Effects of Betaine Supplementation and Choline Deficiency on Folate Deficiency-Induced Hyperhomocysteinemia in Rats

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Summary The effect of betaine status on folate deficiency-induced hyperhomocysteinemia was investigated to determine whether folate deficiency impairs homocysteine removal not only by the methionine synthase (MS) pathway but also by the betaine-homocysteine S-methyltransferase (BHMT) pathway. For this purpose, we investigated the effect of dietary supplementation with betaine at a high level (1%) in rats fed a folate-deprived 10% casein diet (10C) and 20% casein diet (20C). We also investigated the effect of choline deprivation on folate deficiency-induced hyperhomocysteinemia in rats fed 20C. Supplementation of folate-deprived 10C and 20C with 1% betaine significantly suppressed folate deprivation-induced hyperhomocysteinemia, but the extent of suppression was partial or limited, especially in rats fed 10C, the suppression of plasma homocysteine increment being 48.5% in rats fed 10C and 69.7% in rats fed 20C. Although betaine supplementation greatly increased hepatic betaine concentration and BHMT activity, these increases did not fully explain why the effect of betaine supplementation was partial or limited. Folate deprivation markedly increased the hepatic concentration of N,N-dimethylglycine (DMG), a known inhibitor of BHMT, and there was a significant positive correlation between hepatic DMG concentration and plasma homocysteine concentration, suggesting that folate deficiency increases hepatic DMG concentration and thereby depresses BHMT reaction, leading to interference with the effect of betaine supplementation. Choline deprivation did not increase plasma homocysteine concentration in rats fed 20C, but it markedly enhanced plasma homocysteine concentration when rats were fed folate-deprived 20C. This indicates that choline deprivation reinforced folate deprivation-induced hyperhomocysteinemia. Increased hepatic DMG concentration was also associated with such an effect. These results support the concept that folate deficiency impairs homocysteine metabolism not only by the MS pathway but also by the BHMT pathway.

Key Words betaine, folate deficiency, choline deficiency, plasma homocysteine, N,N-dimethylglycine

Homocysteine is a normal intermediate of methionine metabolism (1) (Fig. 1), but a number of studies have suggested that an elevated plasma homocysteine concentration might be an independent risk factor for cardiovascular disease (2–4). Homocysteine has two metabolic fates, i.e., remethylation and transsulfuration. In the remethylation pathway, homocysteine is remethylated to generate methionine using the methyl group of either 5-methyltetrahydrofolate (5-MTHF) or betaine. The former reaction is catalyzed by methionine synthase (MS) and the latter reaction is catalyzed by betaine-homocysteine S-methyltransferase (BHMT). Cystathionine β-synthase (CBS) catalyzes the first step of the transsulfuration pathway, by which sulfur of methionine flows toward cysteine out of the methionine cycle. Plasma homocysteine concentration is affected by various factors including genetic, nutritional, physiological, clinical and lifestyle factors (2–4). Of these factors, genetic and nutritional factors are thought to have a greater influence on plasma homocysteine concentration. For instance, deficiencies of certain vitamins such as folate, vitamin B-6 and vitamin B-12 cause hyperhomocysteinemia, since these vitamins participate in the metabolism of homocysteine as enzyme cofactors (2–4). Furthermore, deprivation of choline, a vitamin-like compound, also induces hyperhomocysteinemia due to betaine deficiency (5). Hyperhomocysteinemia caused by vitamin deficiencies can be easily prevented by...
administration of the deficient vitamins, but other treatments are also required to suppress hyperhomocysteinemia due to genetic defects or mutations of homocysteine-metabolizing enzymes. Results of a number of studies on the plasma homocysteine-lowering effect of betaine in human subjects have been reported (6, 7). The effects of betaine in hyperhomocysteinemic animal models have also been reported (8–12). The efficacy of betaine is based on the mechanism by which the compound increases hepatic betaine concentration and BHMT activity (13) and thereby stimulates homocysteine removal by the betaine-BHMT system.

One of the representative experimental hyperhomocysteinemia models in rodents is a folate deficiency model (14, 15). It has been thought that folate deficiency induces hyperhomocysteinemia by duplicate mechanisms (3, 14): (1) disturbed remethylation of homocysteine due to a decrease in 5-MTHF concentration and (2) decreased transsulfuration due to a decrease in hepatic concentration of S-adenosylmethionine (SAM), which is an activator of CBS (16). However, these explanations raise the question of why a folate deficiency-induced decrease in homocysteine metabolism cannot be fully compensated for by the betaine-BHMT system, the capacity of which is thought to be greater than the capacity of the 5-MTHF-MS system judging from their enzyme activities in the liver of rats (17, 18). This issue appears to be resolved at least in part by the fact that the betaine-BHMT system requires folate, since tetrahydrofolate participates in the metabolism of N,N-dimethylglycine (DMG) and N-methylglycine as a methyl-group acceptor (19), suggesting that folate deficiency might also affect the BHMT pathway. Of interest is that DMG is not only a product of the BHMT reaction but also an inhibitor of BHMT (20, 21).

