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Metabolism of Nonessential ^{15}N-Labeled Amino Acids and the Measurement of Human Whole-Body Protein Synthesis Rates[1]

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ABSTRACT Eight ^{15}N-labeled nonessential amino acids plus $^{15}NH_4Cl$ were administered over a 10-h period to four healthy adult males using a primed-constant dosage regimen. The amount of ^{15}N excreted in the urine and the urinary ammonia, hippuric acid and plasma alanine ^{15}N enrichments were measured. There was a high degree of consistency across subjects in the ordering of the nine compounds based on the fraction of ^{15}N excreted (Kendall coefficient of concordance W=0.83, $P < 0.01$). Protein synthesis rates were calculated from the urinary ammonia plateau enrichment and the cumulative excretion of ^{15}N. Glycine was one of the few amino acids that gave similar values by both methods. J. Nutr. *116*: 1651–1659, 1986.

INDEXING KEY WORDS ^{15}N • nonessential amino acids • protein synthesis

We are interested in the use of ^{15}N-labeled glycine as a tracer for determining the human whole-body protein synthesis rate. There are several variants, the principal ones being *1)* methods based on the enrichment of the urinary ammonia (1,2), *2)* decay curve analyses based on the decay of isotope enrichment in the urine or plasma following a single pulse, (3–5), *3)* from the cumulative excretion ^{15}N excreted (6–8) and *4)* a primed constant infusion-flux method (9).

Although several other methods involving ^{13}C-labeled amino acids have been described, the methods using ^{15}N are particularly suitable for use outside a hospital or clinical research center. The principal advantages of ^{15}N are: *1)* ^{15}N can be used for single dosage–single sampling point methods, *2)* serial sampling is not required and *3)* there is no need to collect and analyze exhaled breath. Although other ^{15}N-labeled amino acids have been used, for example, alanine (10), aspartate (11) and lysine (9, 12), glycine is the most frequently used ^{15}N-labeled amino acid because of its availability and relatively low price (13).

This study was designed to determine whether any of the other nonessential amino acids labeled with ^{15}N offer any advantages over glycine. We compared the glycine-based protein synthesis rates (PSR) using both the urinary ammonia plateau, ^{15}N enrichment and a cumulative excretion method against those found with other ^{15}N-labeled nonessential amino acids. We also compared the relative effectiveness of glycine in transferring ^{15}N to plasma alanine, urinary ammonia and urinary hippuric acid.

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MATERIALS AND METHODS

The subject population consisted of four healthy adult males between 27 and 42 years of age. Informed consent was obtained in accordance with the policies of the Graduate Hospital and the University of Pennsylvania, Philadelphia, PA.

Eight ^{15}N-labeled nonessential L-amino acids plus [^{15}N]ammonium chloride were tested. [^{15}N]glycine (99%) and arginine (96%) were obtained from US Services/ ICONS, Summit, NJ; [^{15}N]alanine (99%); $^{15}NH_4Cl$ (99%) and [^{15}N]glutamic acid (99%) from KOR Isotopes, Inc. (now ICN), Cambridge, MA; [^{15}N]aspartic acid (98%), [^{15}N]serine (99%) and [^{15}N]amido-labeled asparagine (99%) from Merck and Co., St. Louis, MO. and [^{15}N]amido-labeled glutamine (99%) from Cambridge Isotopes, Inc., Cambridge, MA. Chemical and isotopic purity were verified by gas chromatograph-mass spectrometry (GC-MS). The amino acids were converted to their *N*-acetyl isopropyl derivatives, and the spectra were compared with the appropriate spectra from unlabeled compounds.

The total dose of ^{15}N ranged between 20 and 100 mg ^{15}N per subject. For the more expensive amino acids, the first subject took between 35 and 70 mg ^{15}N. The results were then analyzed and a decision made as to the minimum amount needed to give accurate data, and that dose was given to subsequent subjects. The reason for doing so was to minimize the cost of the study. The total dose (20–100 mg ^{15}N) was divided into 13 equal aliquots and placed in gelatin capsules. Each capsule also contained 300 mg of sodium benzoate. Glycine is conjugated with benzoic acid in the liver to give hippuric acid, which is excreted in the urine. Thus the urinary hippuric acid provides a means of sampling the hepatic glycine pool (14).

