Measurement of intracellular sulfur amino acid metabolism in humans

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MacCoss, Michael J., Naomi K. Fukagawa, and Dwight E. Matthews. Measurement of intracellular sulfur amino acid metabolism in humans. Am J Physiol Endocrinol Metab 280: E947–E955, 2001.—Methionine metabolism forms homocysteine via transmethylation. Homocysteine is either 1) condensed to form cystathionine, which is cleaved to form cysteine, or 2) remethylated back to methionine. Measuring this cycle with the use of isotopically labeled methionine tracers is problematic, because the tracer is infused into and measured from blood, whereas methionine metabolism occurs inside cells. Because plasma homocysteine and cystathionine arise from intracellular metabolism of methionine, plasma homocysteine and cystathionine enrichments can be used to define intracellular methionine enrichment during an infusion of labeled methionine. Eight healthy, postabsorptive volunteers were given a primed continuous infusion of [1-13C]methionine and [methyl-3H]methionine for 8 h. Enrichments in plasma methionine, [13C]homocysteine and [13C]cystathionine were measured. In contrast to plasma methionine enrichments, the plasma [13C]homocysteine and [13C]cystathionine enrichments rose to plateau slowly (rate constant: 0.40 ± 0.03 and 0.49 ± 0.09 h⁻¹, respectively). The enrichment ratios of plasma [13C]homocysteine to [13C]methionine and [13C]cystathionine to [13C]methionine were 58 ± 3 and 54 ± 3%, respectively, demonstrating a large intracellular/extracellular partitioning of methionine. These values were used to correct methionine kinetics. The corrections increase previously reported rates of methionine kinetics by ~40%.

Homocysteine has become an important topic of nutritional research since the observation that elevated levels of plasma homocysteine (>15 μM) are an independent risk factor in cardiovascular disease (23). The reason for an elevated plasma homocysteine concentration must lie in the balance between its rates of production and disposal. For this reason, we need knowledge of the rate of homocysteine formation via methionine transmethylation (TM) and the rate of homocysteine disposal via RM of homocysteine to methionine and TS of homocysteine to form cysteine (Fig. 1).

Assessment of dietary amino acid requirements in healthy adults is an important aspect of nutritional research (34, 35). Stable isotope tracers have been used previously to measure methionine oxidation in vivo. These measurements of methionine oxidation have led to our current approximation of dietary methionine requirements (38). The pioneering isotope studies of Storch et al. (25) defined the relative recycling of methionine through the RM of homocysteine. These studies measured the flux of methionine C via a [1-13C]methionine tracer (where the 13C is conserved with homocysteine conversion to methionine) and the flux of methionine via a [methyl-3H]methionine tracer (which loses the methyl-3H during TM). The difference between the fluxes is the rate of RM of homocysteine to methionine (25, 26). Although these studies have advanced our understanding of dietary sulfur amino acid requirements in humans, there is one critical caveat to this method. The tracers are infused into plasma and measured from plasma, yet the metabolism to be measured is occurring inside cells. Storch et al. assumed that the intracellular dilution of methionine was the same as what was observed for intracellular leucine, which in general is 20% lower than plasma leucine enrichment (16). This assumption has been neither confirmed nor tested for methionine.

Recently, we reported an analytical method for measuring homocysteine concentrations and stable isotope tracer enrichments in human plasma with the use of gas chromatography-mass spectrometry (GC-MS) (11). Plasma homocysteine can be derived only from the intracellular homocysteine formed by transmethylation of methionine. During an infusion of labeled me-
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Fig. 1. Sulfur amino acid metabolism in humans. Methionine can either be incorporated into proteins by protein synthesis (S) or undergo transmethylation (TM) where the methyl group is transferred to a methyl acceptor (X), eventually returning homocysteine. Homocysteine can be remethylated (RM) to methionine by one of two pathways or undergo transsulfuration (TS). TS starts with condensation of homocysteine with serine to form cystathionine. Cystathionine is then reduced to form cysteine, with the C-backbone of the original homocysteine being oxidized and only the sulfur being transferred to the resulting cysteine. The reader is referred to a recent review that provides detail about the individual metabolic steps, enzymes involved, and their location/activity in the body (9). THF, tetrahydrofolate.

thionine, the homocysteine enrichment in plasma will be equal to the methionine enrichment inside cells under steady-state conditions. Therefore, the measurement of plasma homocysteine enrichment allows us to determine intracellular methionine enrichment. We have also used cystathionine enrichment during the same infusion to confirm that the labeled homocysteine is equilibrating with total homocysteine (free and disulfide bound). In this report, we present the first direct measurement of TM, RM, and TS in healthy, postabsorptive adults by use of homocysteine and cystathionine enrichments to define intracellular methionine enrichments.