In fact, serum DMG concentration was significantly increased by folate deficiency, but not by vitamin B-12 deficiency, in human subjects (22, 23). McGregor et al. (24) reported that plasma DMG concentration was increased in chronic renal failure patients and that there was a significant positive correlation between plasma DMG concentration and plasma total homocysteine concentration. Based on these findings, they postulated that reduced BHMT activity due to inhibition by DMG might contribute to hyperhomocysteinemia in chronic renal failure patients. This suggests that folate deficiency might impair not only the 5-MTHF-MS system but also the betaine-BHMT system. However, there is little information on the significance of hepatic DMG concentration in folate deficiency-induced hyperhomocysteinemia.

In the present study, we investigated the effect of betaine status on folate deficiency-induced hyperhomocysteinemia to determine whether folate deficiency actually impairs the function of the hepatic betaine-BHMT system. For this purpose, we investigated the effect of dietary supplementation with betaine at a high level (1%) in rats fed folate-deprived low (10%) and standard (20%) casein diets (experiment 1), because folate deficiency-induced hyperhomocysteinemia and the supplemental effect of betaine were anticipated to differ depending on dietary casein level. In addition, we investigated the effect of choline deprivation in rats fed folate-sufficient and folate-deprived standard casein diets to determine whether there is an interacting effect between folate deficiency and choline (betaine) deficiency (experiment 2).

**MATERIALS AND METHODS**

**Chemicals.** Betaine and folic acid were purchased from Sigma-Aldrich (St. Louis, MO). Succinylsulfathiazole was purchased from MP Biomedicals (Irvine, CA). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Sigma-Aldrich and were of analytical grade. Vitamin-free casein, mineral mixture (AIN-93G), vitamin mixture (AIN-93, folate-free), and cellulose powder were purchased from Oriental Yeast Co., Ltd. (Tokyo). Other ingredients of the diet were purchased from Wako.

**Animals and diets.** Six-week-old male rats (120–140 g) of the Wistar strain were obtained from Japan SLIC, Inc. (Hamamatsu, Japan). They were individually housed in hanging stainless-steel wire cages in an isolated room kept at a controlled temperature (23–25°C) and humidity (40–60%). Lighting was maintained on a 12-h cycle (lights on from 07:00 to 19:00 h). Before starting the experiments, all rats were acclimated to the facility for 5 d and given free access to water and a 25% casein diet. In this study, two separate animal experiments were conducted. In experiment 1, rats were randomly assigned to the following six diet groups: 10% casein diet (10C), folate-deprived 10C (10CFD), 10CFD + 1% betaine (10CFDB), 20% casein diet (20C), folate-deprived 20C (20CFD), and 20CFD + 1% betaine (20CFDB). One of the control diets (10C) consisted of

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**Fig. 1.** Metabolism of methionine and homocysteine. BHMT, betaine-homocysteine S-methyltransferase (EC 2.1.1.5); CBS, cystathionine β-synthase (EC 4.2.1.22); DMG, N,N-dimethylglycine; FA, folic acid; MS, methionine synthase (EC 2.1.1.13); 5-MTHF, 5-methyltetrahydrofolate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAM, S-adenosylmethionine; Sar, sarcosine (N-methylglycine); THF, tetrahydrofolate.
the following ingredients (g/kg): vitamin-free casein, 100; cornstarch, 572.26; sucrose, 200; corn oil, 50; mineral mixture (AIN-93G), 35; vitamin mixture (AIN-93, folate-free), 10; choline bitartrate, 2.5; lactose containing folate (33.3 mg/g), 0.24; succinylsulfathiazole, 10; and cellulose powder, 20. In 20C, vitamin-free casein was raised to 200 g/kg at the expense of cornstarch. In folate-deprived diets, folate-free lactose was used. In experiment 2, rats were randomly assigned to the following four diet groups: 20C, choline-deprived 20C (20CCD), 20CFD, and choline-deprived and folate-deprived 20C (20CCDFD). In choline-deprived diets, choline bitartrate was omitted and cornstarch was increased. In addition to the folate-free vitamin mixture and vitamin-free casein, antibiotic succinylsulfathiazole was included in the diet to suppress folate synthesis by intestinal bacteria according to a previous report (25). Folate-sufficient diets (10C, 20C, and 20CCD) contained folate at a level of 8 mg/kg, four fold the level of AIN-93, to make clear the effect of folate deficiency according to a previous report (25). Rats were given free access to the experimental diets and water for 4 wk and killed by decapitation between 10:00 and 11:00 h without prior food deprivation, since it has been shown that dietary treatment did not affect fasting plasma homocysteine concentration in humans (26). This study was approved by the Animal Use Committee of Shizuoka University, and the animals were maintained in accordance with the “Guidelines for the Care and Use of Laboratory Animals” of Shizuoka University.

