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Biochemical Pharmacology 65 (2003) 1565–1574
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Effect of acute betaine administration on hepatic metabolism of *S*-amino acids in rats and mice

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Received 23 November 2002; accepted 5 February 2003

Abstract

Alterations of hepatic glutathione level by betaine were observed previously. In this study effects of betaine administration (1000 mg/kg, i.p.) on *S*-amino acid metabolism in rats and mice were investigated. Hepatic glutathione level decreased rapidly followed by marked elevation in 24 hr. Concentrations of *S*-adenosylmethionine, *S*-adenosylhomocysteine, and methionine were increased whereas cystathionine decreased significantly, suggesting that homocysteine generated in the methionine cycle is preferentially remethylated to methionine rather than being utilized for synthesis of cysteine. Hepatic cysteine concentration declined immediately, but plasma cysteine increased. Effect of betaine on hepatic cysteine uptake was estimated from the difference in cysteine concentration in major blood vessels connected to liver. Cysteine concentration either in the portal vein or abdominal aorta was not altered, however, a significant increase was noted in the hepatic vein, indicating that hepatic uptake of cysteine was decreased by betaine treatment. Activities of glutamate cysteine ligase, cystathionine β-synthase, and cystathionine γ-lyase were elevated in 24 hr. Pretreatment with propargylglycine, an irreversible inhibitor of cystathionine γ-lyase, did not abolish the betaine-induced reduction of hepatic glutathione in 4 hr, however, the elevation at $t = 24$ hr was blocked completely. In conclusion the present results indicate that betaine administration induces time-dependent changes on hepatic metabolism of *S*-amino acids. Betaine enhances metabolic reactions in the methionine cycle, but inhibits cystathionine synthesis and cysteine uptake, leading to a decrease in supply of cysteine for glutathione synthesis. Reduction in glutathione is subsequently reversed due to induction of cysteine synthesis and glutamate cysteine ligase activity.

Keywords: Glutathione; Cysteine; Methionine cycle; Transsulfuration pathway; *S*-adenosylmethionine

1. Introduction

Betaine, an oxidative metabolite of choline, is abundant in animal tissues. This endogenous compound serves as an organic osmolyte that is accumulated or released by the cells in response to hyperosmotic cell shrinkage or hypoosmotic cell swelling, respectively, to maintain cell volume homeostasis in liver and kidney [1,2]. Betaine is a major water-soluble component in *Lycium fructus* that has been used as a traditional remedy for hepatic disorders in Southeast Asia. Recently this compound was approved for a drug in the treatment of homocystinuria by FDA [3].

Betaine is participating in the methionine cycle as a substrate, in combination with homocysteine, for synthesis of methionine (Fig. 1). Betaine homocysteine *S*-methyltransferase (BHMT) mediates this reaction that has an importance role in the maintenance of hepatic methionine concentration in mammals especially when dietary intake of this *S*-amino acid is limited [4]. Methionine is further converted by the activity of methionine adenosyltransferase (MAT) into *S*-adenosylmethionine (SAM), the principal biological methyl donor to a variety of acceptor substrates including nucleic acids, proteins, phospholipids, biological amines and a long list of small molecules. The co-product of transmethylation reaction, *S*-adenosylhomocysteine (SAH),

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Abbreviations: BHMT, betaine homocysteine *S*-methyltransferase; CDO, cysteine dioxygenase; CβS, cystathionine β-synthase; CγL, cystathionine γ-lyase; GCL, glutamate cysteine ligase; γ-GT, γ-glutamyltransferase; GSH, glutathione; GSSG, glutathione disulfide; MAT, methionine adenosyltransferase; MHMT, 5-methyltetrahydrofolate homocysteine *S*-methyltransferase; PPG, propargylglycine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

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doi:10.1016/S0006-2952(03)00115-1

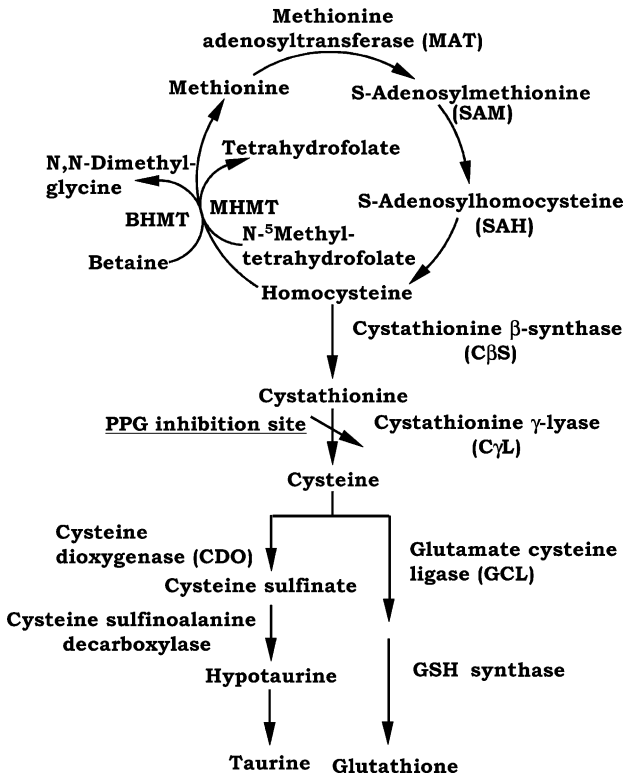

Fig. 1. Metabolic reactions of *S*-amino acids in the methionine cycle and transsulfuration pathway.

is hydrolyzed to yield homocysteine that is either remethylated to methionine or condensed with serine into cystathionine. Cystathionine is converted to cysteine *via* metabolic reactions mediated by cystathionine β-synthase (CβS) and cystathionine γ-lyase (CγL) consecutively. The distribution of homocysteine between the two competitive reactions, therefore, provides a major regulatory locus for the metabolism of *S*-amino acids in liver [5]. When methionine is needed, remethylation of homocysteine to methionine is increased. Conversely, when methionine is in excess, catabolism of homocysteine *via* CβS reaction is enhanced [6,7].

