Tracer-derived total and folate-dependent homocysteine remethylation and synthesis rates in humans indicate that serine is the main one-carbon donor

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Tracer-derived total and folate-dependent homocysteine remethylation and synthesis rates in humans indicate that serine is the main one-carbon donor. Am J Physiol Endocrinol Metab 286: E272–E279, 2004. First published October 14, 2003; 10.1152/ajpendo.00351.2003.—Hyperhomocysteinemia in humans is associated with genetic variants of several enzymes of folate and one-carbon metabolism and deficiencies of folate and vitamins B12 and B6. In each case, hyperhomocysteinemia might be caused by diminished folate-dependent homocysteine remethylation, but this has not been confirmed in vivo. Because published stable isotopic tracer approaches cannot distinguish folate-dependent from folate-independent remethylation, we developed a dual-tracer procedure in which a [U-13C5]-methionine tracer is used in conjunction with a [3-13C]serine tracer to simultaneously measure rates of total and folate-dependent homocysteine remethylation. In young female subjects, plasma [U-13C5]methionine enrichment, a surrogate measure of intracellular [U-13C5]methionine enrichment, reached ~90% of the plasma [U-13C5]methionine enrichment. Methionine-methyl and -carboxyl group fluxes were in the range of 32611–0370 (E-mail jfgy@mail.ifas.ufl.edu).

ELEVATED PLASMA HOMOCYSTEINE CONCENTRATION is considered an independent risk factor for the development of cardiovascular disease (5, 30, 32). Accordingly, many investigators have sought to define the genetic and environmental factors that affect plasma homocysteine concentration. Associations exist between plasma homocysteine concentrations and gene polymorphisms, as well as lifestyle and other environmental factors (5). Strong evidence implicates nutritional deficiencies of folate, vitamins B12 and B6, and the methylenetetrahydrofolate reductase (MTHFR) 677C → T polymorphism as causes of elevated plasma homocysteine concentration (12, 25). Folate and vitamin B6 deficiencies and the MTHFR 677C → T polymorphism are thought to increase circulating homocysteine concentrations by decreasing the availability of 5-methyltetrahydrofolate (5-CH3THF) and thereby inhibiting homocysteine remethylation (24). However, a causal relationship between reduced homocysteine remethylation and hyperhomocysteinemia under these conditions has not been confirmed in humans in vivo.

Steady-state plasma homocysteine concentration is not solely a function of the rate of its removal by remethylation but is also affected by the rates of homocysteine production, catabolism through transsulfuration, and loss in renal excretion (24). Specific measurements of homocysteine metabolism through these individual pathways are needed to clarify why homocysteine concentration is elevated by particular genetic variations as well as by nutritional and other environmental conditions. To test the hypothesis that homocysteine remethylation is compromised when 5-CH3THF availability is reduced, homocysteine remethylation rates must be measured in individuals affected by these homocysteine-elevating factors.

Remethylation rates have been measured in humans in vivo by use of methionine tracers labeled with stable isotopes at both the carboxyl and methyl groups or else by measurements of separate methyl- and carboxyl-labeled methionine tracers in primed constant infusion experiments (18, 26). By use of these methodologies, the effects of dietary sulfur amino acid intake, sex, age, prandial status, one-carbon (1-C) donor intake, and burn injury on the rates of homocysteine remethylation have been defined (7, 8, 11, 18, 27, 28, 36). A complication of the measurement of homocysteine remethylation rate is that the reaction can also be catalyzed in a folate-independent pathway via a second homocysteine methyltransferase, betaine-homocysteine methyltransferase (9). Previously published methods for measuring total body homocysteine remethylation cannot distinguish between the methyl groups transferred by these two homocysteine methyltransferases, which precludes the use of
such methods for measuring the mechanism by which homocysteine remethylation is altered.

We refined published tracer methods to allow simultaneous measurement of total and folate-dependent homocysteine remethylation in humans through the combined use of a universally $^{13}$C-labeled methionine tracer ([U-$^{13}$C$_5$]methionine) and a serine tracer labeled at the three-carbon ([3-$^{13}$C$_3$]serine). With this tracer combination, the remethylated methionine tracer ([13C$_2$]methionine) was measured separately from the [13C$_1$]methionine generated by the folate-dependent homocysteine remethylation pathway from [3-$^{13}$C$_3$]serine. Using this tracer model, we determined that serine is the predominant one-carbon donor for homocysteine remethylation in healthy females with adequate folate, vitamin B$_{12}$, and vitamin B$_6$ nutritional status.

**METHODS**

**Materials**

L-[5,5,5-$^{2}$H$_3$]leucine, L-[3-$^{13}$C$_3$]serine, and L-[U-$^{13}$C$_5$]methionine were purchased from Cambridge Isotope Laboratories (Woburn, MA). Tracer solutions were prepared in isotonic saline, filter sterilized, and analyzed to ensure lack of pyrogenicity and microbial contamination. Pyrogenicity was determined by a commercial laboratory (Focus Technologies, Cypress, CA) using the Limulus amebocyte lysate assay.