Tissue collection and fractionation. Blood plasma was separated from heparinized whole blood by centrifugation at 2,000 ×g for 15 min at 4°C and was stored at −30°C until needed for analysis. After collection of blood, the whole liver was quickly removed, rinsed in ice-cold saline, blotted on filter paper, cut into two portions, weighed, quickly frozen in liquid nitrogen, and stored at −80°C until needed for analysis. One portion of the liver was homogenized in 4 volumes (vol/wt) of ice-cold 0.3 M trichloroacetic acid solution and then centrifuged at 10,000 ×g for 10 min at 4°C. The supernatant of the deproteinized liver homogenate was subjected to assays for methionine metabolites, betaine, dimethylglycine and serine. The other portion of the liver was homogenized in 4 volumes (vol/wt) of a 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M KCl, and the resulting homogenate was centrifuged at 14,000 ×g for 10 min at 4°C. The supernatant was subjected to enzyme assays. For the assay of hepatic triglyceride concentration, an aliquot of the liver homogenate was lyophilized, and total lipids were extracted by the method of Folch et al. (27).

Biochemical analysis. The concentrations of total (protein-bound plus non-protein-bound) homocysteine and cysteine in the plasma and liver were measured by HPLC using the method of Durand et al. (28). The concentration of non-protein-bound homocysteine was measured using deproteinized plasma, and protein-bound homocysteine was estimated by subtracting non-protein-bound homocysteine from total homocysteine. The concentrations of SAM and S-adenosylhomocysteine (SAH) in the liver were measured by HPLC following Cook et al. (29). The concentrations of 5-MTHF in the plasma and liver were measured by HPLC by the method of Shimoda (30). The concentrations of betaine and DMG in the liver were measured by HPLC following Laryea et al. (31) and the concentration of serine in the liver was measured by an amino acid autoanalyzer (Model L-8500; Hitachi). The activity of MS in the liver was measured following Huang et al. (32). The activity of BHMT in the liver was measured following Finkelstein and Mudd (33), but HPLC was used in the assay of the reaction product, DMG, following Laryea et al. (31). The activity of CBS in the liver was measured following Mudd et al. (34), but HPLC was used in the assay of the reaction product, cystathionine, following Einarsson et al. (35). The hepatic triglyceride concentration was measured enzymatically using a commercial kit (Triglyceride E-Test Wako, Wako). The protein concentration was measured according to Lowry et al. (36) using bovine serum albumin as a standard.

Statistical analysis. Each value is expressed as the mean ± SE. Data were analyzed by a one-way ANOVA (experiment 1) or two-way ANOVA (experiment 2), and differences among the experimental groups were analyzed by the Tukey test when the F value was significant. When variances among the experimental groups were not homogeneous, data were logarithmically transformed before ANOVA. Statistical analysis was performed with Mac Tokei-Kaiseki software (version 1.5; Esumi, Tokyo).

RESULTS
Effect of betaine supplementation (experiment 1)
The effects of dietary supplementation with betaine on folate deficiency-induced hyperhomocysteinemia and other variables were investigated in rats fed folate-deprived 10C and 20C. The results are summarized in Table 1. Body weight gain and liver weight were significantly higher in rats fed 20C than in rats fed 10C irrespective of folate deprivation or betaine supplementation, whereas food intake did not differ among the groups. Plasma total homocysteine concentration was significantly increased by folate deprivation in both rats fed 10C and those fed 20C, while the magnitude of the increase was greater in rats fed 10C (126.7%) than in rats fed 20C (93.5%). Betaine supplementation significantly suppressed folate deprivation-induced increase in plasma total homocysteine concentration in rats fed both 10C and 20C, while the extent of increment suppression was smaller in rats fed 10C (48.5%) than in rats fed 20C (69.7%). Plasma homocysteine exists in the form of either protein-bound or non-protein-bound (37). Folate deprivation and betaine supplementation affected the concentrations of both types of homocysteine similarly to that of total homocysteine. Plasma total cysteine concentration was significantly higher in rats fed 20C than in rats fed 10C irrespective of folate deprivation or betaine supplementation. Plasma 5-

Table 1. Effects of dietary supplementation with betaine on plasma homocysteine concentration and other variables in rats fed folate-deprived 10% and 20% casein diets (experiment 1).

