Tissue methionine cycle activity and homocysteine metabolism in female rats: impact of dietary methionine and folate plus choline

Fiona A. Wilson,1 Joost J. G. C. van den Borne,2 A. Graham Calder,1 Niamh O’Kennedy,3 Grietje Holtrop,4 William D. Rees,1 and Gerald E. Lobley1

1The Rowett Institute of Nutrition and Health (RINH), University of Aberdeen, Bucksburn, Aberdeen, United Kingdom;
2Animal Nutrition Group, Department of Animal Sciences, Wageningen University, Wageningen, The Netherlands;
3and 4Provexis plc and 4Biomathematics and Statistics Scotland, RINH, University of Aberdeen, Aberdeen, United Kingdom

Submitted 6 August 2008; accepted in final form 9 January 2009

Wilson FA, van den Borne JJ, Calder AG, O’Kennedy N, Holtrop G, Rees WD, Lobley GE. Tissue methionine cycle activity and homocysteine metabolism in female rats: impact of dietary methionine and folate plus choline. Am J Physiol Endocrinol Metab 296: E702–E713, 2009. First published January 13, 2009; doi:10.1152/ajpendo.90670.2008.—Impaired transfer of methyl groups via the methionine cycle leads to plasma hyperhomocysteinemia. The tissue sources of plasma homocysteine in vivo have not been quantified nor whether hyperhomocysteinemia is due to increased entry or decreased removal. These issues were addressed in female rats offered diets with either adequate or excess methionine (additional methyl groups) with or without folate and choline (impaired methyl group transfer) for 5 wk. Whole body and tissue metabolism was measured based on isotopomer analysis following infusion with either [1-13C,methyl-2H3]methionine or [U-13C]methionine plus [1-13C]homocysteine. Although the fraction of intracellular methionine derived from methylation of homocysteine was highest in liver (0.18–0.21), most was cysteine. Although the fraction of intracellular methionine derived from methylation of homocysteine was highest in liver (0.18–0.21), most was retained. In contrast, the pancreas exported to plasma more of methionine synthesized de novo. The pancreas also exported homocysteine to plasma, and this matched the contribution from liver. Synthesis of methionine from homocysteine was reduced in most tissues with excess methionine supply and was also lowered in liver (P < 0.01) with diets devoid of folate and choline. Plasma homocysteine concentration (P < 0.001) and flux (P = 0.001) increased with folate plus choline (impaired methyl group transfer) for 5 wk. Whole body and tissue metabolism was measured based on isotopomer analysis following infusion with either [1-13C,methyl-2H3]methionine or [U-13C]methionine plus [1-13C]homocysteine. Although the fraction of intracellular methionine derived from methylation of homocysteine was highest in liver (0.18–0.21), most was retained. In contrast, the pancreas exported to plasma more of methionine synthesized de novo. The pancreas also exported homocysteine to plasma, and this matched the contribution from liver. Synthesis of methionine from homocysteine was reduced in most tissues with excess methionine supply and was also lowered in liver (P < 0.01) with diets devoid of folate and choline. Plasma homocysteine concentration (P < 0.001) and flux (P = 0.001) increased with folate plus choline deficiency, although the latter still represented <12% of estimated tissue production. Hyperhomocysteinemia also increased (P < 0.01) the inflow of homocysteine into most tissues, including heart. These findings indicate that a full understanding of hyperhomocysteinemia needs to include metabolism in a variety of organs, rather than an exclusive focus on the liver. Furthermore, the high influx of homocysteine into cardiac tissue may relate to the known association between homocysteinemia and hypertension.

Address for reprint requests and other correspondence: G. E Lobley, Obesity and Metabolic Health Division, The Rowett Institute of Nutrition and Health, Univ. of Aberdeen, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, UK (e-mail: g.lobley@rowett.ac.uk).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of homocysteinemia affected by either increased or decreased supply of methyl groups? These objectives were tackled by stable isotope procedures based on isotopomer analysis. The first involved infusion of bis-[1-13C,methyl-2H3]methionine (36, 37), whereas the second utilized [1-13C]homocysteine in methionine cycle.

**Fig. 1.** The methionine cycle. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DMG, dimethylglycine; Ser, serine; Gly, glycine; BHMT, betaine homocysteine methyltransferase; MS, methionine synthase (N5-methyltetrahydrofolate transferase).

**MATERIALS AND METHODS**


**Animals and Diets**

All rodent studies were approved by the Ethical Review Committee of the Rowett Institute of Nutrition and Health and conducted in accordance with the conditions of the United Kingdom Animal Scientific Procedures Act (1986). The diets (Table 1) were formulated in accordance with AIN-93 regulations (30) as described previously (20, 28). Diets contained the equivalent of 18% protein, provided as 9% casein and a supplement of crystalline amino acids (SpodellFellon, London, UK) in similar amounts to those present in the 9% casein. Methionine was omitted from the supplement to allow subsequent diet manipulation. Similarly, the vitamin mix was prepared devoid of folate, and this was added as a supplement where appropriate. Dietary treatments allowed a 2 × 2 factorial design (experiment 1) with either excess (+M) or adequate (−M) methionine (5.6 and 2.3 g/kg total diet, respectively) and with adequate folate (0.2 g/kg diet) plus choline (2 g/kg diet) either present (+FC) or absent (−FC). To prevent sulfur group deficiency, cysteine was supplemented at 20.2 g/kg diet.

**Experiment 1**

Virgin female group-housed Hooded-Lister (Rowett strain) rats (7.5 wk of age, start body weight 168.2 ± 2.1 g; n = 24) were separated into four equal weight-matched groups. Animals were fed ad libitum for 5 wk on one of the four experimental diets (Table 1).

**Experiment 2**

Virgin female group-housed Hooded-Lister rats (8.5 wk of age, start body weight 182.5 ± 2.0 g, n = 16) were divided into two equal weight-matched groups and offered one of the low-methionine diets detailed above (see Table 1) with either adequate (−M + FC) or devoid (−M − FC) of folate plus choline. These diets were identical to those for experiment 1 except for the addition of cysteine at 3.9 g/kg diet.

**Infusion Procedures**

In pilot studies, groups of six rats fed the study diets with either normal or deficient folate and choline present for the previous 4 wk were given primed infusions of either [1-13C,methyl-2H3]methionine or [U-13C]methionine and [1-13C]homocysteine, and blood samples were taken at intervals up to 6 h from the caudal vein. Pseudo-plateau enrichments were attained in plasma within 3–4 h for both the labeled forms of methionine and homocysteine infused and also these produced as secondary products within tissues and exported to plasma e.g., [U-13C]homocysteine and [1-13C]methionine (data not shown). Attainment of plateau for these secondary metabolites was taken to indicate that isotopic plateau had been achieved within the tissues where these were formed originally.

For experiment 1, after 5 wk on diet, the rats were trained to a commercial restraint tube (Harvard Apparatus, Kent, UK) and then infused via a tail vein for 4 h with [1-13C,methyl-2H3]methionine (2.0 mg/ml saline, flow rate 0.36 ml/h; Mass Trace, Woburn, MA), following an initial priming dose infused over 8 min, equivalent to 1 h of infusion. Infusions were by syringe pumps (Scientific and Research Instruments, Edenbridge, Kent, UK). At the end of the infusion, the rats were killed by stunning and cervical dislocation followed by decapitation, and the trunk blood obtained was collected into heparinized tubes on ice. The liver, pancreas, heart, lung, kidney, small intestine, spleen, reproductive organs, muscle, skin, and brain were removed in sequence quickly (<5 min), washed in ice-cold 0.15 M NaCl, and immediately frozen in liquid N. Samples were then stored at −80°C until analysis.