**Human Subjects**

Subjects were healthy 20- to 26-yr-old female nonsmokers who did not use oral contraceptives or other medications that might interfere with folate metabolism. Subjects also abstained from alcohol consumption during the study period. Informed consent was obtained from all subjects. MTHFR 677C → T genotype was determined by a polymerase chain reaction-restriction fragment length polymorphism procedure (35). Plasma concentrations of folate (11–32 nmol/l), vitamin B$_{12}$ (178–448 pmol/l; Quantaphase II B12/Folate Radiobinding Assay; Bio-Rad Laboratories, Hercules, CA), pyridoxal phosphate (43–81 nmol/l) (31), and homocysteine (6.3–12.1 µmol/l) (20) were acceptable in all subjects. A medical history questionnaire, physical examination, and clinical blood chemistry screening were used to confirm general health, including renal function. This protocol was approved by the University of Florida Institutional Review Board and the General Clinical Research Center (GCRC) Scientific Advisory Committee.

**Dietary Treatments**

All meals were prepared by the metabolic kitchen in the GCRC. Subjects consumed nutritionally adequate meals of controlled amino acid content at the GCRC or in their homes for three days before infusions.

**Infusion Protocol**

Subjects were admitted to the GCRC on the evening before each infusion protocol and consumed no food between 8:30 PM and initiation of the infusion. On the morning of the infusion, a heparin lock was established in one vein of each arm, one for blood collection and one for the tracer infusion. Blood samples were taken before infusion to measure concentrations of background homocysteine, vitamin B$_{12}$, and vitamin B$_6$, as well as for measurement of background isotopic enrichment of amino acids. Infusions were initiated at 8:30 AM with a 5-min 20-ml priming dose that delivered 9.26, 1.62, and 1.87 µmol/kg of [3-$^{13}$C$_3$]serine, [U-$^{13}$C$_5$]methionine, and [H$_3$]leucine, respectively. The 9-h constant infusion followed immediately after the priming dose and delivered 20 ml of infusion solution per hour that contained 9.26, 1.62, and 1.87 µmol/kg of [3-$^{13}$C$_3$]serine, [U-$^{13}$C$_5$]methionine, and [H$_3$]leucine, respectively. Heparinized blood samples were taken at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7.5, and 9 h of the infusion. These samples were placed on ice and centrifuged, and the plasma was stored at −80°C until analysis. Subjects were kept from a catabolic state during the infusion by hourly consumption of a protein-free formulation containing carbohydrate (70% of energy) and fat (30% of energy) that provided 1.24/24 h of the subject’s daily energy requirement per serving (1.25 kcal·kg$^{-1}$·h$^{-1}$).

**Gas Chromatography-Mass Spectrometry Analysis of Amino Acid Isotopic Enrichments**

The methods used for determination of amino acid isotopic enrichment are similar to those previously described (4, 15–17). To isolate free amino acids, 200 µl of plasma from each infusion time point were combined with 400 µl of 1 mol/l trichloroacetic acid to precipitate plasma proteins. After centrifugation (10,000 g for 10 min), supernatants were removed and loaded onto columns packed with ~0.5 ml of AG50W-X8 cation exchange resin (Bio-Rad) in the acid form. Columns were washed with water (10 × 1 ml), followed by elution of amino acids into reaction vials using 3 ml of 3 mol/l NH$_4$OH. Samples were dried in a Speedvac at ambient temperature and then reconstituted in 10% H$_2$O.

Amino acids were derivatized by reacting dry samples with 500 µl of a 5:1 mixture of 1-propanol-acetyl chloride and 10 µl of ethanethiol for 1 h at 110°C. Samples were dried under N$_2$, and the n-propyl amino acid esters were derivatized by reaction with 100 µl of heptafluorobutyric anhydride and 10 µl of ethanethiol for 1 h at 60°C. The reaction mixtures were dried under N$_2$, and the resulting N-heptafluorobutytryl-n-propyl ester derivatives were solubilized in 100 µl of ethyl acetate and stored at −20°C until analysis.

Isotopic enrichment was determined using a Finnigan-Thermoquest Voyager gas chromatograph-mass spectrometer. Chromatography was performed at a He flow rate of 1.0 ml/min using a 30-m poly(5% diphenyl-95%dimethylsiloxane)-fused silica capillary column (Equity 5; Supelco, Bellefonte, PA) and using 0.3- to 2.0-µl injections and an injector temperature of 250°C. The initial oven temperature was held at 95°C for the first 3 min, increased to 130°C at 3.5°C/min, held at 130°C for 10 min, raised to a final temperature of 250°C at 8°C/min, and held at 250°C for 10 min. Negative chemical ionization-mass spectrometry was performed with methane as the reagent gas at a source temperature of 150°C and an electron energy of 70 eV. The abundance of specific ions was determined by selected ion monitoring at the following mass-to-charge ratios: serine (519–520), leucine (349–352), methionine (367–372), homocysteine (549–553), cysteine (535–536), and cystathionine (678–682). Isotopic enrichments are expressed as molar ratios of labeled to nonlabeled isotopomers after correction for the natural abundance of stable isotopes (26).