It was demonstrated in this laboratory that acute betaine treatment to mice induced time-dependent changes in the hepatotoxicity of chloroform [8]. Betaine administered 1–4 hr prior to a chloroform challenge enhanced the hepatotoxicity, but decreased it significantly when given 24 hr earlier. Since hepatic glutathione (GSH) level was reduced rapidly after betaine treatment followed by marked elevations, the reversal of the chloroform-induced hepatotoxicity was attributed to alterations in the availability of hepatic GSH.

In this study the effects of betaine administration on metabolism of *S*-amino acids were evaluated using rats and mice. Since the supply of cysteine is directly related to the synthesis of GSH that has diverse biological functions, it was of particular interest to examine the mechanism of changes in the concentration of this *S*-amino acid in liver of animals treated with betaine.

2. Materials and methods

2.1. Animals and treatments

Adult male ICR mice and Sprague–Dawley rats (Dae-Han Laboratory Animal) were used throughout the study. The use of animals was in compliance with the guidelines established by the Animal Care Committee of this institute. Animals were housed in temperature ($22 \pm 2^{\circ}$) and humidity ($55 \pm 5\%$) controlled rooms with a 12-hr light/dark cycle (light: 07:00–19:00 hr, dark: 19:00–07:00 hr) for 1 week prior to use. Regular laboratory chow and tap water were allowed *ad lib.* Animals were fasted in stainless steel wire-bottomed cages for 16 hr prior to betaine treatment (1000 mg or 8.5 mmol/kg, i.p.) made at 09:00 hr on the following morning. Control animals were treated with an identical volume of saline prepared to be isotonic with the betaine injection used. Propargylglycine (PPG) was dissolved in normal saline and injected into animals i.p. at a dose of 200 μmol/kg.

Animals were sacrificed at predetermined time points, and samples were processed according to the methods described below. The samples were analyzed either immediately or stored at -70° until used.

2.2. Chemicals

Drugs and chemicals such as NADPH, GSSG reductase, betaine anhydrous, various amino acid standards, *S*-adenosylmethionine iodide salt, SAH, cystathionine, GSH and GSSG were purchased from Sigma Chemical. Ethylene dichloride and tetrahydrofuran were obtained from Aldrich-Chemie. All other chemicals and solvents used were reagent grade or better.

2.3. Determination of GSH and amino acids

Mice were killed by decapitation, and their livers and kidneys were homogenized in a cold 1 M perchloric acid solution with 2 mM EDTA or in methanol. Denatured protein was removed by centrifugation at 10,000 *g* for 10 min. Blood was collected in a centrifuge tube; after immediate centrifugation, plasma was mixed with 10% sulfosalicylic acid or cold methanol.

Total GSH concentration was determined using an enzymatic recycling method of Griffith [9]. Betaine levels were measured according to the method of Barak and Tuma [10]. Cysteine and cystine concentrations were determined by the acid-ninhydrin method [11]. Free amino acids, cystathionine and taurine were analyzed after derivatization with *O*-phthaldialdehyde using the method of Rajendra [12]. A HPLC system equipped with a fluorescence detector (FS-980; Applied Biosystem) and a Crest Pak C18S Column (Jasco) was used. The method of She *et al.* [13] was employed to quantify SAM and SAH. The supernatant was directly applied to a HPLC system with a

UV-975 detector (Jasco) and a TSK-GEL ODS-80TM column (4.6 × 250 mm) (Tosoh).

For the estimation of hepatic uptake or release of cysteine and GSH, differences in concentration of these substances between blood immediately prior to entering liver and blood leaving this organ were measured. For this assay rats were employed for the experimental animals. Blood samples were collected in heparinized syringes from the vein of the left hepatic lobe, the portal vein and the abdominal aorta in each rat under ether anesthesia. The difference was calculated by subtracting the concentrations of cysteine or GSH in blood of the hepatic vein from the corresponding values in blood being introduced into liver. The blood flow entering liver was assumed to be 25% from the hepatic artery and 75% from the portal vein [14].

2.4. Enzyme assays

Livers were homogenized in a 3-fold volume of 0.154 M KCl containing 1 mM Tris–HCl (pH 7.4). The supernatant obtained after centrifugation at 10,000 *g* was used for the measurement of glutamate cysteine ligase (GCL) activity [15]. Reaction mixtures contained 0.1 M Tris–HCl buffer (pH 8.0), 150 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM EDTA, 0.2 mM NADH, 17 μg pyruvate kinase, 17 μg lactate dehydrogenase and 0.1 mg protein of enzyme solution in a final volume of 1 mL. Absorbance at 340 nm was monitored for 5 min at 37°. The enzyme activity was calculated using a molar extinction coefficient of 6.3×10^2 mol^{-1} mm^{-1}.

The activity of γ-glutamyltransferase (γ-GT) was measured using whole tissue homogenates. Renal γ-GT activity was assayed kinetically at 37° by employing a spectrophotometrical method [16]. γ-Glutamyl-*p*-nitroanilide was used as a substrate. A nonkinetic method was used to determine hepatic γ-GT activity [17]. Tissue homogenates and the substrate were added to a reaction mixture containing Tris–glycine buffer (pH 8.0) for incubation at 37° for 30 min. Reaction was stopped by addition of 0.25 mL 20% trichloroacetic acid. After centrifugation, absorbance was measured at 410 nm.

