Effects of methyl-deficient diets on methionine and homocysteine metabolism in the pregnant rat

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Wilson FA, Holtrop G, Calder AG, Anderson SE, Lobley GE, Rees WD. Effects of methyl-deficient diets on methionine and homocysteine metabolism in the pregnant rat. Am J Physiol Endocrinol Metab 302; E1531–E1540, 2012. First published March 27, 2012; doi:10.1152/ajpendo.00668.2011.—Although the importance of methyl metabolism in fetal development is well recognized, there is limited information on the dynamics of methionine flow through maternal and fetal tissues and on how this is related to circulating total homocysteine concentrations. Rates of homocysteine remethylation in maternal and fetal tissues on days 11, 19, and 21 of gestation were measured in pregnant rats fed diets with limiting or surplus amounts of folic acid and choline at two levels of methionine and then infused with L-[1-13C,5-2H3]-methionine. The rate of homocysteine remethylation was highest in maternal liver and declined as gestation progressed. Diets deficient in folic acid and choline reduced the production of methionine from homocysteine in maternal liver only in the animals fed a methionine-limited diet. Throughout gestation, the pancreas exported homocysteine for methylation within other tissues. Little or no methionine cycle activity was detected in the placenta at days 19 and 21 of gestation, but, during this period, fetal tissues, especially the liver, synthesized methionine from homocysteine. Greater enrichment of homocysteine in maternal plasma than placenta, even in animals fed the most-deficient diets, shows that the placenta did not contribute homocysteine to maternal plasma. Methionine synthesis from homocysteine in fetal tissues was maintained or increased when the dams were fed folate- and choline-deficient methionine-restricted diets. This study shows that methyl-deficient diets decrease the remethylation of homocysteine within maternal tissues but that these rates are protected to some extent within fetal tissues.

folate; choline; fetal development; stable isotopes

Numerous large-scale clinical trials have clearly established the value of folic acid supplements in pregnancy (23). In addition to a marked reduction in the frequency of neural tube defects and other congenital malformations of the fetus (7, 13), improved folate status is also associated with enhanced fetal growth (17). Elevated plasma total (t-) homocysteine concentration, a marker of changes in methyl metabolism, is also recognized as a risk factor for a number of adverse pregnancy outcomes in humans (11, 24, 27). It has been suggested that folate supplements improve fetal development by increasing the recycling of homocysteine to methionine through the methionine cycle (Fig. 1) (1, 2, 8).

The relationship between the availability of folic acid and associated methyl donors in the maternal diet and the flow through the methionine cycle in the tissues of the mother and the developing fetus is poorly quantified. Studies of pregnant rats show that methyl-deficient diets low in folic acid, choline, and methionine increase t-homocysteine concentrations in maternal and fetal plasma (9). While the enzymes of the methionine cycle are present in maternal and fetal tissues of the rat (25), there is limited information on the dynamics of methionine flow through these tissues and on how this is related to circulating homocysteine concentrations.

The hypothesis tested in the current study was that limitations in methionine and folate plus choline supply would impact methyl group transfers in the mother and fetus. We have sought to monitor methionine cycle activity in maternal and fetal tissues of the pregnant rat by infusing L-[1-13C,5-2H3]-methionine (21, 22). This tracer was infused into rats offered diets differing in folate, choline, and methionine content at various stages of pregnancy (days 11, 19, and 21 of gestation). In our previous study of the virgin female rat (28), we used a similar approach to show that the liver and pancreas were key tissues in the conversion of homocysteine to methionine and that these tissues differ in the metabolic response to deficiencies in methyl group supply.

Methods

Animals and diets

Diets. Experimental diets for a 2 × 2 factorial design were prepared as described previously (9, 28). A diet containing 90 g of casein per kilogram of diet was supplemented with a mixture of synthetic amino acids (Spodefell, London, UK) equivalent to an additional 90 g of casein. Methionine was omitted or included in the amino acid mixture to produce low-methionine (−M, 2.3 mg/kg) or high-methionine (+M, 5.6 mg/kg) diets. The adequate-folate and -choline (+FC) diets contained 2 mg of folic acid + 2 g of choline chloride per kilogram of diet, whereas the folate- and choline-deficient (−FC) diets contained no additional folic acid and only 1 g of choline chloride per kilogram of diet. The methionine, folic acid, and choline content of the +M+FC diet was equivalent to that of the AIN-76 rodent formula (16). All experimental procedures were approved and conducted in accordance with the UK Animal Scientific Procedures Act (1986).

