# Studies on the Roles of Folate and Betaine in the Metabolism of Homocysteine

(ホモシステイン代謝における葉酸とベタインの役割に関する研究)

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## ABBREVIATIONS

| | |
|---|---|
| **10C** | 10% casein diet |
| **10CCD** | choline-deprived 10C |
| **10CFD** | folate-deprived 10C |
| **20C** | 20% casein diet |
| **20CCDFD** | choline and folate-deprived 20C |
| **20CFD** | folate-deprived 20C |
| **25S** | 25% soybean protein diet |
| **25SCD** | choline-deprived 25S |
| **BHMT** | betaine-homocysteine *S*-methyltransferase |
| **CBS** | cystathionine β-synthase |
| **DMG** | *N,N*-dimethylglycine |
| **PC** | phosphatidylcholine |
| **PE** | phosphatidylethanolamine |
| **MS** | methionine synthase |
| **SAH** | *S*-adenosylhomocysteine |
| **SAM** | *S*-adenosylmethionine |
| **5-MTHF** | 5-methyltetrahydrofolate |
| **VLDL** | very low density lipoprotein |

# CONTENTS

# GENERAL INTRODUCTION

Homocysteine is a sulfur-containing amino acid. It is a homologue of the amino acid cysteine, differing by an additional methylene group. The total homocysteine levels in plasma has been reported to be in the range of 5-15μM in healthy individuals (*1*). Of this total, only 1% to 2% occurs as the thiol homocysteine. The remaining 98% is the form of disulfides. Perhaps 75% of the total is bound to protein through disulfide bonds with protein cysteines, mainly in albumin, whereas the remainder occurs in non-protein-bound forms: homocysteine, homocysteine-cysteine disulfide, and more minor amounts of other mixed disulfides, e.g., homocysteine-cysteinylglycine disulfide (*2*). Hyperhomocysteinemia is a medical condition characterized by a high level of homocysteine in human blood. It is defined as mild (15-30μM), moderate (30-100μM) and severe (>100μM) (*1*).

A number of studies have suggested that hyperhomocysteinemia might be an independent risk factor for cardiovascular and peripheral vascular disease, including atherosclerotic, stroke, dementia and Alzheimer's disease (*1*, *3-6*). It has been though that hyperhomocysteinemia influences atherosclerotic process by duplicate mechanisms: (1) Homocysteine increases superoxide anion production in the vascular wall via NADH/NADPH oxidase, which leads to endothelial dysfunction (*7*). (2) Homocysteine auto-oxidized with trace metal ions, generating reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxylradical. This process has also been shown to promote oxidation of LDL (low density lipoprotein). Oxidized LDL has many characteristic that potentially promote atherogenesis (*8-10*). (3) Homocysteine may induce atherosclerosis by affecting endothelial-derived relaxing factor, nitric oxide (NO). Homocysteine enhances lipid peroxidation which may decrease the expression of endothelial NO synthase and directly degrade NO. Decreased NO bioavailability has been shown to be predictive of cardiovascular events (*11-13*). (4) Homocysteine was also shown to produce endothelial dysfunction and stimulate the proliferation of vascular smooth muscle cells and other cells.

It is also increased the formation of extracellular matrix, which may activate or promote the sclerotic process in vessel walls and other tissues (*14-16*).

Homocysteine is a normal intermediate of methionine metabolism (*17*). It is normally metabolized by two pathways, i.e. remethylation and transsulfuration (Fig. 1). Plasma homocysteine concentration is affected by various factors including genetic, nutritional, physiological, clinical and lifestyle factors (*1,3,4*). Of these factors, genetic and nutritional factors are thought to have a greater influence on plasma homocysteine concentration. For instance, genetic defects in homocysteine metabolism may cause hyperhomocysteinemia, such as 5,10-methylenetetrahydrofolate reductase (MTHFR) deficiency affect the remethylation of homocysteine to methionine, which leads to hyperhomocysteinemia (*18*). And an inborn error of homocystinura dues to Cystathionine-β-synthase (CBS) deficiency, which affect the transsulfuration pathway of homocysteine leads to hyperhomocysteinemia (*19*). Furthermore, several reports has been shown that deficiencies of some vitamins such as folate, vitamin B-12 and vitamin B-6 cause hyperhomocysteinemia, since folate and vitamin B-12 are co-factors of methionine synthase (MS) activity and vitamin B-6 is a cofactor of the CBS activity (*1,3,4*). We also have demonstrated that deprivation of choline, a vitamin-like compound, induces hyperhomocysteinemia due to betaine deficiency (*20*). 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. A number of studies on the plasma homocysteine-lowing effect of betaine in human subjects have been reported (*21, 22*). The effects of betaine in hyperhomocysteinemic animal models have also been reported (*23-27*). The efficacy of betaine is based on the mechanism by which the compound increases hepatic betaine concentration and BHMT activity (*28*) and there by stimulates homocysteine removal by the betaine-BHMT system. There also have been a number of studies on the effect of administration or dietary supplementation with folate with the aim of decreasing plasma

homocysteine concentration in humans and animal models (*29-36*). However, the mechanism by which folate supplementation reduces the concentration of homocysteine has not yet been clarified.

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*-methionine synthase (BHMT). Recently, we paid attention to the relationship between the BHMT pathway and the MS pathway. The two pathways are separately but closely related. Some investigations suggested that the BHMT pathway is connected with the MS pathway through *N, N*-dimethylglycine (DMG). It has been reported that the betaine-BHMT system requires folate, since tetrahydrofolate participate in the metabolism of DMG and *N*-methylglycine as a methyl-group acceptor (*37*). DMG is not only a product of the BHMT reaction but also an inhibitor of BHMT (*38,39*). Thus we assumed that folate deficiency might cause decreased consumption of DMG, which might affect the BHMT pathway. In fact, it has been reported that serum DMG concentration was significantly increased by folate deficiency, but not by vitamin B-12 deficiency, in human subjects (*40,41*). However, litter information is currently available concerning that the specific roles and compensatory relationship between the BHMT pathway and the MS pathway.

Therefore, in the present study, we investigated the roles of folate and betaine in the metabolism of homocysteine. For this purpose, we firstly investigated the effects of betaine supplementation and choline deficiency on folate deficiency-induced hyperhomocysteinemia to determine whether folate deficiency-induced hyperhomocysteinemia could be suppressed by betaine through the hepatic betaine-BHMT system. Since folate deficiency-induced hyperhomocysteinemia and the supplemental effect of betaine were anticipated to differ depending on dietary casein level, 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. Then we secondly investigated the effects of dietary supplementation with folate on choline deprivation-induced hyperhomocysteinemia to determine whether the other remethylation pathway (the betaine-BHMT pathway) impaired-induced hyperhomocysteinemia could be suppressed by folate status. Since choline deprivation of low methionine diet such as 10% casein (10C) and 25% soybean protein (25S) diet resulted in obvious hyperhomocysteinemia (*20*), we investigated the effects of dietary supplementation with folate on choline deprivation-induced hyperhomocysteinemia in rats fed 10C and 25S diet with or without serine, a main source of C1 units for 5-MTHF (*42*).

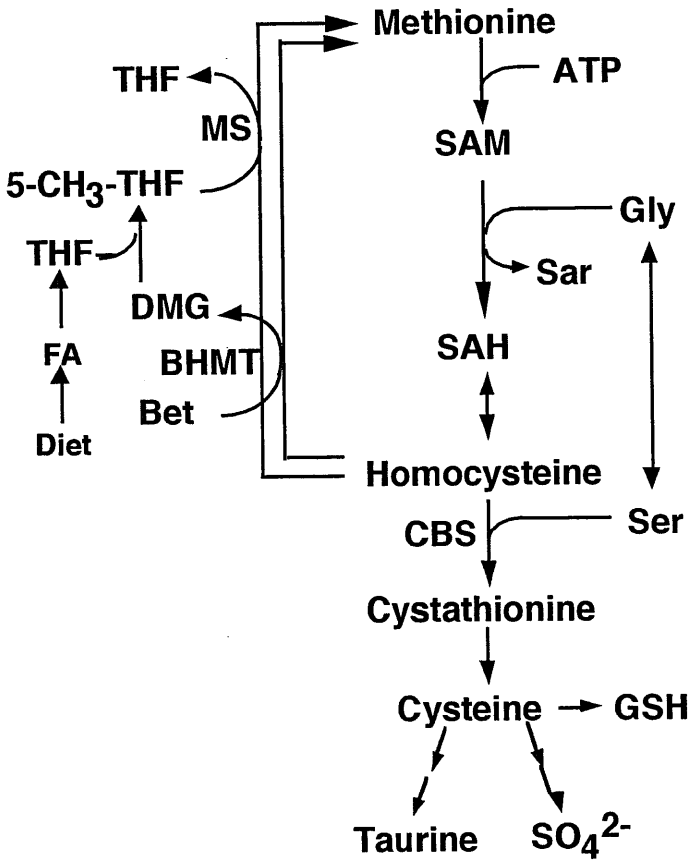


**Fig. 1** Metabolism of methionine and homocysteine. BHMT, betaine-homocysteine *S*-methyltransferase (EC 2.1.1.5); CBS, cystathionine b-synthase (EC 4.2.1.22); DMG, *N*, *N*-dimethylglycine; FA, folic acid; MS, methionine synthase (EC 2.1.1.13); 5-MTHF, 5-methyltetrahydrofolate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; Sar, sarcosine (*N*-methlglycine); Ser, serine; THF, tetrahydrofolate.

# CHAPTER I

## Effects of Betaine Supplementation and Choline Deficiency on Folate Deficiency-induced Hyperhomocysteinemia in rats

## 1.1 Introduction

Homocysteine is a normal intermediate of methionine metabolism (*17*) (Fig. 1.1), but a number of studies have suggested that an elevated plasma homocysteine concentration might be an independent risk factor for cardiovascular disease (*1,3,4*). 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 (*1,3,4*). 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 (*1,3,4*). Furthermore, deprivation of choline, a vitamin-like compound, also induces hyperhomocysteinemia due to betaine deficiency (*20*). 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 (*21,22*). The effects of betaine in hyperhomocysteinemic animal models have also been reported (*23-27*). The efficacy of betaine is based on the mechanism by which the compound increases hepatic betaine concentration and BHMT activity (*28*) and thereby stimulates homocysteine removal by the betaine-BHMT system.

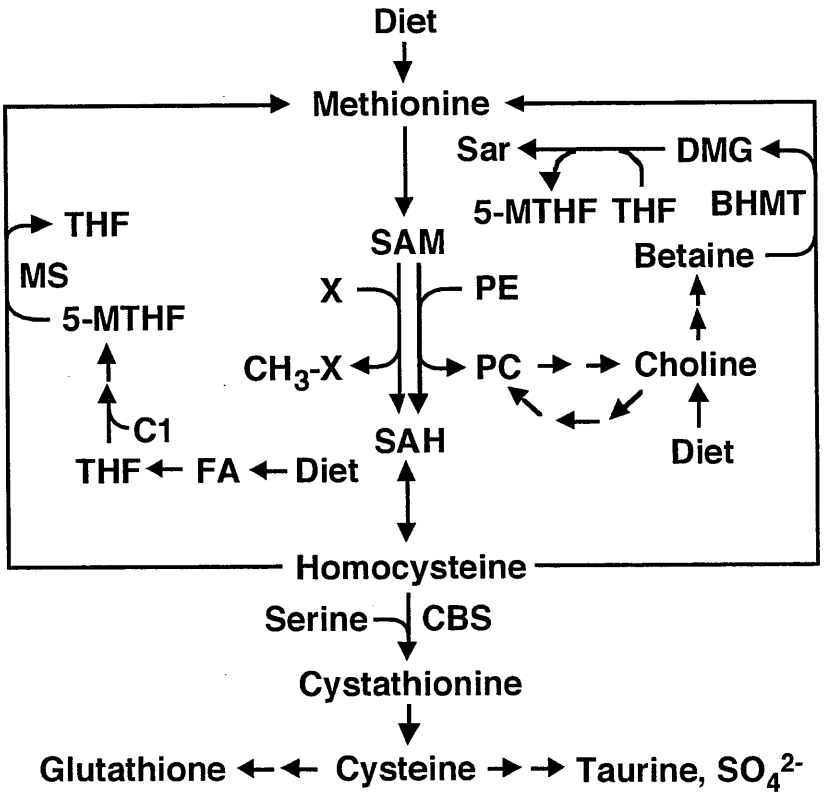


**Fig. 1.1** Metabolism of methionine and homocysteine. BHMT, betaine-homocysteine *S*-methyltransferase (EC 2.1.1.5); CBS, cystathionine b-synthase (EC 4.2.1.22); DMG, *N*, *N*-dimethylglycine; FA, folic acid; MS, methionine synthase (EC 2.1.1.13); 5-MTHF, 5-methyltetrahydrofolate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; Sar, sarcosine (*N*-methlglycine); THF, tetrahydrofolate.