<table>
<thead>
<tr>
<th>Diet</th>
<th>10C</th>
<th>10CFD</th>
<th>10CFDB</th>
<th>20C</th>
<th>20CFD</th>
<th>20CFDB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt gain, g/28 d</td>
<td>75 ± 4a</td>
<td>66 ± 4b</td>
<td>60 ± 4b</td>
<td>100 ± 2c</td>
<td>107 ± 4a</td>
<td>95 ± 5a</td>
</tr>
<tr>
<td>Food intake, g/24 h</td>
<td>460 ± 17</td>
<td>436 ± 13</td>
<td>408 ± 17</td>
<td>461 ± 12</td>
<td>448 ± 19</td>
<td>410 ± 16</td>
</tr>
<tr>
<td>Liver wt, g/100 g body wt</td>
<td>3.55 ± 0.07a</td>
<td>3.69 ± 0.04b</td>
<td>3.64 ± 0.04b</td>
<td>4.28 ± 0.06a</td>
<td>4.31 ± 0.12a</td>
<td>4.25 ± 0.06a</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Hcy, µmol/L</td>
<td>15.1 ± 0.4d</td>
<td>34.1 ± 0.6e</td>
<td>24.9 ± 0.6b</td>
<td>13.7 ± 0.4d</td>
<td>26.5 ± 0.7b</td>
<td>17.6 ± 0.5c</td>
</tr>
<tr>
<td>Unbound Hcy, µmol/L</td>
<td>5.4 ± 0.2d</td>
<td>17.6 ± 0.7a</td>
<td>11.2 ± 0.4b</td>
<td>4.4 ± 0.3d</td>
<td>10.5 ± 0.3b</td>
<td>8.2 ± 0.4c</td>
</tr>
<tr>
<td>Bound Hcy, µmol/L</td>
<td>9.6 ± 0.5b</td>
<td>16.6 ± 0.6e</td>
<td>13.7 ± 0.6b</td>
<td>9.3 ± 0.4c</td>
<td>15.9 ± 0.7ab</td>
<td>9.4 ± 0.5c</td>
</tr>
<tr>
<td>Unbound Hcy, %</td>
<td>36.2 ± 2.0d</td>
<td>51.4 ± 1.7a</td>
<td>45.0 ± 1.8ab</td>
<td>31.9 ± 1.9d</td>
<td>39.9 ± 1.4bc</td>
<td>46.5 ± 2.0ab</td>
</tr>
<tr>
<td>Bound Hcy, %</td>
<td>63.8 ± 2.0ab</td>
<td>48.6 ± 1.7d</td>
<td>55.0 ± 1.8cd</td>
<td>68.1 ± 1.9a</td>
<td>60.0 ± 1.4bc</td>
<td>53.5 ± 2.0cd</td>
</tr>
<tr>
<td>Total Cys, µmol/L</td>
<td>106 ± 3b</td>
<td>112 ± 4b</td>
<td>112 ± 5b</td>
<td>144 ± 3a</td>
<td>137 ± 3a</td>
<td>139 ± 5a</td>
</tr>
<tr>
<td>5-MTHF, nmol/L</td>
<td>202 ± 4.4a</td>
<td>7.7 ± 0.3b</td>
<td>7.3 ± 0.3b</td>
<td>209 ± 4.7a</td>
<td>6.3 ± 0.4b</td>
<td>6.6 ± 0.2b</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>SAM, nmol/g</td>
<td>70.2 ± 1.8c</td>
<td>42.9 ± 1.3d</td>
<td>87.7 ± 1.5b</td>
<td>89.0 ± 1.0b</td>