**Kinetic Analyses**

The [U-$^{13}$C$_5$]methionine and [3-$^{13}$C$_3$]serine tracer paradigms are illustrated in Fig. 1, A and B, respectively. As the methionine tracer is metabolized in the methylation cycle (Fig. 1A), one $^{13}$C atom is lost in the methyltransferase reactions. Consequently, homocysteine produced from this tracer contains four $^{13}$C-labeled carbons. Remethylation of [13C$_4$]homocysteine with an unlabeled methyl group from 5-CH$_3$THF or betaine generates [13C$_4$]methionine. Total homocysteine remethylation is calculated as the in vivo rate of this reaction. As the serine tracer is metabolized in the folate cycle (Fig. 1B), the $^{13}$C-labeled carbon is transferred to tetrahydrofolate, reduced by MTHFR, and subsequently transferred to homocysteine via methionine synthase to generate [13C$_5$]methionine. Folate-dependent remethylation is calculated as the in vivo rate of this reaction.

**Flux calculations.** Plateau enrichments (Ep) for all infused amino acid tracers, determined by visual inspection, were calculated as the mean of the isotopic enrichments for the 5- to 9-h time points for the...
infused [U-^{13}C_5]methionine, [3-^{13}C]serine, and [^{3}H_1]leucine tracers. Plateau enrichments of all labeled products were determined by fitting enrichment data to single exponential curves defined by the equation \( E = E_\infty (1 - e^{-kt}) \). In this equation, \( E \) is the enrichment at time \( t \) (h), and \( E_\infty \) and \( k \) are the enrichment at infinity (i.e., plateau enrichment) and rate constant (h\(^{-1}\)) from the fitted curve, respectively (18).

Steady-state kinetics of amino acid tracers were calculated using standard equations, including correction for overestimation of intracellular enrichment from plasma enrichment data (26). The flux \( Q \) of an amino acid is the rate of appearance of that amino acid from endogenous production (de novo synthesis and protein breakdown) and the tracer infusion and is calculated from the plateau enrichment...
of the corresponding amino acid tracer. Specifically, the flux of leucine \( (Q_{\text{Leu}}) \) in the plasma pool was calculated as

\[
Q_{\text{Leu}} = \left[\text{H}^4\right]\text{leucine infusion rate (I}_{\text{Leu}}) \\
\times \left( \left[\text{H}^4\right]\text{leucine enrichment} \left(\frac{E_{\text{Leu}}}{E_{\text{Ser}}} - 1\right) \right)
\]

Serine flux \( (Q_{\text{Ser}}) \) was calculated from plasma \([3-^{13}C]\)serine enrichment after correction for the overestimation of the intracellular \([3-^{13}C]\)serine enrichment that occurs when the plateau enrichment of the \([3-^{13}C]\)serine tracer from plasma is used. The corrected \([3-^{13}C]\)serine enrichment \( (E'_{\text{Ser}}) \) was calculated by multiplying the observed plasma \([3-^{13}C]\)serine enrichment by a conservative correction factor \((0.4)\) derived from previous serine tracer infusion studies in humans \((4, 10)\)

\[
Q_{\text{Ser}} = I_{\text{Ser}} \times \left[\left(\frac{E_{\text{Ser}}}{E'_{\text{Ser}}} - 1\right)\right]
\]

The flux of the methionine carboxyl group \((Q_c)\) refers to the rate of appearance of the carboxyl moiety of methionine from protein breakdown and the tracer infusion. The flux of the methyl group \((Q_m)\) also includes these sources of methionine as well as the production of methionine via remethylation of homocysteine \((\text{i.e., } Q_m = Q_c + \text{remethylation})\). By use of the kinetic model of Storch et al. \((26)\), calculation of the methionine carboxyl-group flux \((Q_c)\) requires the sum of the Eps of the infused \([U-^{13}C_5]\)methionine and remethylated \([^{13}C_2]\)methionine tracer \((\text{Met}_{\text{1,5}})\) and \(\text{Met}_{\text{1,4}}\), respectively, after adjustment of the former for overestimation of intracellular \([U-^{13}C_3]\)methionine enrichment that occurs when the plateau enrichment of the \([U-^{13}C_3]\)methionine tracer from plasma is used. The corrected \([U-^{13}C_3]\)methionine enrichment \( (E'_{\text{Met}}) \) was calculated by multiplying the observed plasma \([U-^{13}C_3]\)methionine enrichment by a correction factor \((0.8)\)

\[
Q_c = I_{\text{Met}} \times \left[\left(\frac{E_{\text{Met}}}{E'_{\text{Met}}} - 1\right)\right]
\]

Flux of the methionine-methyl group alone \((Q_m)\) requires only the corrected Ep of the infused \([U-^{13}C_3]\)methionine tracer

\[
Q_m = I_{\text{Met}} \times \left[\left(\frac{E_{\text{Met}}}{E'_{\text{Met}}} - 1\right)\right]
\]

The overall rate of homocysteine remethylation \((\text{RM})\) can be calculated as the difference between the fluxes of the methionine carboxyl and methyl groups

\[
\text{RM} = Q_m - Q_c
\]

RM also can be estimated using the plasma homocysteine enrichment \((\text{Hcy}_M)\) as a surrogate measure of the intracellular enrichment of \([U-^{13}C_3]\)methionine \((18)\). In this case, \(\text{EP}_{\text{Hcy}}\) is used in place of \(\text{EP}_{\text{Met}}\) in the calculations of \(Q_m\) and \(Q_c\) listed above.