MATERIALS AND METHODS

Subjects. Eight healthy, normal weight-for-height adults aged 25–34 yr (Table 1) were studied at the University of Vermont Clinical Research Center (CRC). They were screened to ensure an adequate state of health by medical history, physical examination, and blood testing. The subjects were instructed of the purpose, benefits, and risks of the study and gave written consent in accordance with protocols approved by the University of Vermont Institutional Review Board and by the CRC Scientific Advisory Committee. The subjects were paid for their participation in the study.

Infusion protocol. Each volunteer was placed on a standardized diet to provide adequate energy intake on the basis of subjects’ estimated energy expenditure and adequate protein intake (1–1.5 g·kg⁻¹·day⁻¹) for 2 days before admission to the CRC for the infusion study. On the evening before the infusion study, the subjects were admitted to the CRC and given an evening meal before 1900. Thereafter, the subjects consumed only water until completion of the infusion the following day. At 0600 on the infusion day, subjects were awakened, and an intravenous catheter was placed in a forearm vein for infusion of the tracer and in a hand vein for sampling arterialized venous blood by means of the heated-hand box technique. Both catheters were kept patent with a slow infusion of sterile saline. At 0700, a priming dose of [1-¹³C]methionine ([¹³C]methionine; MassTrace, Woburn, MA) and [methyl-²H₃]methionine ([²H₃]methionine; Isotec, Miamisburg, OH) (2.8 and 1.6 μmol/kg, respectively) was administered intravenously, and an infusion of [¹³C]- and [²H₃]methionine (2.4 and 1.4 μmol·kg⁻¹·h⁻¹, respectively) was begun and continued for 8 h by use of an infusion pump. Blood and breath samples were taken just before the start, at hourly intervals for the first 5 h, and at half-hourly intervals during the remainder of the tracer infusion. Blood was placed on ice in tubes containing EDTA. The tubes were promptly centrifuged, and the plasma fraction was removed and frozen at −60°C until analysis. Breath samples were placed in 20-ml evacuated tubes and stored at room temperature until measurement of ¹³CO₂ in the expired air by isotope ratio mass spectrometry. At hourly intervals, CO₂ production was determined using an indirect calorimeter with a flow-through hood (Deltatrak, Sensormedics, Yorba Linda, CA).

Measurement of stable isotope tracer enrichments. Plasma samples for the measurement of [¹³C]homocysteine, [¹³C]methionine, and [²H₃]methionine enrichments were prepared and measured by GC-MS by the method previously described (11). The homocysteine was measured as the S-4-ethylpyridine bis-tert-butyldimethylsilyl (BDMS) homocysteine, and methionine was measured as bis-tBDMS methionine. Separate 1-μl injections of derivatized amino acid samples were injected into a Hewlett-Packard 5971A gas chromatograph-mass spectrometer (Palo Alto, CA) for each amino acid to be measured. The samples were measured by electron ionization at 70 eV. The [M−57]⁺ fragment was measured by selected-ion monitoring for the unlabeled [mass-to-charge (m/z) = 411] and [¹³C]-labeled homocysteine (m/z = 412). The integrated areas of the monitored ions (m/z = 411 and 412) were determined, and the [¹³C]homocysteine enrichments were calculated as previously described (11). The homocysteine was measured as [¹³C]- and [²H₃]methionine enrichments were measured by GC-MS. The integrated areas of the monitored ions were used to calculate the [¹³C]- and [²H₃]methionine enrichments by determining the isotopomer distribution of the infused isotopes by means of a least-squares analysis of the overlapping mass spectra (2, 25).

Samples for measurement of [¹³C]cystathionine were prepared as the N-heptafluorobutyl, n-propyl ester (HFBP)
derivatives from plasma by use of a similar method described previously (15). A 1-ml aliquot of each plasma sample was added to a 13 × 100-mm disposable borosilicate culture tube containing 1 ml of 1 M acetic acid. Each tube was vortexed thoroughly and poured directly onto a disposable column containing 0.5 ml of thoroughly washed cation exchange resin (AG 50W-X8 100–200 mesh, hydrogen form, Bio-Rad Laboratories, Richmond, CA). The cation resin was washed twice with 5 ml of distilled water, and the bound material (including cystathionine) was eluted with 2 ml of 3 M NH₄OH into conical-bottom reaction vials. The vials were dried under a steady stream of N₂. To each reaction vial, 200 µl of propanolic HCl (6.25:1, n-propanol-acetyl chloride) was added, and the vials were capped and heated at 105°C for 30 min. The reagent was evaporated from the vials under N₂. Next, 100 µl of acetylating reagent (1:1 heptafluorobutyric anhydride-ethyl acetate) was added, and the vials were capped and heated at 105°C for 15 min. The mixture was allowed to cool to room temperature, and the reagent was evaporated from the vial. Finally, 250 µl of ethyl acetate were added to the vial as solvent, and the vials were stored in the freezer until measurement by GC-MS.