Experiment 1 (day 19). Forty female rats of the Rowett Hooded strain bred in the Rowett Institute were randomized into four groups at 7.5 wk of age (190 g body wt), group-housed, and fed one of four experimental diets (+M+FC, +M−FC, −M+FC, or −M−FC) ad libitum for a 2-wk adjustment period prior to mating. Females were synchronized by addition of 0.02% progesterone (wt/vol) to the drinking water in the final week before mating and then by administration of serum gonadotropin 1 day before mating with normal males. Vaginal plug detection was denoted day 0. The female rats were maintained on their appropriate diets until the infusion on day 19 of gestation. Because of the stringent requirements of the timed mating, <60% of these animals were pregnant at day 19, so an additional
batch of animals \((n = 17)\) was acclimated to the diets and mated 1 mo later. In total, 36 pregnant animals were infused on day 19 \((n = 9\) \((+M + FC)\), \(n = 11\) \((-M + F C)\), \(n = 8\) \((-M + FC)\), and \(n = 8\) \((-M - FC)\). Eight animals were selected randomly from each group to provide a balanced design for the enrichment analysis.

**Experiment 2 (days 11 and 21).** Forty female Hooded-Lister rats were separated into two groups and fed the \(-M + FC\) or \(-M - FC\) diet. After they were mated, the animals were infused on day 11 or 21 of gestation (20 animals per diet per time point studied in 2 batches of 10 animals per diet per time point 1 mo apart). In total, 12 pregnant animals were analyzed on day 11 and 16 pregnant animals were analyzed on day 21. All other aspects of the protocol were similar to those described for experiment 1.

**Infusion.** The infusion protocol is described elsewhere (28). Briefly, animals were allowed access to the diet until the start of each infusion \((0800–0900)\). Each rat was trained to a commercial restraint tube (Harvard Apparatus, Kent, UK) and infused via a tail vein with L-\([1-13C, 5\text{-methyl-2H}_3\]methionine, L-\([1-13C\]cysteine, and L-\([1-13C\]homocysteine, total cysteine, and total homocysteine concentrations and the determination of enrichments in plasma and tissue homogenates are described elsewhere (28). Briefly, plasma concentrations were determined after addition of an internal standard solution (containing 1-\([1-13C,5\text{-methyl-2H}_3\]methionine, 1-\([1-13C\]cysteine, and 1-\([1-13C\]homocysteine) to a separate aliquot of plasma. Tissue and plasma samples were homogenized in ice-cold water containing 10 mM DTT and deproteinized by addition of sulfosalicylic acid. After centrifugation, the supernatant was applied to a Dowex 50W-X8 resin (mesh size 50–100, \(H^+\) form) column, washed with water, and eluted with 2 M \(NH_2\)OH. Because this approach releases any cysteine and homocysteine complexed with plasma proteins, all subsequent measurements, for enrichments and concentrations, are made on t-cysteine and t-homocysteine.

Deproteinized plasma and tissue samples were dissolved in 10:1 \(n\)-butanol-acetyl chloride, heated to 90°C for 20 min, and dried. A 5% solution of \(N\)-heptafluorobutyrylimidazole in ethyl acetate was added, and samples were left at room temperature for 15 min to form the \(N\)-butyl-heptafluorobutyryl derivative. Samples were then analyzed by gas chromatography-mass spectrometry under negative chemical ionization selective ion monitoring conditions with separation on a 30 \(m\) \(\times\) 0.25 mm \(\times\) 0.25 \(\mu\)m capillary column (model ZB5-MS, Phenomenex, Macclesfield, Cheshire, UK). Fragment ions were measured at mass-to-charge ratios of 381, 382, 385, and 386 for methionine; 563, 564, and 567 for t-homocysteine (for concentration and enrichment); and 549 and 550 for t-cysteine (concentration only).

**Statistics**

**Experiment 1 (day 19 of gestation) consisted of four groups of animals; each group received one of four dietary treatments, with several tissues analyzed per animal. Isotope enrichments in tissues were analyzed by ANOVA, with batch plus methionine status \((+M\) or \(-M\)), folate-choline status \((+FC\) or \(-FC\)), tissue (plasma, liver, pancreas, placenta, and whole fetus and fetal liver), and their various interactions as fixed effects and animal as random effect.**

**Experiment 2 consisted of four groups of animals; each group was assigned to one of two days of gestation (day 11 or 21) and one of two dietary treatments \((-M + FC\) or \(-M - FC\)). Several tissues were analyzed per animal. Isotope enrichments in tissues were analyzed by ANOVA, with batch plus folate-choline status, day of gestation, tissue, and their various interactions as fixed effects and animal as random effect.**

For experiments 1 and 2, batch was initially included as a fixed effect, but this was found to be nonsignificant \((P > 0.10)\) and, therefore, was removed from subsequent analyses. Furthermore, for experiments 1 and 2, significant interactions between tissue and dietary treatment were observed, and data were then analyzed by two-way ANOVA for each tissue separately. Where the main effects or interactions were significant, treatment means were compared by post hoc t-test. Standard error of the differences (SED), presented in Tables 1–6, is “worst case” SED, i.e., that based on comparisons from groups with the fewest animals.