The nine ^{15}N-labeled compounds were given in random order at approximately one per month to the four subjects. The study was started at 0700, after an overnight fast, with the subject voiding and taking a priming dose of four capsules. The remaining nine capsules were taken at hourly intervals thereafter. Each time the subject took a capsule he drank 50 mL of Ensure (Ross Laboratories, Columbus, OH). Ensure is a defined formula diet containing 1 kcal/mL. The 50 kcal/h of Ensure was given because this caloric intake (500 kcal/10 h) approximated the subjects' normal caloric intake for the period 700 to 1700. Subjects were permitted to consume noncaloric beverages (water, coffee or tea but with no cream or sugar) and nothing else from 0700 to 1700. At 1700, 7.0 mL of blood was drawn. Subjects collected a pooled urine for the period 0700 to 1230, after which time urine was collected approximately hourly until 1700. During the 10 h of the study the subjects performed their normal duties, which consisted of a combination of laboratory and office work.

Analytical methodology. Two methods were used for ^{15}N analysis. Plasma alanine enrichments were determined by GC-MS, and optical emission spectroscopy was used for the urinary nitrogen, ammonia and hippuric acid and the plasma urea. The sensitivity of the GC-MS and optical emission methods are ± 0.2 and ± 0.02 atom percent excess, respectively, in the range studied. Optical emission is the simpler technique, but requires a larger sample (3 μg of N_2) than the GC-MS method. Briefly, optical emission spectroscopy involves the irradiation of N_2 gas in a sealed 6-mm Vycor glass tube (Corning, Inc., Corning, NY) at 3 mmHg pressure with radio frequency energy. The N_2 molecules absorb the energy and reemit some of it as mauve light. The intensities of the emitted light at 297.68 nm ($^{14}N_2$) and 298.29 nm ($^{15}N^{14}N$) are compared by a standard monochromator-photomultiplier arrangement. This method requires that all samples be converted to N_2 gas, and this is usually accomplished by conversion to ammonia first and then oxidation to N_2 by alkaline hypobromite (15).

The blood urea nitrogen (BUN) was determined by the urease method using Sigma diagnostic kit #640 (Sigma Chemical Co., St. Louis, MO). For determination of the isotopic enrichment of the BUN, water (1 mL) and urease solution (2.0 mL, 0.21 mg urease/mL in 0.1 M phosphate buffer, pH 6.5) was added to plasma (2.0 mL). After incubating for 30 min at 37°C, K_2CO_3 (5 mL)

and 2-octanol (8 drops) were added. The ammonia was removed by aeration and collected in 0.1 N H_2SO_4 (1 mL). Total urinary nitrogen was measured on 1 mL of urine by the Kjeldahl method. Urinary ammonia was isolated from urine (5 mL) by adding saturated K_2CO_3 (5 mL) and aerating was done as described above for the BUN. The ^{15}N enrichment of the urinary ammonia, total urinary nitrogen for each void (Kjeldahl distillates) and ammonia from the urease reaction on the BUN were determined by optical emission spectroscopy as previously described (15, 16).

Hippuric acid was isolated from the urine by crystallization after adjusting the urine concentration to 30% with sodium chloride (16). Occasionally the hippuric acid did not crystallize out. For those samples the urine (5 mL) was adjusted to pH 1 with concentration. H_2SO_4 and the urine extracted three times with ether (5 mL). The ether extracts were combined and evaporated to dryness. The pale straw residues from either the salt precipitation or the ether extractions were dissolved in minimal hot water. On cooling at 4°C, white needle-shaped crystals of hippuric acid separated out. The hippuric acid was then converted to $(NH_4)_2SO_4$ by Kjeldahl oxidation and analyzed for ^{15}N by optical emission spectroscopy as described above.