Procedures for experiment 2 were similar to those for experiment 1 except that the infusate comprised [U-13C]methionine (1.5 mg/ml saline; flow rate 0.36 ml/h; Cambridge Isotope Laboratories, Andover, MA) plus [1-13C]homocysteine (0.2 mg/ml saline for animals on diet −M + FC and 0.4 mg/ml saline for animals on diet −M − FC), and the infusion period was increased to 5 h. At slaughter, midcolon was added to the list of tissues removed.

All methods for analysis of metabolite concentrations in plasma and determination of enrichments in plasma and tissue homogenates are detailed in the appendix.

**Table 1. Diets for experiments 1 and 2: amounts of methionine, folate, and choline added to basal ration**

<table>
<thead>
<tr>
<th>Diet component</th>
<th>+M + FC</th>
<th>−M + FC</th>
<th>−M + FC*</th>
<th>−M − FC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Methionine, g/kg</td>
<td>2.3</td>
<td>2.3</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Choline chloride, g/kg</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Folate, mg/kg</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

M, methionine; FC, folate + choline. The basal diet included (g/kg) 88.2 casein, 88.2 supplemented amino acids, 189.5 sucrose, 49.1 cellulose, 417.2 corn starch, 98.3 corn oil, 34.4 mineral mix AIN-93, 2.8 potassium phosphate, and 8.3 vitamin mix AIN-93. Amino acid composition (g/kg mixture) was as follows: 26.2 alanine, 36.9 arginine, 67.2 aspartic acid, 97.4 glutamine, 97.4 glutamic acid, 16.8 glycine, 26.1 histidine, 46.3 isoleucine, 84 leucine, 100.7 lysine hydrochloride, 46.2 phenylalanine, 84.3 proline, 46.9 serine, 36.4 threonine, 11.7 tryptophan, 51.2 tyrosine, 56.8 valine, and 20.2 (experiment 1) and 3.9 (experiment 2) cysteine. *Only these diets offered for experiment 2.
Theoretical Background

For experiment 1, the [1-13C,methyl-2H3]methionine (m + 4 methionine) infused into the blood is extracted by tissues and either used for protein synthesis or converted to [1-13C]homocysteine (m + 1 homocysteine), with the loss of the labeled methyl group. Transfer of a methyl group from either 5-methyltetrahydrofolate or betaine to [1-13C]homocysteine yields [1-13C]methionine (m + 1 methionine). Alternatively, the homocysteine can be converted to cysteine and 2-oxo-[1-13C]butyrate (not measured). The ratio of m + 1 to m + 4 methionine in a tissue compared with plasma gives a relative measure of synthesis from homocysteine. Comparison of enrichments of methionine and homocysteine in plasma and tissues allows the relative flows from plasma, protein breakdown (assumed equal to protein synthesis), and methylation of homocysteine to be estimated by a mathematical model (see Appendix) based on the structure shown in Fig. 2.

For experiment 2, where the rats were infused with [U-13C]methionine (m + 5 methionine), this is extracted by the plasma and may be converted to m + 4 homocysteine with subsequent fates as export to plasma, conversion to cysteine and 2-oxo-[U-13C]butyrate (not measured), or methylation to produce m + 4 methionine. The enrichments of these various products give similar information to that obtained in experiment 1 with, other than the m + 5 methionine, all the labeled metabolites generated within the tissues. The [1-13C]homocysteine infused into plasma may enter the cell and be diluted by homocysteine produced by intracellular demethylation of methionine, and this may then be converted to [1-13C]methionine. Together the isotopomers of m + 1 and m + 4 homocysteine within a tissue allow estimation of the relative contribution from the two sources, import from plasma and synthesis from methionine. Similarly, isotopomer analysis of m + 0, m + 1, m + 4, and m + 5 methionine allows the intracellular inflows from plasma, methylation of homocysteine, and release from protein breakdown to be determined. The contributions of tissue-derived homocysteine and methionine to plasma concentrations can also be obtained from the relative enrichments of the isotopomers from those infused in the primary pool (m + 1 homocysteine and m + 5 methionine) and those produced in tissues and then exported (m + 1 and m + 4 methionine and m + 4 homocysteine). In addition, the irreversible loss of whole body homocysteine rate could also be determined.

The various enrichments obtained in both experiments were then used to estimate metabolite flows with the mathematical model described in the Appendix.

Statistics

Experiment 1 (diets −M−FC, −M+FC, +M−FC, +M+FC, n = 8/diet). Technical problems were encountered with three of the infusion procedures in experiment 1; hence, no kinetic data were obtained for one rat fed +M+FC and two offered −M−FC. Consequently, the groups were unbalanced and were analyzed by residual maximum likelihood (REML) procedures. For comparison between tissues, animal was considered as a random effect, with tissue, diet, and their interaction as fixed effects. Because the tissue by diet interaction was significant (P < 0.05), the effect of diet within each tissue was then analyzed by REML with fixed effects for dietary methionine (−M, +M), dietary folate and choline (−FC, +FC), and their interaction. Where any principal effects showed a significant effect at the 5% level, means were compared with post hoc t-test at the 1% significance level.

Experiment 2 (diets −M−FC, −M+FC, n = 8/diet). For comparison between tissues, data were analyzed by ANOVA with animal as a random effect while tissue, diet, and their interaction were regarded as fixed effects. Because the interaction was significant (P < 0.05), data within each tissue were then analyzed by two-sample t-test to test for differences between the two diets (−FC, +FC).

Data were analyzed using Genstat 10th edition (VSN International, Hemel Hempstead, UK). All individual means in the text are presented ± SE while, for comparisons between treatments in the tables, the SED are given.

RESULTS

Experiment 1: Effect of Dietary Methionine and Folate Plus Choline on Whole Body and Tissue Methionine Kinetics

General effects and tissue responses. Neither the level of methionine supplementation nor the presence or absence of dietary folate plus choline affected body weight gain (average 60.0 ± 8.3 g/5 wk). No adverse health effects were noted.

For clarity, data are first compared for all treatments combined to show general effects and differences between tissues. As expected, following infusion of the bis-labeled methionine (m + 4), all tissues showed a lower intracellular trace-to-tracer ratio (TTR) of m + 4 methionine than plasma because of dilution by unlabeled methionine released from protein breakdown (data not shown). The intracellular TTR of m + 1 methionine produced by methylation of homocysteine was lower than plasma for all tissues (P < 0.01; Fig. 3) except pancreas (P < 0.001), which was higher. Homocysteine concentrations obtained by the gas chromatography-mass spectrometry procedure varied markedly between tissues. The largest concentrations were recorded for lung, spleen, muscle, and skin, with m + 1 homocysteine TTR in the lung, indicative of demethylation of m + 4 methionine, not different from plasma while the other three were lower (P < 0.01; Fig. 4). Moderate concentrations, at the limit of confident quantification of TTR, were obtained for liver, small intestine, and pancreas, with the TTR for liver and small intestine lower than plasma (P < 0.01) while the values for pancreas were greater (P < 0.001). For the
other tissues, the homocysteine concentrations were too low to give reliable estimates.

If the ratio of intracellular $m + 1$ to $m + 4$ methionine TTR exceeds that in plasma, then intracellular synthesis of methionine has occurred, as was the case for most tissues (Table 2). Across diets, the greatest increase in ratio was observed for liver followed by pancreas (both $P < 0.001$ compared with the ratio in plasma). Other tissues also converted homocysteine to methionine, but to a lesser extent (all $P < 0.01$), except for brain, where the ratio was similar to plasma.