All statistical analyses were performed in Genstat 13th edition (release 13.2, VSN International, Hemel Hempstead, UK). \(P < 0.05\) was regarded significant, \(P = 0.05–0.10\) is referred to as tendency, and \(P > 0.10\) (nonsignificant) is not reported in detail.

**Kinetic model.** Tracer L-\([1-13C,5\text{-methyl-2H}_3\]methionine infused into the blood is 4 atomic mass units heavier than normal (hereafter designated m+4 methionine). This tracer is extracted by tissues and used for protein synthesis or transmethylated and converted to L-\([1-13C\]homocysteine (1 atomic mass unit heavier and designated m+1 homocysteine). Subsequent transfer of a methyl group from 5-methyltetrahydrofolate or betaine to L-\([1-13C\]homocysteine completes the methionine cycle and yields L-\([1-13C\]methionine (1 atomic mass unit heavier and designated m+1 methionine). The m+1-to-m+4 methionine isomopser (m+1-to-m+4) ratio in intracellular pools provides a measure of methionine synthesis from homocysteine relative to uptake from the plasma (21). Simply, for a tissue that produces methionine from homocysteine, the m+1-to-m+4 ratio will exceed that in the plasma. In tissues where no methylation of homocysteine occurs, the m+1-to-m+4 ratio will be the same as that in the plasma.

The mathematical model used to estimate the fractional inflows into the methionine pools of individual tissues is reported elsewhere (28). The model calculates the fractions derived from the plasma,

![Fig. 1. The methionine cycle. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DMG, dipalmityl glycerol; BHMT, betaine:homocysteine methyltransferase; MS, methionine synthase; 5,10-THF, 5,10-tetrahydrofolate trans-homocysteine; DMG, dimethylglycine; BHMT, betaine:homocysteine methyltransferase; 5-MTHF, N-5-methyltetrahydrofolate trans-ferase.](http://ajpendo.physiology.org/DownloadedFrom)
Table 1. Amino acid concentrations in maternal plasma and methionine ILR on day 19 of gestation

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Diet</th>
<th>+M [n = 5 (batch 1) + 4 (batch 2)]</th>
<th>−M [n = 9 (batch 1) + 2 (batch 2)]</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine, nmol/g plasma</td>
<td>+M [n = 5 (batch 1) + 4 (batch 2)]</td>
<td>70.2*</td>
<td>72.0*</td>
<td>50.8†</td>
</tr>
<tr>
<td>t-Cysteine, nmol/g plasma</td>
<td>+M [n = 5 (batch 1) + 4 (batch 2)]</td>
<td>194.9†</td>
<td>202.5*</td>
<td>137.6†</td>
</tr>
<tr>
<td>t-Homocysteine, nmol/g plasma</td>
<td>+M [n = 5 (batch 1) + 4 (batch 2)]</td>
<td>3.7†</td>
<td>14.4†</td>
<td>4.5†</td>
</tr>
<tr>
<td>ILR for methionine, mmol/h</td>
<td>+M [n = 5 (batch 1) + 4 (batch 2)]</td>
<td>47.0</td>
<td>48.9</td>
<td>44.3</td>
</tr>
</tbody>
</table>

Values are means and standard error of differences (SED); n, number of samples (1 plasma sample was lost from −M−FC group). SED is based on 2-way ANOVA, with methionine status, folate-choline status, and their interaction as fixed effects. †t-Cysteine and t-homocysteine, total cysteine and homocysteine; M, methionine; F, folate; C, choline; ILR, irreversible loss rate. Different symbols (*, †) within rows indicate significant difference (P < 0.05). NS, not significant (P > 0.10).