One of the representative experimental hyperhomocysteinemia models in rodents is a folate deficiency model (*43,44*). It has been thought that folate deficiency induces hyperhomocysteinemia by duplicate mechanisms (*1,43*): (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 (*45*). However, these explanations raise the question of why folate deficiency-induced decrease in homocysteine metabolism cannot be fully compensated 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 (*46,47*). This issue appears to be solved 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 (*37*), 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 (*38,39*). In fact, serum DMG concentration was significantly increased by folate deficiency, but not by vitamin B-12 deficiency, in human subjects (*40,41*). McGregor et al. (*48*) 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). In addition, we investigated the effect of choline deprivation in rats fed folate-sufficient and folate-deprived standard casein diets (experiment 2).

## 1.2 Materials and Method

### 1.2.1 Chemicals

Betaine and folic acid were purchased from Sigma-Aldrich (St. Louis, Mo). Succinylsulfathiazole was purchased from MP Biomedicals (Irvine, Cal). All other chemicals were purchased from Wako Pure Chemical (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 (Tokyo). Other ingredients of the diet were purchased from Wako.

### 1.2.2 Animals and diets

Six-week-old male rats (120-140 g) of the Wistar strain were obtained from Japan SLC (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 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 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 (*49*). Folate-sufficient diets (10C, 20C, and 20CCD) contained folate at a level of 8 mg/kg, a four-fold level of AIN-93, to make clear the effect of folate deficiency according to a previous report (*49*). 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 (*50*). 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.

### 1.2.3 Tissue collection and fractionation

Blood plasma was separated from heparinized whole blood by centrifugation at 2,000 x *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 x *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 x *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.* (*51*).

### 1.2.4 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. (*52*). 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. (*53*). The concentrations of 5-MTHF in the plasma and liver were measured by HPLC by the method of Shimoda et al. (*54*). The concentrations of betaine and *N,N*-dimethylglycine (DMG) in the liver were measured by HPLC following Laryea et al. (*55*) 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. (*56*). The activity of BHMT in the liver was measured following Finkelstein et al. (*57*), but HPLC was used in the assay of the reaction product, DMG, following Laryea et al. (*55*). The activity of CBS in the liver was measured following Mudd et al. (*58*), but HPLC was used in the assay of the reaction product, cystathionine, following Einarsson et al. (*59*). 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. (*60*) using bovine serum albumin as a standard.

### 1.2.5 Statistical analysis

Each value is expressed as the mean ± SEM. 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).

## 1.3 Results

### 1.3.1 Effect of betaine supplementation (experiment 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 (Table 1.1). 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%) (Fig. 1.2, panel A). Betaine supplementation significantly suppressed folate deprivation-induced increase in plasma total homocysteine concentration in both rats fed 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 (*2*). Folate deprivation and betaine supplementation affected the concentrations of both types of homocysteine similarly to that of total homocysteine (Fig. 1.2, panels B and C). Plasma total cysteine concentration was significantly higher in rats fed 20C than in rats fed 10C irrespective of folate deprivation and betaine supplementation (Fig. 1.2, panel D). Plasma 5-MTHF concentration, measured as an index of folate status (*61*), was greatly decreased in rats fed folate-deprived diets irrespective of dietary casein level and betaine supplementation (Fig. 1.2, panel E). Hepatic SAM concentration was significantly decreased by folate deprivation and was restored by betaine supplementation in both rats fed 10C and those fed 20C (Fig. 1.3, panel A). Hepatic SAH concentration was increased or tended to be increased by folate deprivation and was further increased by betaine supplementation (Fig. 1.3, panel B). 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 (Fig. 1.3, panel C). Hepatic homocysteine concentration was significantly increased by folate deprivation and this increase was suppressed or tended to

be suppressed by betaine supplementation (Fig. 1.3, panel D).

Hepatic MS activity was significantly decreased by folate deprivation and unaffected by betaine supplementation in both rats fed 10C and those fed 20C (Fig. 1.4, panel A). Hepatic concentration of 5-MTHF, a substrate of MS, was greatly decreased by folate deprivation and unaffected by betaine supplementation (Fig. 1.4, panel B). Hepatic BHMT activity was unaffected by folate deprivation and significantly increased by betaine supplementation in both rats fed 10C and those fed 20C (Fig. 1.4, panel C). 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 supplementation (Fig. 1.4, panel D). Hepatic CBS activity was significantly higher in rats fed 20C than in rats fed 10C (Fig. 1.4, panel E). Folate deprivation decreased or tended to decrease the enzyme activity, although 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 (Fig. 1.4, panel F). Hepatic DMG concentration was greatly increased by folate deprivation in both rats fed 10C and those fed 20C (Fig. 1.5, panel A). 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 (Fig. 1.5, panel B). 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. 1.5, panel C).

**Table 1.1**

Body weight gain, food intake and liver weight of rats fed the experimental diets (experiment 1)

| Diet | Body wt gain | Food intake | Liver wt |
|---|---|---|---|
| | *g/28 d* | | *g/100 g body wt* |
| 10C | $75 \pm 4^{b,1}$ | $460 \pm 17$ | $3.55 \pm 0.07^{b}$ |
| 10CFD | $66 \pm 4^{b}$ | $436 \pm 13$ | $3.69 \pm 0.04^{b}$ |
| 10CFD + 1% Bet | $60 \pm 4^{b}$ | $408 \pm 17$ | $3.64 \pm 0.04^{b}$ |
| 20C | $100 \pm 3^{a}$ | $461 \pm 12$ | $4.28 \pm 0.06^{a}$ |
| 20CFD | $107 \pm 4^{a}$ | $448 \pm 19$ | $4.31 \pm 0.12^{a}$ |
| 20CFD + 1% Bet | $95 \pm 5^{a}$ | $410 \pm 16$ | $4.25 \pm 0.06^{a}$ |

[1]Each value is the mean ± SEM, $n = 8$. Values without a common letter differ, $P < 0.05$. 10C and 20C, 10% casein diet and 20% casein diet, respectively; 10CFD and 20CFD, folate-deprived 10C and 20C, respectively; Bet, betaine.

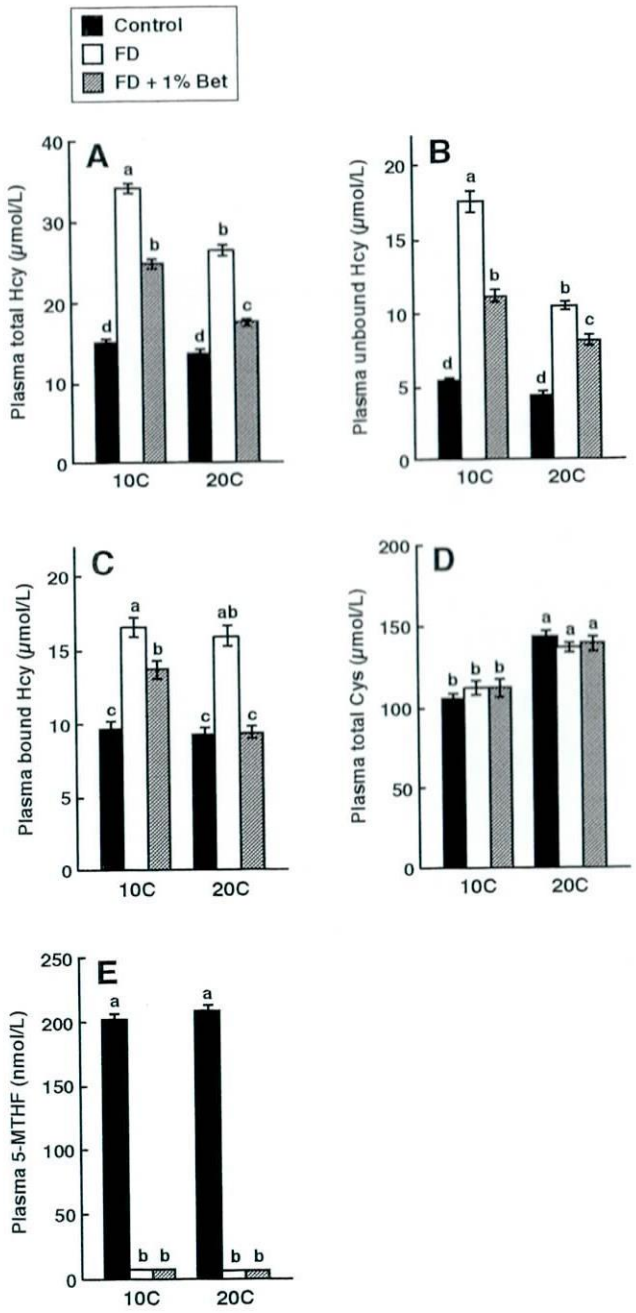


**Fig. 1.2** Effects of supplementation of folate-deprived diets with betaine (1%) on plasma concentration and other variables in rats fed 10% and 20% casein diets (experiment 1). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. 10C and 20C, 10% casein diet and 20% casein diet, respectively; Bet, betaine; bound Hcy, protein-bound homocysteine; FD, folate-deprived; Hcy, homocysteine; 5-MTHF, 5-methyltetrahydrofolate; unbound Hcy, protein-unbound homocysteine.

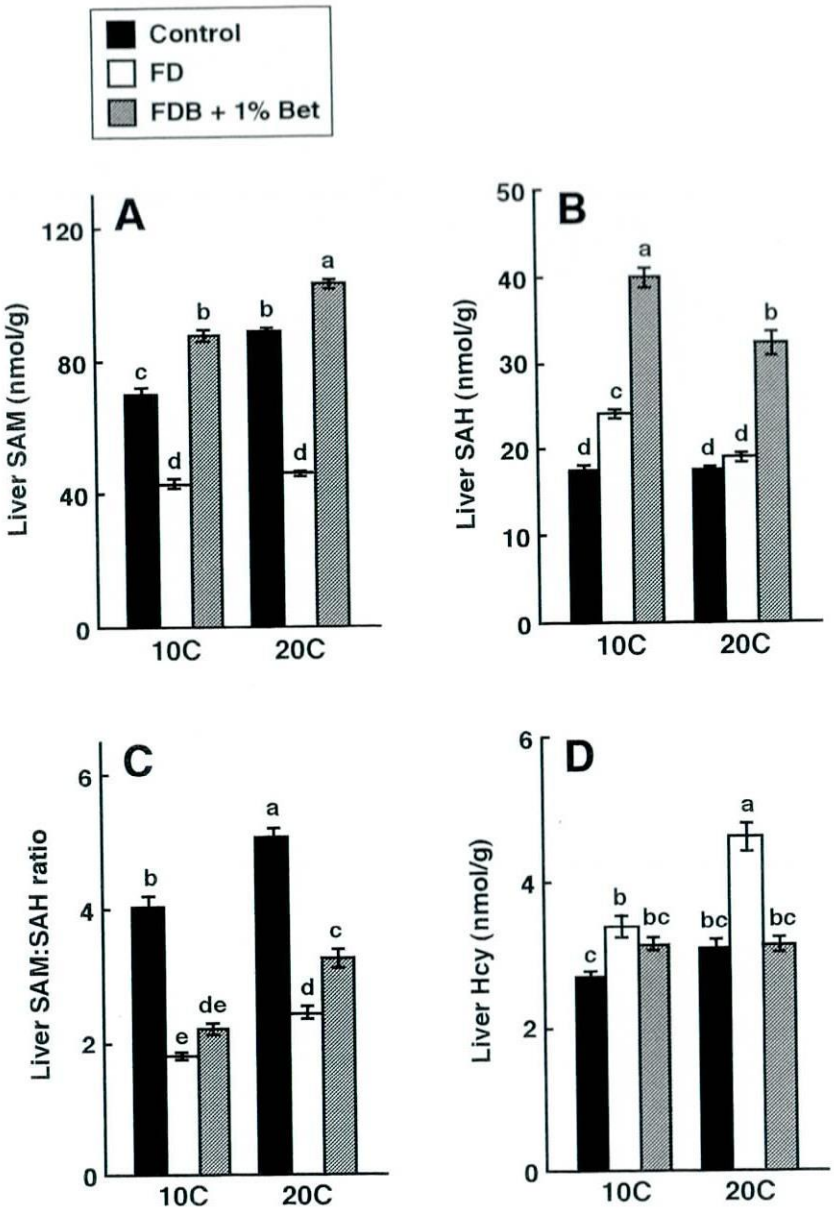


**Fig. 1.3** Effects of supplementation of folate-deprived diets with betaine (1%) on hepatic concentrations of *S*-adenosylmethionine (A), *S*-adenosylhomocysteine (B), their ratio (C), and homocysteine (D) in rats fed 10% and 20% casein diets (experiment 1). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine. See the legend of Fig. 2 for other abbreviations.