<td>46.0 ± 0.8a</td>
<td>104.0 ± 1.5a</td>
</tr>
<tr>
<td>SAH, nmol/g</td>
<td>17.5 ± 0.6d</td>
<td>24.1 ± 0.6e</td>
<td>39.9 ± 1.2a</td>
<td>17.6 ± 0.4d</td>
<td>19.0 ± 0.5d</td>
<td>32.2 ± 1.4a</td>
</tr>
<tr>
<td>SAM : SAH ratio</td>
<td>4.04 ± 0.18b</td>
<td>1.79 ± 0.06e</td>
<td>2.21 ± 0.07de</td>
<td>5.07 ± 0.12a</td>
<td>2.44 ± 0.09d</td>
<td>3.27 ± 0.14a</td>
</tr>
<tr>
<td>Hcy, nmol/g</td>
<td>2.7 ± 0.1c</td>
<td>3.4 ± 0.1b</td>
<td>3.1 ± 0.1bc</td>
<td>3.1 ± 0.1bc</td>
<td>4.6 ± 0.2a</td>
<td>3.1 ± 0.1bc</td>
</tr>
<tr>
<td>MS activity2</td>
<td>0.240 ± 0.007a</td>
<td>0.114 ± 0.006a</td>
<td>0.119 ± 0.002c</td>
<td>0.219 ± 0.006b</td>
<td>0.144 ± 0.003b</td>
<td>0.141 ± 0.003b</td>
</tr>
<tr>
<td>BHMT activity2</td>
<td>0.87 ± 0.04c</td>
<td>0.99 ± 0.04bc</td>
<td>3.00 ± 0.13a</td>
<td>1.36 ± 0.05b</td>
<td>1.37 ± 0.03b</td>
<td>2.74 ± 0.16a</td>
</tr>
<tr>
<td>CBS activity2</td>
<td>4.18 ± 0.13e</td>
<td>3.39 ± 0.12c</td>
<td>3.64 ± 0.15bc</td>
<td>6.01 ± 0.24a</td>
<td>5.39 ± 0.21a</td>
<td>5.72 ± 0.24a</td>
</tr>
<tr>
<td>5-MTHF, nmol/g</td>
<td>18.29 ± 0.38a</td>
<td>1.18 ± 0.08b</td>
<td>1.15 ± 0.05b</td>
<td>16.07 ± 0.62a</td>
<td>1.20 ± 0.09b</td>
<td>2.01 ± 0.05b</td>
</tr>
<tr>
<td>Betaine, µmol/g</td>
<td>2.82 ± 0.12c</td>
<td>1.55 ± 0.07d</td>
<td>9.92 ± 0.22a</td>
<td>1.76 ± 0.11d</td>
<td>1.23 ± 0.08d</td>
<td>6.36 ± 0.30d</td>
</tr>
<tr>
<td>Serine, µmol/g</td>
<td>2.43 ± 0.25e</td>
<td>3.20 ± 0.16c</td>
<td>3.22 ± 0.16a</td>
<td>0.43 ± 0.02c</td>
<td>0.62 ± 0.06c</td>
<td>1.07 ± 0.16c</td>
</tr>
<tr>
<td>DMG, µmol/g</td>
<td>0.14 ± 0.01a</td>
<td>0.90 ± 0.04a</td>
<td>0.63 ± 0.03ab</td>
<td>0.19 ± 0.02c</td>
<td>0.76 ± 0.07ab</td>
<td>0.55 ± 0.01c</td>
</tr>
<tr>
<td>Betaine : DMG ratio</td>
<td>19.9 ± 1.3a</td>
<td>1.8 ± 0.1c</td>
<td>16.0 ± 0.9b</td>
<td>10.0 ± 1.1c</td>
<td>1.7 ± 2.4d</td>
<td>11.6 ± 0.6c</td>
</tr>
</tbody>
</table>