Table 2. Choline, phosphocholine, and triglyceride concentrations in maternal liver on day 19 of gestation

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Diet</th>
<th>+M [n = 9]</th>
<th>−M [n = 8]</th>
<th>SED</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride</td>
<td>+M (n = 9)</td>
<td>42.0*</td>
<td>62.2†</td>
<td>45.6†</td>
<td>88.8‡</td>
</tr>
<tr>
<td></td>
<td>−M (n = 11)</td>
<td>4.22</td>
<td>2.86</td>
<td>3.37</td>
<td>2.61</td>
</tr>
<tr>
<td>Phosphocholine</td>
<td>+M (n = 8)</td>
<td>7.04*</td>
<td>2.92†</td>
<td>5.22†</td>
<td>1.19†</td>
</tr>
</tbody>
</table>

Values (means and SED) are nmol/mg protein; n, number of samples. SED is based on 2-way ANOVA, with methionine status, folate-choline status, and their interaction as fixed effects. Different symbols (*, †, ‡) within columns indicate significant difference (P < 0.05).
The m+1-to-m+4 methionine isotopomer ratio was greater in maternal liver and, to a lesser extent, the pancreas than plasma (both P < 0.001), whereas the ratio in the placenta was similar to that in the plasma (Fig. 3). Within the fetal compartment, the m+1-to-m+4 methionine ratio was greater in the whole fetus and fetal liver than placenta (P < 0.05 and P < 0.001, respectively). This indicates synthesis of methionine from homocysteine within the fetus, notably fetal liver.

Effect of maternal diet. The m+1-to-m+4 methionine isotopomer TTR in maternal plasma was unaffected by the methionine or folic acid and choline content of the diet (Table 3). However, the diet did affect the ratios in maternal tissues. In maternal liver, the m+1-to-m+4 methionine isotopomer ratio was reduced (i.e., hepatic homocysteine remethylation was lower) in animals fed the low-methionine diet compared with animals fed the high-methionine diet (+M vs. −M, P = 0.022). The ratio was also lower in the animals fed diets deficient in folic acid plus choline than in those fed the adequate diet (+FC vs. −FC, P = 0.025). In contrast, the ratio in the pancreas was increased in animals fed the low-methionine diet (+M vs. −M, P = 0.033) but was unaffected by folic acid and choline deficiency. The placental m+1-to-m+4 methionine isotopomer ratio was unchanged by the methionine content of the diets but was markedly increased by diets deficient in folic acid and choline (+FC vs. −FC, P < 0.001). The ratio was further increased in response to folic and choline deficiency when the diet was also low in methionine (M × FC interaction term, P < 0.001).

The m+1-to-m+4 methionine ratio measured in the whole fetus was unchanged by the maternal diet. Nevertheless, the ratio in fetal liver was increased in the fetuses of dams fed diets low in folate and choline compared with those fed the adequate diet (+FC vs. −FC, P < 0.001) and further increased when combined with the low-methionine diet (M × FC interaction term, P < 0.001). The ratios in the fetuses were similar to those in the placenta, i.e., an apparent increase in homocysteine methylation in the livers of fetuses from dams fed the diets with the lowest provision of methyl groups.

The enrichment data were used to calculate the relative proportions of the intracellular methionine pool derived from inflow from the plasma, methylation of homocysteine, and, by difference, release from protein breakdown in maternal liver and pancreas (Table 4). In the liver, 20–25% of the intracellular methionine was derived directly from plasma inflow, regardless of the composition of the diet. Methionine derived from homocysteine accounted for ~14% of the total, and this was reduced in rats fed low-methionine (+M vs. −M, P = 0.002) and low-folate/choline (+FC vs. −FC, P = 0.045) diets. There was also a strong interaction (M × FC interaction term, P < 0.001), such that the proportion derived from homocysteine in the group fed the −M − FC diet was approximately half that in the group fed the +M + FC diet. In contrast, >80% of the free methionine within the pancreas was derived from plasma inflow in the high-methionine groups and still accounted for a substantial proportion (50%) in the low-methionine groups (+M vs. −M, P < 0.001). The folic and choline content of the diet had no effect on plasma inflow or the small fraction (5–6%) of intracellular methionine derived from methylation of homocysteine. These results suggest that the proportion of intracellular methionine derived from intracellular protein breakdown was increased (P < 0.001) in the liver and pancreas of animals fed the low-methionine diets.

**Experiment 2**

The second series of experiments was restricted to examining the effects of low-methionine diets adequate (+FC) or deficient (−FC) in folic acid plus choline, because, in experiment 1, the strongest responses to deficiency were observed in the groups fed low-methionine diets.