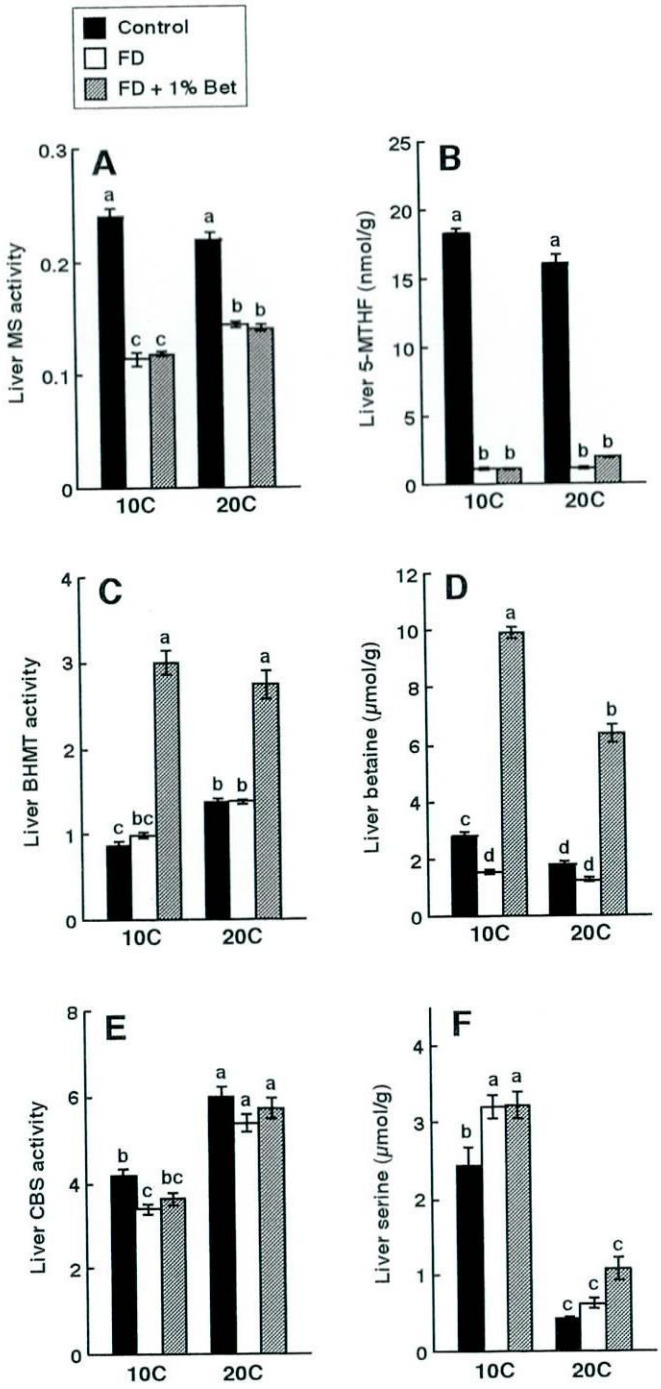


**Fig. 1.4** Effects of supplementation of folate-deprived diets with betaine (1%) on hepatic activities of enzymes of homocysteine metabolism and hepatic concentrations of enzyme substrates in rats fed 10% and 20% casein diets (experiment 1). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. BHMT, betaine-homocysteine *S*-methyltransferase; CBS, cystathionine β-synthase, MS, methionine synthase. See the legend of Fig. 2 for other abbreviations.

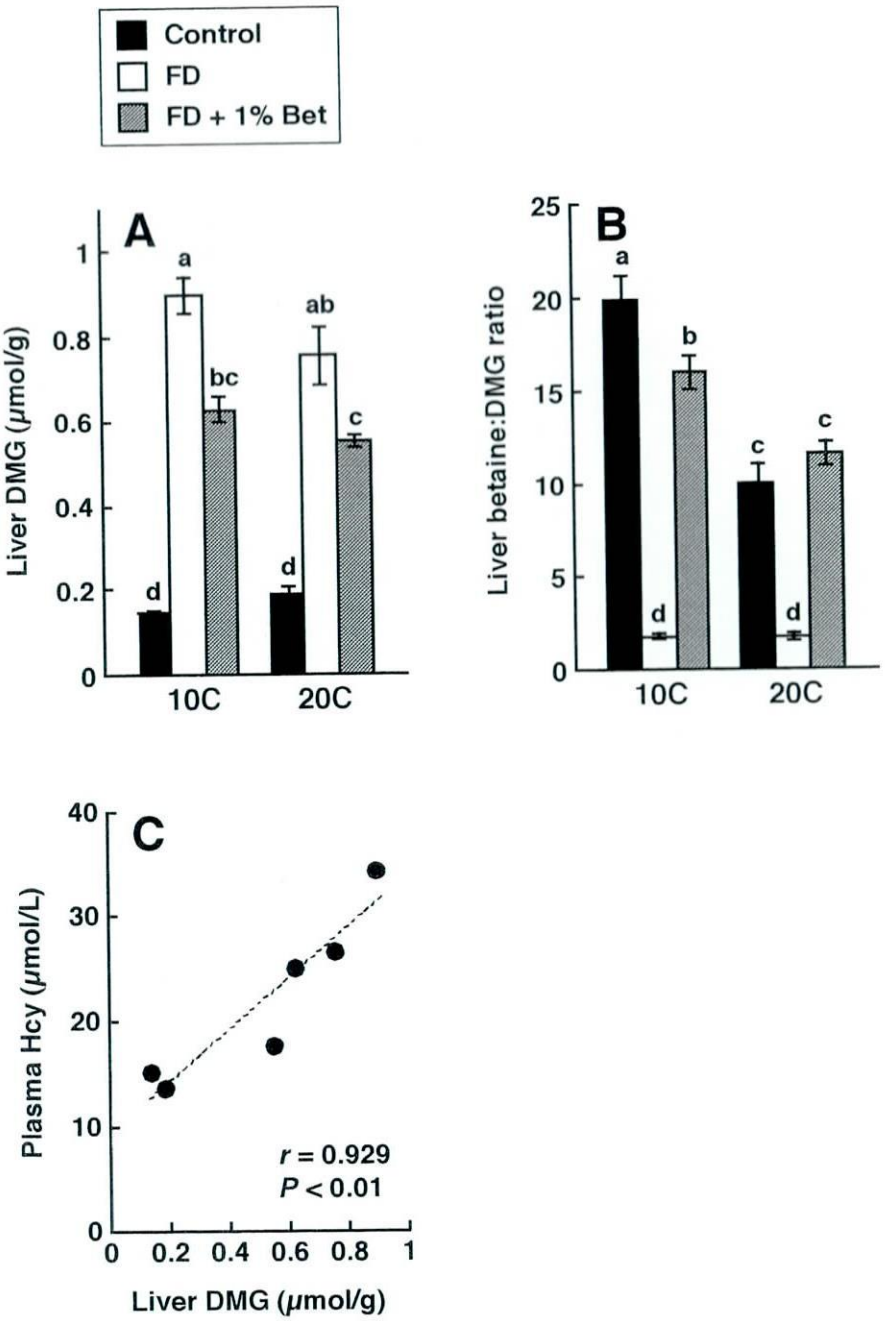


**Fig. 1.5** Effects of supplementation of folate-deprived diets with betaine (1%) on hepatic DMG concentration (A), betaine:DMG ratio (B), and relationship between hepatic DMG concentration and plasma homocysteine concentration (C) in rats fed 10% and 20% casein diets (experiment 1). Each value is the mean ± SEM, $n = 8$ (panels A and B). In panel C, each value represents the mean value. Means in a panel (A and B) without a common letter differ, $P < 0.05$. DMG, *N,N*-dimethylglycine.

### 1.3.2 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. The results are summarized in Table 1.2. Body weight gain and food intake did not differ among the four groups. Liver weight was significantly higher 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 diet 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, SAM:SAH ratio 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 CBS activity was significantly 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. 1.6).

## Table 1.2

Effects of choline and/or folate deprivation on plasma homocysteine concentration and other variables in rats fed a 20% casein diet (experiment 2)

| | Diet | | | | ANOVA[2] |
|---|---|---|---|---|---|
| | 20C | 20CCD | 20CFD | 20CCDFD | |
| Body wt gain, *g/28 d* | 98 ± 4[1] | 107 ± 5 | 105 ± 3 | 99 ± 3 | CF |
| Food intake, *g/28 d* | 452 ± 18 | 467 ± 14 | 452 ± 19 | 454 ± 8 | |
| Liver wt, *g/100 g body wt* | 4.27 ± 0.07$^{ab}$ | 4.19 ± 0.10$^{b}$ | 4.36 ± 0.13$^{ab}$ | 4.62 ± 0.08$^{a}$ | F |
| Plasma: | | | | | |
| Total Hcy, *μmol/L* | 13.5 ± 0.4$^{c}$ | 14.6 ± 0.5$^{c}$ | 26.6 ± 0.8$^{b}$ | 45.4 ± 0.7$^{a}$ | C, F, CF |
| Unbound Hcy, *μmol/L* | 4.3 ± 0.3$^{d}$ | 6.6 ± 0.3$^{c}$ | 10.3 ± 0.2$^{b}$ | 25.4 ± 0.8$^{a}$ | C, F, CF |
| Bound Hcy, *μmol/L* | 9.1 ± 0.3$^{c}$ | 8.0 ± 0.5$^{c}$ | 16.3 ± 0.8$^{b}$ | 20.0 ± 1.0$^{a}$ | F, CF |
| Unbound Hcy, *%* | 32.1 ± 1.9$^{c}$ | 45.3 ± 2.2$^{b}$ | 39.0 ± 1.3$^{ab}$ | 56.0 ± 1.8$^{a}$ | C, F |
| Bound Hcy, *%* | 67.9 ± 1.9$^{a}$ | 54.7 ± 2.2$^{b}$ | 61.0 ± 1.3$^{ab}$ | 44.0 ± 1.8$^{c}$ | C, F |
| Total Cys, *μmol/L* | 146 ± 3$^{a}$ | 131 ± 2$^{b}$ | 136 ± 3$^{ab}$ | 119 ± 2$^{c}$ | C, F |
| 5-MTHF, *nmol/L* | 210 ± 4.1$^{a}$ | 198 ± 4.3$^{b}$ | 6.4 ± 0.4$^{c}$ | 6.6 ± 0.5$^{c}$ | C, F, CF |
| Liver: | | | | | |
| SAM, *nmol/g* | 88.4 ± 1.3$^{a}$ | 49.2 ± 2.4$^{b}$ | 46.4 ± 0.9$^{b}$ | 21.7 ± 1.0$^{c}$ | C, F, CF |
| SAH, *nmol/g* | 17.3 ± 0.4$^{bc}$ | 15.0 ± 0.4$^{c}$ | 19.1 ± 0.5$^{b}$ | 29.8 ± 1.2$^{a}$ | C, F, CF |
| SAM:SAH ratio | 5.13 ± 0.12$^{a}$ | 3.27 ± 0.10$^{b}$ | 2.44 ± 0.09$^{c}$ | 0.73 ± 0.05$^{d}$ | C, F |
| Hcy, *nmol/g* | 3.1 ± 0.1$^{c}$ | 3.1 ± 0.1$^{c}$ | 4.5 ± 0.2$^{b}$ | 7.4 ± 0.4$^{a}$ | C, F, CF |
| MS activity[3] | 0.218 ± 0.06$^{a}$ | 0.201 ± 0.005$^{a}$ | 0.145 ± 0.003$^{b}$ | 0.090 ± 0.004$^{c}$ | C, F, CF |
| BHMT activity[3] | 1.36 ± 0.05 | 1.29 ± 0.03 | 1.37 ± 0.03 | 1.39 ± 0.07 | |
| CBS activity[3] | 6.02 ± 0.24$^{a}$ | 5.26 ± 0.24$^{a}$ | 5.29 ± 0.17$^{a}$ | 3.29 ± 0.13$^{b}$ | C, F, CF |
| 5-MTHF, *nmol/g* | 16.5 ± 0.4$^{a}$ | 15.1 ± 0.5$^{b}$ | 1.1 ± 0.1$^{c}$ | 1.0 ± 0.1$^{c}$ | C, F |
| Betaine, *μmol/g* | 1.72 ± 0.12$^{a}$ | 1.32 ± 0.08$^{b}$ | 1.24 ± 0.07$^{b}$ | 0.84 ± 0.04$^{c}$ | C, F |
| Serine, *μmol/g* | 0.43 ± 0.02$^{b}$ | 0.52 ± 0.07$^{b}$ | 0.62 ± 0.06$^{b}$ | 1.07 ± 0.10$^{a}$ | C, F, CF |
| DMG, *μmol/g* | 0.20 ± 0.02$^{c}$ | 0.19 ± 0.03$^{c}$ | 0.79 ± 0.08$^{b}$ | 1.54 ± 0.05$^{a}$ | C, F, CF |
| Betaine:DMG ratio | 9.38 ± 1.12$^{a}$ | 8.16 ± 1.28$^{a}$ | 1.69 ± 0.21$^{b}$ | 0.55 ± 0.03$^{b}$ | F |
| Triglyceride, *μmol/g* | 23.1 ± 0.4$^{b}$ | 25.7 ± 0.5$^{b}$ | 26.3 ± 0.3$^{b}$ | 61.3 ± 1.9$^{a}$ | C, F, CF |