The contribution of plasma inflow to total tissue methionine flux (inflow + methylation + protein degradation) differed between groups of tissues ($P < 0.01$) and ranged from 0.24 to 0.30 (kidney, liver, small intestine), 0.37 to 0.45 (brain, skin, spleen, reproductive organs), 0.56 to 0.59 (heart, lungs), and 0.69 to 0.75 (muscle and pancreas). The contribution of homocysteine methylation to tissue methionine flux was lower in all tissues and ranged from 0.13, for both liver and pancreas ($P < 0.001$, compared with all other tissues), to <0.02 for skin.

Effect of methionine and folate plus choline supply. Folate concentrations were reduced in plasma (56.0 ± 4.6 vs. 14 ± 1.2 ng/mL, $P < 0.001$) and liver (37.4 ± 3.0 vs. 9.9 ± 0.8 μg/g wet wt, $P < 0.001$) during dietary folate plus choline deficiency but were unaffected by dietary methionine level. Plasma methionine concentrations were not altered by either the reduced dietary methionine supply (52.9 ± 1.2 vs. 54.9 ± 1.4 nmol/g, $P > 0.10$) nor by the removal of folate plus choline (52.6 ± 1.1 vs. 55.3 ± 1.4 nmol/g, $P > 0.10$). Similarly, total plasma free cysteine was unaffected by diet and averaged 224 nmol/g. Total homocysteine concentrations in plasma were not altered by dietary methionine content (13.2 ± 2.9 vs. 18.0 ± 4.4 nmol/g, $P > 0.10$) but were increased from 5.1 ± 0.2 to 26.0 ± 3.0 nmol/g ($P < 0.001$) when folate plus choline were omitted.

Whole body irreversible loss rate (ILR) increased at the lower methionine intake, both when the TTR of either $m + 4$ methionine (36.7 ± 2.0 vs. 29.3 ± 0.9 μmol/h, $P = 0.002$) or $m + 1$ homocysteine (48.3 ± 2.0 vs. 43.2 ± 0.6 μmol/h, $P = 0.02$) in the plasma was used as precursor. In neither case did the amount of folate plus choline in the diet affect the ILR. Across all dietary treatments, the ratio of plasma $m + 1$ homocysteine to $m + 4$ methionine was 0.713 ± 0.019 ($P < 0.001$ different from unity) but was greater for the −M+FC diet (0.834, $P < 0.05$) compared with the other diets (average 0.673).

Both methionine and folate plus choline impacted on the ratio of $m + 1$ to $m + 4$ methionine in plasma (Table 2), an index of intracellular conversion of homocysteine to methionine that is exported to plasma. This ratio decreased in the absence of dietary folate plus choline ($P < 0.001$) but increased with the lower methionine supply ($P = 0.01$), particularly for animals fed the −M+FC diet (M×FC, $P < 0.01$). These plasma data reflect changes in response to diet at the tissue level (Table 2). For example, lower dietary methionine increased the ratio of $m + 1$ to $m + 4$ methionine in liver ($P < 0.001$), small intestine ($P < 0.01$), spleen ($P = 0.001$), and pancreas ($P < 0.05$). In contrast, the omission of folate and choline from the diet decreased the ratio of $m + 1$ to $m + 4$ methionine in liver ($P < 0.001$), muscle ($P < 0.01$), and pancreas ($P < 0.05$), with the effect in liver and muscle greater at the lower methionine intake (M×FC, $P < 0.05$). Low folate plus choline increased the ratio in heart, however ($P < 0.05$).

Experiment 2: Effects of Dietary Folate Plus Choline on Whole Body and Tissue Homocysteine Metabolism

Whole body metabolism. Body weight gain over the duration of the experiment was similar between the two diet groups and averaged 44.9 ± 3.1 g over the 5 wk. Whole body ILR of methionine (protein synthesis plus demethylation) averaged 47.1 ± 1.1 μmol/h, based on the harmonic mean of tissue

---

Fig. 3. Experiment 1: comparison of the tracer-to-trace ratio (TTR) of $m + 1$ methionine (Met) from plasma and tissues following infusion with [1,13C]methyl-[H3]methionine. All data are combined, irrespective of dietary group, and presented as means ± SE. Sample numbers for each tissue are 21 except for skin (20). Different letters denote differences ($P < 0.01$) between tissues and/or plasma based on residual maximum likelihood (REML) analysis with animal as a random effect and presented as means ± SE. Sample numbers for each tissue are 21 except for pancreas and skin (20), small intestine (SI; 18), and liver (15). Hatched bars are for tissues where the areas for plasma inflow to total tissue methionine flux were not statistically different from unity. The contribution of homocysteine methylation to tissue methionine flux was lower in all tissues and ranged from 0.13, for both liver and pancreas ($P < 0.001$, compared with all other tissues), to <0.02 for skin.

Fig. 4. Experiment 1: comparison of the TTR of $m + 1$ homocysteine (Hcy) from plasma and tissues following infusion with [1,13C]methyl-[H3]methionine. All data are combined, irrespective of dietary group, and presented as means ± SE. Sample numbers are 21 for each tissue except for pancreas and skin (20), small intestine (SI; 18), and liver (15). Hatched bars are for tissues where the areas for plasma inflow to total tissue methionine flux were not statistically different from unity. The contribution of homocysteine methylation to tissue methionine flux was lower in all tissues and ranged from 0.13, for both liver and pancreas ($P < 0.001$, compared with all other tissues), to <0.02 for skin.
enrichments of free \( m + 5 \) methionine and was not altered by diet (\( P > 0.10 \)). Lower values were obtained if either plasma \( m + 5 \) methionine or \( m + 4 \) homocysteine was used as the precursor pool (23.3 ± 0.6 and 26.4 ± 0.6 μmol/h, respectively), again with no effect of diet (\( P > 0.10 \)). As with experiment 1, the ratio of plasma TTR for synthesized original methionine (i.e., \( m + 5 \) to \( m + 5 \)) was decreased (0.221 ± 0.008 vs. 0.257 ± 0.008, \( P < 0.01 \)) when the diet was deficient in folate plus choline. Demethylation in both absolute terms (5.5 ± 0.19 vs. 9.0 ± 0.53 μmol/h, \( P < 0.001 \)) and as a fraction of total methionine IRL (0.118 ± 0.006 vs. 0.190 ± 0.007, \( P = 0.001 \)) increased when folate plus choline were absent from the diet. In contrast, resynthesis of methionine was not changed (\( P > 0.10 \)) by diet manipulation, either in absolute terms (9.6 ± 0.5 μmol/h) or as a fraction of IRL (0.203 ± 0.006). These differential responses in methionine demethylation and methylation were reflected in an increased \([1-^{13}C]\)homocysteine IRL (1.3 ± 0.04 vs. 2.8 ± 0.33 μmol/h, \( P = 0.001 \)) when folate and choline were omitted from the diet.

**Tissue responses.** The TTR of \( m + 4 \) homocysteine (Fig. 5), formed by demethylation of \([U-^{13}C]\)methionine, was highest in the pancreas and exceeded that of plasma (\( P < 0.001 \)), similar to the observations in experiment 1. Enrichments in the small intestine, skin, and spleen (all \( P < 0.001 \)) plus colon (\( P < 0.01 \)) were also greater than plasma as was lung (\( P < 0.05 \)). Muscle, liver, and heart TTR values were not different from plasma, whereas kidney and brain were lower (both \( P < 0.001 \)).