The enrichment of the plasma alanine was determined by converting the plasma amino acids to their N-acetyl isopropyl esters as previously described (15). GC-MS analysis was done on a Hewlett-Packard 5992A GC-MS in the selective ion monitoring mode (SIM, ref. 15). The fragments at 186 amu (atomic mass units) and 187 amu were monitored. The parent peak is at 186 amu.

Methods of calculation. Fraction of ^{15}N excreted. The amount of the administered dose excreted ($^*e$) was calculated by summing the ^{15}N excreted in each urine sample collected over the 10-h period and the ^{15}N remaining in the body urea pool at 10 h. The latter was calculated from the blood taken at the end of the experiment. The size of body urea pool (in grams) was estimated from the BUN (in milligrams/100 mL) using an equation derived by Hume and Weyers (17):

$$\text{urea pool} = \text{UDS} \times 10^2 \times \text{BUN}$$

where UDS is urea distribution space and UDS = (0.195 × ht in centimeters) + (0.297 × wt in kilograms) − 14.013. Thus ^{15}N in urea pool = UDS (in liters) × BUN (in grams/liter) × BUN ^{15}N (atom percent excess) × 0.01.

Whole-body protein synthesis rates. The whole-body protein synthesis rate (PSR) was calculated from the urinary ammonia ^{15}N enrichment at plateau (18) and from the total amount of ^{15}N excreted (6–8). From the urinary ammonia, PSR was calculated as follows:

$$Q = {}^*d/A \text{ and } Q = E + S$$

where Q is nitrogen flux in grams N/hour; $^*d$ is rate of ^{15}N glycine administration in grams N/hour; A is ^{15}N abundance in the urinary ammonia at plateau (APE·0.01); E is rate of N excretion in grams N/hour; S is rate of protein synthesis in grams N/hour; and APE is atom percent excess ^{15}N.

From the total ^{15}N excreted (cumulative excretion), PSR was calculated as:

$$S = E_T \cdot ({}^*d/{}^*e - 1)$$

where $^*d$ is amount of ^{15}N given in grams N 10/hour; $^*e$ is amount of ^{15}N excreted (urine + BUN) in grams N 10/hour; E_T is amount of N excretion in grams N 10/hour; and S is rate of protein synthesis in grams N 10/hour.

The total amount of ^{15}N excreted ($^*e$) was defined as described above. In this study, we assumed that there was no change in the BUN during the course of the study, and the amount of ^{15}N in the tissue free amino acid pools at 10 h was negligible (5, 19).

RESULTS

A one-way, repeated-measures design analysis of variance was run on each measure: *1)* % of ^{15}N excreted, *2)* PSR by a cumulative excretion method, *3)* PSR from the urinary ammonia ^{15}N plateau, *4)* urinary NH_3 ^{15}N enrichment at plateau, *5)* plasma alanine ^{15}N enrichment and *6)* the urinary hippuric acid ^{15}N enrichment. The results are summarized in figure 1 and table 1. The Kendall coefficient of concordance *(W)* was determined for each of the above measurements to provide a measure of the degree of consistency across subjects ("intersubject reliability") for the amino acids (table 1, ref.