[1]Each value is the mean ± SEM, $n = 8$. Values without a common letter differ, $P < 0.05$. 20C, 20% casein diet; 20CCD, choline-deprived 20C; 20CFD, folate-deprived 20C; 20CCDFD, choline and folate-deprived 20C. See the legend of Table 1 for other abbreviations.

[2]Two-way ANOVA; C, affected by choline deprivation, $P < 0.05$; F, affected by folate deprivation, $P < 0.05$; CF, interactively affected by choline deprivation and folate deprivation, $P < 0.05$.

[3]Expressed as nmol/(min·mg protein).

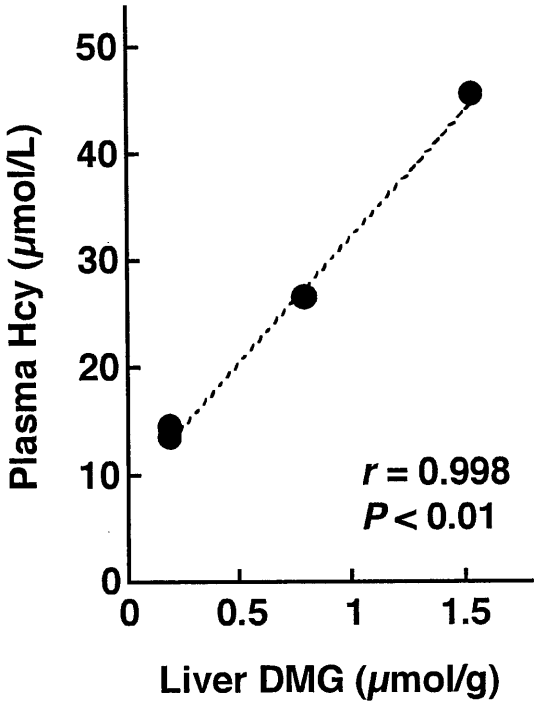


**Fig. 1.6** Relationship between hepatic *N,N*-dimethylglycine concentration and plasma homocysteine concentration in rats fed the experimental diets (experiment 2). Each closed circle represents the mean value of the group. DMG, *N,N*-dimethylglycine.

## 1.4 Discussion

In the present study, we used vitamin-free casein, folate-free vitamin mixture and antibiotic succinylsulfathiazole to induce convenient folate deficiency. The magnitude of hyperhomocysteinemia is defined as mild (15-30 $\mu$M), moderate (30-100 $\mu$M) and severe (> 100 $\mu$M) (*1*). Hence, folate deprivation-induced hyperhomocysteinemia observed in the present study was moderate (34.1 $\mu$M) and mild (26.5 $\mu$M) 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 (*62,63*). Furthermore, guanidinoacetic acid-induced hyperhomocysteinemia was also suppressed by raising dietary casein level (*64*). 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 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 (*65*). In any case, the present study suggests that rats fed diets containing higher levels of casein are less susceptible to folate deficiency.

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 (*1*). 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. (*66*) 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, 1.55 ± 0.07 $\mu$mol/g in the 10CFD group and 1.23 ± 0.08 $\mu$mol/g in the 20CFD group, were still one-order higher than the reported Km value of rat BHMT for betaine, 120 $\mu$M (*67*). 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. (*38*) 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, 0.90 ± 0.04 $\mu$mol/g in the 10CFD group and 0.76 ± 0.07 $\mu$mol/g in the 20CFD group, might strongly inhibit BHMT reaction in vivo, although hepatic DMG concentrations in control groups, 0.14 ± 0.01 $\mu$mol/g in the 10C group and 0.19 ± 0.02 $\mu$mol/g in the 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% (*27*) and that choline deprivation-induced hyperhomocysteinemia was completely suppressed by betaine supplementation at a level of 0.28% (*20*). Therefore, 1% supplementation level of betaine used in the present study appears to be high enough to elicit its effect, although a dose-response experiment is needed to confirm this. 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 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 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 existence of a significant correlation between hepatic DMG concentrations and plasma homocysteine concentrations among the six experimental groups (Fig. 1.5, panel C) supports such a possibility. On the other hand, the present study showed that betaine supplementation completely restored folate deprivation-induced decrease in hepatic SAM concentration and significantly increased hepatic SAH concentration, while hepatic homocysteine concentration was decreased or tended to be decreased (Fig. 1.3). These results suggest that methionine synthesis by the betaine-BHMT system might be significantly enhanced by betaine

supplementation. Hence, the possibility of another mechanism underlying the insufficient effect of betaine supplementation on plasma homocysteine concentration cannot be excluded. This remains to be clarified by further studies.

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 (*68*). 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 SAM:SAH ratio (*69-71*). 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 (*20*). 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 $\mu$M) in rats fed 20C, while choline deprivation alone did not increase plasma homocysteine concentration and folate deprivation alone caused only mild hyperhomocysteinemia (26.6 $\mu$M). This indicates that choline deprivation and folate deprivation synergistically 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 deprivation synergistically affected several variables in the liver, e.g., SAM, SAH and homocysteine concentrations, MS and CBS activities, and DMG and triglyceride

concentrations. Some of these changes are thought to be associated with the increase in plasma homocysteine concentration. For instance, decreased MS and CBS activities are unfavorable for homocysteine metabolism, and increased DMG concentration results in inhibition of BHMT reaction. Although hepatic betaine concentration also decreased in rats fed 20CCDFD, the betaine concentration, 0.84 $\mu$mol/g, was still higher than the Km value of BHMT for betaine as described above. However, the betaine-BHMT system might not be functional in vivo in rats fed 20CCDFD, since hepatic SAM concentration markedly decreased and, conversely, hepatic SAH and homocysteine concentrations significantly increased in such rats. This assumption suggests that hepatic betaine consumption decreased and thereby betaine concentration was maintained at relatively high level in rats fed 20CCDFD. It is likely that higher hepatic DMG concentration inhibited BHMT reaction in vivo in rats fed 20CCDFD as well as in rats fed 20CFD. It is of interest that folate deprivation-induced increase in hepatic DMG concentration was reinforced by choline deprivation, a treatment that decreased hepatic betaine 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 synergistic effect of choline deprivation and folate deprivation. The fact that there existed a significant positive correlation between hepatic DMG concentrations and plasma homocysteine concentrations (Fig. 1.6) suggests that hepatic DMG concentration is one of the key variables for alterations of plasma homocysteine concentration, especially under the condition of folate deficiency.

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 synergistically enhances hepatic triglyceride concentration even when a relatively high level of methionine is contained in the diet. Most of the hepatic fatty infiltrations caused by nutritional treatments, e.g., choline deficiency, are due to PC

deficiency (*62*). 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 (*71*). 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 (*69,70*), 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 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.

# CHAPTER Ⅱ

## Effect of Dietary Supplementation with Folate on Choline Deficiency -induced Hyperhomocysteinemia in rats

## 2.1 Introduction

A number of studies have suggested that an elevated plasma homocysteine concentration is an independent risk factor for cardiovascular disease (*1,3,4*). Plasma homocysteine is also a risk factor for the development of cognitive impairment and Alzheimer's disease (*72*). Of the many factors affecting plasma homocysteine concentration, nutritional and genetic factors are thought to have a greater influence on the concentration (*73*). Homocysteine is remethylated to methionine or condensed with serine to cystathionine (Fig. 2.1) (*17*). Homocysteine is remethylated either by methionine synthase (MS) using the methyl group of 5-methyltetrahydrofolate (5-MTHF) or by betaine-homocysteine *S*-methyltransferase (BHMT) using the methyl group of betaine. Cystathionine synthesis is catalyzed by cystathionine β-synthase (CBS). It has been shown that deficiencies of some vitamins such as folate, vitamin B-12, and vitamin B-6 cause hyperhomocysteinemia, since folate and vitamin B-12 are cofactors of MS and vitamin B-6 is a cofactor of CBS (*1,3,4*). We have demonstrated that choline deprivation of low methionine diets also cause hyperhomocysteinemia mainly due to the deficiency of betaine, which is formed from the vitamin-like compound choline (*20*).

When considering the metabolism of homocysteine, it is of interest to know whether impairment of one of the two remethylation pathways can be compensated by stimulating the other pathway. According to this line, we previously investigated the effect of dietary supplementation with betaine on the hyperhomocysteinemia induced by dietary folate deprivation in rats fed low (10%) casein diet (10C) or standard (20%) casein diet. The results showed that betaine significantly suppressed the hyperhomocysteinemia, but the effect was partial or limited despite supplemented betaine level being relatively high (1%). The results suggested that folate deficiency could not be fully overcome by betaine. There have been a number of studies on the effect of administration or dietary supplementation with folate with the aim of decreasing plasma homocysteine concentration, especially in

humans (*29-34*). However, there is little information on the effect of folate on betaine deficiency-induced hyperhomocysteinemia.

Therefore, in this study we investigated the effects of dietary supplementation with folate on choline deprivation-induced hyperhomocysteinemia in rats fed 10C and 25% soybean protein diet (25S). Since serine is thought to be a main source of C1 units for 5-MTHF (*42*), the effect of serine alone or serine in combination with folate was also investigated.

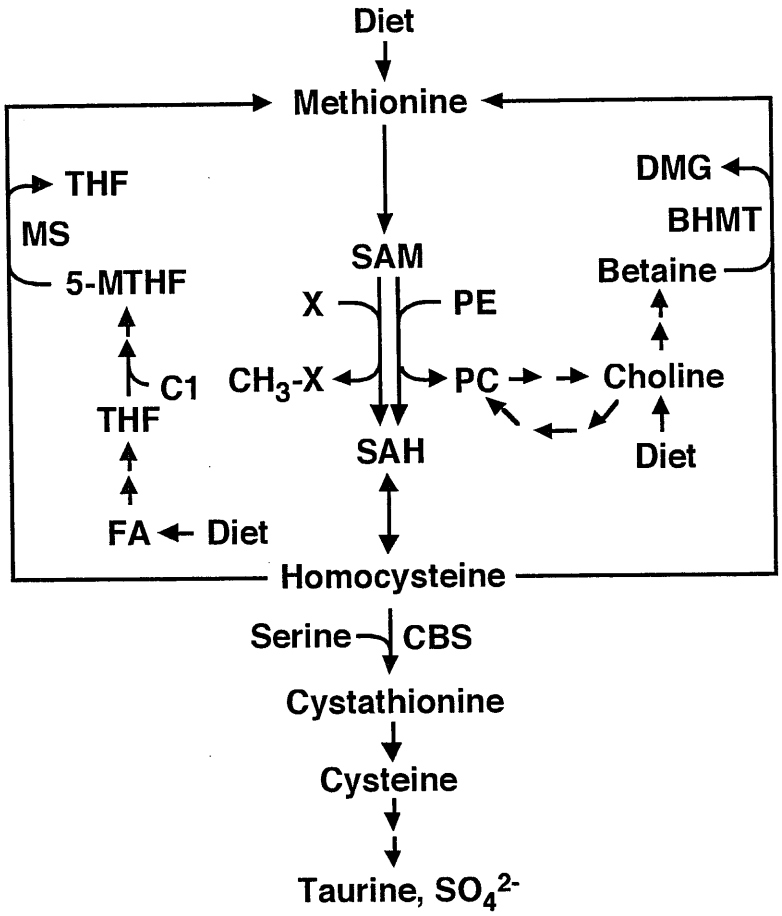


**Fig. 2.1** Metabolism of methionine and homocysteine. BHMT, betaine-homocysteine *S*-methyltransferase (EC 2.1.1.5); CBS, cystathionine β-synthase (EC 4.2.1.22); DMG, *N*, *N*-dimethylglycine; FA, folic acid; MS, methionine synthase (EC 2.1.1.13); 5-MTHF, 5-methyltetrahydrofolate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate.

