Studies on Interactive Effects of Choline and Methionine on the Metabolism of Homocysteine

(ホモシステイン代謝におけるコリンとメチオニンの相互作用的効果に関する研究)

2011

The United Graduate School of Agriculture Science,
Gifu University
Science of Biological Resources
(Shizuoka University)

LIU YI-QUN

Contents

Abbreviations

AAA: acidic amino acids

Asp: aspartic acid

BCAA: branched-chain amino acids

BHMT: betaine-homocysteine *S*-methyltransferase

CBS: cystathionine β-synthase

Cys: cysteine

DMG: *N,N*-dimethylglycine

GAA: guanidinoacetic acid

Glu: glutamic acid

Gly: glycine

Ile: isoleucine

Leu: leucine

Met: methionine

MS: methionine synthase

PC: phosphatidylcholine

PE: phosphatidylethanolamine

SAH: *S*-adenosylhomocysteine

SAM: *S*-adenosylmethionine

Ser: serine

THF: tetrahydrofolate

5-MTHF: 5-methyltetrahydrofolate

Val: valine

Introduction

The sulfhydryl-containing amino acids, or aminothiols, maintain intracellular and extracellular redox homeostasis and are part of the armamentarium of antioxidant defense systems. They are also precursors and intermediates in numerous metabolic pathways and facilitate the removal of noxious compounds. The "good thiols", glutathione and cysteine, are well known and abundant. The intracellular concentration of glutathione is usually in the 1 to 10 mmol/L range, and plasma total cysteine ranges from 200 to 300 μmol/L. Homocysteine is perhaps less well known because it is normally found at relatively low concentrations within the cell ($\leq$ 1 μmol/L) and in the circulation (5 to 15 μmol/L). When excess homocysteine is produced in the body and not readily converted into methionine or cysteine, it is excreted out of the tightly regulated cell environment into the blood. However, elevated plasma total homocysteine has acquired the reputation as the " not-so good thiol" because of its association with cardiovascular disease *(1,2)*, end-stage renal disease *(3,4)*, hypothyroidism *(5,6)*, neural tube defects *(7,8)*, and cognitive dysfunction including Alzheimer`s disease (*9,10*).

Homocysteine occupies a pivotal position in the metabolism of the essential amino acid, methionine. It is at the junction point of the transsulfuration pathway and the formation of cysteine and excretion of sulfur on the one hand and the remethylation of homocysteine to methionine with conservation of the carbon skeleton on the other (*11*). These findings followed the identification in 1932 by Butz and du Vigneaud of homocysteine as a biologically important amino acid (*12*). By heating methionine in sulfuric acid, a compound was isolated and crystalized that had chemical properties similar to those of cysteine and cystine. Using elemental analysis data and other chemical properties, the investigators concluded that they had synthesized "*bis*-(γ-amino-γ-carboxypropyl) disulfide" and suggested that it be called homocystine since it had the structure of the "next higher symmetrical homolog of cystine." They also suggested that homocystine might support growth on cysteine-deficient diets in advance of the discovery of the transsulfuration pathway.

Homocysteine thiolactone was first prepared from methionine by Baernstein (*13*) in 1934

and further characterized by the du Vigneaud group (*14, 15*). L-Homocysteine thiolactone, a very stable form of homocysteine, can be converted to L-homocysteine by alkaline hydrolysis (*13, 16*). L-Homocysteine is thus available for cell culture and other studies. Although homocysteine was discovered in 1932, until in the early 1960s, the two homocystinuric sisters identified by Carson *et al.*(*17*). And in 1964 Mudd and colleagues identified the enzyme defect and established that this form of homocystinuria was due to a deficiency of cystathionine β-synthase (CBS) (*11*). In 1969, the first case of a remethylating disorder was identified by McCully that the vascular complications were a consequence of the elevated homocysteine rather than the result of any of the other complex metabolic changes occurring in cystathionine β-synthase deficiency (*18*).

McCully`s unique training in biochemistry and pathology, along with his inclination to be curious, placed him in a unique position to pioneer a new theory in cardiovascular research. He concluded, as did others, that severely elevated levels of homocysteine were directly responsible for the various vascular lesions in individuals with genetic defects in homocysteine metabolism. He further postulated that moderately elevated homocysteine due to heterozygous mutations in homocysteine-related genes or poor vitamin status would also lead to increased risk of cardiovascular disease in the general population (*18*). Since his new theory questioned the role of cholesterol and other lipids in the genesis of arteriosclerosis, finding acceptance within the mainstream medical community was difficult; eventually, his medical career came to an end when he was unable to "prove" the theory to the satisfaction of some of his colleagues (*19*,20). While employed as a pathologist at the Veterans Affairs Medical Center in Providence, Rhodo Island, he continued his work through the past several decades. By the early 1990s, elevated homocysteine was being considered an independent risk factor for cardiovascular disease *(21-23)*, along with cholesterol and other lipid markers, age *(24)*, gender, smoking status *(25,26)*, obesity, hypertension, and diabetes (*27-29*).

Figure1 shows the basic metabolic pathways concerning homocysteine. Homocyeteine is an intermediate in methionine metabolism, with the methionine derived primarily from dietary protein. This pathway involves the formation of *S*-adenosylmethionine (SAM), which

subsequently transfers a methyl group to any number of several methyl acceptor molecules (DNA, proteins, neurotransmitters) and forms *S*-adenosylhomocysteine (SAH), which is subsequently converted to homocysteine.

Homocysteine is then either converted back to methionine by remethylation or further metabolized to cysteine *via* the trans-sulfuration pathway. Remethylation primarily occurs when a methyl group is transferred from methyltetrahydrofolate (MTHF), the active form of the folic acid, by a methyltransferase enzyme requiring cobalamin (vitamin B_{12}) as a necessary cofactor. A secondary remethylation pathway, active primarily in liver and kidney cells, uses trimethylglycine (betaine) as the methyl donor. The trans-sulfuration pathway requires two enzymatic reactions both of which require the cofactor pyridoxal-5`-phosphate, the active form of vitamin B_6.

The causing mechanisms of various diseases where homocysteine has been seen as a risk factor or marker has been previously reviewed. Table 1 summarizes this information *(30-34)*. Of these factors, nutritional *(35-38)* and genetic factors *(39-42)* are thought to have a greater influence on the plasma homocysteine concentration (*43*). For instance, deficiencies of folate, vitamin B_{12}, and vitamin B_6 cause hyperhomocysteinemia, since folate and vitamin B_{12} participate in the metabolism of homocysteine as cofactors of methionine synthase and vitamin B_6 participates as a cofactor of cystathionine β-synthase. In addition to these vitamins, deficiency of choline also elevates plasma homocysteine concentration.

Choline is important for the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane singaling, and lipid and cholesterol transport and metabolism, because it is a precursor for acetylcholine, phospholipids, and the methyl donor betaine (*44*). Like 5-methyltetrahydrofolate, once choline is oxidized to betaine, it can provide the necessary one-carbon unit in the conversion of homocysteine to methionine, thus generating SAM, the universal methyl donor (*44*). These folate and choline metabolic pathways are closely interrelated (*45, 46*). It has been estimated that 60% of methyl groups

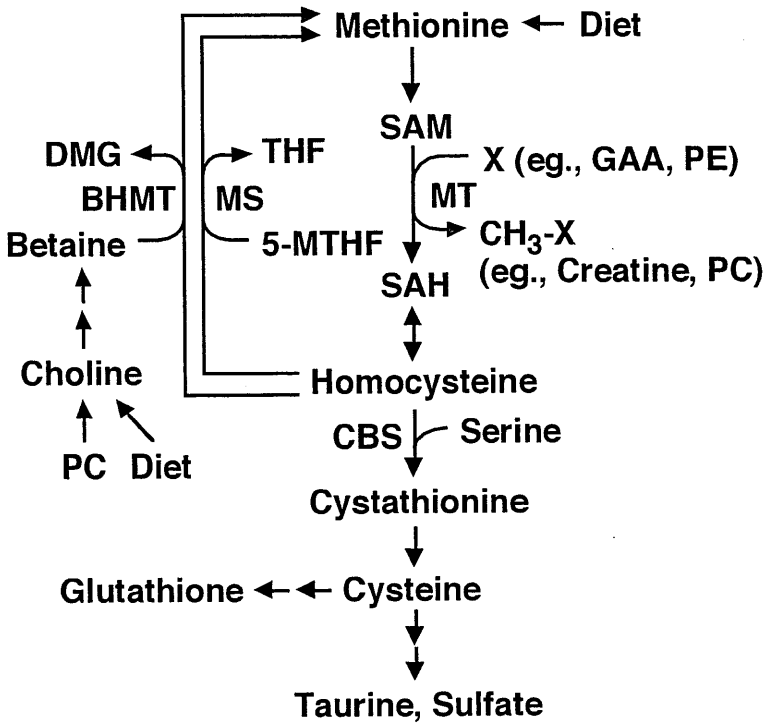

Fig. 1 Metabolism of Methionine and Homocysteine.

BHMT, betaine-homocysteine *S*-methyltransferase (EC 2.1.1.5); CBS, cystathionine β-synthase (EC 4.2.1.22); DMG, *N,N*-dimethylglycine; GAA, guanidinoacetic acid; MS, methionine synthase (EC 2.1.1.13); PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; 5-MTHF, 5-methyltetrhydrofolate.

Table 1. Factors Causing Hyperhomocysteinemia

General

- Increased age
- Male gender
- Menopause (HRT may lower homocysteine)

Lifestyle factors

- Smoking
- High consumption of coffee
- Alcohol consumption (moderate beer intake may be beneficial)

Diet

- Low consumption of fruits and vegetables
- No consumption of multivitamins
- Low intake of folic acid, vitamin B_6, vitamin B_{12}
- High intake of methionine-containing proteins

Diseases or Inherited Causes

- Cystathionine β-synthase deficiency
- 5-MTHFR errors
- Methionine synthase deficiencies
- Chronic renal failure
  - Diabetes
  - Hypothyroidism
  - Psoriasis
  - Certain malignancies
  - Malabsorption syndrome
  - Rheumatoid arthritis
  - Helicobacter pylori infection

Drugs that increase Homocysteine

- Some antiepileptic drugs (phenobarbitol, valproate, phenytoin etc)
- Diuretic therapy
  - Methotrexate
  - Nitrous oxide
  - Cholestyramine
  - Fibric acid derivatives (fenofibrate)
  - Estrogen-containing oral contraceptives
  - Metformin
  - Niacim
  - Theophylline
  - Sulfasalazine

are derived from choline, 20% from methionine, and 10-20% from folate (*46*), which supports the central role of choline as a methyl donor. However, in many mammals, the ingestion of a diet deficient in choline has major consequences, including hepatic, renal, pancreatic, memory, and growth disorders (*44*). A sign of organ dysfunction that occurs when human are deprived of choline is the development of fatty liver (*47, 48*) because a lack of phosphatidylcholine limits the export of excess triacylglycerols from the liver (*49*). Another sign of organ dysfunction that develops in humans who are fed a low-choline diet is an exaggerated increase in plasma total homocysteine after a methionine load (*50*).

Varela-Moreiras et al. (*51*) first demonstrated that choline deprivation in a diet, which contained methionine at a level of 0.2%, increased serum homocysteine concentration. It was also showed that choline deprivation gave rise to hyperhomocysteinemia in rats fed a 10% casein diet (10C) or 25% soybean protein diet, whereas it did not elevate plasma homocysteine concentration in rats fed a 25% casein diet (*52*). It is evident that choline deprivation-induced hyperhomocysteinemia is associated with choline deficiency, since it was accompanied by the development of fatty liver, which is one of the indices of deficiency of phosphatidylcholine (PC) and choline (*53*). PC is synthesized by two pathways, the CDP-choline pathway and phosphatidylethanolamine (PE) N-methylation pathway (*54, 55*). Dietary methionine level or methionine intake affects hepatic *S*-adenosylmethionine (SAM) concentration and thereby influences PC synthesis via the PE *N*-methylation pathway (*53-55*), indicating that choline status within the body is influenced not only by choline intake but also by methionine intake. It is widely recognized that choline deprivation does not cause choline deficiency when diets contain relatively high levels of methionine. The methionine contents of 10C and 25% soybean protein diet are lower than that of 25% casein diet. The elevation of plasma homocysteine in rats fed choline-deprived 25% soybean protein diet could be effectively suppressed by supplementation with a small amount (0.35%) of methionine (*52*). These results suggest that choline deprivation leads to hyperhomocysteinemia only when dietary methionine levels are relatively low and that methionine in dietary proteins acts as anti-hyperhomocysteinemic amino acid under the condition of restricted choline intake. One

of the mechanisms by which choline deficiency induces hyperhomocysteinemia is thought to be a decrease in hepatic concentration of betaine, which suppressed betaine-dependent homocysteine remethylation that is catalyzed by betaine-homocysteine *S*-methyltransferase (BHMT). Since choline is the sole precursor of betaine, hepatic betaine concentration reflects choline status.

However, how in the world choline and methionine metabolisms interrelated and interacted at the point where homocysteine is converted to methionine? The aim of this study, therefore, was to undertaken to elucidate the interactive effects of choline and methionine on the metabolism of homocysteine using rats as experimental animals. Chapter 1 of this thesis describes about the effects of dietary supplementation with 0.5% Met, 2.5% Ser or both on hyperhomocysteinemia induced by deprivation of dietary choline or by dietary addition of 0.5% guanidinoacetic acid (GAA) in rats fed 10C. Chapter 2 of this thesis describes about the mechanisms by which feeding a higher casein diet results in resistance to choline deprivation-induced hyperhomocysteinemia.

Chapter 1

Methionine and Serine Synergistically Suppress Hyperhomocysteinemia Induced by Choline Deficiency, but Not by Guanidinoacetic Acid, in Rats Fed a Low Casein Diet

Methionine prevents fatty liver due to phosphatidylcholine (PC) deficiency by stimulating PC synthesis *via* the phosphatidylethanolamine (PE) *N*-methylation pathway (*47,51,53*). In fact, choline deprivation does not cause fatty liver due to PC deficiency when diets contain relatively high levels of methionine (*51*). Homocysteine is a metabolite in the metabolism of methionine (Fig. 1) (*54*), but it is widely recognized that an elevated plasma homocysteine concentration is an independent risk factor for cardiovascular disease (*2,55,56*). Previously we found that deprivation of dietary choline caused hyperhomocysteinemia as well as fatty liver in rats fed a low (10%) casein diet (10C) or a moderate (25%) soybean protein diet (25S), but not in rats fed a moderate (25%) casein diet (25C) (*50*). The resistivity of 25C against choline deprivation-induced hyperhomocysteinemia might be due to the higher methionine level of the diet, since a methionine content of 25C was higher than that of 10C or 25S. Choline deprivation-induced hyperhomocysteinemia is primarily attributable to a deficiency of betaine, a methyl-group donor for the re-methylation of homocysteine. We also found that hyperhomocysteinemia induced by choline deprivation in rats fed 25S was effectively suppressed by dietary supplementation with methionine at a level of 0.35% (*50*). On the other hand, dietary supplementation with methionine increased the plasma homocysteine concentration in a dose-dependent manner in rats fed 25C (*57*). Hence it is reasonable to assume that methionine has two opposing effects on the plasma homocysteine concentration, *i.e.*, hypohomocysteinemic and hyperhomocysteinemic effects.

Methionine supplementation of choline-deprived 10C at a level of 0.5% to make the methionine level comparable to that of a 30% casein diet (30C) did not suppress hyperhomocysteinemia in rats, while choline-deprived 30C did not enhance the plasma homocysteine concentration (unpublished observation). This unexpected finding suggests that choline and methionine are not equivalent in preventing hyperhomocysteinemia associated with choline deficiency. Serine and its precursor glycine had suppressive effects on hyperhomocysteinemia induced by methionine supplementation in rats fed 25C, probably through stimulation of cystathionine formation (*57*). This interacting effect of methionine and serine or glycine might have nutritional significance in the metabolism of homocysteine. An

elevated plasma homocysteine concentration due to supplementation of 10C with methionine at a level of 0.5% was significantly suppressed by concurrent supplementation with glycine and serine at levels of 0.32% and 0.94% respectively, to make these amino acid levels comparable to those of 30C (*58*), but little information is available about such an interaction effect, especially under conditions of choline deficiency. It was found that guanidinoacetic acid (GAA) caused hyperhomocysteinemia when added to the diet of rats (*59,60*). GAA also decreased the hepatic betaine concentration (*61,62*), indicating that GAA-induced hyperhomocysteinemia is suppressed by dietary supplementation with methionine.

In this study, we investigated the effects of supplementation with methionine, serine, and both on the plasma homocysteine concentration and related variables in hyperhomocysteinemic rat models to determine whether methionine alone or in combination with serine exhibits a hypohomocysteinemic effect, and whether there is an interaction effect between methionine and serine. For this purpose, we used choline deprivation and GAA supplementation to induce experimental hyperhomocysteinemia in rats fed 10C.