**Weight gain, plasma concentrations, and ILR.** There was no difference between the diet groups in the daily weight gain or
average litter size at day 11 or 21 of gestation. Plasma methionine concentrations were unchanged by maternal diet at either time point but, compared with the +FC group, plasma t-homocysteine concentrations were increased in the −FC group on day 11 (4.5 ± 0.1 vs. 18.1 ± 1.5 nmol/g, P < 0.001) and day 21 (3.9 ± 0.5 vs. 17.4 ± 2.9 nmol/g, P < 0.001) of gestation. Compared with the +FC group, methionine ILR was decreased in the −FC group on day 11 (38.8 vs. 27.8 mmol/h, SED 4.94, P = 0.049) but was unaffected on day 21 (39.2 vs. 37.4 mmol/h, SED 4.92, P = 0.72).

Table 3. Ratio of m +1 to m +4 methionine isotopomers in plasma and tissues of the pregnant rat on day 19 of gestation

<table>
<thead>
<tr>
<th>Diet</th>
<th>Diet</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>+M</td>
<td>−M</td>
<td>SED</td>
</tr>
<tr>
<td>+FC</td>
<td>−FC</td>
<td>M</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.169</td>
<td>0.149</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>0.564*</td>
<td>0.553*</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.205*</td>
<td>0.216*</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.098*</td>
<td>0.138*</td>
</tr>
<tr>
<td>Whole fetus</td>
<td>0.227*</td>
<td>0.186†</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>0.240*</td>
<td>0.221*</td>
</tr>
</tbody>
</table>

Values are means (n = 8). SED is based on 2-way ANOVA, with methionine status, folate-choline status, and their interaction as fixed effects. Different symbols (*, †, ‡) within rows indicate significant difference (P < 0.05).

Methionine and homocysteine enrichment in maternal and fetal tissues. The TTR of m +4 methionine was lower in all tissues than plasma (P < 0.001; data not shown) on days 11 and 21 of gestation. Combined across both dietary treatments, the TTR of m +1 homocysteine in maternal tissues on day 19 of gestation (Fig. 2) showed similarities to that on day 11 (Fig. 4), and the TTRs for m +1 methionine were lower in maternal liver, pancreas, and placenta than maternal plasma (P < 0.001). In the fetal compartment on day 21 of gestation, the m +1 methionine TTR was greater in fetal liver (P < 0.001) than placenta or whole fetus.

The m +1-to-m +4 methionine isotopomer ratio on day 11 of gestation (Fig. 6A) was greater in maternal liver (P < 0.001) and pancreas (P < 0.05) than plasma. By day 21 of gestation (Fig. 6B), the methionine isotopomer ratio in the pancreas was similar to that in the plasma, while the ratio in the liver was still higher (P < 0.001). On day 21, the m +1-to-m +4 methionine isotopomer ratio in the placenta was also similar to that in the plasma and whole fetus but was much greater in fetal liver (P < 0.001).

Effect of stage of pregnancy and maternal diet. Stage of pregnancy and diet composition influenced the m +1-to-m +4 methionine isotopomer ratio within tissues (Table 5). The ratio in maternal plasma, liver, and pancreas declined between days 11 and 21 (P < 0.001). The methionine isotopomer ratio was

Table 4. Proportion of intracellular methionine in maternal tissues derived from inflows from plasma, protein breakdown, and methylation of homocysteine on day 19 of gestation

<table>
<thead>
<tr>
<th>Diet</th>
<th>Diet</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>+M</td>
<td>−M</td>
<td>SED</td>
</tr>
<tr>
<td>+FC</td>
<td>−FC</td>
<td>M</td>
</tr>
<tr>
<td>Inflow from plasma</td>
<td>0.252</td>
<td>0.237</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>0.816*</td>
<td>0.821*</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.135*</td>
<td>0.141*</td>
</tr>
<tr>
<td>Methylation of homocysteine</td>
<td>0.030</td>
<td>0.057</td>
</tr>
<tr>
<td>Protein breakdown</td>
<td>0.613*</td>
<td>0.625†</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>0.154*</td>
<td>0.122*</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.613*</td>
<td>0.625†</td>
</tr>
</tbody>
</table>

Values are means (n = 8). SED is based on 2-way ANOVA, with methionine status, folate-choline status, and their interaction as fixed effects. Different symbols (*, †, ‡) within rows indicate significant difference (P < 0.05).
lower in maternal livers of the deficient group (+FC vs. −FC, P = 0.004), with greater impact on day 21 than day 11 (FC × day, P = 0.026). The ratio in the placenta on day 21 of gestation was lower in animals fed the diet deficient in folate plus choline (P = 0.048), unlike day 19 of gestation, when the ratio was increased. Maternal diets deficient in folate plus choline did not change the m+1-to-m+4 methionine isotopomer ratio in the whole fetus or fetal liver.