[¹³C]cystathionine enrichment was measured by injecting a 1-µl aliquot of the derivatized sample into a Hewlett-Packard 5988A gas chromatograph-mass spectrometer operated in splitless mode. Ionization was achieved using electron capture negative chemical ionization, with methane as the reagent gas. Separation of the HFBP-cystathionine was accomplished using a 15 × 0.25-mm-ID, 0.25-µm film ZB-5 capillary column (Phenomenex, Torrance, CA). The [M − HP⁻]⁻ fragment was monitored for the unlabeled (m/z = 678) and [¹³C]-labeled (m/z = 679) ions for each of the plasma samples. The integrated areas of the monitored ions were used to calculate the [¹³C]cystathionine enrichments of the infused isotopes using a least squares analysis of the overlapping mass spectra (2, 25).

Calculations. As previously defined for leucine (12, 16), the gradient between intracellular methionine enrichment and the plasma enrichment is related to the rate of amino acid transport into cells (X) vs. the rate of unlabeled amino acid release from protein breakdown (B). This gradient was calculated for infusion of the [¹³C]methionine tracer, where extracellular enrichment is represented by plasma [¹³C]methionine (E[¹³C-Met]), and intracellular enrichment is represented by plasma [¹³C]homocysteine (E[¹³C-Hcy]).

\[ \frac{E[¹³C-Hcy]}{E[¹³C-Met]} = \frac{X}{X + B} \]  

(1)

Two fluxes, or turnover rates, were calculated for the two methionine tracers: 1) the flux of the methionine C-backbone (determined from [¹³C]methionine) and 2) the flux of the methionine methyl group (determined from [²H₃]methionine). These fluxes were calculated from the stochastic relationship (14)

\[ Q_X = \frac{1}{E_{IC}} \left( \frac{100}{E_{IC}} - 1 \right) \]  

(2)

where \( Q_X \) is the flux (µmol·kg⁻¹·h⁻¹) of methionine determined using either the [¹³C]methionine (\( Q_C \)) or the methyl-[²H₃]-labeled tracer (\( Q_M \)). \( I \) is the intravenous tracer infusion rate (µmol·kg⁻¹·h⁻¹), and \( E_{IC} \) is the intracellular enrichment [mole percent enrichment (MPE)] of the respective ¹³C or ²H₃ tracer. The methionine C flux (\( Q_C \)) (14, 25) was calculated using plasma [¹³C]homocysteine enrichment as the intracellular [¹³C]methionine enrichment (\( E_{IC} = E_{IC-Hcy} \)). In the postabsorptive state with no exogenous source of methionine, the methionine C flux is derived from methionine entering via protein breakdown (B), and the two are synonymous (i.e., \( B = Q_C \)).

The methionine methyl-group flux cannot be calculated using the intracellular surrogate homocysteine because there is no methyl-group on homocysteine to be measured. Although we cannot directly measure the intracellular enrichment of [methyl-²H₃]methionine, we can calculate it on the basis of the measured methionine intracellular/extracellular gradient determined from the [¹³C]methionine tracer (E[¹³C-Hcy]/E[¹³C-Met]), and can use this ratio to adjust the plasma [²H₃]methionine enrichment (E[²H-Met]) to approximate intracellular [²H₃]methionine enrichment

\[ E_{IC}[²H-Met] = E_{IC-Met} \left( \frac{E_{IC-Hcy}}{E_{IC-Met}} \right) \]  

(3)

This value (E[²H-Met]) can then be used to calculate the methionine methyl flux (\( Q_M \)) per Eq. 2 above. The methyl-group methionine flux is greater than the carboxyl flux by the rate of RM of homocysteine (25). Thus the rate of RM is the difference between the carboxyl and methyl fluxes

\[ R_M = Q_M - Q_C \]  

(4)

The release of the methionine ¹³C label as ¹³CO₂ reflects the rate of oxidation (Ox), but this event occurs with TS (25). The rates of TS or oxidation were calculated using the equation

\[ TS = Ox = \frac{F_{¹³CO₂}}{E_{IC-Hcy}} \]  

(5)

where \( F_{¹³CO₂} \) is the rate of ¹³CO₂ excretion in the breath after being adjusted by 0.81 for bicarbonate retention (1).