Materials and Methods

Choline bitartrate and GAA were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals, including L-methionine and L-serine, were purchased from Wako Pure Chemical Industries, (Osaka, Japan) or Sigma-Aldrich, and were of analytical grade. Casein, a mineral mixture (AIN-93G), a vitamin mixture (AIN-93), and cellulose powder were purchased from Oriental Yeast (Tokyo). The other ingredients of the diet were purchased from Wako.

Six-week-old male rats (120-140 g) of the Wistar strain were obtained from Japan SLC (Hamamatsu, Japan). They were housed individually in hanging stainless-steel wire cages in an isolated room kept at 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). Before the start of the experiments, all the rats were acclimated to the facility for 4 d and given free access to water and 25C. Two separate animal experiments were conducted. In experiment 1, 35 rats were randomly divided into five groups, seven rats to each group, and fed one of the following diets: (i) 10C, (ii) choline-deprived 10C (10CCD), (iii) 10CCD + 0.5% L-Met, (iv) 10CCD + 2.5% L-Ser, and (v) 10CCD + 0.5% L-Met + 2.5% L-Ser. In experiment 2, 35 rats were randomly divided into five groups, seven rats to each group, and fed one of the following diets: (i) 10C, (ii) 10C + 0.5% GAA (10CG), (iii) 10CG + 0.5% L-Met, (iv) 10CG + 2.5% L-Ser, and (v) 10CG + 0.5% L-Met + 2.5% L-Ser. The normal diet (10C) consisted of the following ingredients (g/100 g): casein, 10; α-cornstarch, 58.25; sucrose, 20; corn oil, 5; mineral mixture (AIN-93G), 3.5; vitamin mixture (AIN-93), 1; choline bitartrate, 0.25; and cellulose, 2. The addition of amino acids or GAA and the omission of choline bitartrate were adjusted by changing the cornstarch content. Additional levels of methionine (0.5%), serine (2.5%), and GAA (0.5%) were determined according to the results of our previous studies (*52,59,62*). The rats were fed the experimental diets for 10 d and then killed by decapitation between 10:00 and 11:00 without prior starvation. The experimental period of 10 d was sufficient to induce stable hyperhomocysteinemia by choline deprivation (*52*) or the addition

of GAA (*62*). 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.

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 the 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 de-proteinized liver homogenate was subjected to assays for methionine metabolites, betaine, 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 assay. 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 (65).*

The concentrations of homocysteine and cysteine in the plasma and liver were measured by HPLC by the method of Durand *et al.* (*66*). The concentrations of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) in the liver were measured by HPLC following Cook *et al.* (*67*). The concentration of betaine in the liver was measured by HPLC following Laryea *et al.*, (*68*) and the concentration of serine in the liver was measured with an amino acid autoanalyzer (Model L-8500; Hitachi, Tokyo). The activity of betaine-homocysteine *S*-methyltransferase (BHMT) in the liver was measured following Finkelstein *et al.*, (*69*) but HPLC was used in the assay of the reaction product, *N,N*-dimethylglycine, following Laryea *et al.*,(*68*) . The activity of cystathionine β-synthase (CBS) in the liver was measured following Mudd *et al.*, (*16*) but HPLC was used in the assay of the reaction product, cystathionine, following Einarsson *et al.*, (*70*). The hepatic triglyceride concentration was measured enzymatically using a commercial kit (Triglyceride E-Test Wako, Wako). The protein

concentration was measured following Lowry *et al.*, (*71*) using bovine serum albumin as standard.

Each value is expressed as the mean ± SEM. Data were analyzed by a one-way ANOVA, and differences among 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).

Results

Effect on hyperhomocysteinemia induced by choline deprivation (experiment 1)

Choline deprivation and supplementation with serine alone did not affect body weight gain, food intake, or relative liver weight (Table 2). Supplementation with methionine alone or in combination with serine significantly increased or tended to increase body weight gain and relative liver weight, whereas it significantly decreased or tended to decrease food intake. Choline deprivation of 10C significantly increased the plasma homocysteine concentration at from 16.2 ± 0.2 (10C group) to 33.7 ± 0.6 μmol/L (10CCD group) (Fig. 2.1, panel A). Hyperhomocysteinemia induced by choline deprivation was not suppressed by supplementation with methionine alone. In contrast, supplementation with methionine in combination with serine completely suppressed choline deprivation-induced hyperhomocysteinemia, although supplementation with serine alone also partially suppressed it. Plasma cysteine concentrations did not differ among the experimental groups (Fig. 2.1, panel B). Choline deprivation significantly decreased the hepatic SAM concentration and the SAM/SAH ratio and, conversely, increased the hepatic SAH and homocysteine concentrations (Fig. 2.2, panels A-D). Supplementation with methionine alone significantly increased the hepatic SAM concentration, to a level higher than the level in the rats fed 10C, and partially increased the SAM/SAH ratio, whereas it significantly increased or tended to increase the hepatic SAH and homocysteine concentrations to levels higher than those in the rats fed 10CCD. Supplementation with serine alone did not affect these variables. In contrast, supplementation with methionine in combination with serine completely suppressed increases in hepatic SAH and homocysteine concentrations and restored the hepatic SAM concentration and the SAM/SAH ratio to the levels in the rats fed 10C. Choline deprivation decreased the hepatic betaine concentration significantly from 2.64 ± 0.08 (10C group) to 0.32 ± 0.02 μmol/g (10CCD group), together with hepatic BHMT and CBS activities (Fig. 2.3, panels A-C). Supplementation with methionine alone restored these enzyme activities to the levels in the rats fed 10C, but did not increase the hepatic betaine concentration. Supplementation with

serine alone did not affect hepatic BHMT activity, whereas it restored hepatic CBS activity. Supplementation with methionine in combination with serine increased hepatic BHMT activity significantly to a level higher than that in the rats fed 10C and slightly but significantly increased the hepatic betaine concentration. The hepatic serine concentration was markedly decreased by supplementation with methionine alone, but was markedly increased by supplementation with serine alone, and remained at the same level to those in the rats fed 10C and 10CCD under supplementation with methionine in combination with serine (Fig. 2.3, panel D). Choline deprivation significantly increased the hepatic triglyceride concentration and white tissue was visible, indicating the development of fatty liver (Fig. 2.3, panel E). The increase in the hepatic triglyceride concentration was partially suppressed by supplementation with methionine alone, but was unaffected by supplementation with serine alone. In contrast, supplementation with methionine in combination with serine not only completely suppressed the increase in hepatic triglyceride concentration induced by choline deprivation but also significantly decreased hepatic triglyceride concentration to a level lower than that in the rats fed 10C.

Effect on guanidinoacetic acid-induced hyperhomocysteinemia (experiment 2)

The addition of GAA and supplementation with serine alone did not affect body weight gain, food intake, or relative liver weight (Table 2). Supplementation with methionine alone or in combination with serine significantly increased body weight gain and relative liver weight, whereas it significantly decreased or tended to decrease food intake. The addition of GAA increased the plasma homocysteine concentration markedly from 16.0 ± 0.2 (10C group) to 81.2 ± 0.6 μmol/L (10CG group) (Fig. 2.4, panel A). GAA-induced hyperhomocysteinemia was significantly suppressed by supplementation with methionine alone or in combination with serine, but there was no difference between the effect of methionine alone and that of the combination of methionine and serine. Although supplementation with serine alone also significantly decreased the plasma homocysteine concentration, the effect was limited. The plasma cysteine concentration was significantly higher in the rats fed diets supplemented with

methionine irrespective of simultaneous serine supplementation than in those fed the other diets (Fig. 2.4, panel B). The addition of GAA significantly decreased the hepatic SAM concentration and the SAM/SAH ratio and, conversely, increased hepatic SAH and homocysteine concentrations (Fig. 2.5, panels A-D). Supplementation with methionine alone and in combination with serine partially prevented the effects of GAA on the hepatic concentrations of methionine metabolites. In contrast, supplementation with serine alone did not affect these variables, and there was no additive or synergistic effect between methionine and serine. The addition of GAA significantly decreased hepatic BHMT activity and the hepatic betaine concentration (Fig. 2.6, panels A and B). The decrease in BHMT activity was restored by supplementation with methionine alone and in combination with serine, whereas the decrease in the betaine concentration was further reinforced by methionine supplementation. Supplementation with serine alone did not affect these variables. The addition of GAA slightly but significantly decreased hepatic CBS activity, and this decrease was prevented by supplementation with methionine in combination with serine (Fig. 2.6, panel C). The hepatic serine concentration was significantly decreased by supplementation with methionine alone, whereas it was markedly increased by supplementation with serine alone and was maintained at the same level as in the rats fed 10C and 10CG by supplementation with methionine in combination with serine (Fig. 2.6, panel D). The addition of GAA significantly increased the hepatic triglyceride concentration, and this increase was completely prevented by supplementation with methionine in combination with serine, but not by supplementation with methionine alone or serine alone (Fig. 2.6, panel E).

Table 2. Body Weight Gain, Food Intake and Liver Weight of Rats Fed the Experimental Diets (Experiments 1 and 2).

Diet	Body weight gain	Food intake	Liver weight
	g/10 d	*% of body weight*	
Experiment 1			
10C	29.1 ± 2.2^{b}	151 ± 3^{a}	4.29 ± 0.06^{b}
10CCD	28.8 ± 2.1^{b}	152 ± 3^{a}	4.12 ± 0.06^{b}
10CCD + 0.5% L-Met	38.9 ± 1.0^{ab}	136 ± 3^{ab}	4.80 ± 0.06^{a}
10CCD + 2.5% L-Ser	29.6 ± 3.7^{b}	147 ± 5^{a}	4.17 ± 0.05^{b}
10CCD + 0.5% L-Met + 2.5% L-Ser	42.4 ± 2.5^{a}	131 ± 4^{b}	4.98 ± 0.11^{a}
Experiment 2			
10C	31.9 ± 2.2^{b}	153 ± 3^{a}	3.86 ± 0.02^{b}
10CG	24.3 ± 1.2^{b}	143 ± 4^{ab}	3.94 ± 0.05^{b}
10CG + 0.5% L-Met	47.5 ± 2.1^{a}	138 ± 2^{b}	4.46 ± 0.04^{a}
10CG + 2.5% L-Ser	31.2 ± 2.4^{b}	149 ± 2^{ab}	3.88 ± 0.02^{b}
10CG + 0.5% L-Met + 2.5% L-Ser	41.4 ± 1.7^{a}	126 ± 3^{c}	4.47 ± 0.03^{a}

[1]Each value is the mean ± SEM, $n = 7$. Values with different letters are significantly different at $P < 0.05$. 10C, 10% casein diet; 10CCD, choline-deprived 10C; 10CG, 10C + 0.5% guanidinoacetic acid

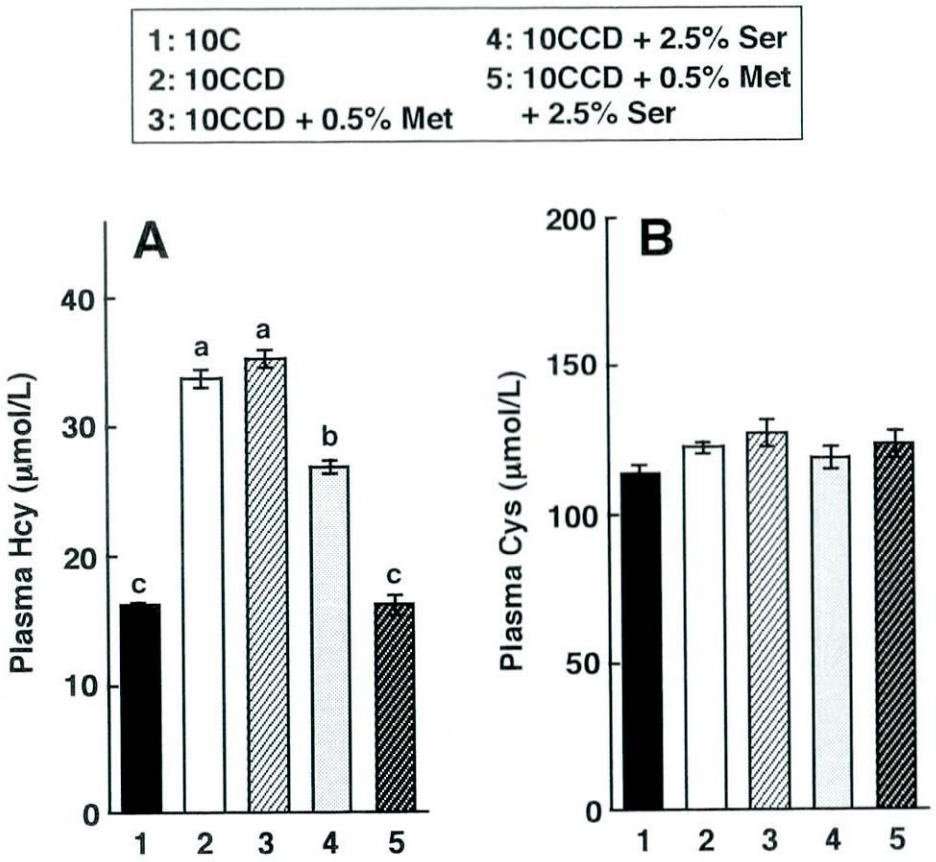

Fig. 2.1 Plasma Homocysteine (A) and Cysteine (B) Concentrations in Rats Fed the Experimental Diets (Experiment 1). Values are mean ± SEM, $n = 7$. Values in a panel without a common letter differ at $p < 0.05$. 10C, 10% casein diet; 10CCD, choline-deprived 10C; Cys, cysteine; Hcy, homocysteine; Met, methionine; Ser, serine. Experimental groups: 1, 10C; 2, 10CCD; 3, 10CCD + 0.5% Met; 4, 10CCD + 2.5% Ser; 5, 10CCD + 0.5% Met + 2.5% Ser.

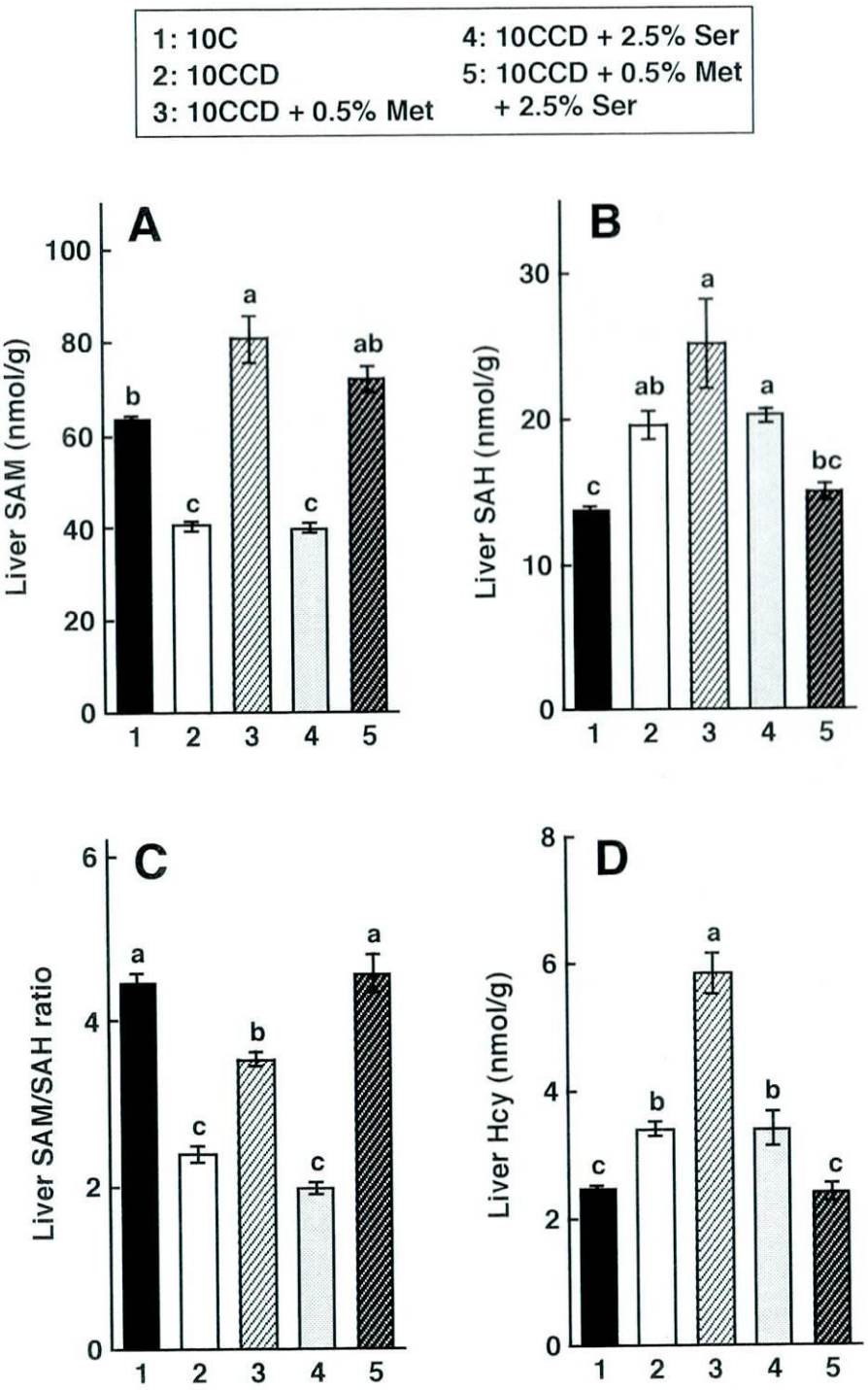

Fig. 2.2 Hepatic Concentrations of *S*-Adenosylmethionine (A), *S*-Adenosylhomocysteine (B), Their Ratio (C), and Homocysteine (D) in the Rats Fed the Experimental Diets (Experiment 1). Values are mean ± SEM, $n = 7$. Values in a panel without a common letter differ at $p < 0.05$. SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine. See the legend to Fig. 2.1 for further abbreviations.