The TTR of synthesized methionine, in both \( m + 4 \) and \( m + 1 \) forms, was higher in the pancreas (21 and 7%, respectively, both \( P < 0.001 \)) than plasma (Fig. 6, A and B). Interestingly, the enrichment of \( m + 1 \) methionine in the heart was even higher than in the pancreas (\( P = 0.001 \); Fig. 6B), indicative of substantial import and methylation of \([1-^{13}C]\)homocysteine from plasma. The higher ratio of \( m + 4 \) to \( m + 5 \) methionine TTR in tissues compared with plasma (Fig. 7A) indicated that most organs converted homocysteine to methionine. As observed in experiment 1, the liver was the most active site, followed by kidney, brain, pancreas, and spleen (Fig. 7A). These latter tissues were more active than liver in utilizing homocysteine imported from blood, as indicated by the high intracellular ratios found for \( m + 1 \) methionine to \( m + 1 \) homocysteine compared with plasma (Fig. 7B).

The model developed (see APPENDIX) allowed estimation of both relative (methionine and homocysteine) and absolute (methionine only) tissue fluxes. For most tissues, diet had no effect on the fractional synthesis rate (%/day), and combined values were used for the model (heart, 12.5; liver, 74.7; lung, 25.1; midcolon, 51.9; muscle, 4.6; pancreas, 162.4; small intestine, 149.4; skin, 14.9; and spleen, 49.5), except for kidney where values were greater when folate and choline were absent from the diet (78.4 vs. 70.1%/day, \( P = 0.044 \)). These data were used to estimate the fractional inflows to the intracellular methionine pool from plasma and methylation of homocysteine (Table 3). The total methionine fluxes (μmol·h⁻¹·g⁻¹ tissue) are also presented, and, from these two sets of data, absolute

---

**Table 2. Experiment 1: ratio of \( m + 1 \) to \( m + 4 \) in free methionine in plasma and tissues following infusion of \([1-^{13}C, methyl-^{2}H_{3}]\)methionine in rats fed either \( +M \) or \( -M \) diet with either \( +FC \) or \( -FC \)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>( +FC )</th>
<th>( -FC )</th>
<th>( +FC )</th>
<th>( -FC )</th>
<th>SED*</th>
<th>( M )</th>
<th>( FC )</th>
<th>( M \times FC )</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma*</td>
<td>0.200†</td>
<td>0.185†</td>
<td>0.256†</td>
<td>0.171†</td>
<td>0.016</td>
<td>0.010</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Heartb</td>
<td>0.246†</td>
<td>0.290†</td>
<td>0.299†</td>
<td>0.304†</td>
<td>0.015</td>
<td>0.006</td>
<td>0.019</td>
<td>0.066</td>
<td></td>
</tr>
<tr>
<td>Kidneybc</td>
<td>0.225</td>
<td>0.252</td>
<td>0.259</td>
<td>0.258</td>
<td>0.012</td>
<td>0.030</td>
<td>0.102</td>
<td>0.099</td>
<td></td>
</tr>
<tr>
<td>Liverd</td>
<td>0.593†‡</td>
<td>0.506†</td>
<td>0.708‡</td>
<td>0.453‡</td>
<td>0.050</td>
<td>0.080</td>
<td>&lt;0.001</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>Lungc</td>
<td>0.217</td>
<td>0.240</td>
<td>0.275</td>
<td>0.238</td>
<td>0.022</td>
<td>0.045</td>
<td>0.736</td>
<td>0.052</td>
<td></td>
</tr>
<tr>
<td>Musclebc</td>
<td>0.259†‡</td>
<td>0.254†</td>
<td>0.322†</td>
<td>0.232†</td>
<td>0.023</td>
<td>0.064</td>
<td>0.008</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>Pancreasf</td>
<td>0.370†‡</td>
<td>0.317†</td>
<td>0.409‡</td>
<td>0.364‡</td>
<td>0.029</td>
<td>0.017</td>
<td>0.019</td>
<td>0.831</td>
<td></td>
</tr>
<tr>
<td>Small intestinebc</td>
<td>0.242†</td>
<td>0.254†‡</td>
<td>0.300‡</td>
<td>0.305‡</td>
<td>0.018</td>
<td>0.004</td>
<td>0.391</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>Spleenbc</td>
<td>0.249†</td>
<td>0.271†‡</td>
<td>0.291‡</td>
<td>0.305‡</td>
<td>0.014</td>
<td>0.001</td>
<td>0.069</td>
<td>0.699</td>
<td></td>
</tr>
</tbody>
</table>

* M, high methionine; +M, marginal methionine; FC, adequate folate + choline; -FC, devoid of folate and choline. Results are means for 5, 6, 6, and 4 rats for \( +M+FC, +M-FC, -M+FC, \) and \( -M-FC \) diets, respectively. Different superscripts (a-e) indicate differences of \( P < 0.01 \) between tissues combined across dietary treatments. Brain was not different from plasma, whereas the reproductive organs and skin gave similar composite values to muscle. *Data from each tissue were analyzed with residual maximum likelihood (REML) with M, FC, and their interaction as fixed effects. Different superscripts († and ‡) indicate \( P < 0.01 \). Other tissues did not show a diet-related effect but had mean ratios of 0.192 (brain), 0.263 (reproductive organs), and 0.240 (skin).
flows (per g tissue) also can be calculated. In liver, methylation of homocysteine and methionine inflow from plasma were approximately equal and, together, represented 40% of hepatic methionine flux. For the other tissues, the methionine inflow from plasma exceeded considerably that derived from methylation of homocysteine (Table 3).

Intracellular homocysteine is derived from two sources, plasma entry and demethylation of methionine. The proportion that arose from plasma entry was <10% for liver and brain (Table 4) compared with skin (89%), heart (68%), and small intestine (38%). Values for muscle, kidney, lung, and pancreas were intermediate and ranged from 17 to 24% (see Table 4; for all tissues, the proportion that arose through demethylation can be obtained by difference).

Effect of dietary folate and choline. The mean plasma concentrations of methionine (77.4 ± 1.4 nmol/g) and total cysteine (222 ± 2.6 nmol/g) were not altered (P > 0.10) by diet, with the latter values similar to those observed in experiment 1 where larger amounts of dietary cysteine were provided. The diet deficient in folate and choline resulted in increased total homocysteine in plasma (from 5.7 ± 0.07 to 14.0 ± 1.17 nmol/g, P < 0.001). Such diets also reduced the proportion of tissue methionine flux derived from methylation of homocysteine (Table 3) in the liver (P < 0.01), whereas, in contrast, there were small increases (P < 0.05) in the proportion for the lung, skin, midcolon, and heart. In the absence of dietary folate and choline, the relative inflow of homocysteine from plasma into liver, pancreas, kidney, midcolon, heart, and muscle increased (P < 0.01; Table 4), whereas there were corresponding decreases for the inflows from demethylation. Tissue homocysteine fluxes were not determined directly, but minimum estimates can be derived from the amount of methionine derived from homocysteine (from Table 4). By this approach then, per unit tissue weight, pancreas had the greatest conversion of plasma-

Fig. 6. Experiment 2: comparison of the TTR of m + 4 methionine from plasma and tissues following infusion with [U-13C]methionine (A) and m + 1 methionine from plasma and tissues following infusion with [1-13C]homocysteine (B). All data are combined, irrespective of dietary group, and presented as means ± SE. Sample numbers are 16 for each tissue except for skin (14). Different letters denote differences (P < 0.01) between tissues and/or plasma obtained by ANOVA with animal as a random effect and diet, tissue, and their interaction as fixed effects.