The calculation of oxidation above assumes that all of the ¹³CO₂ is liberated via the TS pathway. However, some ¹³CO₂ will arise from sequestration of S-adenosylmethionine for polyamine synthesis (3, 5, 9, 17, 18). In this process, the methionine is decarboxylated before its methyl group is transferred. The end product is S-aminopropylthiol adenosine. Although direct measurements of the rate of this reaction in humans are lacking, best estimates based on enzyme activity measurements and polyamine production suggest that <5% of the S-adenosylmethionine goes toward polyamine synthesis (5, 9, 17, 39).

As in previous work (25), the rate of methionine uptake for protein synthesis (S) was calculated from the difference between methionine C flux and oxidation, i.e., \( S = Q_C - Ox \). The rate of TM was calculated from TS and RM: TM = RM + TS, as shown in Fig. 1 and previously described (25).

Statistics. Data are presented as means ± SE. A linear regression analysis was performed for [¹³C]- and [²H₃]methionine enrichments vs. time for each subject’s infusion. Steady state was defined as a mean slope not significantly different from zero for the group (\( P > 0.05 \)). Paired t-tests were used where possible for comparisons of parameters measured within a single individual. The mean [¹³C]homocysteine and [¹³C]cystathionine enrichments were fitted vs. time to a single exponential of the form \( E = E_i (1 - e^{-kt}) \) where \( E \) is the plasma homocysteine or cystathionine [¹³C] enrichment (MPE) at time \( t \) (h), \( E_i \) is the fitted value for the enrichment (MPE) at infinity, and \( k \) is the fitted rate constant (h⁻¹). The fitting of the curves was performed using Origin (version 6.0, Microcal Software, Northampton, MA). The fitting routine computed goodness of fit as \( r^2 \) and standard errors of the fitted parameters, \( E \) and \( k \).
RESULTS

The 8-h time courses of plasma \(^{13}\text{C}\)methionine and \(^{2}\text{H}_3\)methionine enrichments are presented in Fig. 2. Data represent the mean enrichments of the eight subjects. The \(^{13}\text{C}\)- and \(^{2}\text{H}_3\)methionine tracer enrichments reached an isotopic steady state in plasma within the 1st h. The \(^{13}\text{C}\)methionine enrichment values were higher than the corresponding \(^{2}\text{H}_3\)methionine enrichment values, because the \(^{13}\text{C}\)methionine tracer was infused at a faster rate than the \(^{2}\text{H}_3\)methionine to ensure sufficient enrichment in plasma \(^{13}\text{C}\)homocysteine and \(^{13}\text{C}\)cystathionine and \(^{13}\text{C}\)methionine enrichments are shown in Fig. 3. Unlike the \(^{13}\text{C}\)methionine and \(^{2}\text{H}_3\)methionine time courses, the \(^{13}\text{C}\)homocysteine and \(^{13}\text{C}\)cystathionine enrichments rose slowly and did not reach isotopic steady state until after 6 h. This delay is a result of the time necessary for the \(^{13}\text{C}\)methionine tracer to enter into cells, equilibrate with intracellular methionine, be converted to homocysteine, and be transported back into the systemic circulation. The fitted curves in Fig. 3 demonstrate that this delay is represented by a slow rise to plateau without a delay in time of appearance of enrichment into plasma. The best fit of the enrichment vs. time data was to a single exponential pool. There was no significant improvement in adding a second pool or time delay term. As shown from the fitted curves in Fig. 3, there was no difference in the time course of \(^{13}\text{C}\)homocysteine vs. \(^{13}\text{C}\)cystathionine either in rate constant (\(k\)) or in plateau at infinity (\(E_f\)). There were no significant differences between the plateaus at infinity and the plateaus calculated for the last 1.5 h of the infusion for either homocysteine or cystathionine.

The intracellular-extracellular gradient of the infused \(^{13}\text{C}\)methionine tracer can be defined by either the plasma \(^{13}\text{C}\)homocysteine or \(^{13}\text{C}\)cystathionine enrichment at steady state (as the intracellular marker) and plasma \(^{13}\text{C}\)methionine enrichment (as the extracellular value). The steady-state ratios of \(^{13}\text{C}\)homocysteine to \(^{13}\text{C}\)methionine and \(^{13}\text{C}\)cystathionine to \(^{13}\text{C}\)methionine were computed for each individual subject and then averaged. These values were \(0.58 \pm 0.03\) and \(0.54 \pm 0.03\), respectively, and represent the relative rate of methionine transport into cells vs. the rate of methionine release from protein breakdown (Eq. 1). There was no difference in the intracellular-to-extracellular ratio of \(^{13}\text{C}\)methionine with the use of \(^{13}\text{C}\)homocysteine vs. \(^{13}\text{C}\)cystathionine enrichments. Because the two estimates of intracellular methionine were identical, the remaining kinetics are presented using only the \(^{13}\text{C}\)homocysteine enrichments.