The rate of transfer of methyl groups from serine to methionine \((Q_{\text{Ser} \rightarrow \text{Met}})\) is calculated using the rate of appearance of \([^{13}C]\)-labeled methyl groups into methionine \((Q_{\text{Met} \rightarrow \text{Met}})\) and the estimated intracellular plateau enrichment of labeled \([^{13}C]\)serine \((E'_{\text{Ser}})\)

\[
Q_{\text{Met} \rightarrow \text{Met}} = Q_c \times \text{EP}_{\text{Met} \rightarrow \text{Met} + 1}
\]

\[
Q_{\text{Ser} \rightarrow \text{Met}} = Q_{\text{Met} \rightarrow \text{Met}} / \text{EP}_{\text{Ser} \rightarrow \text{Met} + 1}
\]

We also calculated \(Q_{\text{Ser} \rightarrow \text{Met}}\) using the plasma enrichment of \([^{13}C]\)cystathionine as a substitute for intracellular \([^{13}C]\)serine enrichment, which is analogous to the use of plasma \(\alpha\)-ketoisocaproic acid as a measure of intracellular leucine enrichment.

The percent contribution of serine 1-C units to the overall rate of homocysteine remethylation can be determined directly from \(Q_{\text{Ser} \rightarrow \text{Met}}\) and RM

\[
\%\text{RM from serine} = \left(\frac{Q_{\text{Ser} \rightarrow \text{Met}}}{\text{RM}}\right) \times 100
\]

Alternatively, the contribution of serine 1-carbon units to remethylation can be determined as the ratio of the plasma enrichment of \([^{13}C]\)methionine to the intracellular enrichment of \([3-^{13}C]\)serine (or estimated by the plasma enrichment of \([^{13}C]\)cystathionine)

\[
\%\text{RM from serine} = \left(\frac{E_{\text{Met} \rightarrow \text{Met} + 1}}{E_{\text{Ser} \rightarrow \text{Met} + 1}}\right) \times 100
\]

Relative rates of synthesis. Serine and methionine entering into the plasma pool originate from the tracer infusion, endogenous synthesis, and protein breakdown. Because amino acid entry via the tracer infusion is accounted for in the flux calculations, endogenous amino acid synthesis can be estimated after protein breakdown is accounted for. The flux of an essential amino acid, such as leucine, can provide an adjustment factor to account for protein breakdown. Relative fluxes, or relative rates of synthesis \((\text{RRS})\), are fluxes adjusted for protein breakdown that are calculated by dividing serine and methionine fluxes of each subject by the leucine flux calculated for that subject

\[
\text{RRS methionine} = \frac{Q_{\text{Met}}}{Q_{\text{Leu}}}
\]

\[
\text{RRS serine} = \frac{Q_{\text{Ser}}}{Q_{\text{Leu}}}
\]

These equations are analogous to those used in assessing proline synthesis \((13)\). These relative fluxes provide estimates of the synthesis of methionine from homocysteine and of serine from glycine and 3-phosphopyridoxylpyruvate \((34)\).

Fractional synthesis rates. The fractional synthesis rate \((\text{FSR})\) indicates the fraction of a product \((\text{e.g., homocysteine})\) that is produced from its precursor \((\text{e.g., methionine})\) per unit of time. FSRs were calculated from the initial rate of synthesis using early points \((0.5–2\text{ h})\) from the linear portion of the enrichment curves of \([^{13}C]\)methionine, \([^{13}C]\)homocysteine, \([^{13}C]\)cystathionine, and \([^{13}C]\)cystathionine. The FSR of homocysteine from infused methionine was calculated as \((4)\)

\[
\text{FSR}_{\text{Hcy}} = \text{initial rate of Hcy}_{M+1} \text{ enrichment/EP}_{\text{Met} \rightarrow \text{Met} + 5}
\]

where the initial rate of homocysteine enrichment is calculated as the slope of the initial time points \((0.5–2\text{ h})\) of the homocysteine enrichment vs. time curve. Alternately, \(\text{EP}_{\text{Hcy} \rightarrow \text{Met} + 4}\) could be replaced with \(\text{EP}_{\text{Hcy} \rightarrow \text{Met} + 4}\). Similar equations were used to measure FSR of cystathionine and methionine from serine \((\text{Csn}_{\text{Met} + 1} = \text{Mech}_{\text{Met} + 1})\) and of cystathionine from methionine \((\text{Csn}_{\text{Met} + 1} = \text{Met}_{\text{Met} + 1})\).

\[
\text{FSR}_{\text{Csn} + 1} = \text{initial rate of Csn}_{M+4} \text{ enrichment/EP}_{\text{Ser} \rightarrow \text{Met} + 1}
\]

\[
\text{FSR}_{\text{Meth} + 1} = \text{initial rate of Met}_{M+5} \text{ enrichment/EP}_{\text{Ser} \rightarrow \text{Met} + 1}
\]

Statistical Analysis

Data are presented as means ± SE. Some data were log\(_{10}\) transformed before analysis to achieve homogeneous variances. Differences between kinetic parameters were analyzed by \(t\)-test and were considered statistically significant at \(P < 0.05\).