The relative contributions to the intracellular methionine pool (Table 6) from plasma or from protein breakdown in maternal liver and pancreas were unaffected by the stage of pregnancy or the folate and choline content of the diet. In contrast, the proportion of methionine derived from methylation of homocysteine within maternal liver (P < 0.001) and pancreas (P = 0.040) decreased as pregnancy progressed.

**DISCUSSION**

It has long been assumed that diets deficient in folic acid restrict the recycling of homocysteine and that this limits the supply of methyl groups used in a range of reactions essential for maternal metabolism and fetal development (15). This study shows that diets deficient in the key substrates and cofactors of methyl metabolism have very different effects on methionine metabolism in fetal and maternal tissues. The synthesis of methionine from homocysteine in the maternal compartment is centered on maternal liver and is reduced by methyl-deficient diets, but this is counteracted partly by an undefined increase in fetal liver. Despite large changes in plasma t-homocysteine concentrations, methyl-deficient diets produce only modest changes in the methionine isotopomer ratios in maternal plasma. Furthermore, the current results show that the fetus is not a major source of t-homocysteine in maternal plasma, even when the maternal diet is deficient in methyl precursors.

**Methionine Metabolism in Maternal Liver**

Methionine cycling in maternal liver of the pregnant rat declines as gestation progresses, with the proportion of the hepatic intracellular methionine pool produced from t-homocysteine falling from −21% in virgin female rats (28) to 16% by day 11 (Table 6), 14% on day 19 (Table 4), and 11% on day 21 (Table 6) of gestation. In this respect, turnover of methionine in the rat is similar to turnover of other amino acids, such as threonine and serine, the oxidation of which also declines as gestation proceeds (14). These changes may be part of a conservation mechanism that accompanies a mobilization of maternal protein reserves to support the rapid growth of the fetuses during the final stages of gestation.

The observation that methionine cycling in rodents decreases between days 11 and 21 of gestation apparently differs from the findings of studies in humans, where uncomplicated pregnancy is associated with a higher rate of methionine transmethylation in late gestation (6). There are important differences between the studies, even beyond the uncertainty of cross-species comparisons. In the rat, there are two separate phases of protein metabolism during pregnancy (12). During the first 2 wk, there is an anabolic phase, when the dam establishes protein reserves, particularly in the liver. In late gestation, there is a catabolic phase, when these protein reserves are mobilized to support the growth of fetal and mam-
acid and choline, but only when the methionine supply is limited. This situation is similar to that in virgin rats, where the need for methyl group transfer via homocysteine methylation is also reduced by excess dietary methionine (28). The restricted fetal growth and changes in the hepatic proteome observed in companion studies (10) indicate that protein synthesis is reduced in the animals fed the low-methionine diets. This suggests a greater reliance on the recycling of homocysteine when methionine is limiting. Nonetheless, provided adequate amounts of folate acid and choline are available, methionine synthesis from homocysteine in hepatic (and pancreatic) tissues is maintained at rates similar to those observed in animals fed a diet containing the level of methionine recommended by the American Institute for Nutrition (16). This suggests a secondary role for folate acid and choline that only comes into play when the supply of methionine, the direct precursor for S-adenosylmethionine, is restricted.

Methyl-deficient diets adversely affect hepatic lipid metabolism, leading to the accumulation of triglycerides. The hepatic S-adenosylmethionine-dependent phosphatidylethanolamine N-methyltransferase pathway has been suggested to be a major user of methyl groups (20). However, there was only a very weak negative correlation between the fraction of intracellular methionine derived from the remethylation of homocysteine and the triglyceride content of the liver (linear regression $P = 0.040, R^2 = 0.147$). There was a slightly stronger negative correlation between concentrations of phosphocholine, the principal storage form of choline in the liver, and hepatic triglycerides (linear regression $P = 0.017, R^2 = 0.23$), suggesting that the choline supply may be more important. If it is assumed that the development of hepatic steatosis is due to the inability to produce sufficient phosphatidylcholine, these results suggest a complex relationship between choline, the methyl supply, and the accumulation of lipids in maternal liver. Choline is a direct precursor for phosphatidylcholine, as well as a methyl donor via its oxidation to betaine (26), and the relative importance of these two pathways requires further clarification. It is also unclear whether the flow of methyl groups through the hepatic phosphatidylethanolamine N-methyltransferase pathway reflects the general flow for other methyl transfer reactions or whether there is a further prioritization to protect the synthesis of other essential products.