For the measurement of CβS and CγL activities livers were homogenized in a 4-fold volume of 0.1 M phosphate buffer followed by centrifugation at 10,000 *g*. The CβS activity in the supernatant was determined using the method of Kashiwamata and Greenberg [18]. Reaction mixture contained 0.1 M Tris buffer (pH 8.3), 0.1 M D,L-homocysteine, 0.1 M L-serine, 0.12 mM pyridoxal 5-phosphate, 0.5 mM $CuSO_4$ and 1.5 mg protein of enzyme solution in a final volume of 1.0 mL. At the end of incubation for 45 min at 37°, 0.1 mL 50% trichloroacetic acid was added. Color development was measured at 455 nm after addition of the ninhydrin reagent.

The activity of CγL was determined by a modification of the method of Matsuo and Greenberg [19]. Reaction mixture included 32 mM D,L-homoserine, 0.05 mM pyridoxal 5-phosphate, 7.5 mM 2-mercaptoethanol, 7.0 mM EDTA, 0.1 M potassium phosphate buffer (pH 7.5) and 1 mg protein of enzyme solution in a final volume of 1.0 mL. Incubation was initiated by addition of 0.1 mL 320 mM D,L-homoserine and maintained at 37° for 30 min. Reaction was stopped with 2 mL 10% trichloroacetic acid. After centrifugation, the amount of α-ketobutyrate was quantified with 2,4-dinitrophenylhydrazone in an alkaline solution. Color development was measured at 510 nm.

Cysteine dioxygenase (CDO) activity was quantified using the method of Bagley *et al.* [20]. Incubation was carried out for 16 min at 37° in a final volume of 1 mL containing 0.5 mM ferrous ammonium sulfate, 5 mM hydroxylamine HCl, 2 mM NAD^+, 5 mM cysteine and 1 mg protein of enzyme solution. Addition of 2.5 mL ice-cold methanol terminated the reaction. Amount of cysteine sulfinate formed during the enzymatic reaction was measured using a HPLC equipped with a fluorescence detector (FP-920; Jasco) and a 3.5 μm Kromasil C18 column (4.6 × 100 mm) (Eka Chemicals) after pre-column *O*-phthaldialdehyde/2-mercaptoethanol derivatization.

Protein was determined by the method of Lowry *et al.* [21] with bovine serum albumin as a standard.

2.5. Data analysis

All results were expressed as the mean ± SE. Comparison of the means was made using a two-tailed Student's *t*-test. The acceptable level of significance was established at $P < 0.05$ except when otherwise indicated.

3. Results

3.1. Change in concentrations of betaine, GSH, and amino acids

Hepatic concentration of betaine was increased from 2.6 ± 0.2 to 10.1 ± 2.2 μmol/g liver following the treatment in 2 hr, and after that, decreased with a half-life of approximately 6 hr. On the other hand, only a negligible increase in betaine concentration was observed in kidney (data not shown).

Betaine treatment decreased hepatic GSH concentration to approximately 70% of control in 3 hr (Fig. 2). Renal GSH concentration appeared to be slightly decreased. However, GSH concentrations in liver, kidney and plasma were all increased when determined 24 hr following the treatment. The ratio of glutathione disulfide (GSSG) to reduced form (GSH) in liver was nearly a constant, approximately 2.1–2.5% for 24 hr.

The effect of betaine on hepatic GSH concentration was examined at different dose levels ranging from 50 to 1000 mg/kg. Mice were treated with a dose of betaine,

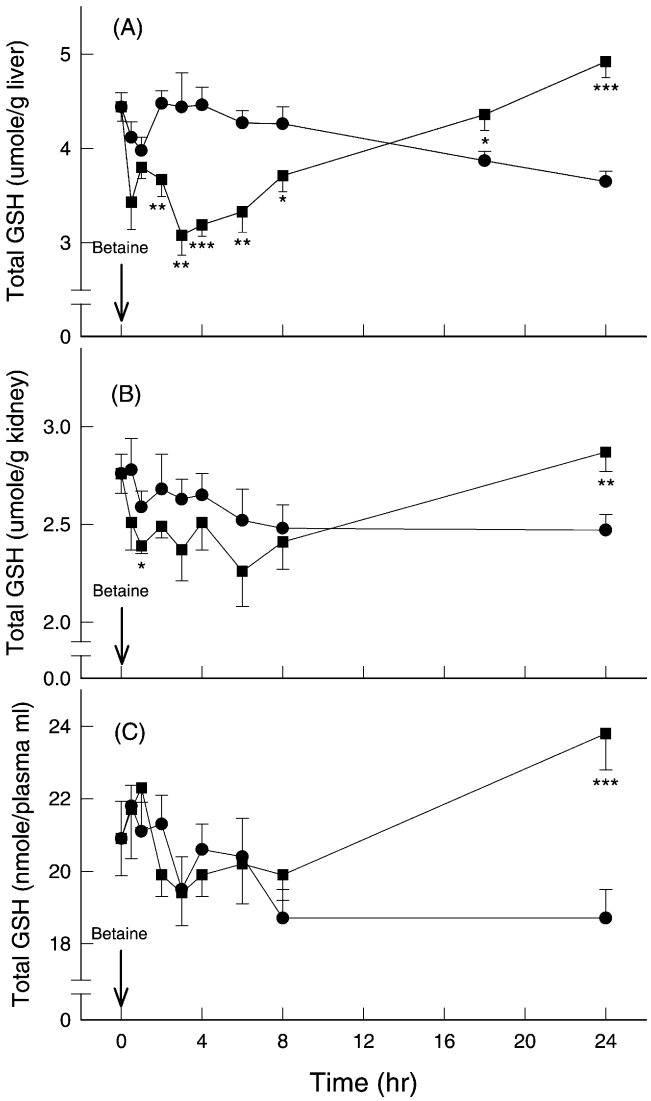

Fig. 2. Changes in GSH concentration in liver (A), kidney (B) and plasma (C) of mice treated with betaine. Filled squares represent animals treated with betaine; filled circles control animals treated with saline. Each value is the mean ± SE for six mice. Asterisk indicates a significant difference from the control (Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

and hepatic GSH was determined at $t = 3$ hr. Betaine did not affect the concentrations of this tripeptide in liver at a dose of up to 100 mg/kg. But hepatic GSH concentration was decreased significantly by a betaine dose of 200 mg/kg (3.7 ± 0.3 μmol/g liver vs. 4.5 ± 0.1 μmol/g liver in control mice, Student's *t*-test, $P < 0.01$). The reduction of hepatic GSH was augmented with increasing doses of betaine.