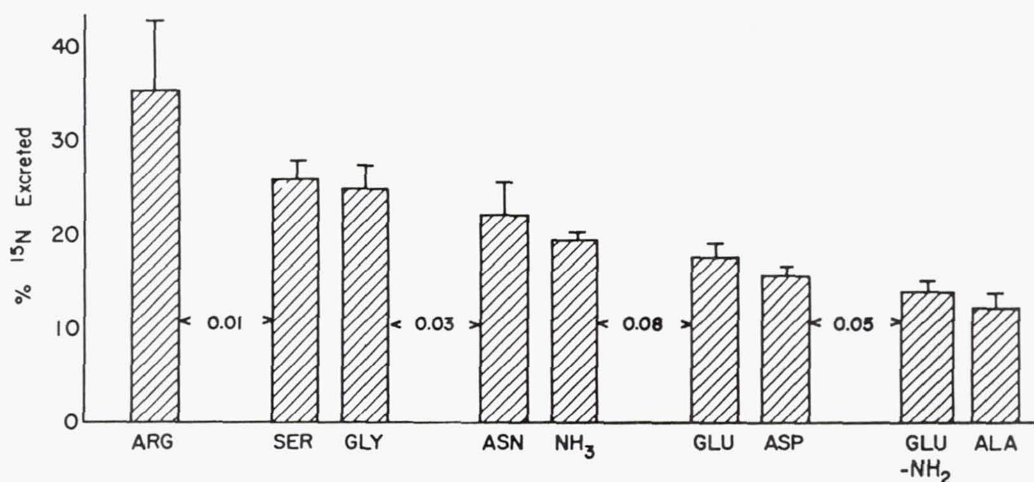

Fig. 1 Percentage of ^{15}N excreted from various nonessential amino acids. Data is mean ± SEM, $n = 4$. Numbers between groups are P-values.

20). The major findings of this study were:

1) There were significant differences in the fraction of ^{15}N excreted among the various amino acids. The nonessential nitrogen compounds studied appeared to fall into five well-defined groups (figure 1).

2) There was high intersubject reliability in the ordering of the nine compounds based on the fraction of ^{15}N excreted ($W = 0.83$, $P < 0.01$). All of the other ^{15}N parameters except alanine also showed high intersubject consistency among subjects (table 1). As expected, parameters reflecting unlabeled nitrogen, such as nitrogen excretion and the BUN showed poor intersubject consistency ($W < 0.35$).

3) There was significantly less transference of ^{15}N from guanido-labeled arginine

TABLE 1

Summary of synthesis rates calculated by flux (PSR_F) and cumulative excretion (PSR_T) methods and ^{15}N enrichment of the urinary ammonia, hippuric acid and plasma alanine[1]

	PSR_T	PSR_F	Urine hippuric acid ^{15}N	Urine ammonia ^{15}N	Plasma alanine ^{15}N
	g protein kg per d			*APE*	
Arginine	2.4 ± 0.7^{a}	27.6 ± 7.5^{a}	0.05 ± 0.02^{a}	0.06 ± 0.01^{a}	$0.67 \pm 0.15^{b,c}$
Serine	$4.4 \pm 0.6^{a,b}$	$6.6 \pm 4.5^{b,c}$	2.03 ± 0.26^{b}	0.26 ± 0.06^{b}	$0.71 \pm 0.29^{a,b}$
Glycine	$4.3 \pm 1.0^{a,b}$	$5.2 \pm 1.9^{c,d}$	1.93 ± 0.31^{b}	0.23 ± 0.06^{b}	1.71 ± 0.89^{a}
Asparagine	$4.8 \pm 2.1^{a,b}$	9.2 ± 2.2^{b}	0.12 ± 0.01^{f}	0.16 ± 0.13^{c}	$0.91 \pm 0.69^{a,b}$
NH_4Cl	$7.2 \pm 2.7^{b,c}$	13.2 ± 2.5^{a}	0.26 ± 0.02^{d}	0.11 ± 0.02^{c}	0.58 ± 0.08^{c}
Glutamate	6.2 ± 1.2^{b}	$7.2 \pm 1.9^{c,d}$	$0.26 \pm 0.04^{c,d}$	0.19 ± 0.03^{b}	$0.89 \pm 0.10^{a,b}$
Aspartate	$6.9 \pm 1.9^{b,c}$	$10.3 \pm 4.5^{a,b}$	$0.23 \pm 0.02^{d,e}$	0.16 ± 0.05^{c}	$0.83 \pm 0.11^{a,b}$
Glutamine	9.4 ± 2.7^{c}	5.1 ± 3.1^{d}	0.17 ± 0.02^{e}	0.25 ± 0.05^{b}	ND
Alanine	10.4 ± 3.3^{c}	9.2 ± 2.4^{b}	0.35 ± 0.02^{c}	0.15 ± 0.03^{c}	0.99 ± 0.42^{a}
Mean	6.1 ± 0.9	10.4 ± 2.6	0.60 ± 0.28	0.23 ± 0.05	1.07 ± 0.34
W (P)	0.79 (0.02)	0.72 (0.05)	0.97 (0.01)	0.84 (0.01)	0.42 (NS)

[1]Values are means ± SEM. ND, no data. Unlike superscripts differ by $P < 0.05$. APE is atom percent excess (^{15}N). W is the Kendall coefficient of concordance (20).

to ammonia or hippuric acid than from any other compound (table 1).

