± 1.03	2.42 ± 0.61	1.06 ± 0.90	12.8 ± 4.2	75.5 ± 5.7
Lys fed	0	0	0	0	0	11.0 ± 1.6
Lys fasted	0	0	0	0	0	11.5 ± 0.6

NOTE. Area under the isotopic enrichment × time curve is presented as moles isotopomer per 100 mol amino acid × h ± 1 SD.
*Not significantly different from zero.
Effect of fasting: †$P < .05$, ‡$P < .01$, §$P < .001$.

tization procedure degrades glutamine to glutamate. It should be noted that because the concentration of plasma glutamine is approximately 10-fold higher than that of glutamate, the labeling patterns reported here will have been dominated by that of glutamine. Glutamine is not only responsible for the transport of a large proportion of the nitrogen moving from skeletal muscle to the viscera, but it is also a potential oxidative substrate and a key biosynthetic precursor in enterocytes and hepatocytes.

Two of the products of these biosynthetic pathways are arginine and proline, both being derived from a common precursor, Δ^1-pyrroline-5-carboxylate, a cyclization product of glutamic semialdehyde.[19,20] This metabolite undergoes one of two fates: reduction to proline, a reaction completed in the mitochondria of intestinal cells, or conversion to ornithine and then to citrulline. The citrulline is then exported in the hepatic portal circulation and subsequently converted to arginine in the kidney.[21] Thus, synthesis of arginine and proline involves a branched reaction pathway within one tissue, and in the case of arginine synthesis, integration of the biosynthetic activities of two organs, the intestine and the kidney.

The synthesis of arginine[16] and proline[22] has been demonstrated in man, and neither amino acid appears to be essential for the maintenance of acute nitrogen equilibrium in healthy adults of omnivorous (as opposed to carnivorous[23]) mammals, including the human.[16,22] However, both amino acids are, from a metabolic perspective, conditionally essential and may become indispensable components of the diet under conditions of trauma and immune activation.[24,25]

Table 4. Plasma Entry Rates of GLX, Proline, Arginine, and Lysine as Calculated From the Kinetics of Their Uniformly Labeled Isotopomer in Four Women in the Fed and Fasted States

	GLX	Proline	Arginine	Lysine
Fed	386 ± 89	128 ± 16	86 ± 11	149 ± 19
Fasted	416 ± 64	95 ± 12*	98 ± 25	141 ± 7

NOTE. Rates are $\mu mol/kg \cdot h^{-1} \pm 1$ SD.
*Effect of fasting, $P < .05$.

Methodologic Considerations

With the notable exception of a recent report involving study of the metabolism of labeled citrulline administered intravenously,[16] previous in vivo investigations of rates of nonessential amino acid synthesis in human beings have been based on the extent to which the ^{13}C, ^{2}H, or ^{15}N in the tracer nonessential amino acid was diluted in excess of the dilution that would have been expected from the entry of that amino acid from the diet and from tissue proteolysis.[12-13] In this method, body protein breakdown is calculated from the kinetics of a labeled essential amino acid administered at the same time as the tracer nonessential amino acid. In the present study, we used the labeling of U-^{13}C-lysine to derive an estimate of whole-body protein breakdown and applied the method to measurements of the labeling of U-^{13}C-glutamate, -arginine, and -proline. Values for the apparent entry rates of GLX,[13] arginine,[16] and proline[22] were compatible with previously published values for which the entry rates were calculated from the plateau isotopic enrichment of the respective plasma amino acids during intravenous infusion of pure tracer forms. The method readily demonstrated the synthesis of GLX and suggested that endogenous synthesis contributed between 20% (fed state) and 50% (fasted state) of the plasma entry rate. Results for arginine and proline from these calculations suggested that there was no de novo synthesis, ie, that their plasma entry rates could be accounted for by the sum of entry from the diet and from body proteolysis. This result stands in contrast to earlier demonstrations of significant but modest rates of synthesis of both amino acids.[16,22]

The "excess isotopic dilution" method of calculating amino acid synthesis is potentially inaccurate for two main reasons. First, it is dependent on the accuracy of the

Table 5. Apparent Rates of De Novo Synthesis of GLX, Proline, and Arginine Calculated From the Difference Between Total Entry Rate and That From the Diet and Body Protein Degradation Measured in Four Women in the Fed and Fasted States

	GLX		Proline		Arginine	
Amino Acid	Fed	Fasted	Fed	Fasted	Fed	Fasted
Entry rate						
Total	53 ± 4	58 ± 3	14 ± 3	11 ± 3	15 ± 3	16 ± 2
From diet	18	0	8	0	3	0
From protein degradation	24 ± 3	30 ± 1	13 ± 2	16 ± 1	15 ± 2	19 ± 1
Synthesis	11 ± 3	28 ± 3†	−7 ± 4*	−5 ± 3*	−3 ± 3*	−3 ± 2*

NOTE. Values are mg amino acid/kg · h^{-1} ± 1 SD.

*Not significantly different from zero.