The concentrations of glutamate, cysteine and glycine, the three building blocks for GSH synthesis, were determined in liver and plasma of mice treated with betaine (Fig. 3). Hepatic concentrations of the three amino acids were decreased immediately following the treatment. The concentration of cysteine in liver appeared to recover in 6 hr, and was significantly elevated at $t = 18$ hr. In plasma cysteine concentration was increased rapidly, but returned to control levels in 6 hr. The concentration of cystine, a major form for this *S*-amino acid in blood, was also increased from $t = 0.5$ to 2 hr. At 24 hr following betaine treatment plasma cysteine concentration was significantly elevated compared to control. Glycine concentration in plasma was reduced and glutamate appeared to be elevated transiently after betaine treatment.

3.2. Effect on methionine, SAM, SAH, cystathionine and taurine concentrations in liver and plasma

Intermediate metabolites in the transsulfuration pathway were determined in liver and plasma following betaine treatment (Table 1). Methionine, SAM and SAH concentrations in liver were elevated to approximately 150% of control when determined at $t = 4$ hr. Hepatic concentration of cystathionine was markedly reduced at this time period. However, all these metabolites returned to control levels in 24 hr. Methionine concentration in plasma was also elevated by betaine from $t = 2$ to 4 hr following the treatment.

Hepatic concentration of taurine, an oxidative product of cysteine, was decreased rapidly, reaching approximately

Table 1
Changes in methionine, SAM, SAH, cystathionine and taurine concentrations in liver and plasma following betaine treatment

Time (hr)	Group	Methionine (nmol/g or mL)	SAM (nmol/g)	SAH (nmol/g)	Cystathionine (nmol/g)	Taurine (μmol/g or mL)
0.5	Control	47 ± 2 (29.7 ± 1.3)	–	–	–	15.0 ± 0.9 (0.42 ± 0.06)
	Betaine	58 ± 4* (30.7 ± 1.3)	–	–	–	11.0 ± 0.8* (1.02 ± 0.09)***
2	Control	48 ± 2 (28.9 ± 1.3)	–	–	–	16.1 ± 1.3 (0.5 ± 0.0)
	Betaine	68 ± 6* (37.2 ± 0.4)***	–	–	–	9.4 ± 0.4** (0.8 ± 0.1)**
4	Control	43 ± 5 (34.3 ± 2.6)	76.3 ± 2.2	30.6 ± 1.9	6.42 ± 0.57	17.3 ± 1.0 (0.5 ± 0.0)
	Betaine	65 ± 4** (60.2 ± 4.2)**	104.0 ± 4.6***	45.8 ± 2.2***	2.97 ± 0.19***	8.6 ± 0.3*** (0.5 ± 0.0)
24	Control	34 ± 2 (31.5 ± 1.0)	64.3 ± 3.6	64.7 ± 3.7	4.63 ± 0.18	18.1 ± 0.8 (0.5 ± 0.1)
	Betaine	39 ± 1 (30.8 ± 2.5)	73.8 ± 2.3	64.5 ± 3.5	4.05 ± 0.46	13.6 ± 0.7** (0.5 ± 0.1)

Each value is the mean ± SE for six mice. Values given in parentheses represent concentrations measured in plasma. Asterisk indicates a significant difference from the corresponding control (Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