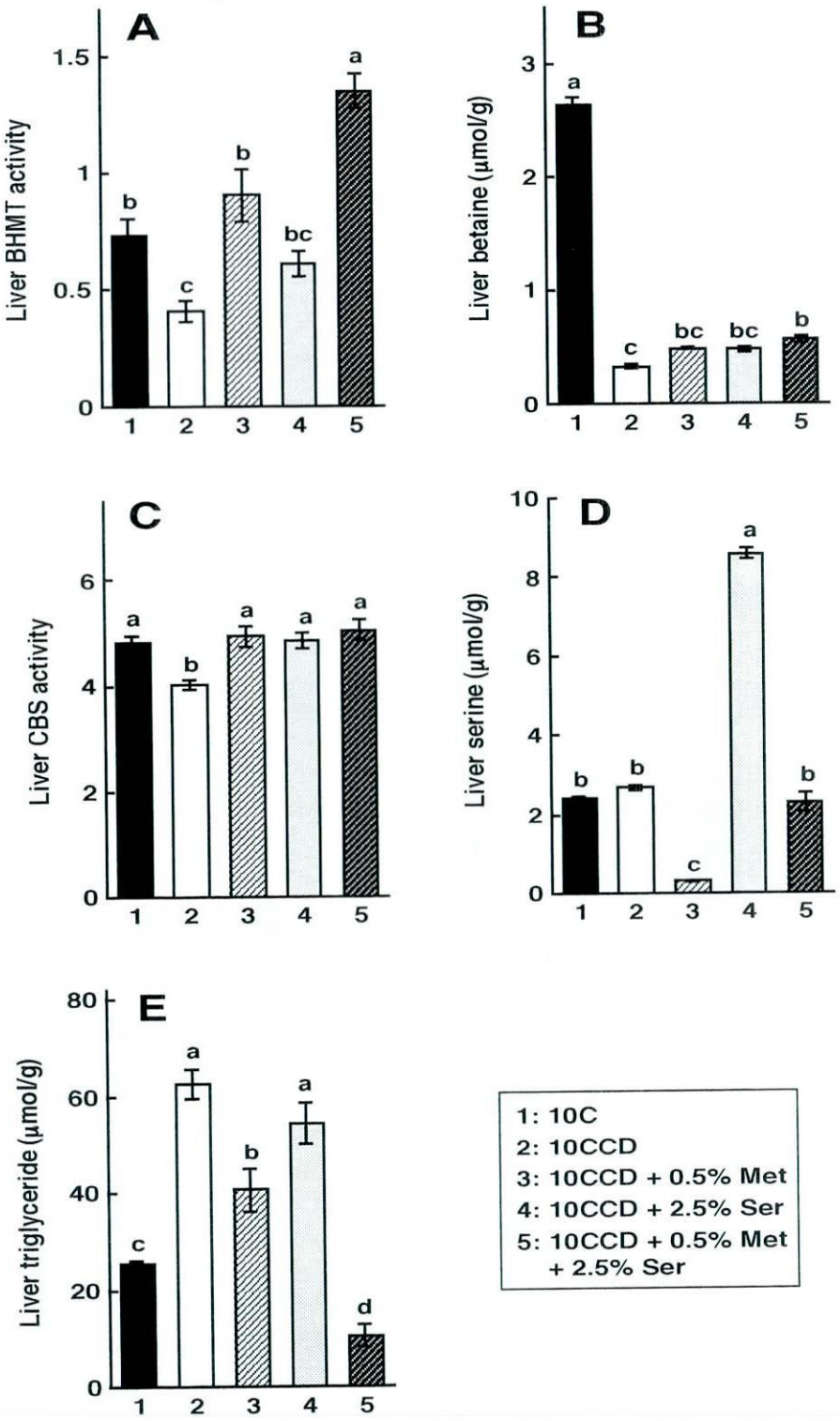

Fig. 2.3 Activities of Betaine-Homocysteine *S*-Methyltransferase (A) and Cystathionine β-Synthase (C), and the Concentrations of Betaine (B), Serine (D), and Triglyceride (E) in the Livers of Rats Fed the Experimental Diets (Experiment 1). Values are mean ± SEM, $n = 8$. Values in a panel without a common letter differ at $p < 0.05$. BHMT, betaine-homocysteine *S*-methyltransferase; CBS, cystathionine β-synthase. See the legend to Fig. 2.1 for further abbreviations.

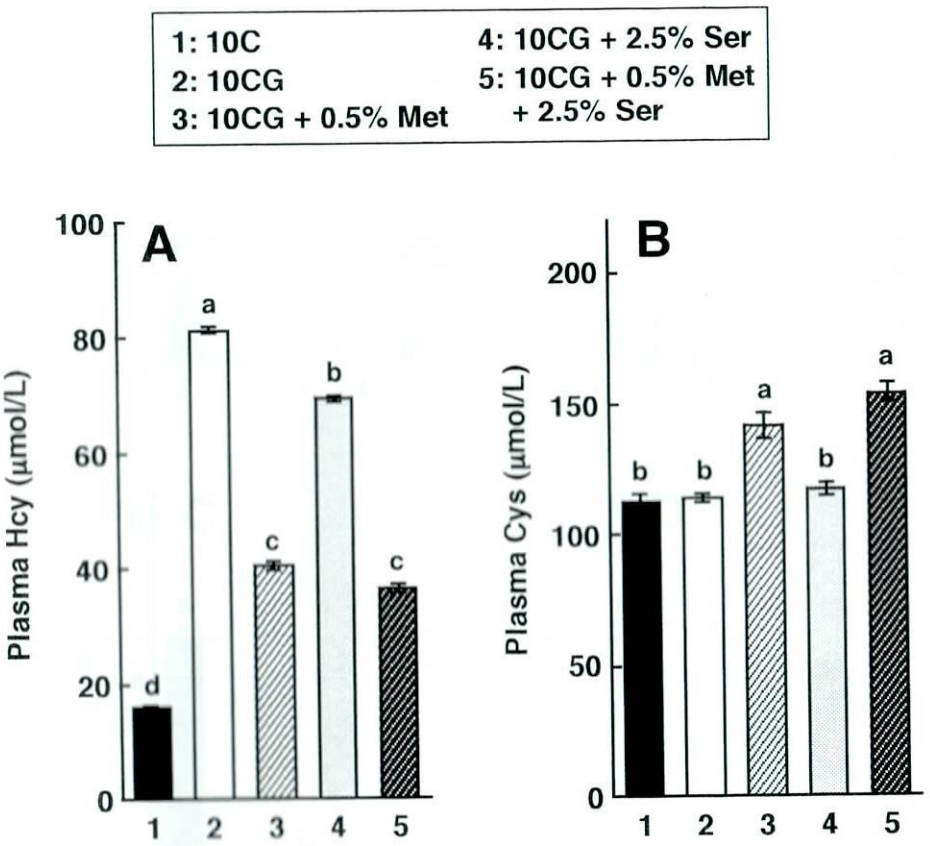

Fig. 2.4 Plasma Homocysteine (A) and Cysteine (B) Concentrations in the Rats Fed the Experimental Diets (Experiment 2). Values are mean ± SEM, $n = 7$. Values in a panel without a common letter differ at $p < 0.05$. 10C, 10% casein diet; 10CG, 10C + 0.5% guanidinoacetic acid. See the legend to Fig. 2 for further abbreviations. Experimental groups: 1, 10C; 2, 10CG; 3, 10CG + 0.5% Met; 4, 10CG + 2.5% Ser; 5, 10CG + 0.5% Met + 2.5% Ser.

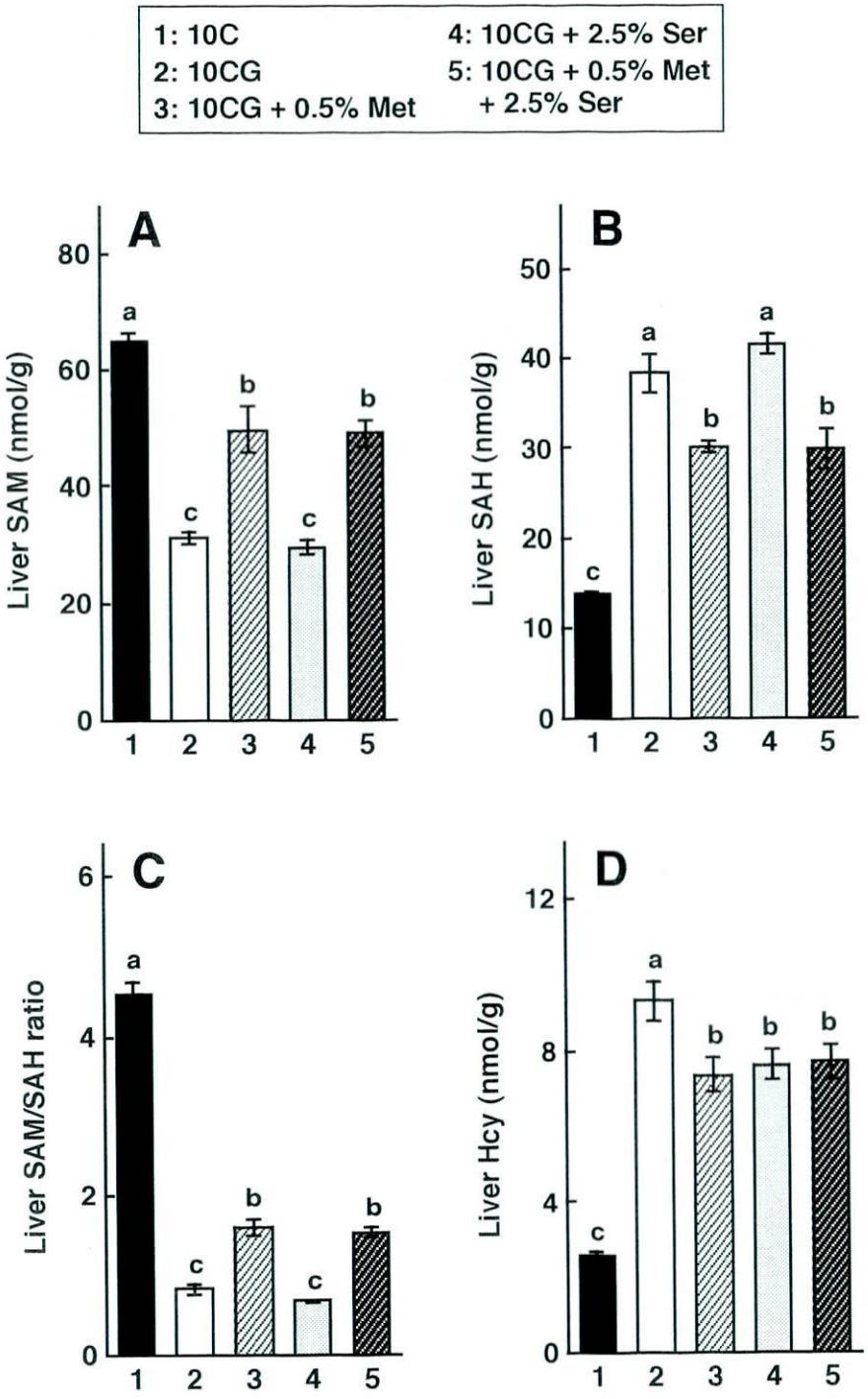

Fig. 2.5 Hepatic Concentrations of *S*-Adenosylmethionine (A), *S*-Adenosylhomocysteine (B), Their Ratio (C), and Homocysteine (D) in the Rats Fed the Experimental Diets (Experiment 2). Values are mean ± SEM, $n = 7$. Values in a panel without a common letter differ at $p < 0.05$. See the legends to Figs. 2.1 and 2.4 for abbreviations.

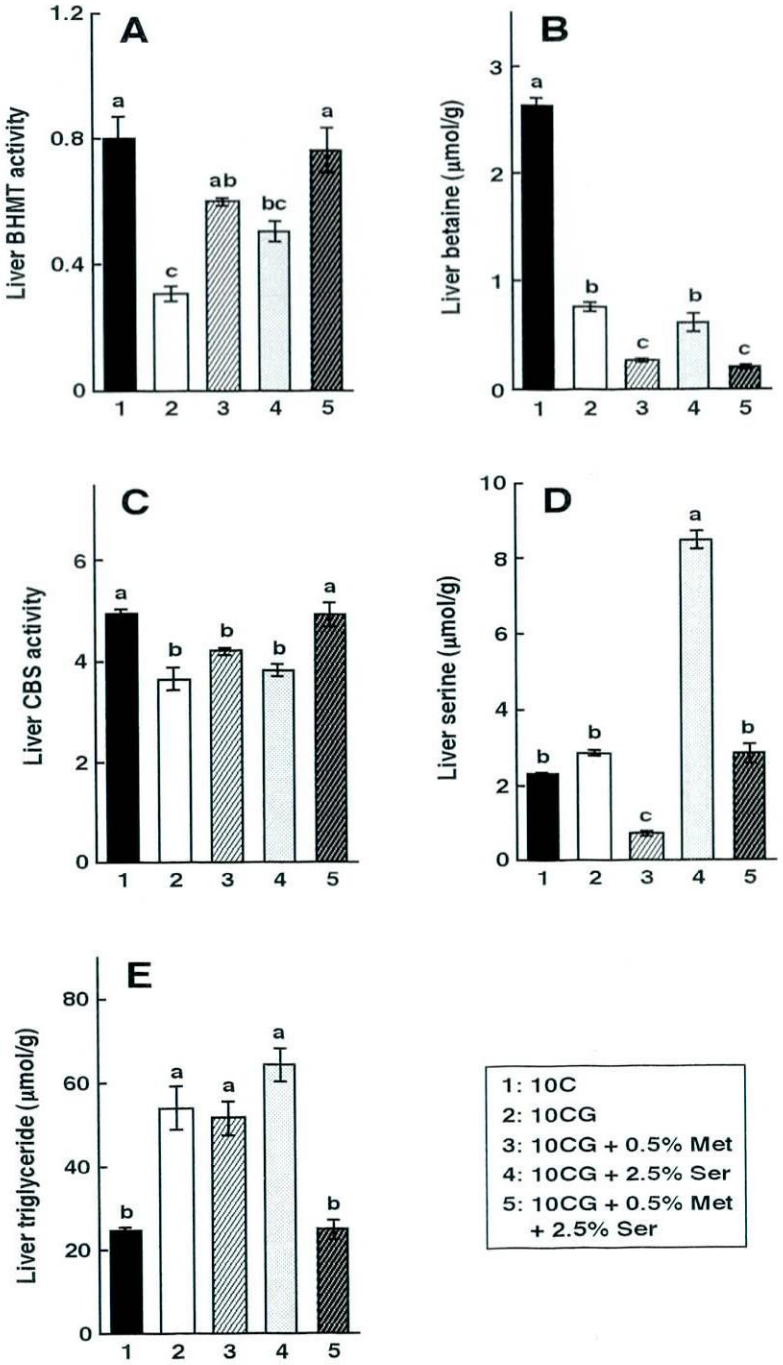

Fig. 2.6 Activities of Betaine-Homocysteine *S*-Methyltransferase (A) and Cystathionine β-Synthase (C), and the Concentrations of Betaine (B), Serine (D), and Triglyceride (E) in the Livers of Rats Fed the Experimental Diets (Experiment 2). Values are mean ± SEM, $n = 7$. Values in a panel without a common letter differ at $p < 0.05$. See the legends to Figs. 2.3 and 2.4 for abbreviations.

Discussion

Choline deprivation-induced hyperhomocysteinemia model

The choline deprivation of low-methionine diets induces hyperhomocysteinemia mainly due to betaine deficiency in the liver (*52*). The dietary methionine level affects choline status within the body, since methionine stimulates the synthesis of the choline moiety of PC *via* the PE *N*-methylation pathway (*49,53,55*). This appears also to be the case for betaine status, because choline is metabolized to betaine (*72*). It is thought that the hepatic SAM concentration, which reflects the dietary methionine level, is critical in the PE *N*-methylation reaction (*49,55*). Hence it is expected that dietary methionine supplementation increases betaine supply in the liver and thereby suppresses the hyperhomocysteinemia associated with betaine deficiency, but the present study indicates that supplementation of choline-deprived 10C with methionine alone at a level of 0.5% did not have any suppressive effect on hyperhomocysteinemia. The methionine content of 10CCD + 0.5% methionine was comparable to that of 30C. In contrast to 10CCD + 0.5% methionine, choline-deprived 30C did not enhance plasma homocysteine concentration or cause the development of fatty liver (unpublished observation). These results suggest that the resistance of rats fed 30C to choline deprivation cannot be attributed solely to the higher methionine content of the diet. A major finding of experiment 1 was that supplementation with methionine in combination with serine completely suppressed hyperhomocysteinemia, although supplementation with serine alone also had a partial effect. This indicates that methionine and serine synergistically suppressed hyperhomocysteinemia.