## 2.2 Materials and Method

### 2.2.1 Chemicals

Folic acid and choline bitartrate were purchased from Sigma-Aldrich (St. Louis, Mo). All other chemicals were purchased from Wako Pure Chemical (Osaka, Japan) or Sigma-Aldrich and were of analytical grade. Vitamin-free casein, mineral mixture (AIN-93G), vitamin mixture (AIN-93), and cellulose powder were purchased from Oriental Yeast (Tokyo). Soybean protein isolate (SPI, Fujipro) was kindly supplied by Fuji Oil (Izumisano, Japan). Other ingredients of the diet were purchased from Wako.

### 2.2.2 Animals and diets

Six-week-old male rats (120-140 g) of the Wistar strain were purchased from Japan SLC (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, forty rats were randomly assigned to the following five diet groups: (1) 10C, (2) choline-deprived 10C (10CCD), (3) 10CCD + folate (20 mg/kg diet), (4) 10CCD + 2.5% L-serine, and (5) 10CCD + folate (20 mg/kg diet) + 2.5% L-serine. In experiment 2, forty rats were randomly assigned to the following five diet groups: (1) 25S, (2) choline-deprived 25S (25SCD), (3) 25SCD + folate (20 mg/kg diet), (4) 25SCD + 2.5% L-serine, and (5) 25SCD + folate (20 mg/kg diet) + 2.5% L-serine. The composition of 10C was as follows (g/kg): vitamin-free casein, 100; α-cornstarch, 582.5; sucrose, 200; corn oil, 50; mineral mixture (AIN-93G), 35; vitamin mixture (AIN-93), 10; choline bitartrate, 2.5; cellulose powder, 20. In 25S, SPI was used at a level of 250 g/kg at the expense of cornstarch. In choline-deprived diets, choline bitartrate was omitted with increase in cornstarch. The

supplementation level of folate (20 mg/kg) was determined as a ten-fold level of AIN-93 (2 mg/kg). Rats were given free access to the experimental diets and water for 14 d and killed by decapitation between 10:00 and 11:00 h without prior food deprivation, since it has been shown that non-fasting plasma homocysteine concentration was liable to be affected by dietary treatment in humans (*50*). 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.

### 2.2.3 Tissue collection and fractionation

Blood plasma was separated from heparinized whole blood by centrifugation at 2,000 x *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 three 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 x *g* for 10 min at 4°C. The supernatant of the deproteinized liver homogenate was subjected to assays for methionine metabolites, betaine and serine. Another 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 x *g* for 10 min at 4°C. The supernatant was subjected to enzyme assays. The third portion of the liver was subjected to analysis of mRNA, and total mRNA was isolated using a kit, ISOGEN (Nippon Gene, Tokyo), according to manufacture's instructions.

### 2.2.4 Biochemical analysis

The concentrations of homocysteine and cysteine in the plasma and liver were measured by HPLC using the method of Durand et al. (*52*). The concentrations of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) in the liver were measured

by HPLC following Cook et al. (*53*). The betaine concentration in the liver was measured by HPLC following Laryea et al. (*55*). The concentrations of 5-MTHF in the plasma and liver were measured by HPLC using the method of Shimoda et al. (*54*). The serine concentration in the liver was measured by an amino acid autoanalyzer. The activity of BHMT in the liver was measured following Finkelstein et al. (*57*), but HPLC was used in the assay of the reaction product, DMG, following Laryea et al. (*55*). The activity of MS in the liver was measured following Huang et al. (*56*). The activity of CBS in the liver was measured following Mudd et al. (*58*), but HPLC was used in the assay of the reaction product, cystathionine, following Einarsson et al. (*59*). The amounts of mRNA for BHMT and CBS relative to β-actin in the liver were measured by quantitative real-time PCR analysis as described previously (*74*). The amount of mRNA for MS was also measured by the same method, where the validated probe and primer for MS (assay identification number: Rn00578368_ml) were pre-designated TaqMan Gene Expression Assay products (Applied Biosystems, Foster City, CA). The protein concentration was measured according to Lowry et al. (*60*) using bovine serum albumin as a standard.

### 2.2.5 Statistical analysis

Each value is expressed as the mean ± SEM. Data were analyzed by a one-way ANOVA, and differences among the experimental groups were analyzed by the Tukey test when the *F* value was significant. Statistical analysis was performed with Mac Tokei-Kaiseki software (version 1.5; Esumi, Tokyo).

## 2.3 Results

### 2.3.1 Effect on hyperhomocysteinemia induced by choline deprivation of 10C (experiment 1)

Body weight gain, food intake and liver weight did not differ among the experimental groups (Table 2.1). Plasma homocysteine concentration was significantly increased by choline deprivation from 15.75 ± 0.38 to 34.28 ± 0.59 μmol/L (Fig. 2.2, panel A). The choline deprivation-induced elevation of plasma homocysteine was significantly suppressed by supplementation with folate alone, serine alone, and folate plus serine to levels of 28.79 ± 0.73, 27.28 ± 0.46, and 25.72 ± 0.47μmol/L, respectively. The extents of increment suppression by folate alone, serine alone, and folate plus serine were 29.6, 37.8, and 46.2%, respectively. Plasma cysteine concentration was slightly higher or tended to be higher in rats fed 10CCD irrespective of supplements than in rats fed 10C (Fig. 2.2, panel B). Plasma 5-MTHF concentration, which is measured as an index of folate status within the body (*61*), was significantly increased by folate supplementation, although supplementation with serine alone also slightly increased the concentration (Fig. 2.2, panel C). Choline deprivation significantly decreased hepatic SAM concentration and SAM:SAH ratio and, conversely, increased hepatic SAH and homocysteine concentrations (Fig. 2.3). The increase in hepatic homocysteine concentration was significantly suppressed by supplementation with folate, serine or both, but choline deprivation-induced changes in SAM and SAH concentrations and SAM:SAH ratio were unaffected. Choline deprivation significantly decreased hepatic activities of BHMT and CBS but not that of MS (Fig. 2.4, panels A, C and E). Supplementation with folate irrespective of serine significantly increased or tended to increase these enzyme activities. Choline deprivation markedly decreased hepatic concentration of betaine, a substrate of BHMT, and this decrease was slightly suppressed by folate supplementation (Fig. 2.4, panel B). Hepatic concentration of 5-MTHF, a substrate of MS, was significantly increased by folate supplementation (Fig. 2.4, panel D). Hepatic concentration of serine, a substrate of CBS, was significantly increased by serine

supplementation (Fig. 2.4, panel F). The relative level of mRNA for MS in the liver was significantly increased by folate supplementation, there was no significant difference in relative levels of mRNA for BHMT and CBS among the experimental groups (Fig. 2.5).

**Table 2.1**

Body weight gain, food intake and liver weight of rats fed the experimental diets

| Diet | Body wt gain | Food intake | Liver wt |
|---|---|---|---|
| | *g/14 d* | *g/100 g body wt* | |
| Experiment 1 | | | |
| 10C | 42 ± 2[1] | 234 ± 7 | 4.08 ± 0.04 |
| 10CCD | 39 ± 3 | 239 ± 8 | 4.15 ± 0.06 |
| 10CCD + FA[2] | 43 ± 4 | 239 ± 7 | 4.18 ± 0.09 |
| 10CCD + Ser[3] | 42 ± 3 | 235 ± 4 | 3.94 ± 0.12 |
| 10CCD + FA[2] + Ser[3] | 40 ± 2 | 232 ± 9 | 3.98 ± 0.08 |
| Experiment 2 | | | |
| 25S | 50 ± 3 | 219 ± 7 | 3.89 ± 0.06[b] |
| 25SCD | 57 ± 2 | 230 ± 6 | 4.17 ± 0.07[a] |
| 25SCD + FA[2] | 53 ± 3 | 229 ± 6 | 4.10 ± 0.05[ab] |
| 25SCD + Ser[3] | 49 ± 2 | 212 ± 5 | 4.04 ± 0.05[ab] |
| 25SCD + FA[2] + Ser[3] | 48 ± 3 | 221 ± 6 | 4.04 ± 0.05[ab] |

[1]Each value is the mean ± SEM, $n = 8$. Values without a common letter differ, $P < 0.05$. 10C, 10% casein diet; 10CCD, choline-deprived 10C; 25S, 25% soybean protein diet; 25SCD, choline-deprived 25S; FA, folic acid.

[2]Supplemented at a level of 20 mg/kg diet.

[3]Supplemented at a level of 25 g/kg.

[3]Supplemented at a level of 25 g/kg diet.

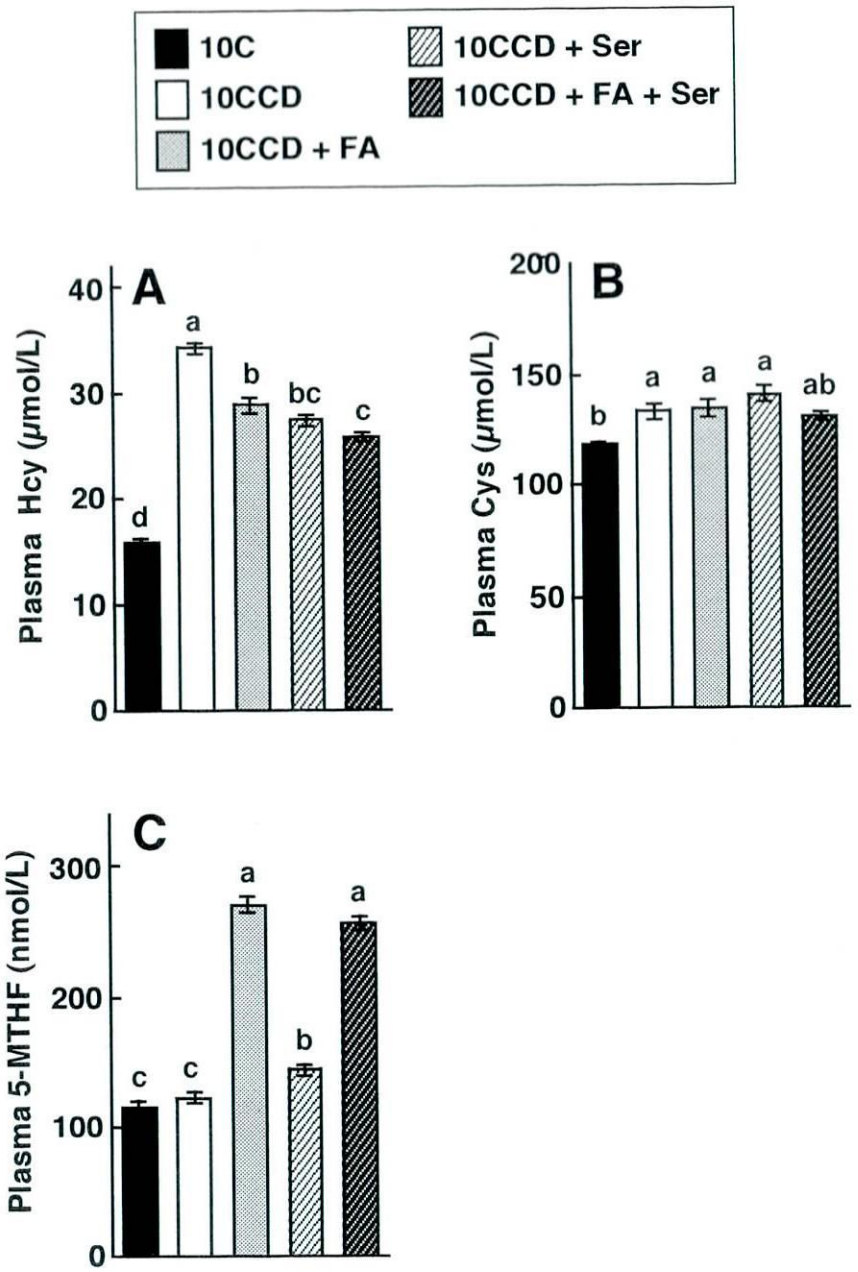


**Fig. 2.2** Effects of supplementation of choline-deprived 10% casein diets with folate, serine or folate plus serine on plasma concentrations of homocysteine (A), cysteine (B) and 5-methylterahydrofolate (C) in rats (experiment 1). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. 10C, 10% casein diet; 10CCD, choline-deprived 10C; FA, folic acid. Folic acid and serine were supplemented to the diet at levels of 20 mg/kg diet and 2.5%, respectively.