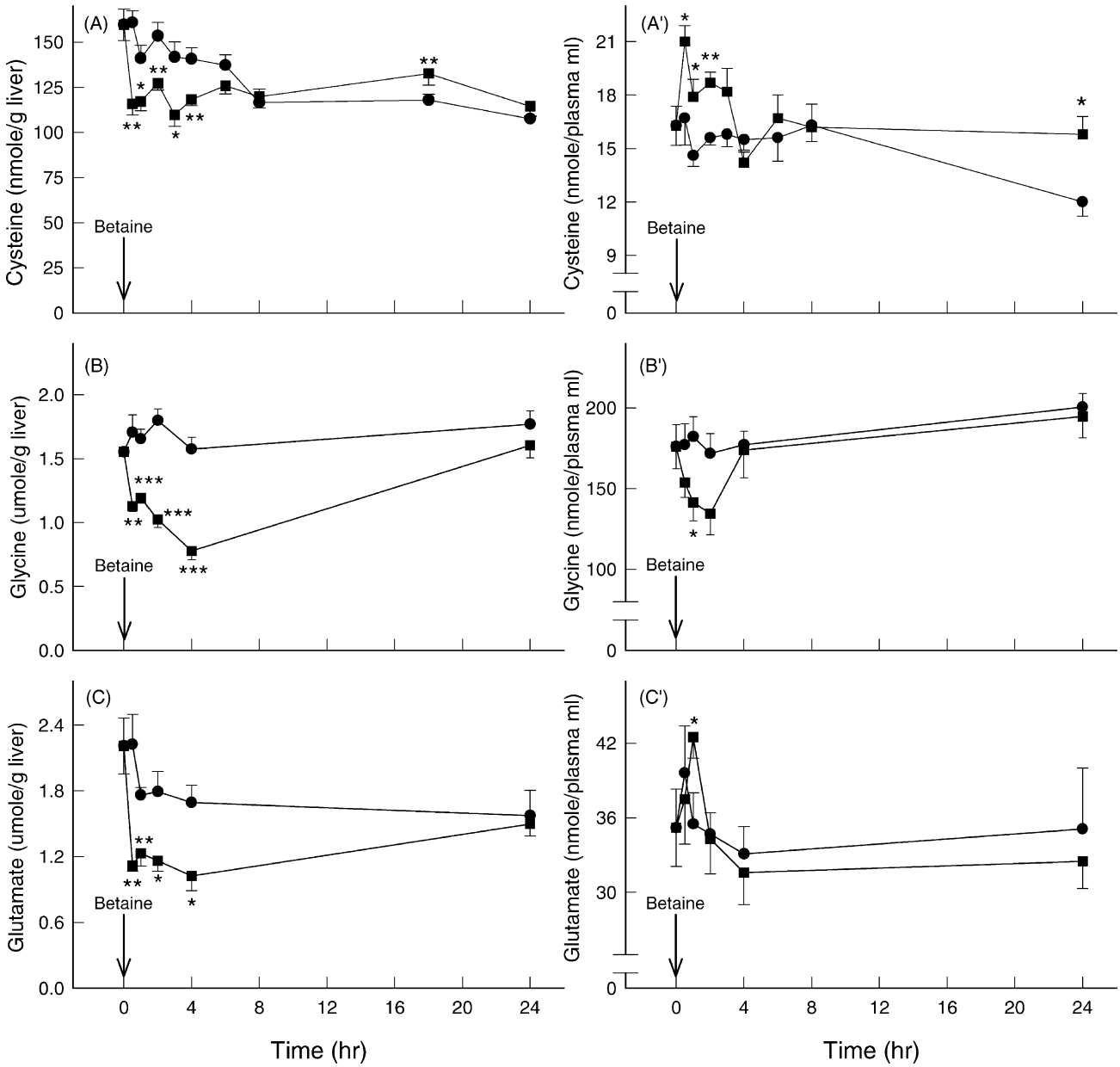

Fig. 3. Changes in cysteine, glycine, and glutamate concentrations in liver (A/B/C) and plasma (A′/B′/C′) of mice treated with betaine. Filled squares represent animals treated with betaine; filled circles controls. Each value is the mean ± SE for six mice. Asterisk indicates a significant difference from the control (Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

50% of control in 4 hr. Plasma taurine concentration was increased from $t = 0.5$ hr, but returned to control levels at $t = 4$ hr.

3.3. Change in hepatic uptake/release of GSH and cysteine

GSH and cysteine concentrations were measured in blood of the portal vein, the abdominal aorta, and of the hepatic vein emerging from the left lobe of a rat (Table 2). Hepatic transport of GSH and cysteine was estimated from a difference in the concentrations of the *S*-containing substances in blood before and after liver.

Plasma GSH concentration was not changed either in the portal vein, abdominal aorta, or hepatic vein at $t = 2$ hr after betaine treatment. Hepatic GSH concentration measured simultaneously decreased from 4.33 ± 0.11 to 3.61 ± 0.14 μmol/g liver (Student's *t*-test, $P < 0.01$).

Hepatic cysteine concentration measured at $t = 2$ hr was significantly lower in the betaine-treated rats than that of the control rats treated with isotonic saline (104.5 ± 4.1 nmol/g liver vs. 132.0 ± 7.2 nmol/g liver; Student's *t*-test, $P < 0.05$). Betaine did not affect the cysteine concentration in blood of either the portal vein or abdominal aorta. However, in blood of the hepatic vein a greater than 50% increase in cysteine concentration was

Table 2
Changes in plasma concentrations of cysteine and GSH in rats treated with betaine

Time (hr)	Group	Hepatic vein (nmol/mL)	Portal vein (nmol/mL)	Abdominal aorta (nmol/mL)	Difference before and after liver (nmol/mL)
2	Control	19.6 ± 0.8 (20.7 ± 0.8)	49.2 ± 2.1 (7.8 ± 0.4)	22.7 ± 1.3 (9.0 ± 0.8)	23.0 ± 1.8 (−12.6 ± 0.5)
	Betaine	31.1 ± 1.5*** (20.2 ± 1.1)	53.8 ± 0.7 (8.1 ± 0.5)	23.9 ± 1.3 (8.8 ± 0.9)	15.3 ± 1.4* (−11.9 ± 1.0)
24	Control	15.8 ± 1.1 (19.6 ± 0.9)	34.1 ± 1.3 (10.0 ± 0.3)	16.6 ± 1.0 (11.6 ± 1.2)	13.9 ± 1.1 (−9.2 ± 1.0)
	Betaine	15.9 ± 0.9 (18.4 ± 0.9)	38.4 ± 1.0 (8.4 ± 0.4)	13.8 ± 1.1 (13.0 ± 0.4)	16.6 ± 1.3 (−8.8 ± 0.6)

Each value is the mean ± SE for five rats. Values given in parentheses represent GSH concentrations in plasma. See the Section 2 for calculation of differences in concentration of each substance before and after liver. Asterisk indicates a significant difference from the corresponding control (Student's *t*-test, $^{*}P < 0.05$, $^{***}P < 0.001$).