4) Two of the subjects excreted a very large proportion of the guanido N from arginine as urea (48 and 44% vs. 23 and 21%).

5) Less ^{15}N from alanine and glutamine were excreted than from any of the other compounds tested (fig. 1).

6) Reasonable urinary ammonia ^{15}N plateaus were obtained for the nine compounds (figure 2).

7) There was no close relationship between the protein synthesis rate based on the urinary ammonia ^{15}N enrichment (PSR_F) and the value derived from the total amount of ^{15}N excreted (PSR_T).

DISCUSSION

Selection of study conditions. The normal dietary intake between 0700 and 1700 of the four subjects ranged from breakfast (toast and coffee) and a meal for lunch to black coffee only. The dietary regimen (50 kcal/h) of Ensure was a compromise designed to approximate the mean normal nutritional intake of the four subjects for that period. During the study, the subjects performed their normal daily duties.

As a proportion of the oral diet given, the test amino acid may have represented up to a 50% increase in the dietary amount of the test amino acid. Such relatively high dosages are routinely used in stable isotope tracer studies because of the relatively high enrichment levels needed for detection with most currently available analytical equipment (9). The "tracer" amino acid is mixed with the much larger pool of amino acids derived from protein breakdown so that the contribution to the amino acid flux approaches a tracer dose (~5%; refs. 9, 18, 19, 21).

Excretion of ^{15}N. There was high intersubject reliability in the ordering of amino acids based on the fraction of ^{15}N excreted (W=0.83, P>0.01). Furthermore, the compounds tested appeared to fall into five well-defined groups (fig. 1): *1)* arginine, *2)* serine and glycine, *3)* asparagine and ammonia, *4)* glutamate and aspartate and *5)* glutamine and alanine (fig. 1). These groupings correspond to *1)* an amino acid which is a very close precursor of urea (arginine), *2)* amino acids which are interconvertible and metabolized via ammonia and one carbon transfer reactions (serine and glycine), *3)* compounds that are metabolized as ammonia (NH_4Cl and asparagine, which is hydrolyzed to ammonia in the gut), *4)* amino acids whose carbon skeletons play key roles in intermediary metabolism (aspartate and glutamate) and *5)* dual-function amino acids where the carbon skeleton is involved in intermediary metabolism and interorgan nitrogen transport (glutamine and alanine).

Although it was expected that a large proportion of the nitrogen from arginine would be metabolized to urea, the magnitude in two of our four subjects was surprising (48 and 44% vs. 21 and 23%). This may be due to human variability (22). Arginine has been reported to be a marginally essential amino acid (23).

Transference of ^{15}N to other nonessential amino compounds. A high degree of consistency across subjects in the ordering of amino acids was found for the ^{15}N enrichment of urinary ammonia and hippuric acid but not for alanine (table 1), possibly because of the lower sensitivity of the GC-MS analysis (±0.2 atom percent excess). Like the alanine and hippuric acid ^{15}N enrichment, the urinary enrichment of ammonia is another indicator of ^{15}N transference but reflects both the enrichment of the free amino acid pools and the proportion of ammonia derived from the carrier amino acid. No other amino acid transferred significantly more ^{15}N to urinary ammonia or plasma than glycine (table 1).