Methionine has two opposing effects on the plasma homocysteine concentration (*52,59*). The hyperhomocysteinemic effect of methionine, usually observed when the rats were fed methionine-supplemented diets (*59,73*), appears to be due to increased homocysteine production, since methionine is the sole precursor of homocysteine. The hypohomocysteinemic effect of methionine, which was observed when the rats were fed choline-deprived 25S (*52*), appears to be due to an increased supply of betaine. On the other

hand, the interacting or synergistical effect of methionine and serine on hyperhomocysteinemia might be explained by stimulation of homocysteine removal, mainly through increased cystathionine synthesis. The reaction of cystathionine synthesis is thought to be a critical step in the metabolism of homocysteine when dietary methionine levels are relatively high (*56,74,75*). Under such conditions, cystathionine synthesis appears to depend on serine supply rather than CBS activity. This assumption is confirmed by the fact that hyperhomocysteinemia caused by methionine supplementation was effectively suppressed by concurrent supplementation with serine or glycine without any increase in CBS activity (*59*). Furthermore, it has been found that methionine supplementation significantly decreases the hepatic serine concentration (*60,74*), suggesting that increases in serine consumption induced by methionine supplementation cannot be fully compensated for by serine synthesis within the body unless serine is provided exogenously. Also, in the present study supplementation of 10CCD with methionine alone decreased the hepatic serine concentration from 2.68 ± 0.07 to 0.31 ± 0.02 μmol/g, and this decrease was restored by concurrent supplementation with serine to 2.30 ± 0.22 μmol/g. Considering the reported *K*m value for serine in rat CBS, about 0.7 mM (*76*), cystathionine synthesis might be reduced in rats fed 10CCD + 0.5% methionine and recovered in rats fed 10CCD + 0.5% methionine + 2.5% serine. Thus it is likely that one of the roles of supplementation with serine in combination with methionine is to stimulate cystathionine synthesis by supplying serine as a substrate for cystathionine synthesis, but not by increasing CBS activity, leading to cancellation of the plasma homocysteine-elevating effect of methionine. The results of the present study confirm that the resistance of the diet to choline deficiency is influenced not only by the dietary methionine level but also by dietary levels of other amino acids such as serine and glycine.

It should be noted that supplementation of 10CCD with methionine alone tended to increase the hepatic SAH concentration, whereas supplementation with methionine in combination with serine decreased the hepatic SAH concentration to the level of the rats fed 10C. The hepatic SAH concentration reflects the hepatic homocysteine concentration, since the reaction catalyzed by SAH hydrolase is reversible and favors the synthesis of SAH (*77*).

The hepatic homocysteine concentration reflects homocysteine removal. Thus it is plausible to assume that the combination of methionine and serine affects not only the hepatic homocysteine concentration, but also the hepatic SAH concentration. This is important in considering PC synthesis *via* PE *N*-methylation, since SAH is known to be an inhibitor of various types of methyltransferase, including PE *N*-methyltransferase (*77,78*). It has been reported that the second and third methylation of PE were inhibited by SAH, with apparent *K*i values of 4.9 and 6.7 μM respectively, when assayed using partially purified rat liver enzyme (*79*). Although these *in vitro* kinetic data cannot be applied directly to the explanation of the *in vivo* phenomenon, it is possible that hepatic PC synthesis *via* PE *N*-methylation is depressed by supplementation with methionine alone through an increased SAH concentration, and de-depressed by supplementation with a combination of methionine and serine through a suppressed SAH concentration. Hence we postulate that an increase in PC synthesis due to supplementation with methionine in combination with serine also contributes to suppression of hyperhomocysteinemia induced by choline deprivation.

It has been reported that the *K*m value for betaine was 120 μM in rat liver purified BHMT (*80*) and 48 μM in rat liver semipurified BHMT (*81*). If these *K*m values are applicable to *in vivo* BHMT, hepatic BHMT might have been saturated with betaine even in the rats fed 10CCD, in which the hepatic betaine concentration was 0.32 ± 0.02 μmol/g, and the BHMT reaction might not have been enhanced by supplementation with methionine in combination with serine even though the betaine concentration was increased to 0.56 ± 0.03 μmol/g. The BHMT reaction is influenced both by BHMT activity and by the concentration of its substrate betaine. It has been found that hepatic BHMT activity is increased by dietary levels of substrate betaine and related compounds such as choline and methionine (*82,83*). This is in contrast to the case of CBS. Furthermore, it must be determined whether the hepatic betaine concentration is the cause, merely the result, or both of the BHMT reaction. The hepatic betaine concentration probably reflects both choline status within the body, which is determined by choline intake and PC synthesis *via* PE *N*-methylation, and the consumption of betaine by the BHMT reaction. Taking into consideration hepatic BHMT activity and the

hepatic betaine concentration, it is apparent that supplementation of 10CCD with methionine in combination with serine strengthened the BHMT reaction.

The hepatic triglyceride concentration is one of the indices of PC status in the liver (*53*), since active synthesis of PC is required for the synthesis and secretion of very low density lipoprotein (*49*). Another major finding of experiment 1 is that fatty liver caused by choline deprivation was not fully suppressed by supplementation with methionine alone, but was completely suppressed by the combination of methionine and serine. A possible explanation of the different effects of methionine alone and the combination of methionine and serine is that PC synthesis *via* the PE *N*-methylation pathway in the rats fed 10CCD + 0.5% methionine was not fully stimulated because of the higher hepatic concentration of SAH. In contrast, it appears that supplemental serine in combination with methionine stimulates PC synthesis through suppression of the hepatic SAH concentration due to increased removal of homocysteine, and thereby prevents fatty liver.

GAA addition-induced hyperhomocysteinemia model

GAA is synthesized in the kidneys and metabolized to creatine with SAM as methyl-group donor, which is catalyzed by GAA *N*-methyltransferase in the liver (*84*). Stead *et al.* (*61*) first reported that dietary addition of GAA increased the plasma homocysteine concentration in rats, and they postulated that GAA increases the plasma homocysteine concentration by accelerating the conversion of SAM to SAH and further to homocysteine due to compulsive metabolism of GAA. In support of this, we found that dietary addition of GAA significantly decreased the hepatic SAM concentration and increased the hepatic SAH and homocysteine concentrations in rats (*62*). In addition, we postulat that betaine deficiency might also contribute to GAA-induced hyperhomocysteinemia, based on the finding that dietary addition of GAA significantly decreases the hepatic betaine concentration (*63,64*). It has been estimated that a major portion (about 75%) of the methyl group of SAM is consumed to synthesize creatine from GAA in humans(*85,86*). Hence the GAA-induced hyperhomocysteinemia model appears to have physiological relevance. The present study

indicates that hyperhomocysteinemia can be suppressed by supplementation with methionine alone, although the effect was limited. No additive or synergistic effect of methionine and serine on plasma homocysteine concentration was detected. This is in contrast to the case of the model used in experiment 1. Consistently with our previous study (*63,64*), dietary addition of GAA markedly decreased the hepatic betaine concentration, indicating that one of the mechanisms by which GAA induced hyperhomocysteinemia was a decrease in the hepatic betaine concentration even when the diet contained choline at a level of 0.1%. There are several possible mechanisms for the GAA-induced decrease in hepatic betaine concentration: (i) increased consumption of betaine due to acceleration of the methionine cycle by GAA loading, (ii) decreased synthesis of PC *via* the PE *N*-methylation pathway due to a decrease in the hepatic SAM concentration, inhibition by an increased SAH concentration, or both, and (iii) decreased synthesis of PC *via* the PE *N*-methylation pathway due to competition between PE *N*-methyltransferase and GAA *N*-methyltransferase for SAM. It appears that methionine supplementation partially restored PC synthesis through the latter two mechanisms, since the action of methionine supplementation significantly increased the hepatic SAM concentration and, conversely, decreased the hepatic SAH concentration is favorable to restoration of decreased PC synthesis *via* the PE *N*-methylation pathway. The reason for the lack of an additive or synergistic effect of methionine and serine on plasma homocysteine concentration is currently unknown, but one possible reason is that the accelerated conversion of SAM to SAH by GAA, which enhances homocysteine production, has a greater effect on the plasma homocysteine concentration than does the increased conversion of homocysteine to cystathionine by serine. Previously, we found that GAA-induced hyperhomocysteinemia could be suppressed by raising the dietary casein level (*63*). The present study indicates the possibility that a higher methionine level contributes to the suppression of GAA-induced hyperhomocysteinemia by a high casein diet, although other mechanisms, *e.g.*, increases in homocysteine-metabolizing enzymes, cannot be ignored.

The present study indicates that dietary addition of GAA caused fatty liver, suggesting that GAA addition brought about PC deficiency in the liver. It also indicates that

GAA-induced fatty liver was completely prevented by supplementation with methionine in combination with serine, while supplementation with methionine alone did not suppress the development of fatty liver. It is difficult, however, to explain why the development of fatty liver was suppressed by the combination of methionine and serine but not by methionine alone, since some hepatic variables that affect PE *N*-methylation, such as SAM and SAH concentrations and the SAM/SAH ratio, did not differ between the rats fed the diet supplemented with methionine alone and the rats fed the diet supplemented with methionine in combination with serine.

Chapter 2

Factors Contributing to the Resistivity of Higher Casein Diet against Choline Deficiency-induced Hyperhomocysteinemia in Rats

In chapter 1, we found an interesting phenomenon that supplementation of choline-deprived 10C with methionine at a level of 0.5% did not suppress choline deprivation-induced hyperhomocysteinemia, whereas supplementation with 0.5% methionine in combination with 2.5% serine completely suppressed the hyperhomocysteinemia (unpublished data). These results suggest that there exist some factors other than methionine to prevent choline deficiency-induced hyperhomocysteinemia.

In this study, we investigated the mechanisms by which rats fed higher casein diet resist choline deprivation-induced hyperhomocysteinemia. At first, we compared the effects of choline deprivation on plasma homocysteine concentration and related variables in rats fed 10C with the effects of choline deprivation in rats fed 30% casein diet (30C) to confirm that diets containing higher levels of casein, e.g., 30C, do not cause choline deprivation-induced hyperhomocysteinemia. Secondly, we investigated the dose-response effects of methionine supplementation (0.1- 0.5%) on plasma homocysteine concentration and related variables in rats fed choline-deprived 10C. Thirdly, we investigated the effects of supplementation of choline-deprived 10C + 0.5% methionine with glycine plus serine, which are known to stimulate homocysteine metabolism (*53-56,90*), and the effects of branched-chain amino acids (valine, leucine and isoleucine) plus acidic amino acid (aspartate and glutamate), which comprise a considerable part (47.1%) of casein (*91*), to determine whether choline deprivation-induced hyperhomocysteinemia can be suppressed when methionine is supplemented in combination with these amino acids.

MATERIALS AND METHODS

Chemicals. Amino acids were purchased from Wako Pure Chemical (Osaka, Japan). All other chemicals were purchased from Wako or Sigma-Aldrich (St. Louis, MO) 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). The other ingredients of the diet were purchased from Wako Pure Chemical.

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, three separate animal experiments were conducted. In experiment 1, rats were randomly assigned to the following four diet groups to investigate the relationship between dietary casein level and effect of choline deprivation: (1) 10% casein diet (10C), (2) choline-deprived 10C (10CCD), (3) 30% casein diet (30C), and (4) choline-deprived 30C (30CCD). In experiment 2, rats were randomly assigned to the following seven diet groups to investigate the dose-dependent effects of methionine supplementation of 10CCD: (1) 10C, (2) 10CCD, (3) 10CCD + 0.1% L-Met, (4) 10CCD + 0.2% L-Met, (5) 10CCD + 0.3% L-Met, (6) 10CCD + 0.4% L-Met, and (7) 10CCD + 0.5% L-Met. In experiment 3, rats were randomly assigned to the following five diet groups to investigate the effects of dietary amino acid level: (1) 10C, (2) 10CCD, (3) 10CCD + 0.5% L-Met (10CCDM), (4) 10CCDM + 1.26% GS (glycine and L-serine) (10CCDMGS), and (5) 10CCDMGS + 3.61% BCAA (branched-chain amino acids) + 4.50% AAA (acidic amino acids) (10CCDMGSBA). The 1.26% GS consisted of 0.32% glycine and 0.94% L-serine, 3.61% BCAA consisted of 1.11% L-valine, 1.51% L-leucine and 0.99% L-isoleucine, and 4.50% AAA consisted of 0.97% L-aspartic acid and 3.53% L-glutamic acid, which corresponded to 30% casein diet with respect to the total amino acid content. The 10C

consisted of the following ingredients (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 and cellulose powder, 20. In 10CCD, choline bitartrate was omitted with an increase in cornstarch content. Amino acids were added to the diet to make their amino acid levels comparable to those of 30C, based on the literature (*91*). Amino acids were added to the 10CCD at the expense of cornstarch. Rats were given free access to the experimental diets and water for 10 d and killed by decapitation between 10:00 and 11:00 h without prior food deprivation. 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 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 post-mitochondrial 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. (*65*). 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 manufacturer's instructions.

Biochemical analysis. The concentrations of homocysteine and cysteine in the plasma and liver were measured by HPLC using the method of Durand et al. (*89*). The method of Cook et al. (*67*) was employed to determine the concentrations of SAM and

S-adenosylhomocysteine (SAH) in the liver by using the HPLC system. The concentration of betaine in the liver from HPLC was determined as described by Laryea et al. (*68*) and the concentration of serine in the liver was measured by an amino acid autoanalyzer (Model L-8500; Hitachi). The activity of BHMT in the liver was measured according to the method of Finkelstein et al. (*69*), but HPLC was used in the assay of the reaction product, DMG, described by Laryea et al. (*68*). The activity of CBS in the liver was determined according to the method of Mudd et al. (*16*), but HPLC was used in the assay of the reaction product, cystathionine, described by Einarsson et al. (*70*). 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 (*60*). 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. (*71*) using bovine serum albumin as a standard.

Statistical analysis. Each value is expressed as the mean ± SEM. Data were analyzed by a two-way ANOVA (experiment 1) or one-way ANOVA (experiments 2 and 3), 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).

RESULTS

Dietary casein level and effects of choline deprivation (experiment 1)

Food intake during the 10-d experimental period was significantly lower or tended to be lower in rats fed 30C and 30CCD than in rats fed 10C and 10CCD, whereas the intake of methionine was reversed (Table 3). Body weight gain and relative liver weight were significantly higher in rats fed 30C and 30CCD than in rats fed 10C and 10CCD. Choline deprivation did not affect these variables. Plasma homocysteine concentration was significantly lower in rats fed 30C than in rats fed 10C. Choline deprivation significantly increased plasma homocysteine concentration in rats fed 10C, but it did not increase plasma homocysteine concentration in rats fed 30C. Plasma cysteine concentration, which was measured for comparison with homocysteine, was significantly higher in rats fed 30C than in rats fed 10C and unaffected by choline deprivation. Hepatic SAM concentration was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation significantly decreased SAM concentration in both rats fed 10C and those fed 30C. Hepatic SAH concentration was slightly higher in rats fed 30C than in rats fed 10C, and choline deprivation significantly increased SAH concentration in rats fed 10C but not in rats fed 30C. Likewise, SAM:SAH ratio was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation significantly increased SAH concentration in rats fed 10C but not in rats fed 30C. Choline deprivation significantly increased hepatic homocysteine concentration in rats fed 10C, but it did not enhance homocysteine concentration in rats fed 30C. Hepatic BHMT activity was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation significantly decreased the enzyme activity in rats fed 10C but not in rats fed 30C. Choline deprivation markedly decreased hepatic concentration of betaine, a substrate of BHMT, in rats fed 10C, whereas the effect of choline deprivation in rats fed 30C was relatively small. Hepatic CBS activity was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation slightly decreased the enzyme activity in both rats fed 10C and those fed 30C. Hepatic concentration of serine, a substrate of CBS, was markedly lower in rats fed 30C than

in rats fed 10C, and choline deprivation did not affect the amino acid concentration. Choline deprivation markedly increased hepatic triglyceride concentration in rats fed 10C, but it did not affect the lipid concentration in rats fed 30C.