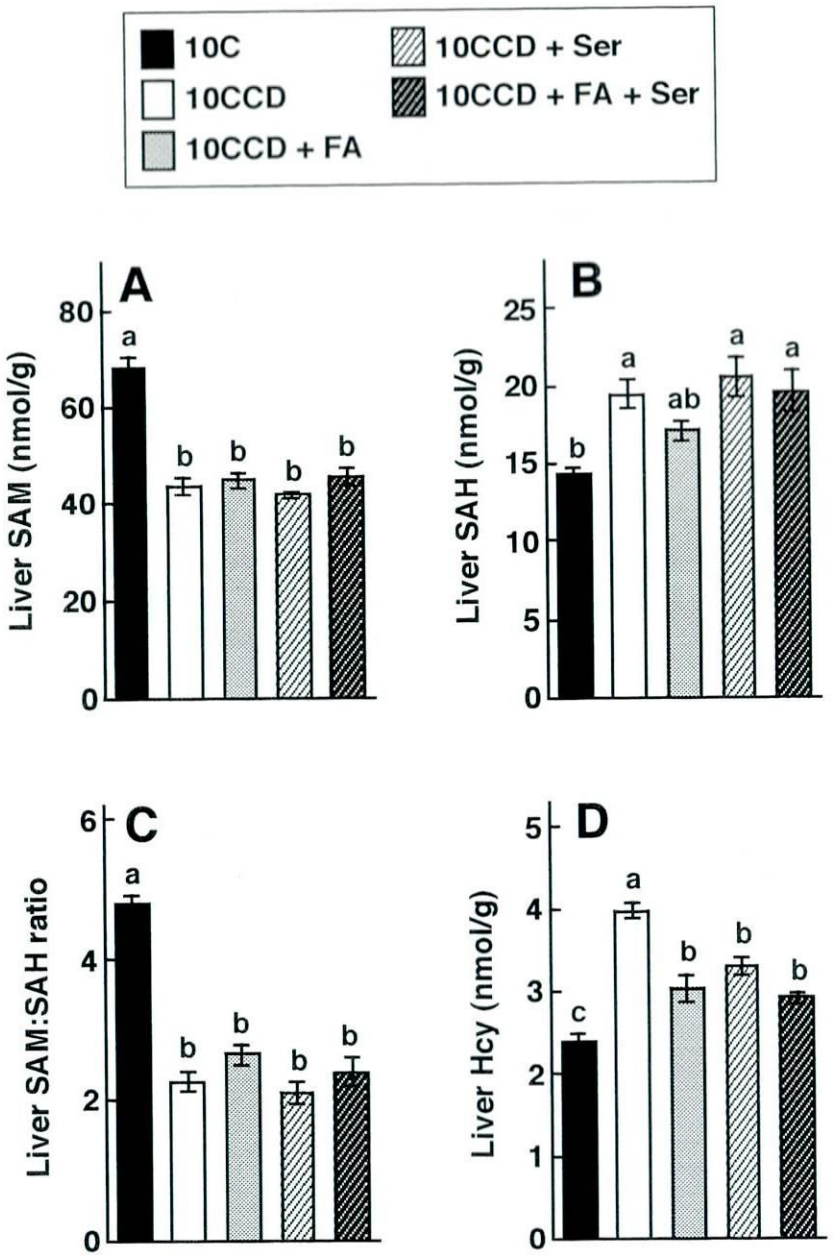


**Fig. 2.3** Effects of supplementation of choline-deprived 10% casein diet with folate, serine or folate plus serine on hepatic concentrations of *S*-adenosylmethionine (A), *S*-adenosylhomocysteine (B), their ratio (C), and homocysteine (D) in rats (experiment 1). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine. See the legend of Fig. 2 for other abbreviations and note.

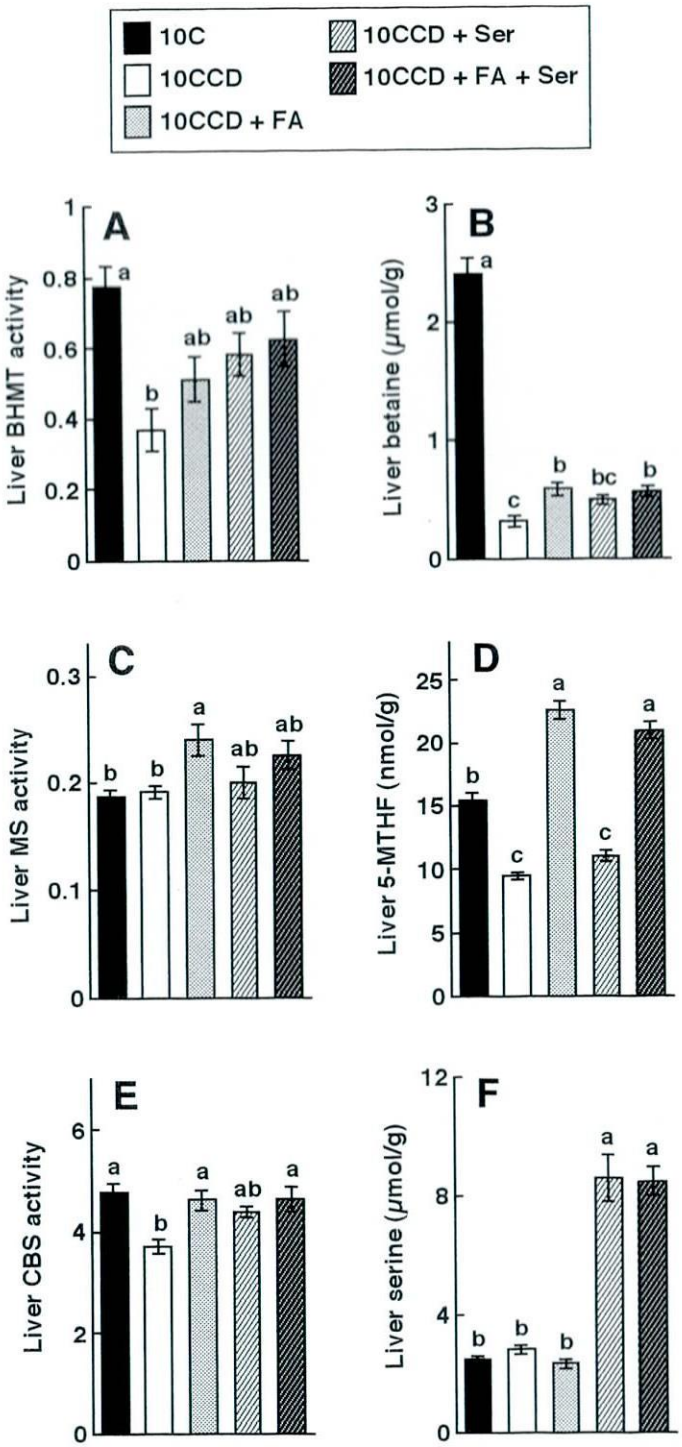


**Fig. 2.4** Effects of supplementation of choline-deprived 10% casein diet with folate, serine or folate plus serine on hepatic activities of betaine-homocysteine *S*-methyltransferase (A), methionine synthase (C) and cystathionine β-synthase (E) and hepatic concentrations of betaine (B), 5-methyltetrahydrofolate (D) and serine (F) in rats (experiment 1). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. BHMT, betaine-homocysteine *S*-methyltransferase; CBS, cystathionine β-synthase; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate.

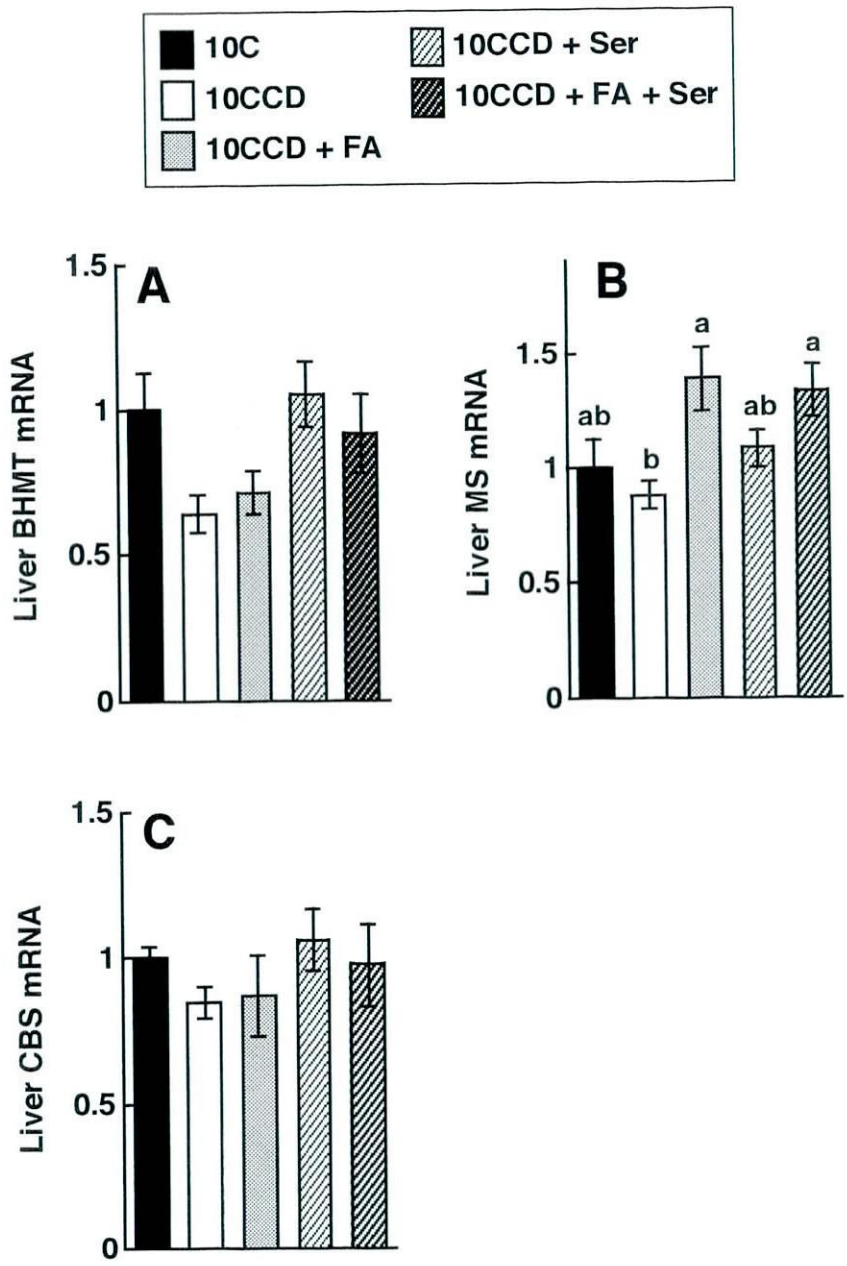


**Fig. 2.5** Effects of supplementation of choline-deprived 10% casein diet with folate, serine or folate plus serine on relative amounts of hepatic mRNA for betaine-homocysteine *S*-methyltransferase (A), methionine synthase (B) and cystathionine β-synthase (C) in rats (experiment 1). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. See the legends of Figs. 2 and 4 for abbreviations and note.