The steady-state plasma tracer enrichments are presented in Table 2 for each of the eight subjects. These enrichments were then used to calculate methionine C and methyl fluxes, \(Q_C\) and \(Q_M\), using the \(^{13}\text{C}\)homocysteine enrichments as a measure of intracellular \(^{13}\text{C}\)methionine. The methyl-methionine flux determined from the \(^{2}\text{H}_3\)methionine tracer was adjusted using the \(^{13}\text{C}\)homocysteine-\(^{13}\text{C}\)methionine gradient, determined for each subject.

Data for the rate of \(^{13}\text{CO}_2\) release from the \(^{13}\text{C}\)methionine tracer are presented in Table 3. We then calculated methionine oxidation from these data and the \(^{13}\text{C}\)homocysteine enrichments. Because methio-
nine $^{13}$C oxidation proceeds via the TS pathway, the oxidation rate data in Table 3 also reflect the rate of TS. The other, nonoxidative route of homocysteine disposal is RM to methionine. RM is the difference between the methionine methyl and C fluxes ($Q_{RM}$) under steady-state conditions. All of these kinetic values were calculated using plasma $^{13}$C homocysteine as an intracellular marker for methionine and correction (where applicable) of plasma $^{2}$H methionine to an intracellular value by use of the $^{13}$C homocysteine-to-$^{13}$C methionine ratio. For completeness, data for the rate of methionine removal for protein synthesis are also shown in Table 3. Of the homocysteine formed via TM, $\sim$45% was remethylated back to methionine, and $\sim$55% was oxidized via TS.

**DISCUSSION**

Sulfur amino acid metabolism is an important aspect of human amino acid metabolism (13, 36), and determining dietary amino acid requirements of sulfur amino acids has been a focus of attention in nutrition research (22, 35, 38). Methionine is particularly important because it is 1) a nutritionally indispensable amino acid that is a necessary substrate for protein synthesis, 2) a precursor for $\alpha$-adenosylmethionine TM reactions, 3) a regulatory outlet for tetrahydrofolate and betaine one-C transfer reactions, and 4) the source of sulfur for cysteine and taurine synthesis (9). Sulfur amino acid metabolism is regulated in humans through the balance of homocysteine production (TM) and disposal (RM + TS) (26). An understanding of homocysteine regulation in humans, how it is regulated, and how its regulation is altered in high risk individuals is essential to understanding the role this amino acid may play in cardiovascular disease.

The measurement of plasma homocysteine enrichment is complicated, because only a fraction of plasma homocysteine exists in a reduced, free form. The great majority of homocysteine in plasma is oxidized and bound to other sulfides, including plasma cysteine and cysteine residues of plasma proteins (31). Our method of measuring homocysteine reduces all homocysteine, and how its regulation is altered in high risk individuals is essential to understanding the role this amino acid may play in cardiovascular disease.

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Table 2. Measured steady-state plasma enrichments and calculated tracer turnover in humans receiving a constant infusion of [1-13C]- and [methyl-2H3]methionine

<table>
<thead>
<tr>
<th>Subject</th>
<th>13C-Met</th>
<th>2H3-Met</th>
<th>13C-Hcy</th>
<th>13C-Hcy/13C-Met (E13C-Hcy/E13C-Met)</th>
<th>Methionine Turnover</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.76</td>
<td>5.78</td>
<td>6.13</td>
<td>0.63</td>
<td>36.4</td>
</tr>
<tr>
<td>2</td>
<td>11.33</td>
<td>6.14</td>
<td>5.59</td>
<td>0.49</td>
<td>40.6</td>
</tr>
<tr>
<td>3</td>
<td>9.64</td>
<td>5.64</td>
<td>5.97</td>
<td>0.62</td>
<td>37.6</td>
</tr>
<tr>
<td>4</td>
<td>10.36</td>
<td>5.39</td>
<td>7.19</td>
<td>0.69</td>
<td>35.7</td>
</tr>
<tr>
<td>5</td>
<td>11.29</td>
<td>5.76</td>
<td>6.91</td>
<td>0.61</td>
<td>45.1</td>
</tr>
<tr>
<td>6</td>
<td>11.46</td>
<td>6.62</td>
<td>6.58</td>
<td>0.57</td>
<td>34.2</td>
</tr>
<tr>
<td>7</td>
<td>10.18</td>
<td>5.24</td>
<td>4.91</td>
<td>0.48</td>
<td>46.2</td>
</tr>
<tr>
<td>8</td>
<td>10.76</td>
<td>6.49</td>
<td>5.55</td>
<td>0.52</td>
<td>40.8</td>
</tr>
<tr>
<td>Means ± SE</td>
<td>10.59 ± 0.25</td>
<td>5.88 ± 0.18</td>
<td>6.10 ± 0.27</td>
<td>0.58 ± 0.03</td>
<td>37.3 ± 1.8</td>
</tr>
</tbody>
</table>