Effects of methionine supplementation at graded levels (experiment 2)

Body weight gain and relative liver weight were significantly increased by methionine supplementation at levels of 0.3% or more, whereas food intake did not differ among the groups (Table 4). The choline deprivation-induced enhancement of plasma homocysteine concentration was significantly suppressed by methionine supplementation in a dose-dependent manner in the range of 0.1 to 0.3%, but the suppressive effect of methionine became smaller with increase in supplementation level in the range of 0.3 to 0.5% (Fig. 3.1, panel A). Choline deprivation or methionine supplementation had little effect on plasma cysteine concentration (Fig. 3.1, panel B). Plasma triglyceride concentration was significantly decreased by choline deprivation and this decrease was restored by methionine supplementation in the range of 0.1 to 0.5% (Fig. 3.1, panel C). The choline deprivation-induced increase in hepatic triglyceride concentration was significantly suppressed by methionine supplementation in a dose-dependent manner in the range of 0.1 to 0.3%, but the suppressive effect of methionine became smaller with increase in supplementation level in the range of 0.3 to 0.5% (Fig. 3.1, panel D). The profile of hepatic triglyceride concentration was similar to that of plasma homocysteine concentration.

The choline deprivation-induced decrease in hepatic SAM concentration was suppressed by methionine supplementation in a dose-dependent manner (Fig. 3.2, panel A). The choline deprivation-induced increase in hepatic SAH concentration was significantly suppressed by methionine supplement in the range of 0.2 to 0.4%, but supplementation with methionine at a level of 0.5% significantly increased SAH concentration to a level higher than the level in the 10CCD group (Fig. 3.2, panel B). The choline deprivation-induced decrease in SAM:SAH ratio was suppressed by methionine supplementation in a dose-dependent manner (Fig. 3.2, panel C). The choline deprivation-induced increase in hepatic homocysteine concentration

was significantly suppressed by methionine supplementation at a level of 0.3%, but methionine supplementation at a level of 0.5% increased homocysteine concentration to a level higher than the level in the 10CCD group (Fig. 3.2, panel D). The profile of hepatic homocysteine concentrations was similar to that of hepatic homocysteine concentrations. The choline deprivation-induced decreases in hepatic BHMT and CBS activities were suppressed by methionine supplementation in a dose-dependent manner (Fig. 3.3, panel A and B). The choline deprivation-induced decrease in hepatic betaine concentration was partially restored by methionine supplementation in a dose-dependent manner (Fig. 3.3, panel C). In contrast, hepatic serine concentration was decreased by methionine supplementation in a dose-dependent manner (Fig. 3.3, panel D).

Effects of supplementation with glycine + serine and dietary amino acid level (experiment 3)

Body weight gain and relative liver weight were significantly higher or tended to be higher in rats fed diets supplemented with methionine irrespective of other supplements, whereas food intake did not differ among the groups (Table 4). The results of experiment 2 showing that choline deprivation-induced increase in plasma homocysteine concentration cannot be suppressed by supplementation with methionine alone at a level of 0.5% were reproduced (Fig. 3.4, panel A). In contrast, choline deprivation-induced increase in plasma homocysteine concentration was completely suppressed by supplementation with methionine + GS. Plasma homocysteine concentration was further decreased by supplementation with BCAA + AAA in addition to methionine + GS. Plasma cysteine concentration was slightly higher in rats fed diets supplemented with GS or GS + BCAA + AAA than in rats fed other diets (Fig. 3.4, panel B). Choline deprivation-induced decrease in plasma triglyceride concentration was restored by supplementation with methionine irrespective of other supplements (Fig. 3.4, panel C). Choline deprivation-induced increase in hepatic triglyceride concentration was partly suppressed by supplementation with methionine alone or methionine + GS and was completely suppressed by supplementation with methionine + GS + BCAA + AAA (Fig. 3.4, panel D).

Choline deprivation-induced decrease in hepatic SAM concentration was completely restored and significantly increased to levels higher than the level in rats fed 10C by supplementation with methionine irrespective of other supplements (Fig. 3.5, panel A). Choline deprivation-induced increase in hepatic SAH concentration was further increased by supplementation with methionine alone, but it was unaffected by supplementation with methionine + GS and was significantly decreased by supplementation with methionine + GS + BCAA + AAA (Fig. 3.5, panel B). Choline deprivation-induced decrease in SAM:SAH ratio was completely restored by supplementation with methionine + GS or methionine + GS + BCAA + AAA (Fig. 3.5, panel C). Choline deprivation-induced increase in hepatic homocysteine concentration was further increased by supplementation with methionine alone or methionine + GS, but it was unaffected by supplementation with methionine + GS + BCAA + AAA (Fig. 3.5, panel D). Choline deprivation-induced decrease in hepatic BHMT activity was completely restored by supplementation with methionine alone and further increased to levels higher than the level in rats fed 10C by supplementation with methionine + GS or methionine + GS + BCAA + AAA (Fig. 3.6, panel A). Choline deprivation-induced decrease in hepatic CBS activity was restored by supplementation with methionine alone or methionine + GS and further increased to a level higher than the level in rats fed 10C by supplementation with methionine + GS + BCAA + AAA (Fig. 3.6, panel B). The profiles of mRNA levels for BHMT and CBS were, on the whole, similar to those of enzyme activities (Fig. 3.6, panels C and D). Choline deprivation-induced decrease in hepatic betaine concentration was partly, but significantly, restored by supplementation with methionine + GS + BCAA + AAA, i.e., from 0.32 ± 0.01 to 0.62 ± 0.05 or 0.66 ± 0.09 μmol/g (Fig. 3.6, panel E). Hepatic serine concentration was markedly decreased by supplementation with methionine alone and this decrease was significantly suppressed or tended to be suppressed by concurrent supplementation with GS or GS + BCAA + AAA (Fig. 3.6, panel F)

Table 3. Effects of choline deprivation on plasma homocysteine concentration and other variables in rats fed 10% and 30% casein diets (experiment 1)

	Diet				ANOVA[2]
	10C	10CCD	30C	30CCD	
Met level in diet, *g/kg*	2.5	2.5	7.5	7.5	
Food intake, *g/10 d*	$171 \pm 8^{ab,1}$	175 ± 6^{a}	148 ± 5^{b}	154 ± 4^{ab}	P
Met intake, *g/10 d*	0.43 ± 0.02^{b}	0.44 ± 0.02^{b}	1.11 ± 0.04^{a}	1.16 ± 0.03^{a}	P
Body wt gain, *g/10 d*	$26 \pm 2^{b,1}$	30 ± 2^{b}	44 ± 2^{a}	47 ± 1^{a}	P
Liver wt, *g/100 g body wt*	4.08 ± 0.04^{b}	4.03 ± 0.07^{b}	4.67 ± 0.10^{a}	4.73 ± 0.08^{a}	P
Plasma:					
Homocysteine, *μmol/L*	18.0 ± 0.2^{b}	32.5 ± 0.5^{a}	12.3 ± 0.3^{c}	12.2 ± 0.4^{c}	C, P, CP
Cysteine, *μmol/L*	104 ± 3^{b}	107 ± 3^{b}	122 ± 4^{a}	124 ± 3^{a}	P
Liver:					
SAM, *nmol/g*	66.1 ± 1.3^{c}	41.8 ± 0.6^{d}	103.4 ± 1.2^{a}	80.3 ± 1.0^{b}	C, P
SAH, *nmol/g*	13.6 ± 0.3^{c}	19.3 ± 0.2^{a}	15.8 ± 0.3^{b}	12.8 ± 0.4^{c}	C, P, CP
SAM:SAH ratio	4.88 ± 0.18^{b}	2.17 ± 0.03^{c}	6.58 ± 0.17^{a}	6.32 ± 0.25^{a}	C, P, CP
Homocysteine, *nmol/g*	2.59 ± 0.04^{b}	3.66 ± 0.06^{a}	2.71 ± 0.04^{b}	2.75 ± 0.03^{b}	C, P, CP
BHMT activity[3]	0.99 ± 0.01^{b}	0.46 ± 0.01^{c}	1.95 ± 0.07^{a}	1.82 ± 0.08^{a}	C, P, CP
BHMT mRNA[4]	1.00 ± 0.02^{c}	0.75 ± 0.04^{c}	1.71 ± 0.12^{a}	1.37 ± 0.10^{b}	C, P
CBS activity[3]	4.86 ± 0.12^{c}	4.12 ± 0.09^{d}	8.50 ± 0.11^{a}	7.93 ± 0.18^{b}	C, P
CBS mRNA[4]	1.00 ± 0.17^{b}	0.91 ± 0.04^{b}	2.11 ± 0.11^{a}	1.79 ± 0.14^{a}	P
Betaine, *μmol/g*	2.69 ± 0.09^{a}	0.33 ± 0.01^{d}	1.22 ± 0.05^{b}	0.94 ± 0.04^{c}	C, P, CP
Serine, *μmol/g*	2.52 ± 0.27^{a}	2.60 ± 0.13^{a}	0.28 ± 0.03^{b}	0.31 ± 0.02^{b}	P
Triglyceride, *μmol/g*	25.7 ± 0.4^{b}	79.4 ± 1.2^{a}	24.3 ± 0.4^{b}	25.5 ± 0.4^{b}	C, P, CP

[1]Each value is the mean ± SE, $n = 8$. Values without a common letter differ, $P < 0.05$. 10C; 10% casein diet; 30C, 30% casein diet; 10CCD, choline-deprived 10C; 30CCD, choline-deprived 30C; BHMT, betaine-homocysteine *S*-methyltransferase; CBS, cystathionine β-synthase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

[2]Two-way ANOVA: C, affected by choline deprivation, $P < 0.05$; P, affected by protein level, $P < 0.05$; CP, interactively affected by choline deprivation and protein level, $P < 0.05$.
effect of choline deprivation and protein level.

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

[4]Values represent BHMT mRNA/β-actin or CBS mRNA/β-actin and are expressed as relative values to the value in the 10C group.

Table 4. Body weight gain, food intake and liver weight of rats fed the experimental diets (experiments 2 and 3)

Diet	Body wt gain	Food intake	Liver wt
	g/10 d		*g/100 g body wt*
Experiment 2			
10C	$27 \pm 2^{bc,1}$	152 ± 3	4.00 ± 0.05^{bc}
10CCD	22 ± 3^{c}	146 ± 6	3.87 ± 0.06^{c}
10CCD + 0.1% Met	35 ± 1^{ab}	152 ± 3	4.11 ± 0.08^{bc}
10CCD + 0.2% Met	36 ± 3^{bc}	144 ± 3	4.27 ± 0.13^{ab}
10CCD + 0.3% Met	41 ± 2^{a}	151 ± 4	4.48 ± 0.04^{a}
10CCD + 0.4% Met	39 ± 1^{a}	143 ± 4	4.51 ± 0.07^{a}
10CCD + 0.5% Met	41 ± 2^{a}	145 ± 5	4.55 ± 0.09^{a}
Experiment 3[2]			
10C	27 ± 2^{b}	174 ± 7	4.14 ± 0.05^{b}
10CCD	28 ± 2^{b}	176 ± 6	4.01 ± 0.06^{b}
10CCD + 0.5% Met (10CCDM)	42 ± 2^{a}	163 ± 6	4.71 ± 0.12^{a}
10CCDM + 0.32% Gly + 0.94% Ser (10CCDMGS)	43 ± 2^{a}	153 ± 5	4.69 ± 0.09^{a}
10CCDMGS + 3.6% BCAA + 4.5% AAA (10CCDMGSBA)	35 ± 2^{ab}	157 ± 3	4.79 ± 0.11^{a}

[1]Each value is the mean ± SE, $n = 8$. Values without a common letter differ, $P < 0.05$. 10C, 10% casein diet; 10CCD, choline-deprived 10C; AAA, acidic amino acids (0.97% Asp + 3.53% Glu); BCAA, branched-chain amino acids (1.11% Val + 1.51% Leu + 0.99% Ile).

[2]In experiment 3, several amino acids were added to the diet to make comparable to the 30% casein diet.

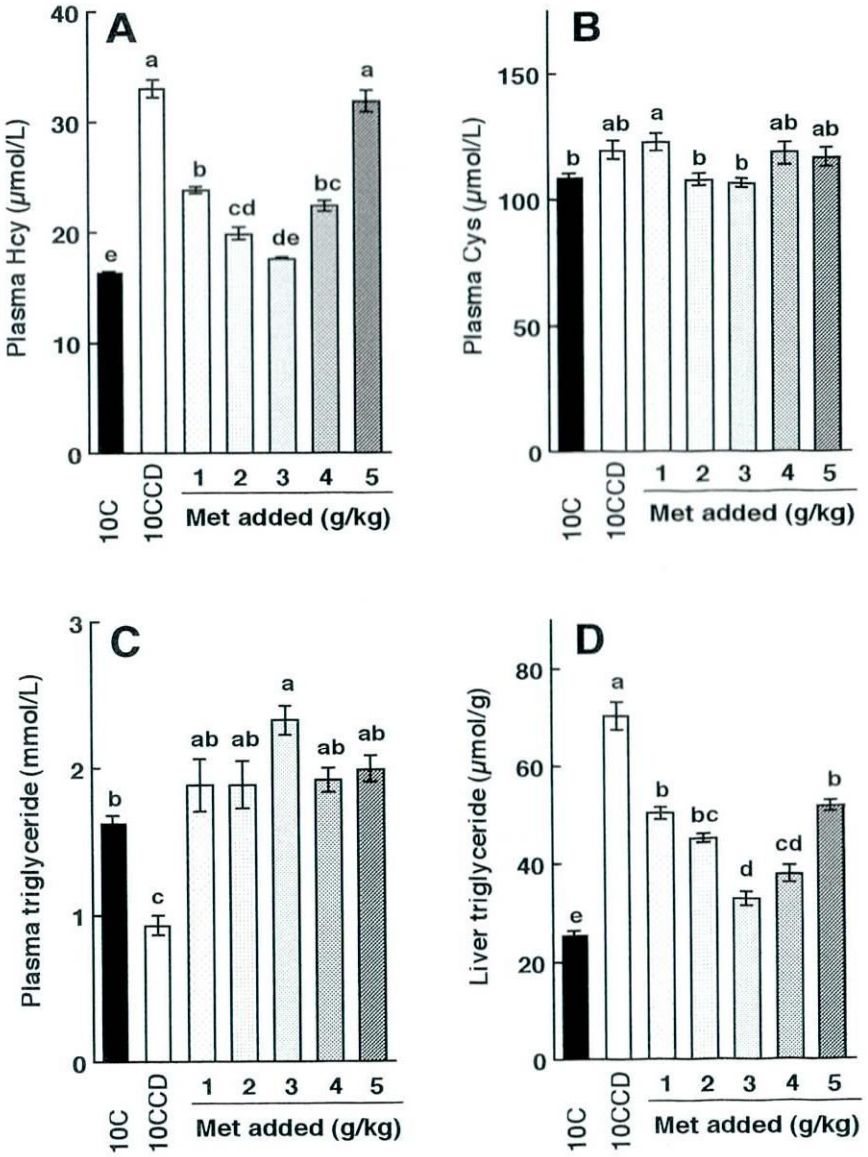

Fig. 3.1 Plasma concentrations of homocysteine (A), cysteine (B), and triglyceride (C) and hepatic triglyceride concentration (D) in rats fed the experimental diets (experiment 2). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. 10C, 10% casein diet; 10CCD, choline-deprived 10C; Cys, cysteine; Hcy, homocysteine.

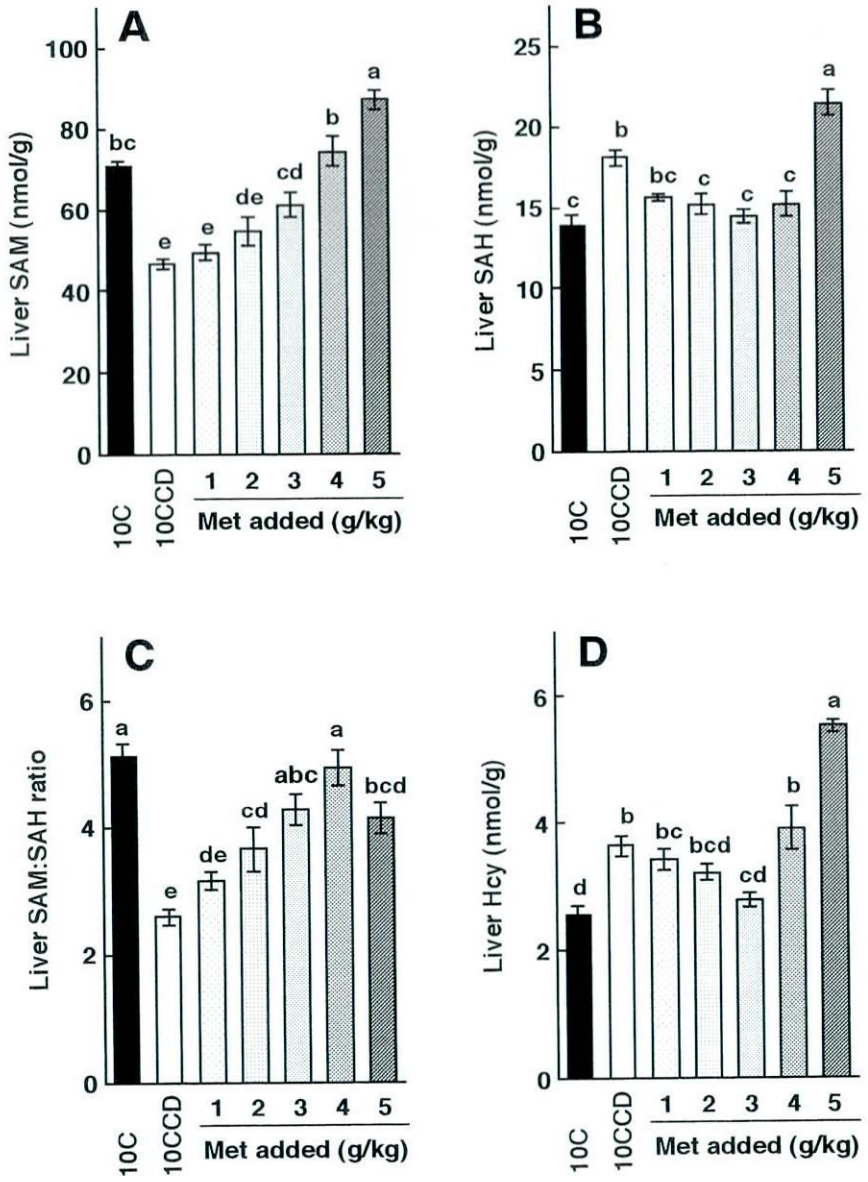

Fig. 3.2 Hepatic concentrations of *S*-adenosylmethionine (A), *S*-adenosylhomocysteine (B), their ratio (C), and homocysteine (D) in rats fed the experimental diets (experiment 2). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine. See the legend of Fig. 3.1 for other abbreviations.