Table 3
Changes in activities of CβS, CγL, GCS, CDO and GGT in liver and kidney

Time (hr)	Group	CβS (μmol/min/g liver)	CγL (μmol/min/g liver)	GCL (μmol/min/g liver)	CDO (μmol/min/g liver)	γGT (nmol/min/g liver)	γGT (nmol/min/g kidney)
4	Control	34.5 ± 1.5 (100)	39.7 ± 2.2 (100)	3.36 ± 0.36 (100)	0.32 ± 0.04 (100)	91 ± 4 (100)	29800 ± 1200 (100)
	Betaine	43.0 ± 0.9*** (125)	37.8 ± 0.4 (95)	4.86 ± 0.21** (145)	0.29 ± 0.02 (91)	98 ± 9 (108)	29700 ± 1800 (100)
24	Control	19.1 ± 0.7 (100)	41.7 ± 1.3 (100)	3.72 ± 0.13 (100)	0.38 ± 0.03 (100)	132 ± 7 (100)	22600 ± 1100 (100)
	Betaine	27.4 ± 1.7** (143)	54.1 ± 2.0** (130)	4.79 ± 0.13*** (129)	0.27 ± 0.02** (71)	135 ± 23 (102)	25600 ± 1100 (113)

Each value is the mean ± SE for six mice. Asterisk indicates a significant difference from the corresponding control (Student's *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$). Value in parenthesis is the percent to control.

observed, indicating that hepatic uptake of cysteine was significantly inhibited by betaine.

3.4. Effect on activities of CβS, CγL, GCL, γ-GT and CDO

Effect of betaine on the activities of major enzymes involved in cysteine and GSH metabolism is shown in Table 3. The hepatic CβS activity was decreased, but γ-GT activity increased spontaneously at 24 hr after the treatment. The change in activities of the enzymes appeared to be associated with reduction of *S*-amino acids supply due to the fasting of animals during the experimental period. Betaine treatment increased the activities of CβS and CγL, each mediating an irreversible step in the synthesis of cysteine from homocysteine. Hepatic GCL activity was rapidly increased by betaine, and still elevated at $t = 24$ hr. Hepatic or renal γ-GT activity, the latter being several hundred-fold greater than the former, was not affected by betaine treatment. The activity of CDO, which has a critical role in the synthesis of taurine from cysteine, was not changed at $t = 4$ hr but decreased significantly at $t = 24$ hr.

3.5. Effect on cysteine and GSH concentrations in PPG-pretreated mice

Effect of betaine on GSH and cysteine concentrations was examined in mice pretreated with PPG. In a preliminary experiment it was shown that the hepatic CγL activity was inhibited almost completely at 4 hr after a PPG challenge. An equivalent magnitude of inhibition in CγL activity was observed in liver of mice treated with a dose of PPG twice, each at 28 and 16 hr before sacrifice.

PPG treatment alone decreased hepatic GSH and cysteine concentrations significantly (Table 4). Betaine reduced hepatic GSH and cysteine concentrations further

Table 4
Effects of betaine on GSH and cysteine concentrations in mice pretreated with PPG

Time (hr)	Group	GSH (μmol/g tissue or nmol/mL plasma)			Cysteine (nmol/g tissue or mL plasma)		
		Liver	Kidney	Plasma	Liver	Kidney	Plasma
4	Control	2.98 ± 0.16 (100)	2.55 ± 0.11 (100)	16.0 ± 1.0 (100)	118.7 ± 4.2 (100)	328.0 ± 8.0 (100)	17.0 ± 0.8 (100)
	Betaine	1.95 ± 0.14** (65)	2.27 ± 0.10 (89)	12.2 ± 0.7* (76)	97.4 ± 3.4** (82)	282.4 ± 7.7** (86)	17.0 ± 1.4 (100)
24	Control	3.20 ± 0.23 (100)	3.10 ± 0.11 (100)	13.0 ± 0.4 (100)	83.6 ± 5.0 (100)	250.7 ± 6.2 (100)	14.6 ± 0.5 (100)
	Betaine	3.61 ± 0.21 (113)	3.22 ± 0.22 (104)	14.5 ± 1.4 (112)	92.1 ± 5.9 (110)	244.7 ± 8.1 (98)	15.4 ± 0.8 (105)

For the 4 hr groups a single dose of PPG (200 μmol/kg, i.p.) was injected to mice 4 hr prior to betaine treatment. For the 24 hr groups an identical dose of PPG was given twice, once at 4 hr prior to and also at 8 hr after betaine treatment. Each value is the mean ± SE for six mice. Asterisk indicates a significant difference from the corresponding control (Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$). Value in parenthesis is the percent to control.

at $t = 4$ hr in mice pretreated with PPG. Cysteine concentration in kidney was also lowered. Plasma GSH concentration at $t = 4$ hr, which was not changed by betaine alone (Fig. 2), was decreased in the PPG-pretreated mice. However, the marked elevation of GSH in liver, plasma, and kidney observed at $t = 24$ hr in mice treated with betaine was abolished by PPG pretreatment.

4. Discussion

In the previous study conducted in this laboratory betaine administration was shown to induce profound changes in the hepatic GSH concentration [8]. A single dose of betaine rapidly depleted GSH in liver followed by elevation beyond its control level in 24 hr. Activity of the major enzymes associated with utilization of GSH, such as GSH *S*-transferases, GSH peroxidase, or GSH reductase, was not altered by betaine treatment. This substance did not interact with GSH *in vitro*, nor was hepatic concentration of GSSG increased in that study.

The maintenance of hepatocellular GSH is a dynamic process. Hepatic GSH level is regulated by a balance of its synthesis, utilization and export to other tissues [22,23]. Since betaine treatment did not alter either the parameters for GSH utilization or concentration of this substance in plasma, its effect on hepatic synthesis was first examined in this study. Synthesis of GSH in liver is limited mostly by two factors, the availability of cysteine and the activity of GCL [24]. Betaine rapidly reduced the hepatic concentrations of cysteine, glycine, and glutamate, the three building blocks for GSH synthesis. Hepatic cysteine concentration measured was close to the Michaelis–Menten constant of GCL [24,25]. Free cysteine pool in liver is at least an order of magnitude less than that of glycine or glutamate, suggesting that the decrease in hepatic cysteine induced by betaine would be responsible for the reduction of GSH synthesis in liver.