Table 3. Rate of [13C]methionine tracer oxidation to $^{13}$CO2 and sulfur amino acid metabolism in postabsorptive humans

<table>
<thead>
<tr>
<th>Subject</th>
<th>Rate of $^{13}$CO2 Excretion, P13CO2 µmol $^{13}$C·kg$^{-1}$·h$^{-1}$</th>
<th>Homocysteine Oxidation and Transsulfuration (TS = Ox)</th>
<th>Homocysteine Remethylation (RM = QO - Qc)</th>
<th>Methionine Transmethylation (TM = RM + TS)</th>
<th>Methionine into Protein (S = Qc - QO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.41</td>
<td>5.8</td>
<td>4.0</td>
<td>9.7</td>
<td>30.7</td>
</tr>
<tr>
<td>2</td>
<td>0.32</td>
<td>5.5</td>
<td>4.9</td>
<td>10.4</td>
<td>35.2</td>
</tr>
<tr>
<td>3</td>
<td>0.26</td>
<td>4.3</td>
<td>1.7</td>
<td>6.0</td>
<td>33.3</td>
</tr>
<tr>
<td>4</td>
<td>0.34</td>
<td>4.2</td>
<td>4.9</td>
<td>9.1</td>
<td>26.5</td>
</tr>
<tr>
<td>5</td>
<td>0.52</td>
<td>7.0</td>
<td>11.4</td>
<td>18.4</td>
<td>25.0</td>
</tr>
<tr>
<td>6</td>
<td>0.33</td>
<td>4.8</td>
<td>0.7</td>
<td>5.5</td>
<td>29.4</td>
</tr>
<tr>
<td>7</td>
<td>0.20</td>
<td>7.1</td>
<td>7.4</td>
<td>11.7</td>
<td>41.9</td>
</tr>
<tr>
<td>8</td>
<td>0.41</td>
<td>7.1</td>
<td>0.2</td>
<td>7.2</td>
<td>33.7</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.35 ± 0.04</td>
<td>5.4 ± 0.4</td>
<td>4.4 ± 1.3</td>
<td>9.7 ± 1.5</td>
<td>32.0 ± 1.9</td>
</tr>
</tbody>
</table>

All values are expressed as µmol·kg$^{-1}$·h$^{-1}$. TS, transsulfuration; RM, remethylation; TM, transmethylation; Ox, oxidation; S, rate of methionine uptake for protein synthesis.
[13C]homocysteine enrichment, of which most of this homocysteine is disulfide-bound homocysteine (Fig. 4). A potential limitation of the use of plasma homocysteine enrichment as a measure of intracellular methionine enrichment is that the equilibration process of free and bound homocysteine may be very slow. If so, then bound homocysteine will not equilibrate with free homocysteine during the time course of our tracer infusion, and our plasma homocysteine enrichment measurements could significantly underestimate significantly the actual intracellular homocysteine enrichments.

To assess this problem, we also measured plasma [13C]cystathionine enrichments in each subject. Cystathionine is produced only inside cells during TS of homocysteine, and like homocysteine, its tracer enrichment in plasma should reflect intracellular homocysteine enrichment (Fig. 4). Unlike homocysteine, cystathionine does not covalently bond with other thiols in plasma.

Figure 3 shows a slow rise toward plateau for both plasma [13C]homocysteine and [13C]cystathionine enrichments for the eight subjects studied. If we had measured only [13C]homocysteine enrichments, we would have been unable to distinguish whether this slow rise toward plateau was due to slow equilibration and exchange of tracer in intracellular pools or whether the rise to plateau was due to equilibration of bound and free plasma [13C]homocysteine. The remarkably similar time courses of plasma [13C]cystathionine and [13C]homocysteine confirm that the slow rise to plateau of the tracers in plasma was due to slow equilibration of the infused methionine tracer with intracellular methionine and intracellular homocysteine pools. These results also show how long the [13C]methionine tracer must be infused to reach isotopic equilibrium in the intracellular compartment. For example, the enrichment would reach 95% of the final plateau after three time constants (inverse of the rate constant, i.e., $k^{-1}$). In Fig. 3, we determined that this period of time would be $3 \div (0.4 \ h^{-1}) = 7.5 \ h$. Thus long infusions of up to 8 h are required to measure intracellular methionine tracer enrichments via plasma homocysteine or cystathionine.