Despite the presence of an active methionine cycle, the enrichment of intrahepatic t-homocysteine was always less than that of the plasma, especially when tissue extracts are corrected for the presence of blood. This situation is the same as that in the virgin rat (28) and suggests that the liver is not the

Table 5. Effect of diet on ratio of $m+1$ to $m+4$ methionine isotopomers on days 11 and 21 of gestation

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Day 11</th>
<th>Day 21</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.227*</td>
<td>0.161‡</td>
<td>0.147‡</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>0.686*</td>
<td>0.506†</td>
<td>0.455†</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.245*</td>
<td>0.269*</td>
<td>0.151†</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.141*</td>
<td>0.078†</td>
<td>0.141†</td>
</tr>
<tr>
<td>Whole fetus</td>
<td>0.134</td>
<td>0.075</td>
<td>0.134</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>0.255</td>
<td>0.235</td>
<td>0.255</td>
</tr>
</tbody>
</table>

Values are means. SED is based on 2-way ANOVA, with folate-choline status, day of gestation, and their interaction as fixed effects. Different symbols (*, †, ‡) within rows indicate significant difference ($P < 0.05$).
dominant contributor to plasma t-homocysteine. Even when the diet is deficient in methyl donors, liver homocysteine is preferentially remethylated to methionine or converted to cysteine, rather than exported to the plasma. If this situation also pertains to human studies, caution is needed when changes in plasma t-homocysteine concentrations are related to intraorgan metabolism.

**Methionine Metabolism in Maternal Pancreas**

The pancreas is also a major site of methionine metabolism in the pregnant and the virgin female rat (28). However, unlike the liver, most of the intracellular methionine in the pancreas is imported directly from the plasma. The methylation of homocysteine accounts for only a very small proportion (3–6%) of the total. These low rates of homocysteine methylation, which are insensitive to folate and choline status, are likely to be of limited biological importance. The enrichment of homocysteine was greater in the pancreas than plasma at all stages of gestation, suggesting that, as in the virgin rat, the export of homocysteine from the pancreas is an important contributor to plasma concentrations. Throughout gestation, the pancreas appears to use methionine as a major methyl donor, with the homocysteine produced exported for methylation within other tissues. Pancreatic methyl metabolism appears to be essential for the secretion of digestive enzymes by the exocrine pancreas (4), although an effect on endocrine functions cannot be excluded.

**Methionine Metabolism in the Placenta**

The placenta, as the interface between maternal and fetal circulation, plays a key role in regulating fetal methionine supply. Although the folate-dependent methionine synthase is expressed in the rat (25) and human (19) placenta, the present data show that a relatively small proportion (probably <5%) of total free intracellular methionine is derived from the methylation of homocysteine within the placenta. Maternal diets deficient in folic acid and choline had no effect on this low rate of homocysteine methylation, suggesting that, as with the pancreas, this reaction is of limited biological importance.

Because the placenta receives homocysteine from maternal and fetal plasma and because the latter was not quantified in this study, the actual contribution from each source cannot be accurately quantified. However, the enrichment of homocysteine was much lower in the placenta than maternal plasma, suggesting that only a small proportion was of maternal origin. Instead, the enrichment of homocysteine in the placenta was similar to that in the whole fetus and also the sum of enrichments from m+4 and m+1 methionine in the placenta. This suggests that placental homocysteine is derived from intracellular and/or fetal sources. Unfortunately, in the absence of a sample of fetal plasma, this issue cannot be resolved. Regardless of source, placental homocysteine enrichment was unaffected by the methionine content of the diet but was increased slightly when the maternal diets were deficient in folic acid and choline. However, even in the animals fed the most-deficient diets, the enrichment of homocysteine was less in the placenta than maternal plasma, showing that the placenta is not contributing homocysteine to maternal plasma.

**Methionine Metabolism in the Fetus**

Although a more complete analysis of methionine metabolism in the fetal compartment is hampered by the lack of reliable fetal plasma samples, considerable information can be obtained from the various isotopomer enrichments from tissues. First, the m+1-to-m+4 methionine isotopomer ratio was higher in the whole fetus than placenta (Fig. 2), showing the synthesis of methionine from homocysteine within the fetal compartment. The much higher m+1-to-m+4 methionine isotopomer ratio in fetal liver than whole fetus suggests that, as in the mother, fetal liver is the dominant site of methionine synthesis from homocysteine. This observation is consistent with the finding that methionine synthase activity was similar in fetal and maternal liver (25). However, methionine synthase activity may also be present in other fetal tissues, including the brain (3), and the present data cannot rule out the possibility that these tissues also make a contribution. This tissue-specific methylation may play vital, but as yet unidentified, metabolic roles.