Fig. 7. Experiment 2: comparison of the ratios of m + 4 to m + 5 methionine from plasma and tissues following infusion with [U-13C]methionine (A) and m + 1 methionine to m + 1 homocysteine (Hcy) from plasma and tissues following infusion with [1-13C]homocysteine (B). All data within a tissue are combined, irrespective of dietary group, and presented as means ± SE. Sample numbers are 16 for each tissue except for skin (14). Different letters denote differences (P < 0.01) between tissues and/or plasma obtained by ANOVA with animal as a random effect and diet, tissue, and their interaction as fixed effects.
Table 3. Experiment 2: total methionine flux in tissues and fp and fh in rats fed either +FC or −FC diet

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Origin</th>
<th>+FC</th>
<th>−FC</th>
<th>SED</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>fp</td>
<td>0.481</td>
<td>0.461</td>
<td>0.016</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.121</td>
<td>0.120</td>
<td>0.011</td>
<td>0.93</td>
</tr>
<tr>
<td>Heart</td>
<td>fp</td>
<td>0.676</td>
<td>0.647</td>
<td>0.017</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.060</td>
<td>0.086</td>
<td>0.007</td>
<td>0.002</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>0.605</td>
<td>0.583</td>
<td>0.043</td>
<td>0.61</td>
</tr>
<tr>
<td>Kidney</td>
<td>fp</td>
<td>0.369</td>
<td>0.316</td>
<td>0.017</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.082</td>
<td>0.082</td>
<td>0.005</td>
<td>0.99</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>1.609</td>
<td>1.635</td>
<td>0.063</td>
<td>0.69</td>
</tr>
<tr>
<td>Liver</td>
<td>fp</td>
<td>0.238</td>
<td>0.240</td>
<td>0.009</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.210</td>
<td>0.180</td>
<td>0.010</td>
<td>0.007</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>1.974</td>
<td>1.770</td>
<td>0.143</td>
<td>0.17</td>
</tr>
<tr>
<td>Lung</td>
<td>fp</td>
<td>0.668</td>
<td>0.635</td>
<td>0.035</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.040</td>
<td>0.066</td>
<td>0.007</td>
<td>0.003</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>1.130</td>
<td>1.075</td>
<td>0.088</td>
<td>0.57</td>
</tr>
<tr>
<td>Midcolon</td>
<td>fp</td>
<td>0.458</td>
<td>0.436</td>
<td>0.017</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.051</td>
<td>0.064</td>
<td>0.004</td>
<td>0.011</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>1.285</td>
<td>1.354</td>
<td>0.094</td>
<td>0.47</td>
</tr>
<tr>
<td>Muscle</td>
<td>fp</td>
<td>0.650</td>
<td>0.640</td>
<td>0.015</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.020</td>
<td>0.020</td>
<td>0.003</td>
<td>0.98</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>0.186</td>
<td>0.171</td>
<td>0.014</td>
<td>0.51</td>
</tr>
<tr>
<td>Pancreas</td>
<td>fp</td>
<td>0.796</td>
<td>0.788</td>
<td>0.023</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.084</td>
<td>0.092</td>
<td>0.007</td>
<td>0.29</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>20.34</td>
<td>18.72</td>
<td>4.72</td>
<td>0.74</td>
</tr>
<tr>
<td>Small intestine</td>
<td>fp</td>
<td>0.374</td>
<td>0.375</td>
<td>0.019</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.032</td>
<td>0.037</td>
<td>0.003</td>
<td>0.12</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>3.233</td>
<td>3.113</td>
<td>0.126</td>
<td>0.36</td>
</tr>
<tr>
<td>Skin</td>
<td>fp</td>
<td>0.452</td>
<td>0.407</td>
<td>0.030</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.043</td>
<td>0.059</td>
<td>0.005</td>
<td>0.007</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>0.368</td>
<td>0.376</td>
<td>0.067</td>
<td>0.81</td>
</tr>
<tr>
<td>Spleen</td>
<td>fp</td>
<td>0.408</td>
<td>0.406</td>
<td>0.023</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>fn</td>
<td>0.048</td>
<td>0.057</td>
<td>0.008</td>
<td>0.099</td>
</tr>
<tr>
<td>Flux†</td>
<td></td>
<td>1.135</td>
<td>1.170</td>
<td>0.069</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Units for flux are μmol·h⁻¹·g⁻¹. fp Fraction of intracellular methionine derived from plasma inflow; fn fraction of intracellular methionine derived from from methylation of homocysteine. Results are means of 8 rats/dietary group, except for skin [n = 6 for +FC group for methylation of homocysteine (MH)]. For each tissue, the origins of intracellular methylome fp and fh are defined as fp = Min/Mflux, and fn = MH/Mflux, where Flux = Mflux, where Min is methionine inward transport in tissue; MH, methionine flux through tissue, and MH is MH in tissue. Full details on Min, MH, and Mflux, are given in MATERIALS AND METHODS. The third heart of intracellular methe- nione is protein breakdown (PB), and this was assumed to be similar to protein synthesis. The contribution of PB to flow through the intracellular methylome pool is 1 - (fp + fn), and this was not significantly different (P > 0.10) between the dietary treatments for any tissue. For each tissue, data were analyzed by two-sample t-test. Flux was not determined for brain. *Flux (μmol·g wet tissue⁻¹·h⁻¹) through the tissue intracellular methylome pool was calculated assuming that PB is equal to the rate of protein synthesis (μmol·h⁻¹·g tissue⁻¹), calculated as described in the APPENDIX), which was then divided by 1 - (fp + fn).

Discussion

Despite the accepted importance of the methionine cycle for the transfer of methyl groups for a variety of key metabolic reactions (21, 39) and the relationship between plasma homocysteine, a cycle intermediate, and aspects of cardiovascular risk (2, 6, 13), relatively few data are available on the dynamic relationships between tissues in both the production and utilization of methionine cycle metabolites. The current study used isotopomer analysis of labeled forms of both methionine and homocysteine to unravel some of the interactions between tissues and how these are altered by either excess or deficient methyl group provision.

Source of Plasma Homocysteine

The liver is considered a major site of methionine cycle activity, based on the high activities of both MS and BHMT in the liver (12, 21, 41). Indeed, this is confirmed by the current dynamic data where the fraction of intracellular methionine flux derived from methylation of homocysteine was much greater for liver than any other tissue. The liver has also been assumed to play a major role in regulation of plasma homocysteinemia (25, 35). Support for this concept arises from observations that release of homocysteine from hepatocytes in vitro is sensitive to methylation demand, including production of serine, creatine, guanidinoacetate, and phosphatidylethanol (26, 35, 34, 40). Indeed, the rates observed in vitro (1.29–2.17 mmol·mg dry wt⁻¹·h⁻¹) (34), if extrapolated to the liver in vivo (4% body weight) (29), would more than accommodate (2.0–3.3 μmol/h) the plasma homocysteine flux of 1.3 μmol/h found for rats fed the complete diets in the current study.