Hepatic uptake from plasma and generation *via* the transsulfuration pathway are two major sources of cysteine supply [22,23,26]. Betaine treatment markedly elevated the hepatic concentrations of metabolites in the methionine cycle, such as methionine, SAM, and SAH, when determined at $t = 4$ hr. However, concentrations of cystathionine and cysteine were decreased significantly at this time period. Remethylation to methionine and condensation with serine into cystathionine are each other competitive in aspect of homocysteine utilization [5,27]. It is therefore suggested that a high concentration of hepatic betaine, by attracting more homocysteine into remethylation process, would decrease the supply of this substance for synthesis of cystathionine and cysteine. This view is supported by the clinical observation that betaine therapy is effective for homocystinuric patients [28]. It is also in accordance with the study results showing that hepatic remethylation of homocysteine to methionine was stimulated by addition of betaine in liver slices of rats and sheep [29,30].

In liver the other major source for cysteine supply is the membrane transport of this *S*-amino acid from plasma [23]. In this study, a concomitant increase in the plasma cysteine concentration was observed with the reduction of cysteine in liver. It was also demonstrated that the cysteine concentration was significantly elevated only in blood of the hepatic vein emerging from the left lobe, but not in the abdominal aorta or the portal vein that supplies this amino acid to liver. Comparison of cysteine concentration in blood before and after liver indicated that the cysteine uptake into liver was depressed to approximately 2/3 of control by betaine treatment.

An amino acid transport system is not usually selective and various amino acids are carried by more than one single transporter [31]. Subsequently amino acids transported by the same system(s) may competitively inhibit each other from being transported across the membrane. Betaine, being an *N*-methylated amino acid, is transported by amino acid transport systems, mostly by the betaine γ-aminobutyric acid transport and also by the amino acid transport system A [2,32,33]. It was suggested that cysteine uptake in isolated rat liver cells be mainly governed by the Na^+-dependent system ASC [34]. But the K_m determined in this system for cysteine is ~2 mM, a concentration at least two orders of magnitude greater than that normally found in plasma. It has been shown later that the Na^+-dependent cysteine uptake is mediated by at least three saturable carriers, system A, system ASC, and a yet unknown system with a high affinity for cysteine, in both human and rat liver [35,36]. Therefore, it is suspected that betaine at high concentrations in blood could competitively inhibit the uptake of cysteine into liver. In fact it was shown that betaine decreased the membrane transport of several amino acids, such as serine, glycine and alanine, and conversely, the betaine transport was inhibited by the amino acids [37,38].

For the experiment determining the change in plasma cysteine and GSH concentrations in the blood vessels connected to liver, rats, instead of mice, were employed. Extensive literature survey failed to reveal the nature of amino acid transport systems in mice. However, preliminary experiments conducted in this laboratory indicated that all the significant findings observed in mice could also be reproduced with rats, including the betaine-induced change in hepatic cysteine and GSH concentrations, which suggests that the potential of species difference would be a minimum for the scope of this study.

The effect of betaine on the hepatic availability of cysteine was examined in mice pretreated with PPG, an irreversible inhibitor of CγL [39,40]. PPG treatment alone reduced GSH and cysteine levels in liver significantly indicating that the generation of cysteine from the transmethylation pathway was inhibited. But the reduction of hepatic GSH and cysteine induced by betaine appeared not

to be diminished by PPG pretreatment. This observation, in combination with the result obtained from the experiments measuring cysteine concentration in blood of the hepatic vessels, strongly suggests that the inhibition of cysteine uptake would play an important role in the reduction of this *S*-amino acid in liver of animals treated with betaine. On the other hand, PPG pretreatment abolished the increment of GSH in liver, kidney, and plasma at 24 hr following betaine treatment. The abolition of increase in GSH concentrations by PPG was attributed to its inhibitory effect on CγL.

Condensation of homocysteine and serine to cystathionine, an irreversible step in the transsulfuration pathway, and subsequent release of cysteine from cystathionine is mediated by CβS and CγL, respectively. Betaine rapidly induced the activity of CβS in 4 hr when the concentrations of cystathionine and cysteine were reduced. Induction of the CβS activity could be accounted for by accumulation of SAM and SAH, both shown to be allosteric activators for this enzyme [41,42]. However, the CβS and CγL activities were still elevated when all the metabolites measured, such as SAM, SAH, methionine, cysteine, and cystathionine, returned to control levels in 24 hr following betaine treatment. It has been reported that the activity of CβS is regulated by changes in composition of *S*-amino acids in diet. A cystine- or methionine-supplemented diet was shown to induce marked changes in the hepatic CβS activity [7,43]. In this study, the rapid fluctuations in the balance of various *S*-containing metabolites observed at earlier time points appeared to be associated with the changes of this enzyme activity at $t = 24$ hr.

The other critical determinant for GSH synthesis is the activity of GCL. GCL, which catalyzes a rate limiting step in the GSH synthesis, is regulated by feedback inhibition of GSH and also by availability of its substrate, cysteine [44,45]. Betaine induced the hepatic GCL activity both at 4 and 24 hr following the treatment. GCL is a heterodimer made up of a catalytic (heavy, 73 kDa) and a regulatory (light, 30 kDa) subunit [46]. It was shown that induction of GCL activity by a GSH depleting agent, such as 2(3)-*tert*-butyl-4-hydroxyanisole, diethylmaleate, or phorone, was associated with elevation of mRNA for the heavy subunit [47]. Therefore, the rapid depletion of hepatic GSH appeared to be responsible for the increase in GCL activity observed at $t = 4$ hr. The mechanism for induction of this enzyme activity following the treatment remains to be studied.

Taurine level in liver was rapidly decreased by betaine treatment, which was accompanied with elevation of this substance in plasma. Taurine, an end product of catabolism of *S*-amino acids, is mainly synthesized in liver and secreted as such or bile salt-related forms [48]. In liver both taurine and betaine are important organic osmolytes, either accumulated or released depending upon changes in external osmotic concentrations [2,32,33]. Therefore, it appeared that the rapid elevation in hepatic betaine was directly responsible for efflux of taurine from liver leading to accumulation of this substance in plasma.