Calculation of protein synthesis rates. Although all estimates of the human body protein synthesis rate are based on the three-pool compartmental model developed by San Pietro and Rittenberg (5), there are many variants, each of which is based on slightly different sets of assumptions (18, 19, 24, 25). From all of these methods a consensus has emerged that the daytime human whole-body PSR is in the range of 1.5 to 6 g protein/kg per d. In this study we obtained values from the urinary ammonia plateau ^{15}N enrichment and from total amount of ^{15}N excreted. There was no apparent relationship between the whole-body PSR values derived

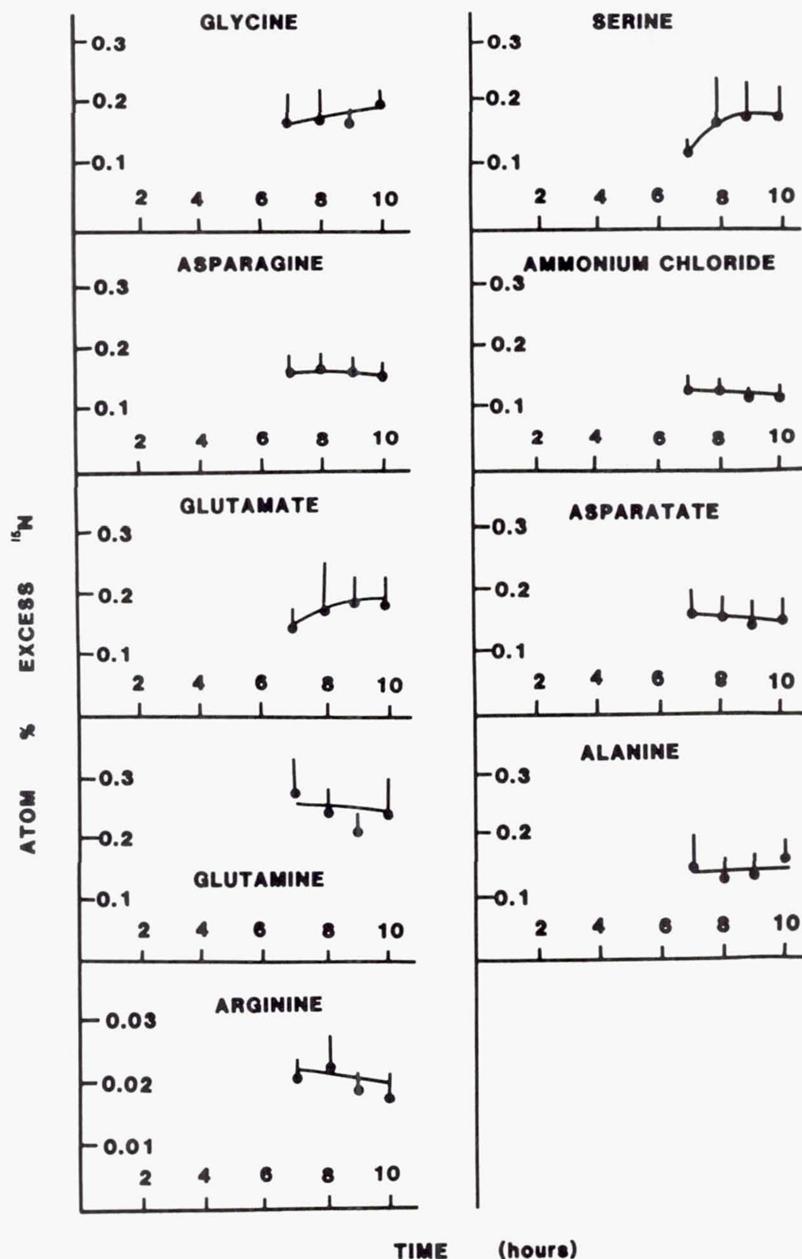

Fig. 2 Urinary ammonia enrichments for the urine specimens collected between 1230 and 1700. Data not normalized.

from the urinary ammonia and total excretion of ^{15}N.