Nonetheless, other data within the present study do not support a dominant role for the liver as the source of plasma homocysteine. First, much of the homocysteine generated within the liver appears to be methylated to reform methionine. Second, the enrichment of homocysteine derived from demethyla- tion of methionine within the liver was less than that observed in plasma. Indeed, this difference would be amplified further if allowance had been made for the blood present in the liver sample. Therefore, other tissues with intracellular homocysteine enrichments greater than in plasma, namely pancreas, small intestine, and spleen, probably also contribute to the homocysteinemia. The highest intracellular enrichment for homocysteine was observed for pancreas, a tissue that requires methyl groups for both the packaging and release of digestive enzymes, such as amylase (3), and to support the considerable glycine methyltransferase activity for synthesis of sarcosine (31). The demand of these various mechanisms in the pancreas may account for both the large intracellular folate stores, second only to those in liver (4), and the high rate of methio-

Table 4. Experiment 2: fraction of intracellular homocysteine derived from plasma inflow for rats fed either +FC or −FC diet

<table>
<thead>
<tr>
<th>Tissue</th>
<th>+FC</th>
<th>−FC</th>
<th>SED</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.050</td>
<td>0.091</td>
<td>0.022</td>
<td>0.065</td>
</tr>
<tr>
<td>Heart</td>
<td>0.581</td>
<td>0.776</td>
<td>0.037</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.190</td>
<td>0.283</td>
<td>0.029</td>
<td>0.007</td>
</tr>
<tr>
<td>Liver</td>
<td>0.050</td>
<td>0.086</td>
<td>0.096</td>
<td>0.002</td>
</tr>
<tr>
<td>Lung</td>
<td>0.144</td>
<td>0.297</td>
<td>0.053</td>
<td>0.012</td>
</tr>
<tr>
<td>Mid-colon</td>
<td>0.087</td>
<td>0.174</td>
<td>0.017</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.172</td>
<td>0.295</td>
<td>0.020</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.122</td>
<td>0.227</td>
<td>0.019</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.328</td>
<td>0.430</td>
<td>0.048</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Skin</td>
<td>0.998</td>
<td>0.795</td>
<td>0.097</td>
<td>0.054</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.154</td>
<td>0.203</td>
<td>0.041</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Results are means of 8 rats/dietary group. The fraction of intracellular homocysteine produced from demethylation of methionine is obtained by 1 – fractional plasma inflow. For each tissue, data were analyzed by 2-sample t-test.
nine cycle activity observed in the current study. The pancreas, along with liver, was also sensitive to folate and choline deficiency with less of intracellular homocysteine methylated, and this may then be exported and contribute to the elevated plasma homocysteinemia.

 Regulation of Homocysteinemia

Plasma homocysteinemia represents the balance between tissue export and import, and these two processes may respond differently between individual organs to challenges (7), including the folate and choline deficiency induced in the present study. In these young female rats, plasma homocysteine enrichment achieved plateau within 3 h of a primed infusion, compatible with rate constants of 1.5–1.7/h, a half-life of <30 min estimated from the observed ILR, and an assumed homocysteine content in total body water based on plasma concentration. A similar rapid equilibration (3 h) was observed in some human studies (38) but not others (19).

In response to the deficiency in folate and choline, plasma homocysteine concentrations increased by two- to fivefold, whereas ILR doubled. The latter represents a greater flow through the plasma of homocysteine from tissues, and this greater export may or may not be linked to additional intracellular homocysteine synthesis. The experimental design did not allow intracellular demethylation to be determined directly, although the insensitivity of tissue methionine fluxes to folate and choline deficiency might suggest that any effect on homocysteine synthesis was limited. A more probable cause of the increased ILR was a change in the proportion of homocysteine exported from cells. Indeed, only a small fraction of homocysteine produced within cells is normally exported to plasma. For example, based on the assumption that most of the dietary methionine intake (50 mg/day) was catabolized via the trans-sulfuration pathway, with homocysteine as an intermediate, this would only represent <12% of the plasma ILR of homocysteine. Therefore, under control dietary conditions, the majority of homocysteine in the cell is either methylated to methionine or converted to cystathionine. When one of these pathways is inhibited, as when dietary folate and choline are deficient, then a greater fraction of intracellular homocysteine may be released into the blood.

Hyperhomocysteinemia would also occur if there was a reduced removal from the plasma. This is not the reason in the current study, however, because, under conditions of isotope equilibrium, entry and exit through a pool will be equal, so that the higher plasma ILR observed also reflects increased removal. This is supported by the observation that, for many tissues, the proportion of intracellular homocysteine derived from plasma import increased with diets devoid of folate and choline. For tissues such as liver, pancreas, heart, and muscle, where uptake from plasma increased but methylation to methionine decreased and then increased, flow through the trans-sulfuration pathway must have occurred, although this was insufficient to restore plasma homocysteinemia to control values.

As might be expected, tissues with high methionine cycle activity, such as liver and pancreas, showed a relatively low contribution of inward transport (7 and 17%, respectively) to intracellular homocysteine. In contrast, for skin, almost all intracellular homocysteine was derived from inward transport; therefore, little of the cysteine needed to support hair growth came from intracellular methionine (42). Interestingly, >50% of homocysteine flux within heart tissue was through entry from the plasma, and this may link to the impaired endothelium-dependent vasodilation (10) observed in other tissues of the cardiovascular system. In addition, the fractional inflow from plasma into kidney increased (from 0.19 to 0.28) with folate and choline deficiency, compatible with the role this organ plays in terms of net removal of homocysteine in vivo (5, 15) accompanied by conversion to cystathionine rather than methionine (14).

Impact of Dietary Methionine and Synthesis De Novo

The respective rates of demethylation of methionine and methylation of homocysteine reflect the physiological and metabolic demand for methyl groups and the adequacy, or otherwise, of required cofactors. The current data confirm earlier observations (11, 31) that dietary methionine provides a source of methyl groups that can reduce the need for C-1 transfers via folate and betaine to methylate homocysteine. This effect was particularly noticeable for heart, pancreas, small intestine, and spleen but whether this is due to a greater sensitivity to methionine supply per se or the interaction with either folate and/or choline supply is unclear. Furthermore, in the present study, no attempt was made to resolve the separate impacts of folate and choline, but previous observations (32) have shown that deficiency of the former leads to greater export of homocysteine to the plasma but with little effect on the synthesis of methionine, whereas diets devoid of choline have a smaller impact on homocysteine export but inhibit synthesis of methionine. Originally, it was proposed that methionine synthesized within the liver was exported but retained when produced in other tissues (32), but the current data indicate that the reverse is true, with little of the synthesized methionine that appears in plasma actually of hepatic origin. This is compatible with data from other species where methionine transfers across the liver are dominated by inward, rather than outward, transport (17). Rather, in the current study, at least 25–50% of synthesized methionine that appeared in the plasma originated from the pancreas.

The mass isotopomer analysis of both synthesized methionine and homocysteine that appears in plasma has implications for isotope kinetic studies. Both have been used as surrogate measures of intracellular methionine enrichments (18, 19, 37, 38). The current findings indicate that tissues such as liver and pancreas may make a disproportionately large contribution to plasma enrichments of homocysteine compared with other tissues, whereas plasma-based estimates of methionine derived from methylation of homocysteine will underestimate the impact of hepatic metabolism on whole body kinetics. This potential problem is further exacerbated when the differential effects on tissue metabolism of dietary excess and deficiency as observed in the current study are also considered.