10C, 10% casein diet; 10CFD, folate-deprived 10C; 10CFDB, 10CFD+1% betaine; 20C, 20% casein diet; 20CFD, folate-deprived 20C; 20CFDB, 20CFD+1% betaine; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine β-synthase; DMG, N,N-dimethylglycine; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

Each value is the mean ± SE, n = 8. Values without a common letter differ, p < 0.05.

Expressed as nmol/(min·mg protein).

MTHF concentration, measured as an index of folate status (38), was greatly decreased in rats fed folate-deprived diets irrespective of dietary casein level or betaine supplementation. Hepatic SAM concentration was significantly decreased by folate deprivation and was restored by betaine supplementation in both rats fed 10C and those fed 20C. Hepatic SAH concentration was increased or tended to be increased by folate deprivation and was further increased by betaine supplementation. The SAM : SAH ratio was significantly lower in rats fed folate-deprived diets than in rats fed the control diets irrespective of betaine supplementation, although betaine supplementation slightly increased or tended to increase the ratio. Hepatic homocysteine concentration was significantly increased by folate deprivation and this increase was suppressed or tended to be suppressed by betaine supplementation.

Hepatic MS activity was significantly decreased by folate deprivation and unaffected by betaine supplementation in both rats fed 10C and those fed 20C. Hepatic concentration of 5-MTHF, a substrate of MS, was greatly decreased by folate deprivation and unaffected by betaine supplementation. Hepatic BHMT activity was unaffected by folate depletion and significantly increased by betaine supplementation in both rats fed 10C and those fed 20C. Hepatic concentration of betaine, a substrate of BHMT, was significantly decreased or tended to be decreased by folate deprivation and was greatly increased by betaine supplementa-
The effect of choline deprivation on folate deprivation-induced hyperhomocysteinemia was investigated in order to determine whether there exists an interacting effect between choline deprivation and folate deprivation. The results are summarized in Table 2. Body weight gain and food intake did not differ among the four groups. Liver weight was significantly lower in rats fed the choline- and folate-deprived diet than in rats fed the choline-deprived diet. Although folate deprivation alone significantly increased plasma total homocysteine concentration, choline deprivation alone did not affect plasma total homocysteine concentration. Choline and folate deprivation markedly enhanced plasma total homocysteine concentration. The profiles of plasma non-protein-bound and protein-bound homocysteine concentrations were similar to that of total homocysteine. The plasma total cysteine concentration was significantly lower in rats fed the choline- and folate-deprived diets than in rats fed other diets. Plasma 5-MTHF concentration was markedly lower in rats fed folate-deprived diets irrespective of choline deprivation. Hepatic SAM concentration was significantly decreased by choline deprivation alone or folate deprivation alone and was further decreased by deprivation of both choline and folate. Hepatic SAH concentration was significantly increased by choline deprivation and folate deprivation. Consequently, the SAM: SAH ratio was a significant positive correlation between the two variables. The extent of the effect was partial. Betaine: DMG ratio was suppressed the increase in DMG concentration, but the magnitude of the effect was relatively small. Hepatic concentration of serine, a substrate of CBS, was significantly lower in rats fed 20C than in rats fed 10C. Hepatic DMG concentration was greatly increased by folate deprivation in both rats fed 10C and those fed 20C. Betaine supplementation significantly suppressed the increase in DMG concentration, but the extent of the effect was partial. Betaine: DMG ratio was markedly lower in rats fed folate-deprived diets without betaine supplementation. Since the profile of plasma total homocysteine concentration was similar to that of hepatic DMG concentration, the correlation coefficient was estimated using mean values of six groups. There was a significant positive correlation between the two variables (Fig. 2).

Effect of choline and folate deprivation (experiment 2)

The effect of choline deprivation on folate deprivation-induced hyperhomocysteinemia was investigated in order to determine whether there exists an interacting effect between choline deprivation and folate deprivation...
was markedly decreased by choline and folate deprivation. Hepatic homocysteine concentration was increased by folate deprivation alone and was further increased by choline and folate deprivation.

Hepatic MS activity was decreased by folate deprivation alone and was further decreased by choline and folate deprivation. Hepatic 5-MTHF concentration was markedly decreased by folate deprivation irrespective of choline deprivation. Hepatic BHMT activity did not differ among the four groups. Hepatic betaine concentration was decreased by choline deprivation and folate deprivation and was further decreased by choline and folate deprivation. Hepatic serine concentration was significantly higher in rats fed the choline- and folate-deprived diet than in rats fed other diets. Hepatic DMG concentration was significantly increased by folate deprivation alone and was further increased by choline and folate deprivation. Consequently, the betaine:DMG ratio was significantly lower in rats fed folate-deprived diets than in rats fed folate-sufficient diets. Since fatty infiltration was visible in rats fed the choline- and folate-deprived diet, hepatic triglyceride concentration was measured. Hepatic triglyceride concentration was significantly increased only in rats fed the choline- and folate-deprived diet. There was a significant positive correlation between hepatic DMG concentration and plasma total homocysteine concentration among the four experimental groups (Fig. 3).