†Effect of fasting, $P < .001$.

estimate of body protein degradation. This, in turn, can suffer from two problems, both of which will lead to systematic underestimates of the entry rate of essential amino acid tracer. The first is the failure to measure accurately the intracellular dilution of the tracer. The second, applicable to studies during which the subjects are fed, is the failure to account for first-pass metabolism of the dietary amino acid in the splanchnic bed.[25] Both these underestimates will lead to an overestimate of the rate of synthesis of a nonessential amino acid. It could be argued that our use of an oral route of administration may have minimized the second source of error, inasmuch as first-pass metabolism of the oral tracer should correct, at least partially, for that of the dietary amino acid. It is noteworthy that the rates of whole-body protein degradation (5.28 in the fed state and 6.67 g/kg · d^{-1} in the fasted) calculated from data of the present study are higher than many estimates made with intravenous 1-^{13}C-leucine as tracer, but are similar to those obtained with oral tracer ^{15}N-lysine (5.8 g/kg · d^{-1}).[26]

As emphasized previously by others,[13,16] a further problem in estimating nonessential amino acid synthesis arises from the likelihood of substantial tissue and intracellular compartmentation of their metabolism. The main effect of this is to lead to an underestimate of the entry rate,[13,16] and the phenomenon presumably underlies the fact that when we applied the "excess isotopic dilution method" to calculations of arginine and proline synthesis we obtained numerically negative values. In fact, similar calculations of the data in a recent publication concerned with arginine synthesis,[16] in which leucine was used as the essential amino acid tracer, give rates of arginine entry that are less than the sum of arginine intake and entry from proteolysis, ie, apparently negative rates of arginine synthesis. Nevertheless, in this report,[16] parallel measurements of the incorporation of labeled citrulline into plasma arginine demonstrated clearly the presence of significant but limited (between 5% and 10% of flux) biosynthesis of arginine. It should also be noted that citrulline flux apparently exceeded the synthesis of arginine from citrulline by a factor of two, and the investigators developed a strong argument in favor of substantial compartmentation of arginine metabolism.

In the present report, we also measured the incorporation of ^{13}C from other precursors administered simultaneously with the U-^{13}C-tracer amino acid. In our study, the labeled precursors were given orally and consisted of a mixture of U-^{13}C-amino acids and carbohydrates. This combination of potential substrates enables us to assess the ^{13}C incorporated via endogenous biosynthesis against a baseline provided by U-^{13}C-amino acid absorbed from the gut lumen. Unfortunately, in the present study, measurement of ^{13}C incorporation can only be used in a qualitative way because (1) we were unable to quantify the isotopic enrichment of amino acid biosynthetic precursors and (2) labeling of the [M + 1] − [M + 3] isotopomers was still substantial even after 8 hours labeling.

Nevertheless, the presence of substantial labeling in plasma arginine attests to the presence of biologically significant rates of biosynthesis. Even this method indicated that there was no proline biosynthesis in the fed state, although significant isotope incorporation into plasma proline occurred during the fasting phase of the study. This observation supports the contention[22] that proline biosynthesis in man is not only limited in scope, but is under strict dietary regulation.

Comments on the Significance of Mass Isotopomer Enrichment Patterns

Although we argue that the incorporation method we have adopted is a sensitive means of detecting amino acid biosynthesis, the method, as applied here, is qualitative. Nevertheless, the relative enrichments and kinetics of the various isotopomers can be used to draw some inferences about the pathways leading to amino acid synthesis and the possible sources of the precursors that are used for this purpose.

Glutamate/glutamine. In the present study, ^{13}C can become incorporated into GLX via two main routes. Each pathway will give different isotopomer patterns.

The first pathway involves the net synthesis of glutamate as an end product of the catabolism of arginine (ornithine), proline, and histidine. Under the conditions of this experiment, the catabolism of uniformly labeled precursor amino acids yields [M + 5]-glutamate. Thus, a proportion of the [M + 5]-glutamate detected in the plasma could have arisen not by absorption from the diet, but from label entry via the catabolism of arginine, proline, and histidine.

Label also enters glutamate as a result of its rapid equilibration with α-ketoglutarate. Because this keto acid is an intermediate in the tricarboxylic acid (TCA) cycle, it can become labeled with ^{13}C from many sources. However, whatever the ultimate origin of the label, ^{13}C effectively

enters the TCA cycle at only two points: acetyl coenzyme A and oxaloacetate. This pathway yields only [M + 1] – [M + 3]-glutamate, and we were surprised to find significant quantities of the [M + 4] isotopomer.

There is no simple explanation for the appearance of [M + 4]-glutamate, since such a labeling pattern could not have occurred via the TCA cycle activity of the subject.[27] One possibility is that it represents labeling from the small amount of [M + 4]-glutamate present in the *Spirulina* that the subjects ingested. However, this isotopomer accounted for no more than 3% of the administered labeled material. A second possible explanation is that [M + 4]-glutamate was formed by random association of acetyl coenzyme A and oxaloacetate derived from metabolism by intestinal cells of the highly ^{13}C-enriched dietary carbohydrate. However, labeling via this route would have been transient, and significant quantities of [M + 4]-glutamate were detected for many hours (Fig 1A).