Conclusions

The isotopomer approach adopted in the current study confirms the dynamic nature of the methionine cycle in vivo, particularly in the liver. Nonetheless, hepatic sources are not the dominant origin of either homocysteine or synthesized methionine present in the plasma. Other tissues, including the pancreas, make important contributions in young female rats.
Only a small fraction of homocysteine produced within cells is exported to the plasma, and increased plasma homocysteinemia, as induced by dietary folate and choline deficiency, probably reflects increased efflux from tissues rather than increased synthesis. Although removal of homocysteine from the plasma is also increased at the same time, this is insufficient to maintain concentrations at control values. A considerable proportion of intracellular homocysteine is imported from plasma in tissues such as kidney, skin, and heart, and this may reflect different metabolic outcomes, including production of cystathionine and regulation of cardiovascular function. Tissues also varied in the responses to reduced folate and choline provision, and this may have implication for both whole body regulation of methyl group supply and metabolism within specific organs. Such metabolic responses may be magnified under conditions of increased demand for methyl group transfer, such as occurs in pregnancy and where improved C-1 metabolism is widely believed to underlie the reduction in the occurrence of neural tube defects following folate supplementation of foods (16).

APPENDIX

Synthesis of Homocysteine

The L-[1-13C]homocysteine was prepared from L-[1-13C]methionine via (4S)-1,3-thiazane-2,4-dicarboxylic acid hydrochloride [(4S)-TDC·HCl] in a modified procedure (33). Briefly, L-[1-13C]methionine (1.25 mmol) was dissolved in concentrated HCl (2.5 ml) at room temperature. Dichloroacetic acid (5 mmol) was added, and the mixture was heated under reflux for 12 h at 150°C. The HCl was removed in vacuo at 60°C, and the residue was suspended in ice-cold absolute ethanol. After standing overnight at 4°C, precipitated (4S)-TDC·HCl (yield ~60%, >97% diastereomeric excess) was filtered and washed with absolute ethanol and tetrahydrofuran. To a suspension of (4S)-TDC·HCl (0.034 g) in absolute ethanol (3.7 ml, TDC·HCl-to-ethanol ratio 1:25) containing the pH indicator bromothymol blue, triethylamine was then added dropwise until solution pH rose to ~7 and the (4S)-TDC·HCl was completely dissolved. The solution was heated under reflux at 120°C. Once refluxing was established, 0.3 ml of 0.15 mM/l ethanolic hydroxylamine hydrochloride was added as five aliquots of 0.06 cm³, at intervals of 10 min. Immediately after each addition, the solution was maintained at pH 7–8 with triethylamine. Refluxing was maintained for 1 h after the final addition. The precipitated L-[1-13C]homocysteine (yield ~80%, >99% isomeric excess) was collected by filtration, washed thoroughly with ethanol, checked for absence of triethylamine, and air-dried before storage at –40°C. Mass spectrometry analysis by the chiral column indicated that the product contained only the L-isomer while melting point and nuclear magnetic resonance spectra proved the purity of the product.

Analysis of Plasma and Tissues Concentrations

To a weighed volume (100 μl) of plasma or tissue supernatant was added a weighed 100 μl of internal standard, containing 5 nmol of S-[methyl-2H]methionine, 4 nmol [1,1,13C]cystine, and 3 nmol [1-13C]homocysteine for plasma and 5 nmol of S-[methyl-1-13C]2H₃methionine, 4 nmol [1,1,13C]cystine, and 0.3 nmol [1-13C]homocysteine for tissue supernatant. These samples were then processed according to the methods detailed below for experiments 1 and 2 and analyzed as described previously (8). Plasma folate, phosphocholine, choline, and triglyceride concentrations were analyzed as previously described (20, 23).

Analysis of Plasma and Tissue Enrichments by GC-MS

All reagents were obtained from Sigma Aldrich (Poole Dorset, UK), Fisher Scientific (Loughborough, Leicestershire, UK), or VWR International (Lutterworth, Leicestershire, UK).

For experiment 1, tissues (0.5 g) were homogenized by Ultra Turrax (IKA-Werke, Staufen, Germany) in 1.5 ml of ice-cold water containing 10 mM dithiothreitol (DTT). Samples were left on ice for 20 min and then 0.5 ml of 35% (wt/vol) 5-sulfosalicylic acid dihydrate (SSA) was added and mixed, and samples were centrifuged for 15 min at 700 g at 4°C. The supernatants were then centrifuged for a further 5 min at 9,500 g until clear. Supernatants were stored at ~80°C until derivatization and analysis.

Plasma samples (100 μl) were thawed on ice, and 50 μl of 100 mM DTT were added, vortex mixed, and incubated at room temperature for 20 min. This was to release cysteine and homocysteine from protein and dinucleotide complexes. Samples were deproteinized by addition of 70 μl 35% (wt/vol) SSA, vortex mixed, and centrifuged at 9,500 g for 5 min at room temperature.

Approximately 100 μl of the supernatant from a plasma or tissue sample were applied to 13 mm of Dowex 50W×8 resin (50–100 mesh, H⁺ form) in a 1 ml disposable pipette tip packed with 2 mm cotton wool. The resin was then washed with 2 × 1.2 ml water, and the sample was eluted with 0.5 ml of 2 M NH₄OH. This eluate was then dried under a gentle stream of nitrogen at 85°C, dissolved in 10 μl 100 mM N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide-acetonitrile (1:1 vol/vol) at 85°C for 20 min (12). Samples were analyzed by GC-MS under electron impact selective ion monitoring conditions with separation on a 60 m × 0.25 mm x 0.25 μm EIC capillary column (Alltech, Carrnforth, Lancashire, UK). The M-57 fragment ions were monitored at mass-to-charge ratio (m/z) 320.2, 321.2, and 324.2 for methionine, 420.2 and 421.2 for homocysteine, and 406.3 and 407.3, respectively.

The samples were derivatized according to procedures described for experiment 2. For preparation of tissues, the low amounts of homocysteine present necessitated techniques that improved GC-MS sensitivity and to prevent enzyme reactions occurring during the preparation stage. The procedures were similar to experiment 1 except that the homogenized tissue samples (0.5 g) were left on ice for 20 min before addition of the 0.5 ml 35% (wt/vol) SSA. The pellet was retained for protein-bound enrichment, whereas the supernatant was put through a desalting procedure where 4 mm of AG 50W-X8 resin (mesh size 100–200, H⁺ form) was placed in the modified pipette tip, and this was washed with 2 × 1 ml water, and the sample was eluted with 0.4 ml of 2 M NH₄OH into a v-vial containing 10 μl 100 mM DTT and derivatized immediately (see below). The protein pellet was thoroughly washed four times with 7% SSA (3 ml on each occasion), and then the equivalent of ~20 mg of protein were hydrolyzed in sealed tubes in 6 M hydrochloric acid at 105°C for 18 h in the presence of 5 mM DTT. The protein-bound methionine enrichment was then determined as the (BDMS) derivative. For the preparation of plasma, to 100 μl of plasma, 20 μl of 100 mM DTT was added, mixed, and incubated at 4°C for 20 min. Samples were deproteinized with 100 μl 15% (vol/vol) SSA and centrifuged at 9,500 g for 5 min. The supernatant was then desalted and treated as detailed for tissues.