**DISCUSSION**

In the present study, we used vitamin-free casein, a folate-free vitamin mixture and antibiotic succinylsulfathiazole to induce convenient folate deficiency. The magnitude of hyperhomocysteinemia is defined as mild (15–30 μM), moderate (30–100 μM) and severe (>100 μM) (3). Hence, the folate deprivation-induced hyperhomocysteinemia observed in the present study was moderate (34.1 μM) and mild (26.5 μM) in rats fed 10C and 20C, respectively, indicating that increasing dietary casein level led to resistance against folate deprivation-induced elevation of plasma homocysteine concentration. Consistent with this, we previously demonstrated that diets containing higher levels of casein or soybean protein did not increase but rather decreased plasma homocysteine concentrations (39, 40). Furthermore, guanidinoacetic acid-induced hyperhomocysteinemia was also suppressed by raising the dietary casein level (41). There are several possible reasons for the phenomenon of plasma homocysteine concentration being significantly lower in rats fed 20CFD than in rats fed 10CFD despite the intake of Met, the sole precursor of homocysteine, being higher in rats fed 20CFD than in rats fed 10CFD. One possible reason is that vitamin-free casein might contain a small amount of folate and rats fed 20CFD ingested about a two-fold larger amount of folate than did rats fed 10CFD. However, this may not be a major reason since plasma 5-MTHF concentration did not differ between the two rat groups. Another possible reason is that 20CFD, compared with 10CFD, increased or tended to increase the activities of three enzymes that participate in the metabolism of homocysteine. The third possible reason is that plasma cysteine concentration was increased in rats fed 20CFD and thereby reduced plasma homocysteine, because cysteine elicits its hypohomocysteinemic effect through the enhancement of plasma cysteine (42). In any case, the present study suggests that rats fed diets containing higher levels of casein are less susceptible to folate deficiency. The present study also showed that plasma concentration of protein-unbound homocysteine was significantly lower in rats fed 20CFD than in rats fed 10CFD. It is thought that protein-unbound homocysteine is taken up and metabolized in the kidney. We previously demonstrated that renal CBS activity was increased by raising the dietary casein level (40). Hence, it is possible that clearance activity of plasma protein-unbound homocysteine is greater in rats fed 20CFD than in rats fed 10CFD. In contrast, hepatic homocysteine concentration was significantly higher in rats fed 20CFD than in rats fed 10CFD. The hepatic homocysteine concentration is thought to be determined by both homocysteine production from methionine and the metabolism of homocysteine by three enzymes. Methionine intake was significantly higher in rats fed 20CFD than in rats fed 10CFD, whereas hepatic activities of MS, BHMT and CBS were significantly higher or tended to be higher in rats fed 20CFD than in rats fed 10CFD. Under the condition of a relatively large amount of dietary methionine, cystathionine formation is thought to be important in the metabolism of homocysteine (17). Of three enzyme substrates, only hepatic cysteine concentration was significantly lower in rats fed 20CFD than in rats fed 10CFD. Hence, it can be considered that decreased cysteine concentration limited the metabolism of homocysteine in the liver.

Folate deficiency is generally thought to induce hyperhomocysteinemia mainly by decreasing the concentration of 5-MTHF, a methyl-group donor for MS, and thereby suppressing homocysteine remethylation.
(3). The present study demonstrated that folate deprivation decreased not only hepatic 5-MTHF concentration but also hepatic MS activity, indicating that the 5-MTHF-MS system was greatly depressed. Kim et al. (43) showed that severe folate deficiency caused secondary depletion of hepatic choline and phosphocholine in rats. This appears to be also the case for hepatic betaine concentration, since choline is easily metabolized to betaine, especially in rats. In fact, the present study showed that hepatic betaine concentration was significantly decreased or tended to be decreased by folate deprivation. However, it is uncertain whether the decrease in hepatic betaine concentration contributed to the induction of hyperhomocysteinemia by folate deprivation, since decreased hepatic betaine concentrations in the 10CFD group and 20CFD group were still one-order higher than the reported Km value of rat BHMT for betaine, 120 μM (44). On the other hand, we demonstrated that the hepatic concentration of DMG, a reaction product and also an inhibitor of BHMT, was markedly increased by folate deprivation. Although the Ki value of rat BHMT for DMG has not been reported, Finkelstein et al. (20) reported that the reaction of rat BHMT was inhibited by DMG by 19% at 0.02 mM, by 76% at 0.1 mM, and by 90% at 1 mM. Hence, the increased hepatic DMG concentrations in the 10CFD group and 20CFD group might strongly inhibit BHMT reaction in vivo, although hepatic DMG concentrations in control groups in the 10C group and 20C group were also considerably high. In addition to DMG concentration, the betaine : DMG ratio may also have some significance in the BHMT reaction in a similar fashion to the SAM : SAH ratio in SAM-dependent transmethylation reactions, where SAH is known as an inhibitor of various types of methyltransferase. Thus, we report here, for the first time to our knowledge, that folate deprivation markedly increased hepatic DMG concentration, supporting the concept that folate deprivation might impair not only the 5-MTHF system but also the betaine-BHMT system and thereby induce hyperhomocysteinemia.

One of the objectives of the present study was to examine whether folate deficiency-induced hyperhomocysteinemia can be suppressed by betaine supplementation. An important finding of the present study is that betaine supplementation could suppress folate deficiency-induced hyperhomocysteinemia, but the effect was partial, especially in rats fed a low casein diet. It appears unlikely that the limited effect of betaine is solely due to the supplementation level of betaine. We previously demonstrated that guanidinoacetic acid-induced hyperhomocysteinemia was almost completely suppressed by betaine supplementation at a level of 0.34% (12) and that choline deprivation-induced hyperhomocysteinemia was completely suppressed by betaine supplementation at a level of 0.28% (5). Therefore, the 1% supplementation level of betaine used in the present study appears to be relatively high, although a dose-response experiment is needed to confirm the effect of betaine. It is reasonable to assume that the hypohomocysteinemic effect of betaine is due to an increase in hepatic betaine concentration, BHMT activity, or both. Indeed, betaine supplementation markedly increased both betaine concentration and BHMT activity in the liver. This also suggests that the 1% supplementation level of betaine was not insufficient. Nevertheless, the effect of betaine on plasma homocysteine concentration was partial or limited, suggesting that the actual reaction catalyzed by BHMT in vivo may not be fully stimulated by betaine supplementation under the condition of folate deficiency. One possible reason for the phenomenon is that a folate deprivation-induced increase in hepatic DMG concentration might interfere with the effect of betaine, despite hepatic betaine concentration and BHMT activity being enhanced. The metabolism of DMG, an inhibitor of BHMT, to N-methylglycine requires folate (19). Hence, it is likely that folate deficiency increased hepatic DMG concentration and the resultant inhibition of the betaine-BHMT system by DMG could not be fully mitigated even by betaine supplementation. The existence of a significant correlation between hepatic DMG concentrations and plasma homocysteine concentrations among the six experimental groups supports such a possibility.