Figure 3 also demonstrates that homocysteine in blood rapidly equilibrates between the free and bound forms. That is, the exchange of homocysteine between free and disulfide forms must be faster than the rate of intracellular equilibration of the methionine tracer. The rate constant of equilibration is $k = 0.4 \ h^{-1}$ is equal to a half-life of 1.7 h for homocysteine. This value defines the upper limit for plasma homocysteine equilibration between bound and free forms, i.e., the half-life of homocysteine equilibration between bound and free forms must be considerably less than 1.7 h in plasma.

All previous measurements of methionine kinetics have made assumptions regarding the intracellular dilution of methionine tracers in vivo. Because plasma homocysteine is derived from intracellular homocysteine that is formed from intracellular methionine, plasma homocysteine can be used to follow intracellular methionine. In this study, we found that the homocysteine-to-methionine [13C]enrichment ratio was $58 \pm 3\%$ (range: 48–69%) in the eight subjects studied (Table 2). This ratio of 58% is significantly lower than the 80% α-ketoisocaproate-to-leucine ratio (12, 16) previously used to study methionine kinetics (25, 26). The [13C]enrichment ratio of plasma homocysteine to methionine of 58% indicates that the inflow of plasma methionine into cells comprises 58% of the intracellular methionine inflow, with the remaining 42% coming from the release of methionine via protein breakdown. That is, the rate of methionine inflow into cells (X) is $X/B = 0.58/0.42 = 1.4$ times faster (Eq. 1) than methionine release from protein breakdown (B). We presume that most of the homocysteine production from the hydrolysis of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolyase occurring in the liver (28–30). In contrast, leucine transamination occurs largely in the skeletal muscle (16). We assume that we observe a greater intracellular dilution of the methionine tracer than branched-chain amino acid tracers because the rate of proteolysis is greater in hepatic tissues than in skeletal muscle (13, 33).

Why have we stressed measurement of plasma homocysteine as an intracellular marker of methionine metabolism over cystathionine? Figure 3 demonstrates the equivalence of plasma homocysteine and cystathionine as intracellular markers. The primary reason that we have stressed plasma homocysteine vs. cystathionine measurement is an analytical one. Although homocysteine is not an abundant amino acid in plasma ($\sim$5–10 μM), cystathionine is present in plasma in very low levels ($\sim$0.1 μM), and the [13C]tracer is >10-fold less in concentration. Although we have developed a GC-MS assay sensitive enough to measure cystathionine and its [13C]label in tracer amounts in plasma, the measurement of cystathionine is naturally more
“noisy” and more difficult to perform overall. However, if there is ever a case where the measurement of plasma homocysteine and its enrichment is questionable, then cystathionine measurement can be performed to confirm the homocysteine results with respect to tracer enrichments.

We performed this study for the specific purpose of testing our ability to measure homocysteine and cystathionine enrichments in plasma. These measurements have been desired ever since Storch et al. (25, 26) reported their first measurements of methionine kinetics in 1988 and 1990. At that time, they acknowledged that interpreting methionine kinetics requires an intracellular tracer enrichment measurement and that they were “exploring this difficult problem by attempting to measure the enrichment of homocysteine or cystathionine in plasma and possibly in urine.” Over a decade passed before this goal was accomplished. The present work is based upon our method for measuring homocysteine and its enrichment in plasma by GC-MS (11). This method and the one described for cystathionine were used to make the measurements reported here. When we began this study, we were not sure how enriched the plasma homocysteine and cystathionine would be. Because precision of enrichment measurement is related to the level of enrichment, we chose an infusion rate of [13C]methionine tracer at the high end of a tracer dose (~10 MPE, Table 2) to ensure adequate enrichment in methionine and, therefore, in homocysteine and cystathionine. This higher infusion rate for [13C]methionine gave us the precision of measurement required to define accurately and precisely the intracellular/extracellular gradient of infused [13C]methionine tracer via homocysteine and cystathionine. As has already been discussed by others, low infusion rates are critical for studies where the flux of the amino acid to be measured has been altered due to dietary restriction. In these cases, the tracer infusion itself may become the predominant exogenous input and act in its own right as a substantial source of methionine intake (37).

The ultimate limitation as to how small the infusion rate of methionine tracer can be is not the measurement of plasma homocysteine enrichment but the measurement of 13CO2 in exhaled air. The rate of TS and, therefore, oxidation of the homocysteine carboxyl 13C to 13CO2 is small (Table 3) compared with total CO2 production. Thus the 13C enrichment in breath is very low. The breath 13CO2 enrichment at plateau was 0.0037 ± 0.0004 atom percent excess (APE) in this study. Natural abundance variations of 13C and variability of 13C plateaus in breath samples are both in the range of ~0.001 APE 13C. Thus the methionine 13C tracer infusion rate must be at least ~1.5–2 μmol·kg−1·h−1 to keep measurement error of methionine oxidation within a tolerable range.