Both the plasma and tissue sample extracts in the alkaline DTT eluate were incubated for 30 min at room temperature and then dried under nitrogen at 90°C. Once dry, 80 μl of a 10:1 mix (vol/vol) of n-butanol and acetyl chloride were added, and samples were heated at 90°C for 20 min followed by drying under nitrogen at 45°C. Next, an appropriate volume, usually between 50 and 100 μl of 5% (vol/vol) N-heptamfluorobutyrimidazole in ethyl acetate,
was added, and samples were left at room temperature for 15 min to form the N-butyl-heptafluorobutyrate derivative. This was necessary to enhance the peak areas for the small amount of homocysteine present in the tissue extracts but sufficiently mild to avoid chemical demethylation of methionine to homocysteine that was found to occur when the more common acylation reagent heptafloorobutyril anhydride was used, which yielded relatively large areas for homocysteine and at enrichments similar to the tissue methionine. Failure to recognize this problem during the pilot study meant that higher infusion rates of [1-13C]homocysteine were employed during experiment 2 than were necessary. Samples were then analyzed by GC-MS under negative chemical ionization-selective ion monitoring conditions with separation on a 30 m × 0.25 mm × 0.25 μm ZB5-MS capillary column (Phenomenex, Macclesfield, Cheshire, UK). Fragment ions were measured at m/z 381, 382, 385, and 386 for methionine, 563, 564, and 567 for homocysteine (for concentration and enrichment), and 549 and 550 for cysteine (concentration only). For the model described below, it is essential that neither the dissection procedures nor the subsequent analytical techniques altered metabolite concentrations or enrichments. Therefore, for both experiments 1 and 2, appropriate tests were performed with fresh liver homogenates, including addition of various isotope mixtures, to ensure that the collection and processing procedures did not alter either the concentrations or isotopomer ratios of the various metabolites.

Mathematical Models of Whole Body and Tissue Metabolism for Methionine and Homocysteine

For the mathematical models, all enrichments are expressed as tracer-to-tracee ratios (TTR), where tracee includes naturally occurring 13C and naturally occurring 13C species. All flows and infusion rates are expressed in micromoles per hour. Rates of infusion of labeled methionine and homocysteine are designated as IM and IH, respectively. Methionine and homocysteine enrichments are denoted by EM and EH, respectively. Subscripts “p” and “t” refer to plasma and tissue, respectively. Subscripts 1, 4, and 5 refer to m + 1, m + 4, and m + 5 molecules, respectively. The models are described first for the more complex isotope procedures used in experiment 2 (infusion of both labeled methionine and homocysteine), and then the simplified versions used for experiment 1 are detailed. The approaches adopted include allowance for the presence of labeled molecules in the infusates that are also formed by metabolism through the methionine cycle, e.g., the bis-[1-13C,methyl-2H3]methionine infused contained 5.3% [1-13C]methionine, the product of methylation of homocysteine for the TTR of intracellular methionine (38), analogous to the use of keto-isocaprate for leucine kinetics (22).

For whole body calculations, the various irreversible loss rates (ILR) were calculated as

\[ \text{ILR} = \text{tracer infusion rate}/E_i \]

where \( E_i \) is the TTR of the appropriate plasma metabolite (methionine or homocysteine). Minimum estimates of ILR were obtained when \( E_i \) was in the same label form as the infusate (e.g., \( m + 5 \) methionine or \( m + 1 \) homocysteine in experiment 2 and \( m + 4 \) methionine in experiment 1). Alternatively, labeled homocysteine generated within tissues and exported to plasma (\( m + 1 \) for experiment 1 and \( m + 4 \) for experiment 2) were used as surrogates for the TTR of intracellular methionine (38), analogous to the use of keto-isocaprate for leucine kinetics (22).

Let PS, PB, DM, OX, and ABS denote, at the whole body level, protein synthesis, protein breakdown, methionine demethylation, oxidation and absorption, respectively, while MH represents methylation of homocysteine. Then the inflows and outflows for tracee methionine are given by

\[ \text{PB} + \text{MH} + \text{ABS} = \text{PS} + \text{DM} \]

and the inflow and outflows for tracee homocysteine correspond to

\[ \text{DM} = \text{MH} + \text{OX} \]

then, for experiment 2:

\[ IM_5 = EM_{5p} (PS + DM) \]  

This solves for PS + DM. In addition:

\[ EH_{1i} \text{MH} = EM_{i} (PS + DM) \]

which solves for MH. Finally,

\[ IH_t + EM_{i} DM = EH_{1i} (MH + OX) \]

Assuming that MH + OX = DM, this simplifies to

\[ IH_t = (EH_{1i} - EM_{i}) DM \]

providing an estimate for DM.

For experiment 1, Eqs. 1 and 2 were replaced with

\[ IM_4 = EM_{4p} (PS + DM) \]

\[ IM_4 + EH_{1i} MH = EM_{i} (PS + DM) \]

For experiment 1, it was not possible to estimate DM.

For tissue calculations, the model shown in Fig. 2 was used. For intracellular methionine kinetics, the relative inflows from plasma methionine, intracellular homocysteine, and protein breakdown (enrichment assumed to be 0) can be determined by isotope dilutions. If the absolute rate of any of the three inflows is known, then rates can be calculated for the other two. For this model, it was assumed that protein degradation was the same as protein synthesis that was measured directly and expressed as micromoles per hour per gram, and this allowed inflows from plasma methionine and homocysteine to be estimated as the same units. Under steady-state conditions, the following equalities hold:

\[ M_{flux_t} = Min_t + MH_t + PB_t = Mout_t + DM_t + PS_t \]

\[ H_{flux_t} = Hin_t + DM_t = Hout_t + MH_t + OX_t \]

where \( M_{flux} \) and \( H_{flux} \) are the methionine and homocysteine fluxes through tissue t, \( Min_t \) and \( Hin_t \) are methionine and homocysteine inward transport into tissue t, and \( Mout_t \) and \( Hout_t \) are methionine and homocysteine outward transport from tissue t.

For experiment 2, flows of \( m + 5 \) methionine through tissue t are given by

\[ EM_{5p} Min_t = EM_{5t} M_{flux_t} \]

This gives \( Min/M_{flux} \). From the labeled methionine derived from the infusion of labeled homocysteine (experiment 2), it follows that

\[ EM_{1i} Min_t + EH_{1i} MH_t = EM_{1t} M_{flux_t} \]

This then gives \( RM/M_{flux} \) and \( PB/M_{flux} = 1 - Min/M_{flux} - MH/M_{flux} \). Based on protein synthesis measurements, and assuming that \( PS = PB, M_{flux} \), and \( MH \) can then be calculated.

PS was obtained as follows. First, the fractional rate of tissue protein synthesis (FSR; %/day) was calculated in experiment 2 from

\[ \text{FSR} = \text{TTR}_{prot}/\text{TTR}_{mt} \times 1/\text{day} \times 100\% \]

where “prot” refers to protein-bound and “mt” to tissue free pool \( m + 5 \) methionine TTR, and day is time of infusion in days. Second, these values were converted to micromoles per hour per gram tissue methionine used for protein synthesis by
EM1p, Mint, + EM1p, DM1 = EH1p, Hflux1,
which gives DM1/Hflux1 and Hint/Hflux1.
For experiment 1, Eqs. 3 and 4 are replaced with
EM1p, Min, = EM1p, Mflux1
EM1p, Min, + EH1p, MF1 = EM1p, Mflux1
There is no equivalent to Eq. 5 for experiment 1, i.e., DM/Hflux and Hint/Hflux cannot be calculated.

ACKNOWLEDGMENTS

The expertise of the staff of the Biosources Reseach Unit for care and attention of the animals is gratefully acknowledged.
Current address for F. A. Wilson: Dept. of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, PA.

GRANTS

J. G. van den Borne was supported as part of a Marie Curie Training Site grant (MScSchool) from the European Union to The Rowett Institute of Nutrition and Health. This work was funded as part of the core budget to The Rowett Institute of Nutrition and Health and to Biomathematics and Statistics Scotland by the Rural and Environment Research and Analysis Directorate of the Scottish Government.

REFERENCES