The third possibility is the carboxylation of a uniformly labeled 4-carbon precursor. As far as we are aware, there is no pathway of this nature present in mammals. This suggests the intriguing possibility that [M + 4]-glutamate derives from α-ketoglutarate synthesized by carboxylation of [M + 4]-succinate (derived in turn from labeled carbohydrate metabolism) by the intestinal anaerobic flora. This pathway has been demonstrated as being of importance to the synthesis of glutamate by rumen bacteria.[28] Whether this occurs in bacteria that colonize the human intestine is not known, but it is likely and is worthy of further investigation.

Arginine. Arginine synthesis ultimately involves the formation of arginosuccinate from aspartate and citrulline. These derive from two sources.

The first source is the urea cycle in the liver. This does not lead to net synthesis of arginine, although this pool of arginine must have a high rate of turnover (at least as great as urea synthesis, which in the present study would have approximated the intake of protein N, ie, 400 μmol/kg · h^{-1}). This obviously greatly exceeds our estimate of plasma arginine flux (85 to 95 μmol/kg/h), and it is likely that the arginine formed during hepatic urea synthesis is almost entirely segregated from the plasma arginine pool.[16]

The other source of arginine synthesis is citrulline, synthesized ultimately from glutamate (via Δ^1-pyrolline-5-carboxylate) and carbamoyl phosphate in the enterocyte. The isotopomers of arginine showed two patterns of labeling: [M + 5]-arginine was rapidly labeled and had very similar kinetics to those of [M + 6]-arginine. Because of the kinetic behavior of this isotopomer, we would propose that it arises from rapid metabolism by enterocytes of lumenal [M + 5]-glutamate. In the present study, [M + 5]-arginine achieved an average isotopic enrichment of 21% (fed) and 35% (fasted) of [M + 5]-glutamate. More prolonged labeling of arginine mass isotopomers of [M + 3] and less was also observed, and although because of their low isotopic enrichments, the errors associated with these measurements were greater, by and large the ratios of the average isotopic enrichments of the respective isotopomers of arginine and glutamate were similar to the ratio of the enrichments of [M + 5]-arginine and [M + 5]-glutamate. We interpret these results to suggest that (1) as shown by others,[21] the major site of citrulline synthesis for net arginine production is the gut; (2) on the basis of the kinetics of the different isotopomers, both lumenal (rapid and transient labeling of [M + 5]) and arterial (slower labeling of [M + 1] ... [M + 3]) GLX contribute to this synthetic pathway; and (3) between 15% and 25% of the plasma arginine flux derives from this pathway.

Proline. Proline is the other main product of Δ^1-pyrolline-5-carboxylate in intestinal cells, and despite our earlier results in a hen (a species in which proline is nutritionally essential), we expected the labeling pattern of proline and arginine to be similar. This was clearly not so, especially in the fed state in which the only isotopomer of proline to be labeled was [M + 5]. It should be emphasized that one problem with the present approach is that proline synthesis from U-^{13}C-glutamate could lead to the appearance of [M + 5]-proline, and this cannot be distinguished from [M + 5]-proline derived directly from the diet. Thus, although the very low or near-zero enrichments of isotopomers of proline less than [M + 5] suggests virtually no proline synthesis from *systemic* glutamate, we cannot exclude the possibility that lumenal glutamate was used for proline synthesis. In fact, we suspect that this is so because the isotopic enrichment of [M + 5]-proline was substantially higher (approximately 55% and 110% higher in the fed and fasted state, respectively) than would have been predicted from the known proline intake and the calculated rate of body protein breakdown. Resolution of this issue will require studies involving oral administration of U-^{13}C-glutamate in the absence of U-^{13}C-proline.

Conclusion

The present results confirm the findings of others,[16,22] ie, human beings are capable of net synthesis of both arginine and proline. They also suggest that the two pathways were regulated differentially by the short-term feeding status of the subject. With regard to arginine, the results are compatible with the idea that nutritionally significant de novo synthesis of ornithine and citrulline destined for arginine synthesis may occur from both dietary and systemic glutamate. On the basis of label incorporation into isotopomers less than [M + 6], fasting increases the contribution of arginine synthesis to plasma arginine flux by approximately twofold.

These results contrasted with those obtained with proline. In the fed state, there was apparently little or no proline synthesis from systemic glutamate, although we strongly suspect that significant proline synthesis occurred by first-pass metabolism of dietary glutamate. On the basis of label incorporation into proline isotopomers less than [M + 5], proline synthesis from systemic glutamate increased by a considerable proportion of the rate in the fed state. Even so, proline synthesis from systemic glutamate accounted for no more than 7% of plasma entry rate. Again, we suspect that proline synthesis from dietary

glutamate also increased. It appears, then, that proline synthesis is either (1) tightly regulated by the presence of proline,[22] as reported for some cells in culture[29]; (2) rigorously compartmentalized, and enters the circulation only in the fasted state; or (3) subject to the condition that the only nutritionally significant precursor of plasma proline synthesis is dietary glutamate. Whatever the ultimate explanation, the demonstration that there is either close regulation or substantial tissue compartmentation of proline metabolism suggests that its role in metabolism may be more important than has hitherto been supposed.

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