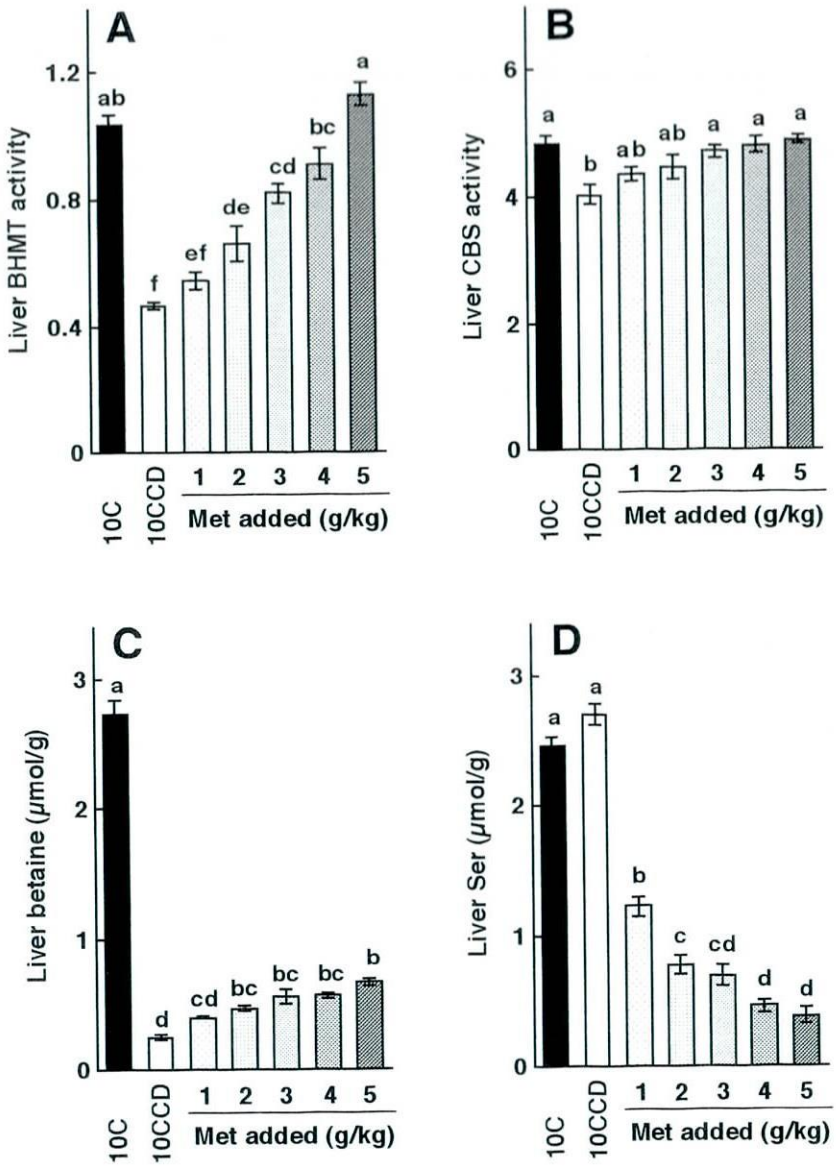

Fig. 3.3 Activities of betaine-homocysteine *S*-methyltransferase (A) and cystathionine b-synthase (B) and concentrations of betaine (C) and serine (D) in the liver of rats fed the experimental diets (experiment 2). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. BHMT, betaine-homocysteine *S*-methyltransferase; CBS, cystathionine β-synthase. See the legend of Fig. 3.1 for other abbreviations. Enzyme activities are expressed as nmol/(min·mg protein).

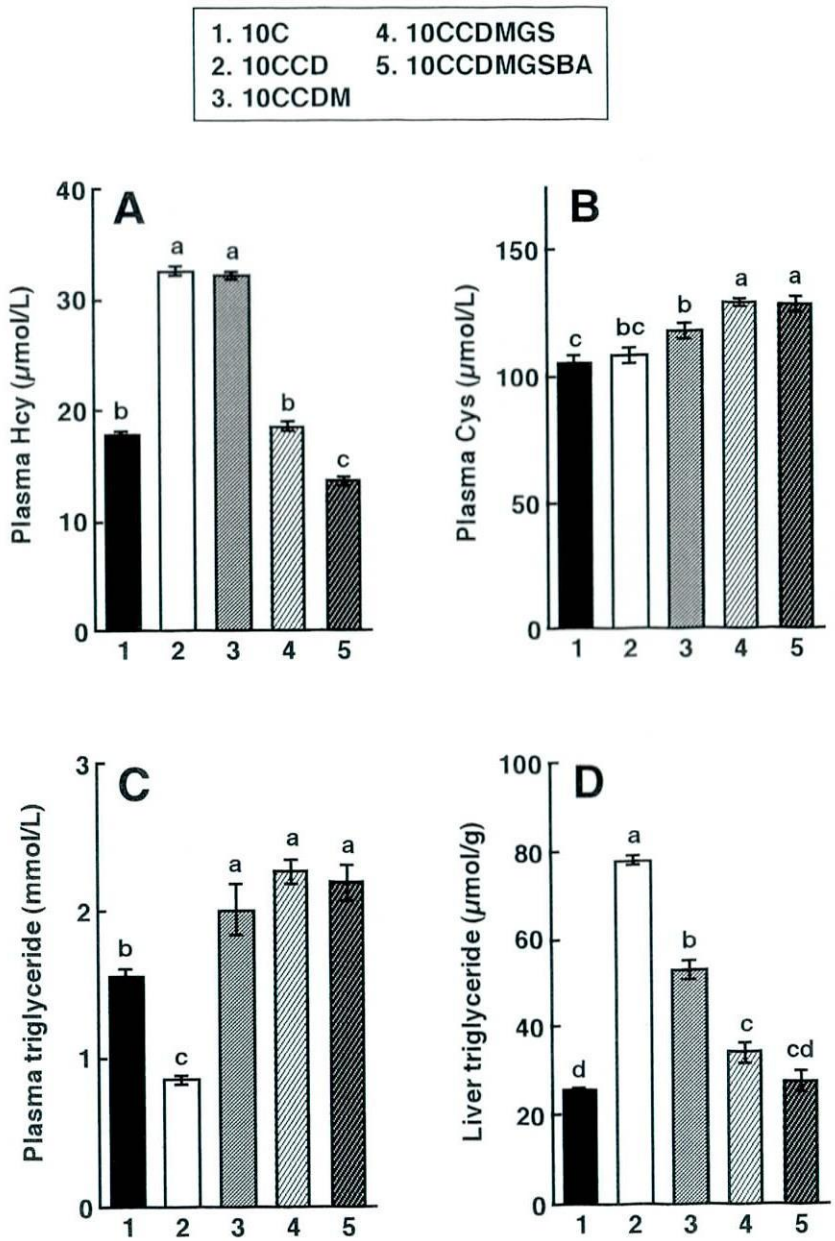

Fig. 3.4 Plasma concentrations of homocysteine (A), cysteine (B), and triglyceride (C) and hepatic triglyceride concentration (D) in rats fed the experimental diets (experiment 3). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. 10C, 10% casein diet; 10CCD, choline-deprived 10C; 10CCDM, 10CCD + 0.5% L-methionine; 10CCDMGS, 10CCDM + 0.32% glycine + 0.94% L-serine; 10CCDMGSBA, 10CCDMGS + 3.61% branched-chain amino acids + 4.5% acidic amino acids. See the legend of Fig. 3.1 for other abbreviations.

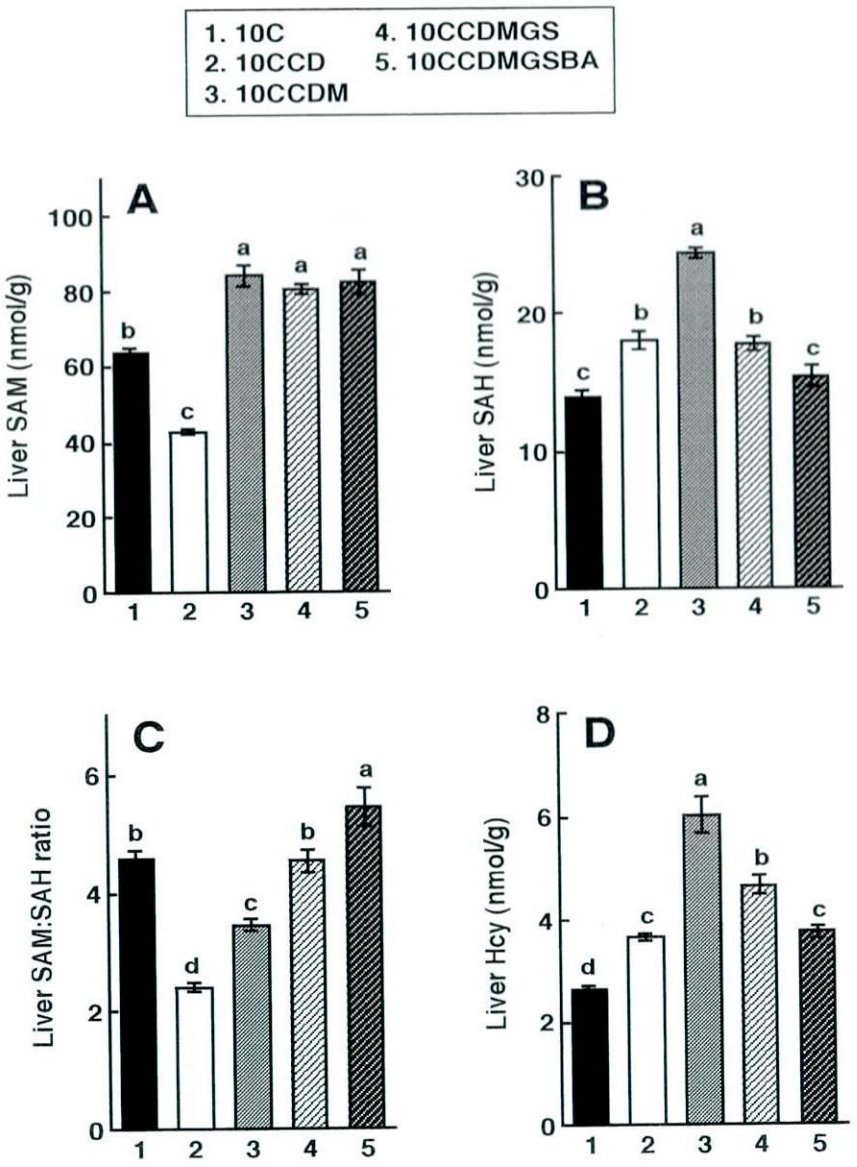

Fig. 3.5 Hepatic concentrations of *S*-adenosylmethionine (A), *S*-adenosylhomocysteine (B), their ratio (C), and homocysteine (D) in rats fed the experimental diets (experiment 3). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. See the legends of Figs. 3.2 and 3.4 for abbreviations.

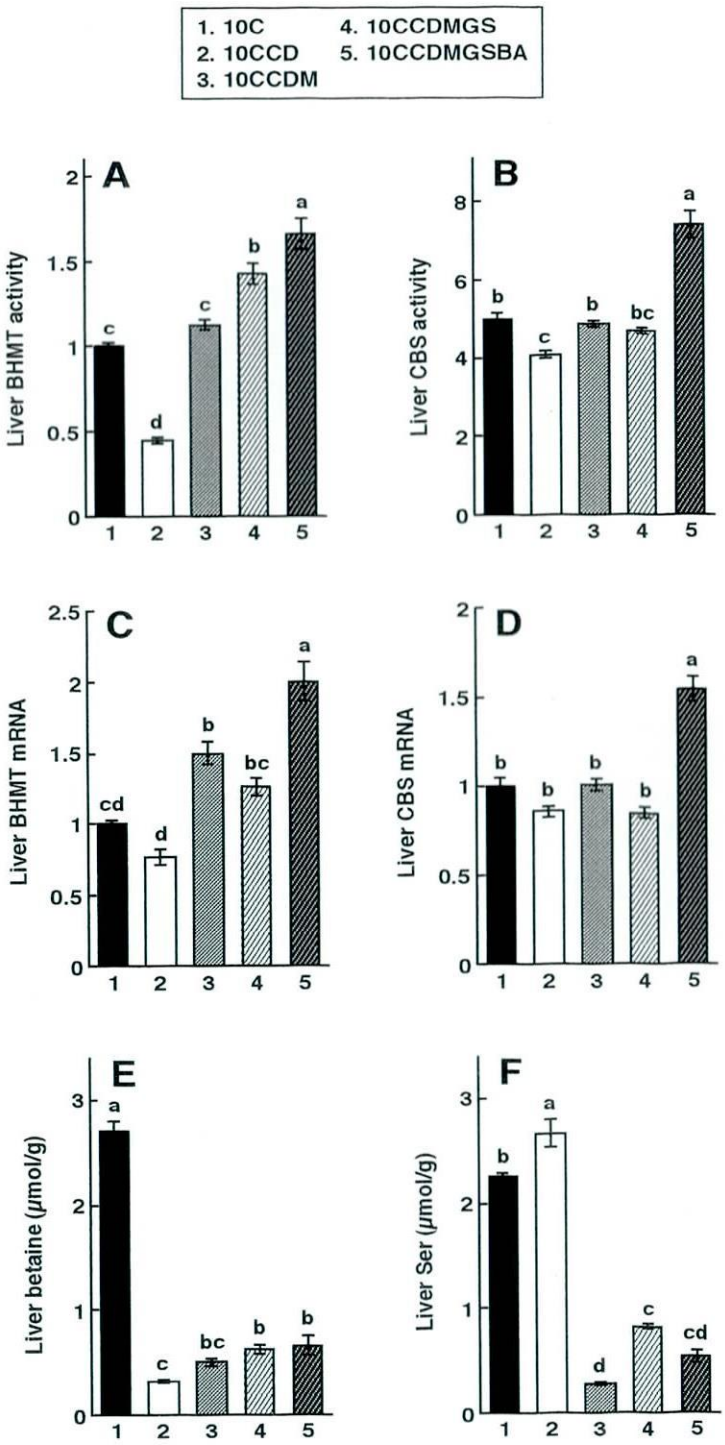

Fig. 3.6 Relative levels of mRNA for betaine-homocysteine *S*-methyltransferase (A) and cystathionine β-synthase (B) and hepatic concentrations of betaine (C) and serine (D) in rats fed the experimental diets (experiment 3). Each value is the mean ± SEM, $n = 8$. Means in a panel without a common letter differ, $P < 0.05$. Enzyme activities are expressed as nmol/(min·mg protein). In panels C and D, values represent BHMT mRNA/β-actin and CBS mRNA/β-actin, respectively, and are expressed as relative values to the value in the 10C group. See the legends of Figs. 3.3 and 3.4 for abbreviations.

DISCUSSION

Our previous study demonstrated that choline deprivation significantly enhanced plasma homocysteine concentration when rats were fed a low casein diet, whereas it did not enhance plasma homocysteine concentration when rats were fed a higher casein diet (*50*). This was confirmed in experiment 1 of the present study. It appears that the different responses of rats fed 10C and rats fed 30C to choline deprivation were associated with different choline status, because fatty liver, which is one of the indices of deficiency of PC or choline (*51*), developed only in rats fed choline-deprived 10C. The results also support the assumption that one of the mechanisms underlying choline deprivation-induced hyperhomocysteinemia is due to deficiency of betaine, since hepatic betaine concentration was markedly decreased by choline deprivation in rats fed 10C, in contrast to only a limited decrease in betaine concentration in rats fed 30C. Betaine status is closely linked to choline status. Choline status is determined by intake of both choline and methionine. Hepatic SAM concentration, which reflects methionine intake, is thought to be a critical factor in PC synthesis via the PE *N*-methylation pathway (*52,53*). Therefore, it is reasonable to assume that choline deprivation does not cause deficiencies of choline-containing compounds such as PC, choline, and betaine under the condition of higher levels of dietary methionine. However, this does not necessarily mean that the resistivity of rats fed higher casein diets is solely attributable to higher methionine levels of diets. This assumption is based on the finding in our previous study that choline deprivation-induced hyperhomocysteinemia in rats fed 10C could not be suppressed by methionine supplementation at a level of 0.5% despite the dietary methionine level of 10C + 0.5% methionine being approximately comparable to that of 30C (unpublished data). This prompted us to investigate the possibility that some factors other than dietary methionine level contribute to the resistivity of rats fed 30C to choline deprivation-induced hyperhomocysteinemia.