RESULTS

All infused tracers reached plateau enrichment in the plasma compartment rapidly, the \([3-^{13}C]\)serine and \([U-^{13}C_3]\)methionine tracers somewhat after the \([\text{H}^4\text{H}]\)leucine tracer (Fig. 2A). The \([3-^{13}C]\)serine and \([U-^{13}C_3]\)methionine tracers reached \(~7–8\%\) enrichment, whereas the \([\text{H}^4\text{H}]\)leucine tracer reached \(~2\%\) enrichment (Table 1).

Plateau enrichments of metabolic products of the infused methionine \(([^{13}C]\)homocysteine, \([^{13}C]\)cystathionine, and \([^{13}C]\)cystathionine) and serine tracers \(([^{13}C]\)methionine and \([^{13}C]\)cystathionine) were determined by fitting enrichment
data to single exponential curves defined by the equation $E = E_0(1 - e^{-kt})$ (Fig. 2, B and C, and Table 1). $[^{13}C_4]$homocysteine reached an average plateau enrichment of 6.5%, which was ~90% of the enrichment of plasma $[^{13}C_5]$methionine. Enrichment of remethylated methionine ($[^{13}C_4]$methionine) reached ~43% of infused $[^{13}C_5]$methionine enrichment. The mean plasma enrichment of $[^{13}C_4]$cystathionine was ~81% of the enrichment of plasma $[^{13}C_5]$methionine. $[^{13}C_1]$Cystathionine enrichment in plasma reached approximately one-fifth the enrichment of infused plasma $[^{3}C]$serine tracer enrichment and 50% of the corrected plasma $[^{13}C]$serine enrichment. No enrichment of $[^{13}C_1]$cysteine was detected.

Leucine and methionine fluxes (Table 1) were similar to those in previous reports from similar protocols (4, 18, 26). Serine fluxes and relative rates of synthesis calculated from corrected plasma $[^{13}C_4]$serine enrichments were also similar to those of previous reports after the correction factor was taken into consideration (4, 10). Serine fluxes and relative rates of synthesis calculated on the basis of plasma $[^{13}C_4]$cystathionine enrichments were 2.2-fold those calculated from $[^{3}C_4]$serine enrichment ($Q_{ser} = 664 ± 119 vs. 299 ± 10.2 μmol·kg⁻¹·h⁻¹; RRS Ser = 8.0 ± 1.1 vs. 3.7 ± 0.13, P < 0.001). The relative rate of methionine synthesis did not differ when measured from the corrected plasma enrichment of the $[^{13}C_5]$methionine tracer (0.32 ± 0.04) or the enrichment of $[^{13}C_4]$homocysteine (0.30 ± 0.030). These results were similar to previously published results (4) after the methionine enrichment correction factor is taken into account. Homocysteine remethylation rate (Table 2) was calculated using the original model proposed by Storch et al. (26) as well as by the modification of MacCoss et al. (18). Because the adjusted plateau enrichment of $[^{13}C_5]$methionine (5.90 ± 0.314) was similar to the plateau enrichment of $[^{13}C_4]$homocysteine (6.20 ± 0.696), estimates of methionine methyl group flux, methionine carboxyl group flux, and total homocysteine remethylation rates did not differ between the Storch et al. and MacCoss et al. models. Homocysteine re-

### Table 1. Plateau enrichments of stable isotope-labeled amino acids in plasma and amino acid fluxes of subjects during a primed constant infusion of $[^{13}C_5]$methionine, $[^{13}C_4]$serine, and $[^{3}H_2]$leucine

<table>
<thead>
<tr>
<th>Labeled Amino Acid</th>
<th>Plateau Enrichment, mol % excess</th>
<th>Flux ($Q$), μmol·kg⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{3}H_2]$Leucine</td>
<td>2.25 ± 0.117</td>
<td>82.0 ± 4.20 ($Q_{leu}$)</td>
</tr>
<tr>
<td>$[^{13}C_4]$Serine</td>
<td>7.58 ± 0.320</td>
<td>299 ± 10.2 ($Q_{ser}$)</td>
</tr>
<tr>
<td>$[^{13}C_5]$Methionine</td>
<td>1.38 ± 0.0508</td>
<td></td>
</tr>
<tr>
<td>$[^{13}C_4]$Cystathionine</td>
<td>1.44 ± 0.0667</td>
<td></td>
</tr>
<tr>
<td>$[^{13}C_5]$Methionine</td>
<td>7.37 ± 0.380</td>
<td></td>
</tr>
<tr>
<td>$[^{13}C_4]$Homocysteine</td>
<td>6.20 ± 0.696</td>
<td>26.3 ± 1.54 ($Q_{hm}$)</td>
</tr>
<tr>
<td>$[^{13}C_4]$Methionine</td>
<td>2.51 ± 0.128</td>
<td>24.4 ± 2.60 ($Q_{hm}$)</td>
</tr>
<tr>
<td>$[^{13}C_5]$Cystathionine</td>
<td>4.86 ± 0.791</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5, except for $[^{13}C_4]$cystathionine (n = 4). Serine and methionine flux values were calculated from corrected plateau enrichments. See METHODS section for details. $[^{13}C_4]$methionine, $[^{13}C_5]$methionine, $[^{13}C_4]$homocysteine, $[^{13}C_4]$cystathionine, and $[^{3}H_2]$leucine plateau enrichments were derived by fitting enrichment data to the single exponential curve $E = E_0(1 - e^{-kt})$. See METHODS section for details.