### 2.3.1 Effect on hyperhomocysteinemia induced by choline deprivation of 25S (experiment 2)

Body weight gain and food intake did not differ among the experimental groups, whereas relative liver weight was significantly higher or tended to be higher in rats fed choline-deprived diets irrespective of supplements than in rats fed 25S (Table 2.1). Plasma homocysteine concentration was significantly increased by choline deprivation from 14.79 ± 0.58 to 34.61 ± 0.80 μmol/L (Fig. 2.6, panel A). The choline deprivation-induced elevation of plasma homocysteine was significantly suppressed by supplementation with folate alone, serine alone, and folate plus serine to levels of 29.21 ± 0.58, 27.35 ± 0.71, and 26.12 ± 0.71 μmol/L, respectively. The extents of increment suppression by folate alone, serine alone, and folate plus serine were 27.2, 36.6, and 42.8%, respectively. The profile of plasma homocysteine concentration was similar to that in experiment 1. Plasma cysteine concentration did not differ among the experimental groups (Fig. 2.6, panel B). Plasma 5-MTHF concentration was significantly increased by folate supplementation, although supplementation with serine alone slightly decreased the concentration (Fig. 2.6, panel C). Similar to the results in experiment 1, choline deprivation significantly decreased hepatic SAM concentration and SAM:SAH ratio and, conversely, increased hepatic SAH and homocysteine concentrations (Fig. 2.7). These changes induced by choline deprivation were not affected by supplementation with folate, serine, or both, although the increase in hepatic homocysteine concentration tended to be suppressed by supplements. Choline deprivation significantly decreased hepatic CBS activity, but it did not affect BHMT and MS activities (Fig. 2.8, panels A, C and E). The decrease in CBS activity was slightly suppressed by supplementation with folate, serine, or both. Choline deprivation markedly decreased hepatic betaine concentration and this decrease was unaffected by supplementation with folate, serine, or both (Fig. 2.8, panel B). Hepatic concentration of 5-MTHF was significantly increased by folate supplementation and hepatic serine concentration was significantly increased by serine supplementation (Fig. 2.8, panels D and F).

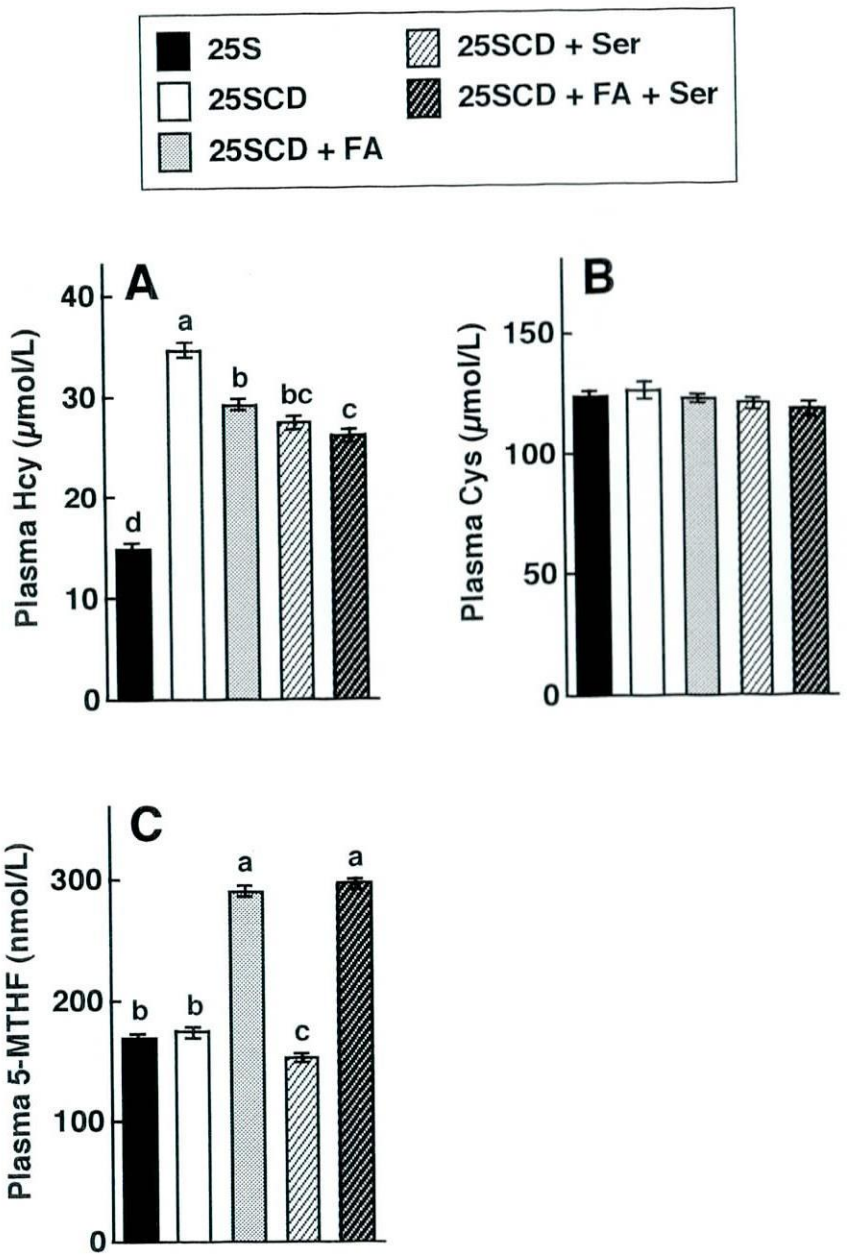


**Fig. 2.6** Effects of supplementation of choline-deprived 25% soybean protein diet with folate, serine or folate plus serine on plasma concentrations of homocysteine (A), cysteine (B) and 5-methyltetrahydrofolate (C) in rats (experiment 2). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. See the legend of Fig. 2 for abbreviations and note.

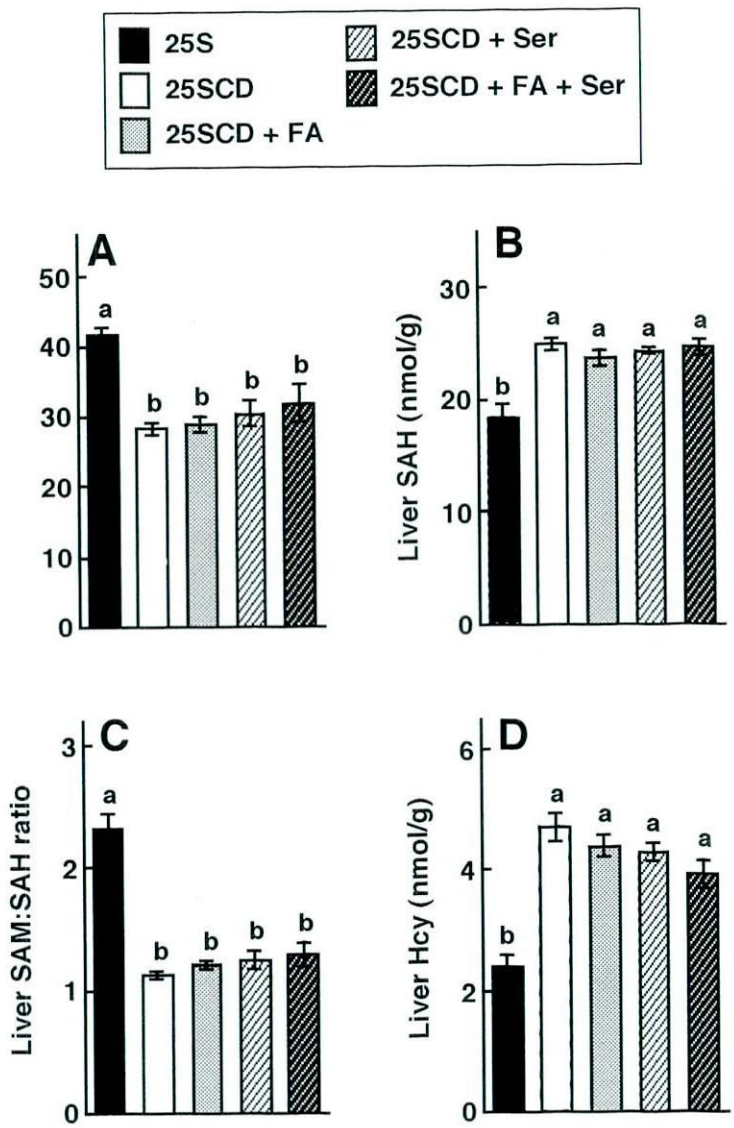


**Fig. 2.7** Effects of supplementation of choline-deprived 25% soybean protein diet with folate, serine or folate plus serine on hepatic concentrations of *S*-adenosylmethionine (A), *S*-adenosylhomocysteine (B), their ratio (C), and homocysteine (D) in rats (experiment 2). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. See the legends of Figs. 2 and 3 for abbreviations and note.

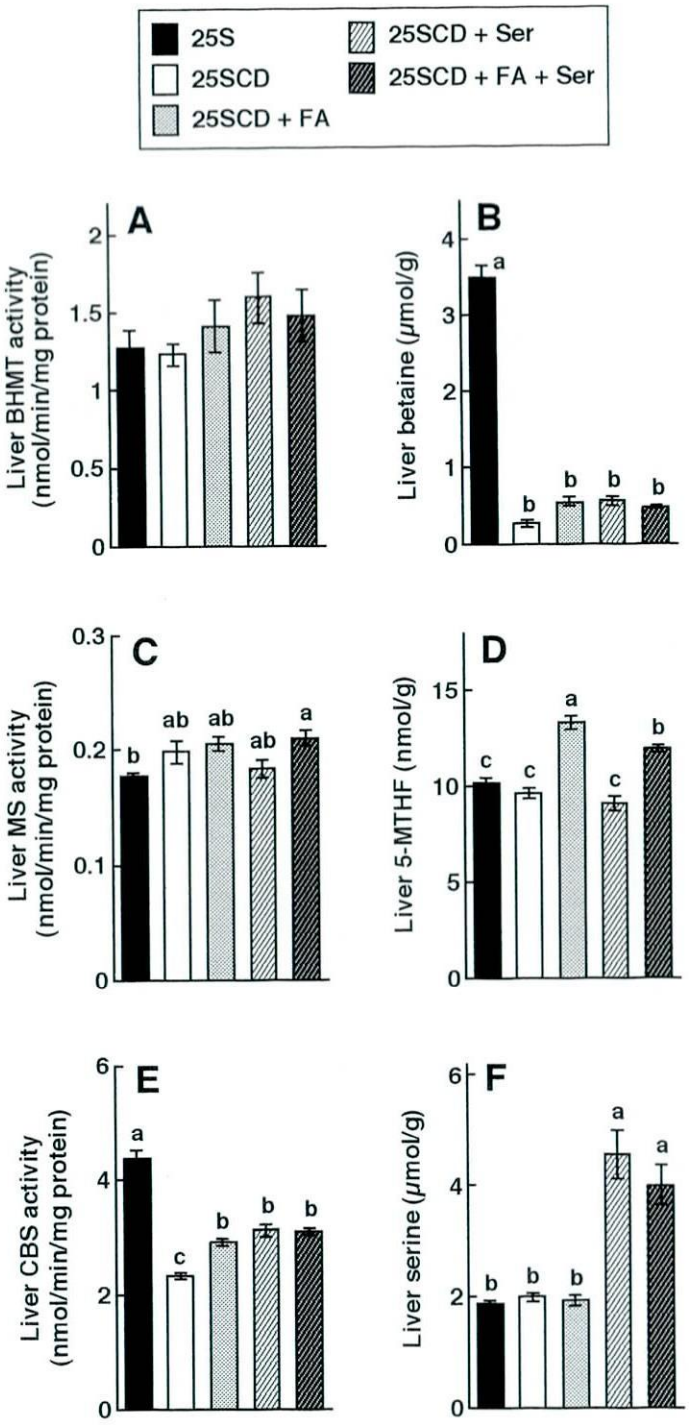


**Fig. 2.8** Effects of supplementation of choline-deprived 25% soybean protein diet with folate, serine or folate plus serine on hepatic activities of betaine-homocysteine *S*-methyltransferase (A), methionine synthase (C) and cystathionine β-synthase (E) and hepatic concentrations of betaine (B), 5-methyltetrahydrofolate (D) and serine (F) in rats (experiment 2). Each value is the mean ± SEM, $n$ = 8. Means in a panel without a common letter differ, $P < 0.05$.