One of the important findings obtained in experiment 2 is that methionine had two opposing effects on plasma homocysteine concentration, which were dependent on the

supplementation level of methionine, i.e., plasma homocysteine-lowering effect of methionine in the supplementation range of 0.1 to 0.3% and plasma homocysteine- elevating effect in the supplementation range of 0.3 to 0.5%, as shown in Fig. 3.1. It appears that the former effect of methionine is mainly ascribed to stimulation of PC synthesis via the PE *N*-methylation pathway and resulting increase in betaine supply. On the other hand, the latter effect might be mainly ascribed to the augmented production of homocysteine, since methionine is the sole precursor of homocysteine. In support of this, dietary supplementation with methionine increased plasma homocysteine concentration in a dose-dependent manner in rats fed a 25% casein diet, although the diet contained choline at a level of 0.1% (*57*). Hence, the effect of methionine supplementation of choline-deprived 10C on plasma homocysteine concentration is the result of the sum of the two opposing effects of methionine. The results showing that methionine supplementation at around 0.3% led to the lowest plasma homocysteine concentration suggest that the plasma homocysteine-lowering effect of methionine at this supplementation level was greater than the plasma homocysteine-elevating effect of methionine in rats fed choline-deprived 10C. The fact that methionine supplementation at a level of 0.5% did not have any suppressive effect on choline deprivation-induced hyperhomocysteinemia indicates that 10C + 0.5% methionine is definitively distinct from 30C despite the methionine contents of the two diets being comparable. Interestingly, hepatic triglyceride concentration was influenced by methionine supplementation in a manner similar to that of plasma homocysteine concentration despite hepatic betaine concentration being increased with increase in methionine supplementation level. The results suggest that supplementation of choline-deprived 10C with methionine at a relatively high level, e.g., 0.5%, might not fully stimulate PC synthesis via the PE *N*-methylation pathway. The reason for the inadequate effect of methionine in suppressing the development of choline deprivation-induced fatty liver is unclear. However, one possibility is that higher hepatic SAH concentration in rats fed 10CCD + 0.5% methionine inhibited PC synthesis via the PE *N*-methylation pathway, since SAH is known as an inhibitor of various types of methyltransferase, including PE *N*-methyltransferase (*75,76*). This may be related to the fact

that active synthesis of PC is required for the secretion of very low density lipoprotein from liver cells (*47*). These assumptions, however, do not appear to account for the significant increase in hepatic betaine concentration by methionine supplementation.

The results obtained in experiment 3 clearly demonstrate that choline deprivation-induced hyperhomocysteinemia has been effectively suppressed by methionine when supplemented in combination with GS alone or with GS + BCAA + AAA. The effect of methionine + GS is essentially consistent with our previous finding that choline deprivation-induced hyperhomocysteinemia was completely suppressed by supplementation with 0.5% methionine + 2.5% serine in rats fed 10C, although in contrast to the present study, 0.5% methionine + 2.5% serine decreased hepatic triglyceride concentration to a level lower than the level in rats fed 10C (unpublished data). It is assumed that GS stimulated cystathionine synthesis by supplying serine, a substrate of CBS, and thereby diminished the plasma homocysteine-elevating effect of methionine, since supplementation with GS did not increase hepatic CBS activity. The results showing that hepatic SAH and homocysteine concentrations were significantly lower in rats fed 10CCD supplemented with methionine + GS than in rats fed 10CCD supplemented with methionine alone support the idea of GS-stimulated homocysteine removal. An important finding in experiment 3 is that BCAA + AAA had a plasma homocysteine-lowering effect when supplemented in combination with methionine + GS. We added BCAA + AAA to the diet so as to raise the dietary amino acid level, since these amino acids comprise a major part of total amino acids of casein. The addition of BCAA + AAA was found to increase hepatic CBS and BHMT activities and their gene expression, which are favorable for the removal of homocysteine. It is thought that cystathionine formation is critical in homocysteine metabolism under the condition of relatively high levels of dietary methionine (*72,88*). Therefore, the effect of BCAA + AAA on plasma homocysteine concentration appears to be explained mainly by increased CBS activity, although the increase in BHMT activity cannot be ignored. It has been shown that CBS activity increased in response to dietary casein level (*58,89*), but it did not respond to dietary methionine level within the nutritional range (*58*). The latter phenomenon is unexpected,

since CBS is a member of the family of sulfur-containing amino acid-metabolizing enzymes. It is known that BCAA, especially leucine, stimulates muscle protein synthesis and decreases muscle protein proteolysis, and their mechanisms have been extensively studied (*90,91*). On the other hand, Zhong et al. (*92*) have shown that hepatic serine dehydratase activity and its gene expression were significantly increased by dietary addition of a large amount (12.7%) of leucine, but not isoleucine and valine, in rats fed 10C, indicating the possibility that amino acid-metabolizing enzymes other than serine dehydratase are induced by leucine. However, it is unlikely that the increased CBS activity and its gene expression are solely attributable to BCAA, since our previous study showed that hepatic CBS activity was also significantly increased not only by dietary addition of 12% BCAA but also by dietary addition of 12% amide amino acids (glutamine + asparagines) (unpublished data). Therefore, it seems reasonable to assume that hepatic CBS activity was induced by enhancing the dietary level of total amino acids regardless of the type of amino acid. Bella et al. (*93*) have shown that some enzymes participating in the metabolism of cysteine, such as cysteinesulfinate decarboxylase, cysteine dioxygenase, and γ-glutamylcysteine synthetase, were influenced not only by dietary addition of sulfur-containing amino acids (0.96% methionine or 0.78% cystine) but also by dietary addition of a large amount (30%) of non-sulfur amino acid mixture, although sulfur-containing amino acids had stronger effects than non-sulfur amino acid mixture. In any case, the results obtained in experiment 3 indicate that a higher casein diet results in resistance to choline deficiency-induced hyperhomocysteinemia by at least three mechanisms: (i) supply of methionine, which increases hepatic SAM concentration and thereby stimulates PC synthesis via the PE *N*-methylation pathway, (ii) supply of glycine and serine, which stimulate homocysteine metabolism as an indirect or direct substrate of CBS, and (iii) supply of a relatively large amount of amino acids, which induces CBS and thereby enhances homocysteine metabolism.

We previously demonstrated that plasma homocysteine concentration was lower in rats fed casein or soybean protein diets containing higher levels of protein than in rats fed casein or soybean protein diets containing lower levels of protein (*58,94-96*). Since higher protein diets

inevitably increase methionine intake, such a phenomenon seems to be paradoxical. One of the possible mechanisms by which higher protein diets decrease plasma homocysteine concentration is that higher protein diets increase CBS and BHMT activities and thereby effectively enhance homocysteine removal despite homocysteine production being augmented (*96*). The present study also demonstrated that plasma homocysteine concentration was significantly lower in rats fed 30C than in rats fed 10C irrespective of choline deprivation (experiment 1). Raising dietary amino acid content by adding BCAA + AAA in combination with methionine + GS was found to decrease plasma homocysteine concentration to a level lower than the level in rats fed 10C (experiment 3). These results suggest that higher amino acid content itself contributes to lower plasma homocysteine concentration in rats fed 30C even under the condition of choline deprivation. We previously demonstrated that the plasma homocysteine-elevating effect of dietary supplementation with 0.5% methionine was smaller in rats fed 30C than in rats fed 10C (*94*). Dietary addition of 0.5% guanidinoacetic acid did not increase plasma homocysteine concentration in rats fed a 40% casein diet, while it markedly enhanced plasma homocysteine in rats fed 10C (*61*). Furthermore, folate deficiency-induced enhancement of plasma homocysteine concentration was significantly smaller in rats fed a 20% casein diet than in rats fed 10C (unpublished data). These results indicate that higher casein diets generally cause resistance to various types of hyperhomocysteinemic treatment. Some of the factors contributing to the resistivity to choline deficiency, as presented here, may also be associated with the suppressive effects of higher casein diets on hyperhomocysteinemia induced by methionine, guanidinoacetic acid, or folate deficiency. However, this remains to be clarified experimentally in further studies.

It is concluded that there are at least three factors contributing to the resistivity of rats fed 30C to choline deprivation-induced hyperhomocysteinemia, i.e., higher methionine intake, higher glycine + serine intake, and higher amino acid intake. This is also the case for choline deprivation-induced development of fatty liver. The information provided here might be useful for studies on prevention of hyperhomocysteinemia and fatty liver.

Conclusion

In this study, we primarily investigated the effects of supplementation with methionine, serine, and both on the plasma homocysteine concentration and related variables in hyperhomocysteinemic rat models to determine whether methionine alone or in combination with serine exhibits a hypohomocysteinemic effect, and whether there is an interaction effect between methionine and serine. Secondly, we investigated the mechanisms by which rats fed higher casein diet resist choline deprivation-induced hyperhomocysteinemia.

In chapter 1, one of an interesting finding of experiment 1 was that supplementation of choline-deprived 10C with methionine alone at a level of 0.5% did not have any suppressive effect on hyperhomocysteinemia, and supplementation with methionine in combination with serine completely suppressed hyperhomocysteinemia, although supplementation with serine alone also had a partial effect. This indicates that methionine and serine synergistically suppressed hyperhomocysteinemia. This synergistical effect of methionine and serine on hyperhomocysteinemia, at first, might be explained by stimulate cystathionine synthesis by supplying serine as a substrate for cystathionine synthesis, but not by increasing CBS activity, leading to cancellation of the plasma homocysteine-elevating effect of methionine. The results of the present study confirm that the resistance of the diet to choline deficiency is influenced not only by the dietary methionine level but also by dietary levels of other amino acids such as serine and glycine. Secondly, an increase in PC synthesis due to supplementation with methionine in combination with serine also contributes to suppression of hyperhomocysteinemia induced by choline deprivation. Thirdly, considering hepatic BHMT activity and the hepatic betaine concentration, it is apparent that supplementation of 10CCD with methionine in combination with serine strengthened the BHMT reaction.

Another major finding of experiment 1 is that fatty liver caused by choline deprivation was not fully suppressed by supplementation with methionine alone, but was completely suppressed by the combination of methionine and serine. A possible explanation of the

different effects of methionine alone and the combination of methionine and serine is that PC synthesis *via* the PE *N*-methylation pathway in the rats fed 10CCD + 0.5% methionine was not fully stimulated because of the higher hepatic concentration of SAH. In contrast, it appears that supplemental serine in combination with methionine stimulates PC synthesis through suppression of the hepatic SAH concentration due to increased removal of homocysteine, and thereby prevents fatty liver.

However, in model of GAA addition- induced hyperhomocysteinemia, we found that hyperhomocysteinemia can be suppressed by supplementation with methionine alone, although the effect was limited. No additive or synergistic effect of methionine and serine on plasma homocysteine concentration was detected. This is in contrast to the case of the model used in experiment 1. The reason for the lack of an additive or synergistic effect of methionine and serine on plasma homocysteine concentration is currently unknown, but one possible reason is that the accelerated conversion of SAM to SAH by GAA, which enhances homocysteine production, has a greater effect on the plasma homocysteine concentration than does the increased conversion of homocysteine to cystathionine by serine. The present study indicates that a higher methionine level contributes to the suppression of GAA-induced hyperhomocysteinemia by a high casein diet, although other mechanisms, *e.g.*, increases in homocysteine-metabolizing enzymes, cannot be ignored. It also indicates that GAA-induced fatty liver was completely prevented by supplementation with methionine in combination with serine, while supplementation with methionine alone did not suppress the development of fatty liver. It is difficult, however, to explain why the development of fatty liver was suppressed by the combination of methionine and serine but not by methionine alone, since some hepatic variables that affect PE *N*-methylation, such as SAM and SAH concentrations and the SAM/SAH ratio, did not differ between the rats fed the diet supplemented with methionine alone and the rats fed the diet supplemented with methionine in combination with serine.

In chapter 2, we once again confirmed it in experiment 1 of the present study that choline deprivation significantly enhanced plasma homocysteine concentration when rats were fed a low casein diet, whereas it did not enhance plasma homocysteine concentration when rats

were fed a higher casein diet. The result prompted us to investigate the possibility that some factors other than dietary methionine level contribute to the resistivity of rats fed 30C to choline deprivation-induced hyperhomocysteinemia. One of the important findings obtained in experiment 2 is that methionine had two opposing effects on plasma homocysteine concentration, which were dependent on the supplementation level of methionine, i.e., plasma homocysteine-lowering effect of methionine in the supplementation range of 0.1 to 0.3% and plasma homocysteine- elevating effect in the supplementation range of 0.3 to 0.5%, as shown in Fig. 3.1. It appears that the former effect of methionine is mainly ascribed to stimulation of PC synthesis via the PE *N*-methylation pathway and resulting increase in betaine supply. On the other hand, the latter effect might be mainly ascribed to the augmented production of homocysteine, since methionine is the sole precursor of homocysteine. The results obtained in experiment 3 clearly demonstrate that choline deprivation-induced hyperhomocysteinemia has been effectively suppressed by methionine when supplemented in combination with GS alone or with GS + BCAA + AAA. The results indicate that a higher casein diet results in resistance to choline deficiency-induced hyperhomocysteinemia by at least three mechanisms: (i) supply of methionine, which increases hepatic SAM concentration and thereby stimulates PC synthesis via the PE *N*-methylation pathway, (ii) supply of glycine and serine, which stimulate homocysteine metabolism as an indirect or direct substrate of CBS, and (iii) supply of a relatively large amount of amino acids, which induces CBS and thereby enhances homocysteine metabolism.

These results in the study indicate that higher casein diets generally cause resistance to various types of hyperhomocysteinemic treatment. Some of the factors contributing to the resistivity to choline deficiency, as presented here, may also be associated with the suppressive effects of higher casein diets on hyperhomocysteinemia induced by methionine, guanidinoacetic acid, or folate deficiency. Thus, the present study demonstrated that some other amino acids, such as Gly + Ser, BCAA + AAA, are required to elicit suppressing effect of Met on choline deprivation-induced hyperhomocysteinemia. The information provided here might be useful for studies on prevention of hyperhomocysteinemia and fatty liver.

Acknowledgements

My study at the United graduate School of Agricultural Science, Gifu University will soon come to the end and, at the completion of my graduation thesis, I wish to express my sincere appreciation to all those who have offered me invaluable help during the five years of my study and research period.

My deepest gratitude goes first and foremost to Prof. Kimio Sugiyama of Laboratory of Food and Nutritional Biochemistry, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, the supervisor and tutor of my study and also my life. With his constant encouragement and guidance, I can walk through all the stages of the five years of life in Japan and finally complete this thesis smoothly. I clearly remember that, at the beginning of my research period, I did not perform well and was not a qualified researcher, but it was Prof. Kimio Sugiyama 's serious scientific attitude and rigorous scholarship that deeply influenced me, and it was his patience and encouragement that greatly touched me. He leads me, step by step, to the correct way to scientific field. Besides, Prof. Kimio Sugiyama is also very supportive and concerned with my life, helping me to solve the difficulties of my daily life, which brings me great confidence and courage. His consistence, illuminating instruction and warm-hearted concern are the most precious wealth for me in my life.

Secondly, I would like to express my heartfelt thanks to Prof. Makoto Mori of Department of Applied Biological Chemistry, Shizuoka University, for his kindness to permit me to use ABI 7000 Sequence Detection System for mRNA measurements. Without his instruction or help, I can not handle the experiment devices so well and process my experiment so smoothly. Moreover, he offered me the chance as being a teaching assistant so that I got many opportunities to learn from him in many ways, from the method of research work to daily life experience. For example, he once took me to the high school to communicate with the high school students, which helped me to know deeply about the Japanese life and the society.

Last but not the least, my gratitude also extends to Dr. Takashi Hayakawa, Professor of

Laboratory of Food and Nutritional Biochemistry, Department of Food and Life Science, Faculty of Applied Biological Sciences, Gifu University, Dr. Tatsuya Morita, Professor of Laboratory of Food and Nutritional Biochemistry, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, who have been assisting, supporting and caring for me with my research as well as my life.

I also thank all members of Laboratory of Food and Nutritional Biochemistry, who helped me directly or indirectly during the course of this study.

REFERENCES

1) Cattaneo M. 1999. Hyperhomocysteinemia, atherosclerosis and thrombosis. *Thromb Haemost* 81: 165-176.