### Table 2. Homocysteine remethylation-related kinetics calculated from the plasma enrichments of the methionine tracer (Storch method) or homocysteine (MacCoss method)

<table>
<thead>
<tr>
<th>Remethylation Parameter</th>
<th>Flux, μmol·kg⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_m$</td>
<td>26.3 ± 1.54</td>
</tr>
<tr>
<td>$Q_{cys}$</td>
<td>17.9 ± 0.874</td>
</tr>
<tr>
<td>$Q_{hm}$</td>
<td>8.39 ± 0.750</td>
</tr>
<tr>
<td>$Q_{max} + 1$</td>
<td>0.263 ± 0.0284</td>
</tr>
<tr>
<td>$Q_{ser} - Q_{max}$</td>
<td>8.66 ± 0.949</td>
</tr>
<tr>
<td>%RM from serine</td>
<td>100 ± 5.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5. See METHODS for definitions.
methylation accounted for ~31% of the total methionine methyl group flux.

Folate-dependent homocysteine remethylation flux (Q_{Ser→Met}) was calculated using plasma [\textsuperscript{13}C\textsubscript{1}]cystathionine or corrected plasma [\textsuperscript{3-13}C]serine enrichments as estimates of the intracellular [\textsuperscript{3-13}C]serine enrichment (Table 2). Because the plateau enrichment of [\textsuperscript{3-13}C]serine in plasma is greater than that of [\textsuperscript{13}C\textsubscript{1}]cystathionine, the Q_{Ser→Met} is 2.3 times larger when calculated using plasma [\textsuperscript{13}C\textsubscript{1}]cystathionine enrichment than when calculated using plasma [\textsuperscript{3-13}C]serine enrichment (19.7 vs. 8.66 \textmu mol kg\textsuperscript{-1} h\textsuperscript{-1}). Q_{Ser→Met} calculated from [\textsuperscript{13}C\textsubscript{1}]cystathionine data is twice the overall remethylation rate, indicating that this measure is not a useful indicator of total body serine metabolism. The percentage of homocysteine remethylation from the 3-C of serine was measured from Q_{Ser→Met} as a fraction of the total remethylation rate. By use of corrected plasma [\textsuperscript{3-13}C]serine enrichment data, 100 and 110% of 1-C units used for homocysteine remethylation are derived from serine when calculated using corrected [U-\textsuperscript{13}C\textsubscript{5}]methionine enrichments and [\textsuperscript{13}C\textsubscript{1}]homocysteine enrichments, respectively. The proportion of serine flux used for homocysteine remethylation was calculated to be ~2.80%.

The FSR of homocysteine from the [\textsuperscript{13}C\textsubscript{4}]methionine tracer (0.18 ± 0.014 and 0.17 ± 0.029 using Ep_{\textsubscript{Hcy} M+4} for precursor enrichment, respectively; n = 4 for both measures) indicates that approximately one-fifth to one-sixth of the homocysteine pool was synthesized from methionine per hour. The FSR of methionine from the [\textsuperscript{3-13}C]serine tracer (0.12 ± 0.021; n = 5) indicates that one-eighth of the methionine pool is produced from serine per hour.

**DISCUSSION**

The tracer model described in this study was designed to add a measure of folate-dependent homocysteine remethylation rates to existing stable-isotope tracer models of homocysteine metabolism (18, 26). This addition is necessary to assess the effects of genetic and environmental perturbations on homocysteine remethylation, because subtle alterations of folate-dependent homocysteine remethylation rate might be compensated for by induction of folate-independent homocysteine remethylation via betaine-homocysteine methyltransferase (1, 33). Such a scenario would be undetectable with previous research models. Although the folate-dependent homocysteine remethylation pathway can also use histidine, glycine, sarcosine, dimethylglycine, and formate as 1-C donors, labeled [\textsuperscript{3-13}C]serine was chosen to measure folate-dependent homocysteine remethylation rate because serine is thought to be the major source of 1-C units for homocysteine remethylation in most human tissues (3). Data from studies of human serine metabolism support this viewpoint. Metabolism of a [\textsuperscript{3}H\textsubscript{2}]serine tracer by cytoplasmic and mitochondrial serine hydroxymethyltransferase enzymes produces [\textsuperscript{3}H\textsubscript{2}] and [\textsuperscript{2}H\textsubscript{2}]serine species. If there were quantitatively significant entry of 1-C units into the 5,10-methylenetetrahydrofolate pool from formate, glycine, dimethylglycine, or sarcosine, [\textsuperscript{3}H\textsubscript{2}]serine enrichment should be lower than [\textsuperscript{2}H\textsubscript{2}]serine enrichment. Two recent studies have shown that this is not the case (4, 10).