In experiment 2, we investigated the effect of choline deprivation on folate deprivation-induced hyperhomocysteinemia. Choline deprivation does not cause choline deficiency under the condition of relatively high levels of dietary methionine, since choline status is determined not only by choline intake but also by methionine intake (45). The basis of this phenomenon is that phosphatidylethanolamine (PE) N-methylation using SAM can synthesize the choline moiety of phosphatidylcholine (PC) and the PE N-methylation is regulated mainly by hepatic SAM concentration, which responds to methionine intake or the SAM : SAH ratio (46–48). In support of this, we previously demonstrated that choline deprivation did not cause hyperhomocysteinemia when rats were fed a 25% casein diet, while it caused marked hyperhomocysteinemia when rats were fed low-methionine diets such as 10C and 25% soybean protein diet (5). We used 20C as the control diet to avoid induction of hyperhomocysteinemia by choline deprivation alone, since it was considered that choline deprivation and folate deprivation may give rise to an interacting effect under such a condition. The results clearly showed that the combination of choline deprivation and folate deprivation caused moderate hyperhomocysteinemia (45.4 μM) in rats fed 20C, while choline deprivation alone did not increase plasma homocysteine concentration and folate deprivation alone caused only mild hyperhomocysteinemia (26.6 μM). This indicates that choline deprivation and folate deprivation enhanced plasma homocysteine concentration. In other words, the hyperhomocysteinemic effect of choline deprivation appeared under the condition of folate deprivation and, conversely, the hyperhomocysteinemic effect of folate deprivation was reinforced by choline deprivation. The present study also demonstrated that the combination of choline deprivation and folate depri-
PC synthesis mainly depends on the PE pathways for PC synthesis, i.e., the CDP-choline pathway. Decreased PC synthesis, therefore, active synthesis of PC is essential for the major surface phospholipid of the lipoprotein particle. Lipoprotein (VLDL), which contains PC as an exclusively secreted from the liver in the form of very low density lipoprotein (VLDL) and triglyceride concentration, whereas betaine supplementation significantly decreased DMG concentration as shown in experiment 1. The mechanism by which hepatic betaine status influences DMG concentration is currently uncertain, but it seems reasonable to assume that such a mechanism is associated with the effect of combination of choline deprivation and folate deprivation. The fact that there existed a significant positive correlation between hepatic DMG concentrations and plasma homocysteine concentrations suggests that hepatic DMG concentration is an important variable for alterations of plasma homocysteine concentration, especially under the condition of folate deficiency.

Most of the hepatic fatty infiltrations caused by nutritional treatments, e.g., choline deficiency, are due to PC deficiency (39). So, we measured hepatic triglyceride concentration as an index to determine whether choline deprivation and folate deprivation interactively affect the status of PC or its precursor choline. The combination of choline deprivation and folate deprivation induced development of fatty liver, while single deprivation of choline or folate did not, indicating that deprivation of both choline and folate enhances hepatic triglyceride concentration even when a relatively high level of methionine is contained in the diet. Triglyceride is secreted from the liver in the form of very low density lipoprotein (VLDL), which contains PC as an exclusively major surface phospholipid of the lipoprotein particle. Therefore, active synthesis of PC is essential for the secretion of VLDL (48). The fatty liver observed in rats fed 20CCDFD appears to be also explained by the depression of PC synthesis. Although there are two pathways for PC synthesis, i.e., the CDP-choline pathway and PE N-methylation pathway (46, 47), hepatic PC synthesis mainly depends on the PE N-methylation pathway when choline is not supplied from the diet. Hence, it is reasonable to assume that marked decreases in hepatic SAM concentration and the SAM : SAH ratio and an increase in SAH concentration in rats fed 20CCDFD might suppress PC synthesis via the PE N-methylation pathway and thereby cause development of fatty liver.

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