2) Refsum H, Ueland PM, Nygard O, Vollset SE. 1998. Homocysteine and cardiovascular disease. *Annu Rev Med* **49**: 31-62.

3) Bostom AG, Lathrop L. 1997. Hyperhomocysteinemia in endstage renal disease: Prevalence, etiology, and potential relationship to arteriosclerotic outcomes. *Kidney int* 52: 10-20.

4) Dennis VW, Robinson K. 1996. Homocysteinemia and vascular disease in end-stage renal disease. *Kidney int* 50 suppl. 57: S11-S17.

5) Hussein WI, Green R, Jacobsen DW, Faiman C. 1999. Normalization of hyperhomocysteinemia with L-thyroxine in hypothyroidism. *Ann Intern Med* 131: 348-351.

6) Nedrebo BG, Ericsson UB, Nygard O, Refsum H, Ueland PM, Aakvaag A, Aanderud S, Lien EA. 1998. Plasma total homocysteine levels in hyperthyroid and hypothyroid patients. *Metabolism* 47: 89-93.

7) Eskes TKAB. 1998. Open or closed? A world of difference: A history of homocysteine research. *Nutr Rev* 56: 236-244.

8) VanAerts LAGJM, Blom HJ, Deabreu RA, Trijbels FJM, Eskes TKAB, Peereboom-Stegeman JHJC, Noordhoek J. 1994. Prevention of neural tube defects by and toxicity of L-homocysteine in cultured postimplantation rat embryos. *Teratology* 50: 348-360.

9) Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. 1998. Folate, vitamin B_{12}, and serum total homocysteine levels in confirmed Alzheimer disease. *Arch Neurol* 55: 1449-1455.

10) Lehmann M, Gottfries CG, Regland B.1999. Identification of cognitive impairment in

the elderly: Homocysteine is an early marker. *Dementia* 10: 12-20.

11) Mudd SH, Finkelstein JD, Irreverre F, Laster L. 1964. Homocystinuria: An enzymatic defect. *Science* 143: 1443-1445.

12) Butz LW, du Vigneaud V. 1932. The formation of a homologue of cystine by the decompensation of methionine with sulfuric acid. *J boil Chem* 99: 135-142.

13) Clarke R, Woodhouse P, Ulvik A, Frost C, Sherliker P, Refsum H, Ueland PM, Khaw KT. 1998. Variability and determinants of total homocysteine concentrations in plasma in an elderly population. *Clin Chem* 44: 102-107.

14) Du Vigneaud V, Patterson WI, Hunt M. 1938. Opening of the ring of the thiolactone of homocysteine. *J Biol Chem* 126: 217-231.

15) Riegel B, du Vigneaud V. 1935. The isolation of homocysteine and its conversion to a thiolactone. *J Biol Chem* 112: 149-154.

16) Mudd SH, Finkelstein JD, Irreverre F, Laster L. 1965. Transsulfuration in mammals. Microassays and tissue distribution of three enzymes of the pathway. *J Biol Chem* 240: 4382-4392.

17) Cavallini D, De Marco C, Dupre S, Rotilio G. 1969. The copper catalyzed oxidation of cysteine to cystine. *Arch Biochem Biophys* 130: 354-361.

18) McCully KS. 1969. Vascular pathology of homocysteinemia: Implication for the pathogenesis of arteriosclerosis. *Am J Pathol* 56: 111-128.

19) The Homocysteine Revolution. Kilmer McCully. McGraw-Hill Press; 1999.

20) The Heart Revolution: The B vitamin Breakthrough That Lowers Homocysteine, Cuts Your Risk of Heart Disease, and Protects Your Health. Kilmer McCully, Martha McCully. Diane Pub Co Press: 1999.

21) Stampfer MJ, et al. 1992. A prospective study of plasma homocysteine and risk of myocardial infarction in US physicians. *JAMA* 268(7): 877-881.

22) Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. 1995. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA* 274(13): 1049-1057.

23) Jacobsen DW. 1998. Homocysteine and vitamins in cardiovascular disease. *Clin Chem* (8 pt 2): 1833-1843.

24) Haynes WG. 2002. Hyperhomocysteinemia, vascular function and atherosclerosis: effects of vitamins. *Cardiovasc Drugs Ther* 16(5): 391-399.

25) Clarke R, Stansbie D. 2001. Assessment of homocysteine as a cardiovascular risk factor in clinical practice. *Ann Clin Biochem* 38(Pt 6): 624-632.

26) Auer J, Berent R, Eber B. 2001. Homocysteine and risk of cardiovascular disease. *J Clin Basic Cardiol* 4: 261-264.

27) Herrmann W. 2001. The importance of hyperhomocysteinemia as a risk factor for diseases: an overview. *Clin Chem Lab Med* 39(8): 666-674.

28) Homocysteine in Health and Disease. Carmel R, Jacobsen D. Cambridge University Press; UK Press: 2001.

29) Lentz SR, Sobey CG, et al. 1996. Vascular dysfunction in monkeys with diet-induced hyperhomocysteinemia. *J Clin Invest* 98(1): 24-29.

30) Wolters M, Hermann S, Hahn A. 2003. B vitamin status and concentrations of homocysteine and methylmalonic acid in elderly German women. *Am J Clin Nutr* 78(4): 765-772.

31) Ward M. 2001. Homocysteine, folate, and cardiovascular disease. *Int J Vitam Nutr Res* 71(3): 173-178.

32) Mizrahi EH, Jacobsen DW, et al. 2003. Plasma total homocysteine levels, dietary vitamin B_6 and folate intake in AD and healthy aging. *J Nutr Health Aging* 7(3): 160-165.

33) Durand P, Prost M, Loreau N, Lussier-Cacan S, Blache D. 2001. Impaired homocysteine metabolism and atherothrombotic disease. *Lab Invest* 81(5): 645-672.

34) Sunder-Plassmann G, Fodinger M. 2003. Genetic determinants of the homocysteine level. *Kidney Int Suppl* (84): S141-S144.

35) Lievers KJ, Kluijtmans LA, Blom HJ. 2003. Genetics of hyperhomocysteinemia in cardiovascular disease. *Ann Clin Biochem* 40(pt 1): 46-59.

36) Cortese C, Motti C. 2001. MTHFR gene polymorphism, homocysteine and

cardiovascular disease. Public Health Nutr 4(2B): 493-497.

37) Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. 2001. Biological and clinical implications of the MTHFR C677T polymorphism. *Trends Pharmacol Sci* 22(4): 195-201.

38) Ma J, Stampfer MJ, et al. 1996. Methylenetetrahydrofolate reductase polymorphism, plasma folate, homocysteine, and risk of myocardial infarction in US physicians. *Circulation* 94(10): 2410-2416.

39) Malinoe MR, et al. 1997. The effects of folic acid supplementation on plasma total homocysteine are modulated by multivitamin use and methylenetetrahydrofolate reductase genotypes. *Arterioscler Thromb Vasc Biol* 17(6): 1157-1162.

40) Klerk M, Verhoef P, et al. 2002. 677C-->T polymorphism and risk of coronary heart disease: a meta-analysis. *JAMA* 288(16): 2023-2031.

41) Graham IM, O'Callaghan P. 2002. Vitamins, homocysteine and cardiovascular risk. *Cardiovasc Drugs Ther* **16**: 383-389.

42) Zeisel SH, Blusztajn JK. 1994. Choline and human nutrition. *Annu Rev Nutr* 14: 269-296.

43) Niculescu MD, Zeisel SH. 2002. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. *J Nutr* 132: 2333S-5S.

44) Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin, and choline. Washington, DC: The National Academy Press: 1998.

45) Zeisel SH, da Costa K-A, Franklin PD, et al. 1991. Choline, an essential nutrient for humans. *FASEB* J 5: 2093-2098

46) Buchman A, Dubin M, Moukarzel A, et al. 1995. Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. *Hepatology* 22: 1399-1403.

47) Yao Z, Vance DE. 1988. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem* **263**: 2998-3004.

48) da Costa KA, Gaffney CE, Fischer LM, Zeisel SH. 2005. Choline deficiency in mice and humans is associated with increased plasma homocysteine concentration after a methionine load. *Am J Clin Nutr* 81: 440-444.

49) Varela-Moreiras G, Ragel C, de Miguelsanz JP. 1995. Choline deficiency and methotrexate treatment induces marked but reversible changes in hepatic folate concentrations, serum homocysteine and DNA methylation rates in rats. *J Am Coll Nutr* **14**: 480-485.

50) Setoue M, Ohuchi S, Morita T, Sugiyama K. 2008. Choline deprivation induces hyperhomocysteinemia in rats fed low methionine diets. *J Nutr Sci Vitaminol* **54**: 483-490.

51) Zeisel SH. 1990. Choline deficiency. *J Nutr Biochem* **1**: 332-349.

52) Tijburg LBM, Geelen MJH, van Golde LMG. 1989. Regulation of the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. *Biochim Biophys Acta* **1004**: 1-19.

53) Pelech SL, Vance DE. 1984. Regulation of phosphatidylcholine biosynthesis. *Biochim Biophys Acta* **779**: 217-251.

54) Stipanuk MH. 2004. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Ann Rev Nutr* **24**: 539-577.

55) Selhub J. 1999. Homocysteine metabolism. *Annu Rev Nutr* **19**: 217-246.

56) De Bree A, Verschuren WM, Kromhout D, Kluijtmans LA, Blom HJ. 2002. Homocysteine determinants and the evidence to what extent homocysteine determines the risk of coronary heart disease. *Pharmacol Rev* **54**: 599-618.

57) Fukada S, Shimada Y, Morita T, Sugiyama K. 2006. Suppression of methionine-induced hyperhomocysteinemia by glycine and serine in rats. *Biosci Biotechnol Biochem* **70**: 2403-2409.

58) Ohuchi S, Morita T, Mori M, Sugiyama K. 2009. Hepatic cystathionine β-synthase activity does not increase in response to methionine supplementation in rats fed a low casein diet: association with plasma homocysteine concentrations. *J Nutr Sci Vitaminol*

55: 178-185.

59) Stead LM, Au KP, Jacobs RL, Brosnan ME, Brosnan JT.2001. Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. *Am J Physiol Endocrinol Metab* **281**: E1095-E1100.

60) Fukada S, Setoue M, Morita T, and Sugiyama K. 2006. Dietary eritadenine suppresses guanidinoacetic acid-induced hyperhomocysteinemia in rats. *J. Nutr.*, **136**, 2797-2802 (2006).

61) Ohuchi S, Matsumoto Y, Morita T, Sugiyama K. 2008: High-casein diet suppresses guanidinoacetic acid-induced hyperhomocysteinemia and potentiates the hypohomocysteinemic effect of serine in rats. *Biosci Biotechnol Biochem* **72**: 3258-3264.

62) Setoue M, Ohuchi S, Morita T, and Sugiyama K. 2008. Hyperhomocysteinemia induced by guanidinoacetic acid is effectively suppressed by choline and betaine in rats. *Biosci Biotechnol Biochem* **72**: 1696-1703.

63) Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**: 497-509.

64) Durand P, Fortin LJ, Lussier-Cacan S, Davignon J, and Blache D. 1996. Hyperhomocysteinemia induced by folic acid deficiency and methionine loading-applica- tions of a modified HPLC method. *Clin Chim Acta* **252**: 83-93.

65) Cook RJ, Horne DW, Wagner C. 1989. Effect of dietary methyl group deficiency on one-carbon metabolism in rats. *J Nutr* **119**: 612-617.

66) Laryea MD, Steinhagen F, Pawliczek S, Wendel U. 1998. Simple method for the routine determination of betaine and *N,N*-dimethylglycine in blood and urine. *Clin Chem* **44**: 1937-1941.

67) Finkelstein JD, Mudd SH. 1967. Trans-sulfuration in mammals: the methionine-sparing effect of cystine. *J Biol Chem* **242**: 873-880.

68) Einarsson S, Josefsson B, Lagerkvist S. 1983. Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *J Chromatogr* **282**: 609-618.

69) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275.

70) Zeisel SH. 1981. Dietary choline: Biochemistry, physiology and pharmacology. *Annu Rev Nutr* **1**: 95-121.

71) Zhou J, Moller J, Danielson CC, Bentzon J, Ravn HB, Austin RC, and Falk E. 2001. Hyperhomocysteinemia promotes the development of collagen-rich and stable plaques in apoE-deficient mice. *Arterioscler Thromb Vasc Biol* **21**: 1470-1476.

72) Finkelstein JD, Martin JJ. 1986. Methionine metabolism in mammals: adaptation to methionine excess. *J Biol Chem* **261**: 1582-1587.

73) Finkelstein JD. 1990. Methionine metabolism in mammals. *J Nutr Biochem* **1**: 228-237.

74) Kimura H and Nakagawa H. 1971. Studies on cystathionine synthetase: Characteristics of purified rat liver enzyme. *J Biochem* **69**: 711-723.

75) Stipanuk MH. 1986. Metabolism of sulfur-containing amino acids. *Annu Rev Nutr* **6**:179-209

76) Kerr SJ. 1972. Competing methyltransferase systems. *J Biol Chem* **247**: 4248-4252

77) Schneider WJ and Vance DE. 1979. Conversion of phosphatidylethanolamine to phosphatidylcholine in rat liver. Partial purification and characterization of the enzymatic activities. *J Biol Chem* **254**: 3886-3891.

78) Lee KH, Cava M, Amiri P, Ottoboni T, and Lindquist RN. 1992. Betaine:homocysteine methyltransferase from rat liver: purification and inhibition by a boronic acid substrate analog. *Arch Biochem Biophys* **292**: 77-86.

79) Finkelstein JD, Harris BJ, and Kyle WE. 1972. Methionine metabolism in mammals: Kinetic study of betaine-homocysteine methyltransferase. *Arch Biochem Biophys* **153**: 320-324.

80) Finkelstein JD, Harris BJ, Martin JJ, and Kyle WE. 1982. Regulation of hepatic betaine homocysteine methyltransferase by dietary methio nine. *Biochem Biophys Res Commun* **108**: 344- 348.

81) Finkelstein JD, Harris BJ, Martin JJ, and Kyle WE. 1983. Regulation of hepatic

betaine-homocysteine methyltransferase by dietary betaine. *J Nutr* **113**: 519-521.

82) da Silva RP, Nissim I, Brosnan ME, and Brosnan JT. 2009. Creatine synthesis: hepatic metabolism of guanidinoacetate and creatine in the rat in vitro and in vivo. *Am J Physiol Endocrinol Metab* **296**: E256-E261.

83) Mudd SH and Poole JR. 1975. Labile methyl balances for normal humans on various dietary regimens. *Metabolism* **24**: 721-735.

84) Mudd SH, Ebert MH, and Scriver CR. 1980. Labile methyl group balances in the human: the role of sarcosine. *Metabolism* **29**: 707-720.

85) Verhoef P, Steenge GR, Boelsma E, van Vliet T, Olthof MR, Katan MB. 2004. Dietary serine and cystine attenuate the homocysteine-raising effect of dietary methionine: a randomized crossover trial in humans. *Am J Clin Nutr* **80**: 674-679.

86) Block RJ, Weiss KW. 1956. Amino acid handbook, pp. 341-346. Charles C. Thomas Pub., Springfield, Illinois.

87) Durand P, Lussier-Cacan S, Blache D. 1997. Acute methionine load-induced hyperhomocysteinemia enhances platelet aggregation, thromboxane biosynthesis, and macrophage-derived tissue factor activity in rats. *FASEB J* **11**: 1157-1168.

88) Finkelstein JD, Martin JJ. 1984. Methionine metabolism in mammals: distribution of homocysteine between competing pathways. *J Biol Chem* **259**: 9508-9513.

89) Yamamoto N, Tanaka T, Noguchi T. 1996. The effect of a high-protein diet on cystathionine beta-synthase activity and its transcript levels in rat liver. *J Nutr Sci Vitaminol* **42**: 589-593.

90) Nair KS, Short KR. 2005. Hormonal and signaling role of branched-chain amino acids. *J Nutr* **135**: 1547S-1552S.

91) Kimball SR, Jefferson LS. 2006. Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. *J Nutr* **136**:227S-231S.

92) Zhong B, Sakai S, Saeki T, Kanamoto R. 2007. Excess leucine intake induces serine dehydratase in rat liver. *Biosci Biotechnol Biochem* **71**: 2614-2617.

93) Bella DL, Hahn C, Stipanuk MH.1999. Effects of nonsulfur and sulfur amino acids on the regulation of hepatic enzymes of cysteine metabolism. *Am J Physiol* **227**: E144-E153.

94) Okawa H, Morita T, Sugiyama K. 2006. Increased plasma homocysteine concentration in rats from a low casein diet. *Biosci Biotechnol Biochem* **70**: 3050-3053.

95) Okawa H, Morita T, Sugiyama K. 2008. Effect of dietary soybean protein level on the plasma homocysteine concentration in rats. *Biosci Biotechnol Biochem* **72**: 1607-1610.

96) Ohuchi S, Matsumoto Y, Morita T, Sugiyama K. 2009. High casein diet decreases plasma homocysteine concentration in rats. *J Nutr Sci Vitaminol* **55**: 22-30.