A limitation of single-pool models to measure whole body amino acid kinetics in humans is the uncertainty of the true intracellular tracer enrichment. Initial studies of methylation cycle kinetics used a correction factor of 0.8 to compensate for the overestimation of intracellular methionine tracer enrichment that occurs when measured plasma methionine tracer enrichment is used for kinetic calculations (26). MacCoss et al. (18) recently used plasma homocysteine enrichment as a measure of intracellular methionine enrichment and found the ratio of the plasma enrichments of homocysteine and the infused methionine tracer to be ~0.6. The results obtained on the basis of [\textsuperscript{13}C\textsubscript{1}]homocysteine enrichment in the present investigation indicate a correction factor of ~0.8, which is more consistent with the original estimate (26). This difference between our findings and those of MacCoss et al. is likely due to the fact that homocysteine enrichment measured at the M + 4 ion is subject to far less background abundance than the M + 1 ion.

Although our methionine-methyl (Q_{\text{M}}) and -carboxyl (Q_{\text{C}}) fluxes were in the range of those previously reported, total homocysteine remethylation rates in this investigation were twice those found in young, fed females by means of other methionine tracer models (7, 8, 18). These values did not differ when measured using the corrected [\textsuperscript{13}C\textsubscript{5}]methionine enrichment or the [\textsuperscript{13}C\textsubscript{1}]homocysteine enrichment. The difference between these results and the results of other studies might reflect greater precision in our measurements, due to the lower natural abundances of the [\textsuperscript{13}C\textsubscript{1}]homocysteine and [\textsuperscript{13}C\textsubscript{5}]methionine that we measured compared with the [\textsuperscript{13}C\textsubscript{5}]homocysteine and [\textsuperscript{13}C\textsubscript{5}]methionine that were measured by the other investigators. Furthermore, subjects in this study were maintained in the fed state during the infusion by consuming a formulation that provided energy but negligible amino acids. In contrast, subjects in the study of MacCoss et al. were kept in the postabsorptive state. This is an important distinction, because methylation cycle kinetics are more rapid in the fed than in the postabsorptive state (26). Results from this investigation indicate that homocysteine remethylation accounts for ~30% of total methionine flux, which is substantially greater than previous estimates (10–15%) (18, 26), and suggests a much greater role for homocysteine remethylation in methionine metabolism than previously appreciated. The remaining 70% of methionine flux under these conditions represents methionine derived from protein breakdown.

Whole body folate-dependent remethylation rate from the 3-C of serine was measured in humans for the first time in this investigation. An assumption of this tracer model is that the measured [\textsuperscript{13}C\textsubscript{1}]methionine enrichment is derived only from the infused [\textsuperscript{3-13}C]serine tracer via folate-dependent remethylation. This is an approximation, because three minor metabolic pathways also can generate [\textsuperscript{13}C\textsubscript{1}]methionine from the [U-\textsuperscript{13}C\textsubscript{5}]methionine tracer. The polyamine synthesis pathway can produce [\textsuperscript{13}C\textsubscript{1}]methionine from the [U-\textsuperscript{13}C\textsubscript{5}]methionine tracer through salvage of the \textsuperscript{13}C-labeled thiomethyl moiety back into the folate pool (2). Mudd and Poole (19) estimated that this pathway uses <5% of the labile methyl groups, which is small enough to be considered insignificant in this model. The methyl group of [U-\textsuperscript{13}C\textsubscript{5}]methionine can also be used for production of [\textsuperscript{13}C\textsubscript{1}]sarcosine (14), and the [\textsuperscript{13}C\textsubscript{1}] methyl group can be recycled back into the folate pool (3). However, this is
known to occur in folate deficiency, when hepatic S-adenosylmethionine concentrations are elevated, or with specific hormonal stimuli. Phosphatidylcholine (PC) synthesis could generate [13C]betaine and subsequently [13C]methionine (29). However, this pathway synthesizes only ~30% of hepatic PC (6, 22), the 13C enrichment of choline produced by this pathway would be diluted upon mixing with membrane PC pools and the betaine pool, and betaine is a relatively minor contributor of 1-C groups for remethylation. Thus, for the purposes of this report, we assume that the measured [13C]methionine enrichments are not significantly different from those generated by folate-dependent remethylation from the [3-13C]serine tracer.