A second finding involves the sensitivity of homocysteine methylation within the fetus to the maternal diet, a feature reflected in data from the whole fetus and fetal liver. Importantly, fetal tissues showed similarities to and differences from

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Table 6. Proportion of intracellular methionine in maternal tissues derived from inflows from plasma, protein breakdown, and methylation of homocysteine on days 11 and 21 of gestation

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Inflow from plasma</th>
<th>Methylation of homocysteine</th>
<th>Protein breakdown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC</td>
<td>Value</td>
<td></td>
</tr>
<tr>
<td>Maternal liver</td>
<td>(+) 0.257</td>
<td>0.161*</td>
<td>0.582</td>
</tr>
<tr>
<td>Pancreas</td>
<td>(+) 0.753*</td>
<td>0.135†</td>
<td>0.014*</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>(+) 0.254</td>
<td>0.122</td>
<td>0.634</td>
</tr>
<tr>
<td>Pancreas</td>
<td>(+) 0.660†</td>
<td>0.008†</td>
<td>0.355</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>(+) 0.279</td>
<td>0.065‡</td>
<td>0.657</td>
</tr>
<tr>
<td>Pancreas</td>
<td>(+) 0.768*</td>
<td>–0.019*</td>
<td>0.271</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>(+) 0.020</td>
<td>0.011</td>
<td>0.025</td>
</tr>
<tr>
<td>Pancreas</td>
<td>(+) 0.043</td>
<td>0.014</td>
<td>0.044</td>
</tr>
</tbody>
</table>

Values are means. SED is based on 2-way ANOVA, with methionine status, folate-choline status, and their interaction as fixed effects. Different symbols (*, †, ‡) within rows indicate significant difference (\(P < 0.05\)).
methyl group metabolism in maternal organs. When methio-
nine intake was high, there was a minimal effect of folate plus
choline deficiency on methylation of homocysteine by mater-
nal and fetal liver. This result suggests that methionine is used
as a primary methyl donor in the same way by both tissues.
However, the response of maternal and fetal liver differs when
the maternal diet is low in methionine and deficient in folate
plus choline. While the methylation of homocysteine within
maternal liver is reduced, methionine synthesis in fetal liver in
late gestation is maintained (day 21) or even enhanced (day
19). This suggests that there is a mechanism to protect the fetus
during nutritional deficiency, albeit at the expense of maternal
metabolism. Indeed, earlier studies with similar diets showed
that although the folic acid content declined in fetal and
maternal liver, there is a preferential redistribution of the
residual folate to the fetus, such that levels were maintained at
~30% of those in the animals fed the complete diet (9). The
current data clearly show that even this reduced folic acid is
sufficient to support the synthesis of methionine from homo-
cysteine in the fetus. This implies that there is no effect of
dietary deficiencies on the products of methyl transfer reactions
in the fetus. However, it is not clear from the current data if the
products of the methylation reactions in fetal liver are able to
replace quantitatively those normally produced by maternal
liver. Resolution of this issue and determination of absolute,
rather than relative, changes require additional measurements,
including enrichment of fetal plasma methionine and the rate of
protein breakdown (or synthesis) within fetal liver. Such in-
formation has been obtained for adult animals previously, and
an extended mathematical model was applied (28), but the
failure to obtain a reliable fetal plasma sample precludes
implementation of this approach here. Fetal development may
still be dependent on products such as phospholipids (5) from
maternal liver, and an improvement in the biosynthetic capac-
ity of the maternal compartment may underlie the benefits of
folate supplementation in human pregnancy.

In conclusion, this study shows that there is a high rate of
methionine synthesis from homocysteine in maternal liver and
that these reactions are sensitive to the availability of folic acid
and choline in the diet only for animals fed a methionine-
limited diet. However, methionine synthesis from homocys-
teine in fetal tissues is maintained or increased when dams are
fed folate- and choline-deficient methionine-restricted diets,
suggesting that these reactions are protected to some extent in
fetal tissues.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

F.A.W., G.E.L., and W.D.R. are responsible for conception and design of
the research; F.A.W., A.G.C., S.E.A., and G.E.L. performed the experiments;
F.A.W., G.H., A.G.C., S.E.A., G.E.L., and W.D.R. analyzed the data; F.A.W.,
G.H., G.E.L., and W.D.R. interpreted the results of the experiments; F.A.W.
and W.D.R. prepared the figures; F.A.W., G.H., and W.D.R. drafted the
manuscript; F.A.W., G.H., G.E.L., S.E.A., G.E.L., and W.D.R. approved the
final version of the manuscript; G.H., G.E.L., and W.D.R. edited and revised
the manuscript.

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