Cumulative ^{15}N excretion. The basic assumption underlying the end point approach is that administered ^{15}N is partitioned between end product (urea) and protein in the same proportion as total nitrogen, most of which is derived from endogenous protein breakdown. Although this issue has been addressed by several authors

previously, it is still not resolved for ^{15}N (7, 8, 13). The partitioning coefficient, or synthesis rates derived therefrom, should be independent of the route of isotope administration. Unless they are, one or both routes of isotope administration cannot reflect the composite endogenous nitrogen metabolism. Thus this study yields data on two criteria: *1)* the calculated PSR should be in reasonable agreement with the value derived by other (carbon-labeled) methods, and *2)* there should be reasonable agreement between the intravenous and orally derived values.

Most of the PSRs calculated by the cumulative excretion method in the present study are within the "consensus range" of 1.5 to 6 g protein/kg per d. The oral values found for glutamine and alanine (10.4 and 9.4 g protein/kg per d) in this study were high, although rates within the "consensus range" have been reported with the intravenous route (2.8 and 3.6 g protein/kg per day), respectively (10, 24). The oral aspartate and glutamate values are also elevated (table 1). These four amino acids are, as pointed out above, dual-function amino acids, and the second function can affect the partitioning of nitrogen. Previously, Tarunga (26) reported that essential amino acid nitrogen was unsuitable as a tracer because the nitrogen metabolism reflected the metabolism of the carbon skeleton. This study extends that conclusion to nonessential amino acids where the metabolism of the amino acid is dependent on both the partitioning between synthesis and excretion and the role in intermediary metabolism of the deaminated amino acid. Glycine meets the criteria of having no other major metabolic role if serine is regarded as part of the glycine pool. Glycine gives the same values orally or intravenously (25).

Urinary ammonia. There are at least two routes by which ^{15}N can be transferred to ammonia and then excreted in the urine. One is by entering, mixing and equilibrating with the body's free amino acid pools, a key assumption of both the flux and end product approaches. The other is by serving as a precursor to urinary ammonia without having attained isotopic equilibration in the tissue free amino acid pools. Different amino acids contribute differently to the urinary ammonia, although the major precursor is plasma glutamine, and this may explain the lack of correlation between the methods (table 1, ref. 24). The values derived for the two methods were more similar for glycine than for any other ^{15}N compound tested.

The cumulative excretion method is much less dependent on the urinary ammonia enrichment because more than 90% of the ^{15}N is excreted as urea. These uncertainties, the lack of correlation with the more theoretically sound total-excretion-based values and the range and scatter in the flux-based values suggest that the cumulative excretion of total ^{15}N is the preferable approach.

Use of ^{15}N-labeled nonessential amino acids as tracers. There is some question as to how widely applicable single-isotope whole-body methods are. Anomalously high PSRs have been reported with glycine (8, 27), tyrosine (28) and leucine (29) in situations where there was hepatic insufficiency. The liver is particularly important in the mixing and equilibrating processes. Unexpectedly low (negative values) have been found with [1-^{13}C]leucine in human subjects on low protein diets and during a simulated triathlon (30, 31). The fact that apparently anomalous results are found with several tracers suggests that the effect is not specific to the amino acid used but a failure of the model where liver insufficiency or other serious perturbations may result in compartmentation of the tissue free amino acid pools (27). The presence of any of these situations limits the applicability of single-isotope whole-body methods for the measurement of human whole-body protein synthesis rates.

The use of [^{15}N]glycine. The PSR values derived from the two methods used in this study were closer for glycine than for any other amino acid and are similar to those found with ^{13}C-labeled amino acids. No other nonessential ^{15}N-labeled amino acid has been shown to be superior to glycine. [^{15}N]glycine has several distinct advantages for studying human protein metabolism: *1)* Methods involving [^{15}N]glycine are technically the simplest (e.g., single-point assays (2); *2)* [^{15}N]Glycine is relatively cheap and readily available in a pure state; *3)* More is

known about the metabolism of [^{15}N]glycine than any other ^{15}N-labeled amino acid; *4)* Only [^{15}N]glycine offers the possibility of simultaneously measuring liver-originated plasma protein synthesis rates by using the urinary hippuric acid to determine the enrichment of the liver intracellular glycine pool (16).

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