Another complication of the measurement of folate-dependent remethylation from serine is that we cannot directly measure the true intracellular [3-13C]serine enrichment. Because the majority of serine flux (~95%) is from de novo synthesis (21), we expect that the serine tracer will be more highly diluted in the intracellular serine pool than most amino acids. Two human studies confirm this assumption. Hepatic serine enrichment, estimated from the plateau enrichment of serine in VLDL apoB-100, was ~23% of the plasma serine enrichment after infusion of [2,2,3-2H3]serine in a human (10). Also, the enrichments of plasma [3H2]serine and [3H1]serine, both products of intracellular metabolism of the [2,2,3-2H3]serine tracer in all tissues, were ~35% of the plasma [2,2,3-2H3]serine enrichment in humans (4). The basis of this evidence, a correction factor for intracellular serine enrichment from plasma serine enrichment would be in the range of 0.2–0.4. By use of the upper end of this range as a conservative estimate of intracellular dilution of the [3-13C]serine tracer, the serine 3-C was found to provide all of the 1-C units used for total body homocysteine remethylation. This is greater than the ~50% of homocysteine remethylation from the serine 3-C as measured in rat liver (23). Although these results might indicate that we underestimated intracellular serine enrichment and therefore overestimated the rate of remethylation using the 3-C of serine, these data strongly support the assumption that serine is the primary 1-C donor. In fact, even if our assumption of intracellular serine enrichment is 20% lower than actual enrichment, 80–90% of 1-C units would still derive from the 3-C of serine. Consequently, glycine, histidine, betaine, sarcosine, and formate are minor sources of 1-C units for remethylation under these experimental conditions. Despite the strong dependence of the remethylation process on serine as a 1-C donor, only 2.8% of the serine flux was used for remethylation.

Another possible measure of intracellular serine enrichment is the plasma enrichment of cystathionine, which is an amino acid produced in liver, kidney, pancreas, brain, and intestine as an intermediate in the transsulfuration pathway (34). Cystathionine was recently used as an estimate of intracellular methionine enrichment with infusions of carboxyl-labeled methionine (18). However, in this study the QSer->Met values measured from [13C]cystathionine enrichments were twice the total homocysteine remethylation rates, indicating that this measure is not useful for calculations of total body serine metabolism.

FSRs of homocysteine from methionine were measured in addition to homocysteine remethylation rates to provide a more complete view of homocysteine metabolism. These results suggest that one-fifth to one-sixth of the total homocysteine pool is synthesized per hour. This is a greater fraction than previously reported in humans (4), but the difference could be due to the different serine and methionine tracers used. We previously measured the FSR of homocysteine from the enrichments of [1-13C]homocysteine and [1-13C]methionine, and there was greater uncertainty in estimating M + 1 enrichments than in the measured M + 4 enrichments of this study. Moreover, we previously overestimated intracellular [1-13C]methionine enrichment by ~10% by using plasma [1-13C]methionine plateau enrichment as an estimate. Finally, measurement of [1-13C]methionine enrichment was confounded (and overestimated) by the production of [3H1]methionine from remethylation of homocysteine using 2H1-labeled 1-C units from the infused [2,3,3-2H3]serine tracer. Consequently, we underestimated the actual intracellular methionine enrichment and underestimated the FSR of homocysteine from methionine.

Transsulfuration flux was not measured via 13CO2 expiration in this study, due in part to the complication presented by oxidation of the [3-13C]serine tracer to 13CO2. Therefore, we were unable to estimate transmethylation (TM) as the sum of RM and transsulfuration (TS) (26). However, the FSR (Hcy from Met) provides an indirect measure of TM, since all TM reactions produce S-adenosylhomocysteine, the precursor of homocysteine. Thus FSR (Hcy from Met) can be used to compare TM rates among groups. Specifically, an elevated FSR of homocysteine would indicate a greater overall TM rate.

In conclusion, the [U-13C5]methionine tracer model allows measurement of total homocysteine remethylation rate by using plasma [13C4]homocysteine enrichment as a sensitive measure of intracellular methionine enrichment. These results confirmed that intracellular methionine enrichment is ~10% lower than the plasma methionine tracer enrichment. Homocysteine remethylation rates were twice as large as in previous reports, which suggests that homocysteine remethylation is a more significant fraction of methionine kinetics than previously appreciated. The 3-C of serine is estimated to provide all of the 1-C groups used for homocysteine remethylation under these experimental conditions. More precise measures of the rate of 1-C transfer from serine to methionine will be possible only when a more accurate measure of intracellular [3-13C]serine enrichment is identified. This model is currently being applied to investigate the effects of vitamin B6 or folate deficiency, and the MTHFR 677C→T polymorphism on folate-dependent and total homocysteine remethylation rates, and to determine the relative contributions of serine, glycine, and betaine in homocysteine remethylation in humans.

ACKNOWLEDGMENTS

We thank the staff of the University of Florida General Clinical Research Center (GCRC) for many contributions to this study. Thanks are also extended to David R. Maneval for technical expertise, Dr. Andrew Hanson for critical review of the manuscript, and Karen Novak for recruitment, screening, and management of subjects.
GRANTS
This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-56274 (J. F. Gregory) and DK-42033 (B. Shane), GCRC M01 RR-00082, and US Department of Agriculture-National Research Institute Grant 00–35200–9113 (J. F. Gregory). This paper is Florida Agricultural Experiment Station Journal Series No. R-09756.

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