We chose to infuse L-[1-13C]methionine and L-[methyl-2H3]methionine as separate tracers. Most infusion studies have used a combined L-[1-13C; methyl-2H3]methionine dilabeled tracer (6–8, 10, 20, 21, 26, 27, 32, 38). The problem for this study is that we need to compare plasma [13C]methionine enrichment against [13C]homocysteine enrichment in plasma. When the dilabeled tracer is used, there are two 13C-labeled methionine species in plasma: an isotopomer with a 4-mass increase, [1-13C; methyl-2H3]methionine (M4) and an isotopomer with a 1-mass increase, [1-13C]methionine (M1). The latter is derived from intracellular methionine RM. We avoided the dilabeled tracer for this study because measuring two 13C species for methionine adds noise and uncertainty to the measurement of the methionine and, hence, the intracellular/extracellular gradient. Now that this preliminary study has been completed and the gradient has been established, a dilabeled tracer can be used in the future.

The calculations of methionine kinetics with the use of plasma [13C]homocysteine as a surrogate measure for intracellular enrichment of methionine do change when the dilabeled [1-13C; methyl-2H3]methionine tracer is used. When the dilabeled tracer is being used, Eq. 1 needs to include both isotopomers in the denominator (i.e., M4 + M1). Calculation of methionine C flux (Eq. 2) is unchanged because [13C]homocysteine enrichment is used. The enrichment of the [2H3]methionine is the M4 isotopomer, and it is adjusted as indicated in Eq. 3; the remaining equations are unchanged.

What happens when we try to adjust values of methionine kinetics in the literature for our measured intracellular/extracellular gradient of 58%? First, a number of reports in the literature studied subjects on crystalline amino acid diets where sulfur amino acid intake was manipulated (8, 10, 20, 21, 26, 38). These reports would be difficult to compare with our results with the use of a normal antecedent diet. Second, the range of reported values for methionine C and methyl fluxes is broad. Third, most studies using the dilabeled tracer do not report enrichment values for the M4 and M1 isotopomers. Finally, many of the reports gave no clear definition of how calculations were performed or how specific enrichment values were adjusted to define the kinetic parameters reported (10, 19, 26, 27). The most straightforward set of results comes from the original report of Storch et al. (25). Converting their kinetic values from an assumed gradient of 80% to one of 58% produces flux, RM and oxidation (TS) and TM values of QM = 36 ± 1, QC = 33 ± 1, RM = 4 ± 1, TS = 6 ± 1, and TM = 10 ± 1 μmol·kg−1·h−1. Adjusting the kinetic values from the report of van Guldener et al. (32) gives QM = 37 ± 1, QC = 31 ± 1, RM = 4 ± 1, TS = 5.2 ± 0.2, and TM = 9 ± 1 μmol·kg−1·h−1, and the report of Fukagawa et al. (7) gives QM = 31 ± 1, QC = 26 ± 1, RM = 7 ± 1, TS = 5 ± 1, and TM = 12 ± 1 μmol·kg−1·h−1. The values we report in Tables 2 and 3 are at the higher end of the methionine C and methyl fluxes, but the rates of RM, TS, and TM are similar.

Epidemiological studies have shown that an elevated concentration of plasma homocysteine (>15 μM) is a risk factor for the development of cardiovascular disease (24). However, measurement of a plasma homocysteine concentration that is high does not give suffi-
cient information as to why it is high. Plasma homocysteine concentration is defined by the balance of homocysteine production and disposal. A high homocysteine concentration could be due to an elevated production rate, a decreased rate of TS, a decreased rate of RM to methionine, or any of these three. Administration of an oral methionine load (~0.1 g/kg) with the subsequent measure of the time course of plasma homocysteine concentration has been used as a method to measure an individual’s ability to dispose of methionine through the TS pathway (4). Although measurement of a plasma homocysteine concentration after a methionine load is an improvement over the static measurement of homocysteine concentration, the administration of a load method does not define homocysteine kinetics or the regulation of homocysteine disposal through TS and RM. Use of methionine tracers with measurement of plasma homocysteine enrichment gives a direct assessment of these rates. This approach can be combined with administration of a load to assess an individual’s ability to dispose of methionine in amounts greater than basal.

In summary, we present the combination of a previously reported dual-isotope tracer method (25) with measurement of homocysteine and cystathionine stable isotope tracer enrichments for assessing sulfur amino acid metabolism in humans. The advantage of this approach is that we can measure methionine metabolism without making assumptions regarding the intracellular dilution of the infused tracer. The results presented here allow for correction of previous reports of methionine kinetics in humans. Furthermore, the identical 13C enrichment measurements determined with plasma homocysteine and cystathionine give confidence in the measurements presented and give the researcher the opportunity to use either of these markers of intracellular methionine-homocysteine metabolism.

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