## 2.4 Discussion

Our previous study showed that choline deprivation of low methionine diets such as 10C and 25S resulted in hyperhomocysteinemia in rats (*20*). In the present study, we therefore used both 10C and 25S as basal diets to induce hyperhomocysteinemia by choline deprivation, because the supplemental effect of folate alone or in combination with serine may differ depending on the dietary protein level. It appears that choline deprivation-induced hyperhomocysteinemia is mainly caused by decreased homocysteine removal via the BHMT pathway due to a decrease in hepatic betaine concentration. Under the condition of choline deficiency, hepatic SAM concentration decreases and, conversely, hepatic SAH concentration increases (*75*). This results in depression of the synthesis of phosphatidylcholine (PC), which provides choline and further betaine endogenously, via the phosphatidylethanolamine (PE) *N*-methylation pathway, since the reaction of PE *N*-methylation depends on hepatic SAM concentration and SAH inhibits the reaction (*69,70*). Thus, dietary choline deprivation accelerates betaine deficiency in the liver. The objective of the present study was to determine whether choline deprivation-induced depression in the BHMT pathway can be compensated by stimulation of the MS pathway. To stimulate the MS pathway, choline-deprived diets were supplemented with folate alone or in combination with serine, since serine is thought to be a major source of C1 units for 5-MTHF (*42*). The results obtained in the present study demonstrated that choline deprivation-induced hyperhomocysteinemia could be significantly suppressed by dietary supplementation with folate alone, serine alone, or folate plus serine. The profiles of plasma homocyseine concentrations were similar in rats fed 10C and rats fed 25S, indicating that the effects of folate and/or serine on choline deprivation-induced hyperhomocysteinemia were not dependent on dietary protein levels. In our preliminary experiment, supplementation of 10CCD with folate at levels of 5, 10, and 20 mg/kg diet suppressed choline deprivation-induced elevation of plasma homocysteine concentration in a dose-dependent

manner (data not shown). This suggests that supplementation level of folate higher than 20 mg/kg diet may bring about a further effect. However, it has been shown that a supraphysiological dose of folate (e.g., 20 times the requirement, 40 mg/kg diet) tended to have a harmful effect on colorectal carcinogenesis in rats, while modest doses of folate (4-10 times the requirement, 8-20 mg/kg diet) suppressed the carcinogenesis (*76*). Hence, in the present study, we used folate at a level of 20 mg/kg diet, which is considered to be the maximal dose within the nutritional range. Although the hypohomocysteinemic effect of folate plus serine was significantly greater than the effect of folate alone, there was no significant difference between the effects of folate plus serine and serine alone in both experiments 1 and 2. These results indicate that folate and serine had little additive effect on the plasma homocysteine concentration. Judging from the results shown in Figs. 2.4 and 2.8, it is probable that folate supplementation decreased plasma homocysteine concentration, though only partially, by increasing hepatic 5-MTHF concentration together with MS and CBS activities. On the other hand, serine supplementation might decrease plasma homocysteine concentration by increasing hepatic serine concentration rather than by increasing hepatic 5-MTHF concentration, since hepatic 5-MTHF concentration was not increased by supplementation with serine. However, it is uncertain whether increased serine concentration actually stimulated CBS reaction, since CBS appears to be saturated with serine even in rats fed serine-unsupplemented diets when the reported Km value of CBS for serine, about 0.7 mM (*77*), is taken into consideration.

It should be stressed that the effect of folate or serine was only partial or limited even in the case of the combination of folate and serine, which tended to exhibit the maximal effect. It has been shown that the activity of MS was lower than the activity of BHMT in the liver of rats (*78,46,47*), although these enzyme activities were also influenced by dietary conditions. This is also the case for the present study, supporting the concept that the capacity of the MS pathway for homocysteine metabolism is far lower than the capacity of the BHMT pathway. This might be one of the reasons for the insufficient effect of

supplementation with folate alone or in combination with serine. If so, there is the question of why folate deficiency generally causes hyperhomocysteinemia despite the capacity of the MS pathway being small. Although several mechanisms for the folate deficiency-induced elevation of plasma homocysteine concentration have been proposed, the most likely mechanism is that folate deficiency might impair not only the MS pathway but also the BHMT pathway (*41*). This mechanism is based on the fact that folate deficiency increases the plasma concentration of *N,N*-dimethylglycine (DMG) in human subjects (*41*). DMG is a product of BHMT reaction but also an inhibitor of BHMT (*38*). Tetrahydrofolate (THF) is required for the metabolism of DMG as a methyl-group acceptor (*37*), indicating that activities of both the MS and BHMT pathways are influenced by folate deficiency. In our previous study, we demonstrated that folate deprivation-induced hyperhomocysteinemia could not be fully suppressed by dietary supplementation with betaine even at a relatively high level, 1%, in rats (unpublished data). One of the reasons for the insufficient effect of betaine might be that folate deficiency impaired BHMT reaction by increasing hepatic DMG concentration, based on the fact that there was a significantly positive correlation between hepatic DMG concentrations and plasma homocysteine concentrations. Thus, our previous and present studies support the notion that the two pathways for homocysteine removal by remethylation, MS and BHMT pathways, cannot be fully compensated mutually.

It has been shown that dietary addition of guanidinoacetic acid (GAA) increased plasma homocysteine concentration in rats (*79,80*). At least two mechanisms are considered for the GAA-induced hyperhomocysteinemia: (i) accelerated conversion of SAM to SAH and homocysteine due to compulsive metabolism of GAA to creatine (*38,39*) and (ii) betaine deficiency due to decreased PC synthesis via the PE *N*-methylation pathway (*27,64*). The latter mechanism resembles that of choline deprivation-induced hyperhomocysteinemia. In fact, GAA-induced hyperhomocysteinemia could be effectively suppressed by dietary supplementation with choline or betaine (*27*), but it was not suppressed by folate supplementation (unpublished data). These results, together with the results in the present

study, suggest that folate deficiency causes obvious hyperhomocysteinemia, whereas folate supplementation has no more than a partial or limited effect on several types of hyperhomocysteinemia, except for folate deficiency-induced hyperhomocysteinemia.

There have been several reports on the distinct features of MS and BHMT and the roles of the MS pathway and BHMT pathway. The most striking difference is the Km value for homocysteine. In rats, the Km value of hepatic MS for homocysteine was 1.7 μM (*81*), whereas the Km value of hepatic BHMT for homocysteine was 12 μM (*38*). Under normal conditions, the hepatic homocysteine concentration in rats is relatively low, e.g., approximately 4 nmol/g (*82*), which is considerably lower than the Km value of BHMT. Another difference is the response to dietary methionine level. The activity of hepatic BHMT increased as the dietary methionine level was increased in rats, although methionine restriction also increased the enzyme activity (*46,83*). In contrast, the activity of hepatic MS decreased as the dietary methionine level was increased (*46*). Furthermore, hepatic BHMT activity increased in response to dietary levels of choline or betaine (*28*). Based on these facts, Finkelstein et al. (*28,46,78,83*) have postulated that homocysteine remethylation by the MS pathway might contribute to maintenance of the basal methionine level and that homocysteine remethylation by the BHMT pathway might function as a pathway for catabolism of choline and betaine in addition to removal of homocysteine. Furthermore, it should not be ignored that the MS pathway regenerates THF and, conversely, the BHMT pathway provides C1 units, which are accepted by THF in the metabolism of DMG and sarcosine. These features and roles characteristic of the MS or MS pathway and the BHMT or BHMT pathway appear to be reconciled with the fact that impairment of one pathway could not be fully compensated by another pathway.

# CONCLUSION

In this study, we investigated the roles of folate and betaine in the metabolism of homocysteine. For this purpose, we firstly investigated the effect of betaine stauts on folate deficiency-induced hyperhomocysteinemia to determine whether folate deficiency-induced decrease in homocysteine metabolism can be fully compentated by the betaine-BHMT system. Then we secondly investigated the effect of choline deprivation on folate deficiency-induced hyperhomocysteinemia in rats fed standard casein diet to determine whether there is a synergtic effect between folate deficiency and choline deficiency. We thirdly investigated the effects of folate status on choline deficiency-induced hyperhomocysteinemia to determine whether choline deficiency-induced decrease in homocysteine metabolism can be compensated by the 5-MTHF-MS system.

Firstly, we investigated the effect of supplementation of folate-deprived diets with 1% betaine in rats fed 10% and 20% casein diets to find out that folate deficiency-induced decrease in homocysteine remethylation cannot be fully compensated by the betaine supplementation. The present study demonstrated that folate deficiency-induced hyperhomocysteinemia mainly by decreasing the concentration of 5-MTHF and the hepatic MS activity. Even hepatic betaine concentration and the BHMT activity markedly increased by 1% betaine supplementation, the extent of suppression was partial or limited. In fact, 0.25% betaine is high enough to suppress folate deficiency-induced hyperhomocysteinemia (unpublished data). Thus 1% supplementation level of betaine in the present study was high enough to elicit its effect. One possible reason for the phenomenon is that folate deprivation-induced increase in hepatic DMG concentration might interfere with the effect of betaine, despite hepatic betaine concentration and the BHMT activity being enhanced. The metabolism of DMG, an inhibitor of BHMT, to *N*-methylglycine requires folate (*37*). Hence, it is likely that folate deficiency increased hepatic DMG concentration and resultant inhibition of betaine-BHMT system by DMG could not be fully mitigated even by betaine

supplementation.

Then we investigated the effect of choline deprivation on folate deprivation-induced hyperhomocysteinemia in rats fed standard casein diet (20C) to find out that choline deprivation and folate deprivation synergistically enhanced plasma homocysteine concentration. Choline deprivation does not cause choline deficiency under the standard casein diet, but choline deprivation cause choline deficiency under the condition of folate-deprived standard casein diet and folate deprivation induced hyperhomocysteinemia also reinforced under the choline deprivation diet. The present study demonstrated that the combination of choline deprivation and folate deprivation synergistically affected several variables in the liver. For instance, hepatic MS and CBS activities significantly decreased, which suggested that homocysteine metabolism are unfavorable, hepatic SAM and betaine concentrations markedly decreased, conversely hepatic SAH and homocysteine concentrations significantly increased, which suggested that betaine-BHMT system might not be functional to remethylate homocysteine, and hepatic DMG concentration significantly increased, which might result in inhibition of the BHMT activity. These changes are thought to be associated with the synergistic increase in plasma homocysteine concentration. There is also an important finding that only the combination of choline deprivation and folate deprivation induced hepatic triglyceride concentration increased, while single deprivation of choline or folate did not, indicating that deprivation of both choline and folate induced choline deficiency in standard casein diet. Since hepatic triglyceride concentration was measured as an index to represent PC deficiency or its precursor choline deficiency. These findings support the concept that folate deficiency impairs homocysteine metabolism not only by the MS pathway but also by the BHMT pathway.

Finally, we investigated the effects of dietary supplementation with folate, serine, or both on choline deprivation-induced hyperhomocysteinemia in rats fed 10% casein (10C) or 25% soybean protein (25S) diet to find out that choline deprivation-induced depression in

the BHMT pathway cannot be fully compensated by stimulation of the MS pathway. In fact, in the present study, we used folate at a maximal dose within the nutritional range, and folate and serine had little additive effect on the plasma homocysteine concentration, even folate supplementation alone or with serine significantly increased hepatic 5-MTHF concentration together with the MS activity. One possible reason for the insufficient effect of supplementation with folate alone or in combination with serine was that the activity of MS was lower than the activity of BHMT in the liver of rats, although these enzyme activities were also influenced by dietary conditions.

In conclusion, the present study investigated the roles of folate and betaine in the metabolism of homocysteine to demonstrate that folate deprivation markedly increased hepatic DMG concentration. This finding might be one reason for incomplete effectiveness of betaine supplementation in suppressing folate deficiency-induced hyperhomocysteinemia. The combination of choline deficiency and folate deficiency synergistically enhanced plasma homocysteine concentration. These findings support the concept that folate deficiency might impair not only the 5-MTHF-MS system but also the betaine-BHMT system. Furthermore, it has been found that the effect of folate supplementation on choline deprivation-induced hyperhomocysteinemia was also partial or limited even when folate was supplemented with serine, probably because of the lower capacity of 5-MTHF-MS system compared with betaine-BHMT system. The two pathways for homocysteine removal by remethylation, the MS pathway and the BHMT pathway are separately but closely related, the impairment of one pathway could not be fully compensated by another pathway.

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