Hepatic taurine remained at significantly lower concentrations for 24 hr following betaine treatment. It has been known that the cysteine availability is a major factor determining the metabolic fate of cysteine to either taurine, sulfate or GSH in hepatocytes [49]. That is, low cysteine availability favors its utilization for synthesis of GSH whereas partitioning of cysteine for catabolism to taurine is enhanced under greater cysteine availability. In fact, it was shown that hepatic taurine concentration and CDO activity were correlated well with supply of *S*-amino acids in diet [50]. The present results suggest that the increased demand of cysteine for GSH synthesis would be responsible for the prolonged decrease in taurine concentration in liver.

In summary, the present study demonstrates that a dose of betaine could induce profound alterations in the generation of metabolic intermediates and products in the transsulfuration pathway. Betaine treatment rapidly elevates the levels of metabolites in the methionine cycle, such as methionine, SAM, and SAH. However, the supply of homocysteine for cystathionine synthesis would be depressed due to accelerated extraction of homocysteine into remethylation reaction to form methionine. Subsequently the decrease in generation of cystathionine and cysteine would result in the reduction of GSH synthesis. Betaine also reduces the cysteine supply into liver by inhibiting its uptake as evidenced by the increase in plasma cysteine concentration in the hepatic vein. In the meanwhile the increases in the activities of GCL, a key enzyme for GSH synthesis, and the enzymes CγL and CβS, mediating the generation of cysteine in the transsulfuration pathway, appear to account for the elevation of hepatic GSH concentration in 24 hr following betaine treatment.

Betaine is widely distributed both in animal tissues and in plants. Thus, intake of this compound at high levels would be a common practice to a large number of human populations. The present results suggest that the regulation of hepatic *S*-amino acid metabolism could be affected by chronic betaine uptake, which is being studied in this laboratory. In patients with homocystinuria, an inborn error of methionine metabolism, a daily dose of 6–9 g betaine is indicated [28]. The dose of betaine mostly used in this study was 1000 mg/kg body weight. However, the changes in hepatic SAM, cysteine and GSH were significant at a dose of 100–200 mg/kg that is comparable to the dose of betaine employed in human. The results suggest that the homocysteine concentration in liver and plasma would be decreased while hepatic betaine is maintained at high levels, which serves as a rationale for the use of this substance in the treatment of homocystinuria. However, a dose of betaine could induce rapid alterations in the hepatic concentrations of the critical metabolites in the transsulfuration pathway such as SAM and GSH that have diverse biological functions in body. Furthermore, a subsequent decline in betaine

concentration would be accompanied with an abrupt increase in homocysteine level as suggested by the elevation in hepatic and plasma cysteine concentrations. This warrants a more careful evaluation on the use of betaine in clinical situations.

Acknowledgments

This work was supported in part by a grant (02-PJ2-PG3-21605-0001) from the Ministry of Health and Welfare, Korea.

References

[1] Garcia-Perez A, Burg MB. Renal medullary organic osmolytes. Physiol Rev 1991;71:1081–115.
[2] Wettstein M, Weik C, Holneicher C, Hussinger D. Betaine as an osmolyte in rat liver: metabolism and cell-to-cell interactions. Hepatology 1998;27:787–93.
[3] Anonymous. Prescription Drug Product List. 16th ed. Washington, DC: US Food and Drug Administration; 1996.
[4] Finkelstein JD, Harris BJ, Martin JJ, Kyle WE. Regulation of hepatic betaine-homocysteine methyltransferase by dietary methionine. Biochem Biophys Res Commun 1982;108:344–8.
[5] Finkelstein JD, Martin JJ. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J Biol Chem 1984;259:9508–13.
[6] Eloranta TO, Martikainen V, Smith TK. Adaptation of adenosylmethionine metabolism and methionine recycling to variations in dietary methionine in the rat. Proc Soc Exp Biol Med 1990;194: 364–71.
[7] Finkelstein JD, Martin JJ. Methionine metabolism in mammals. Adaptation to methionine excess. J Biol Chem 1986;261:1582–7.
[8] Kim SK, Kim YC, Kim YC. Effects of singly administered betaine on hepatotoxicity of chloroform in mice. Food Chem Toxicol 1998; 36:655–61.
[9] Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 1980; 106:207–12.
[10] Barak AJ, Tuma DJ. A simplified procedure for the determination of betaine in liver. Lipids 1979;14:860–3.
[11] Gaitonde MK. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. Biochem J 1967;104:627–33.
[12] Rajendra W. High performance liquid chromatographic determination of amino acids in biological samples by pre-column derivatization with *O*-phthaldialdehyde. J Liq Chromatogr 1987;10:941–55.
[13] She QB, Nagao I, Hayakawa T, Tsuge H. A simple HPLC method for the determination of *S*-adenosylmethionine and *S*-adenosylhomocysteine in rat tissues: the effect of Vitamin B6 deficiency on these concentrations in rat liver. Biochem Biophys Res Commun 1994;205: 1748–54.
[14] Garcia RA, Stipanuk MH. The splanchnic organs, liver and kidney have unique roles in the metabolism of sulfur amino acids and their metabolites in rats. J Nutr 1992;122:1693–701.
[15] Sekura R, Meister A. γ-Glutamylcysteine synthetase. Further purification, ''half of the sites'' reactivity, subunits, and specificity. J Biol Chem 1977;252:2599–605.
[16] Orlowski M, Meister A. γ-Glutamyl-*p*-nitroanilide: a new convenient substrate for determination and study of L- and D-glutamyltranspeptidase activities. Biochim Biophys Acta 1963;73:676–9.
[17] Hinchman CA, Ballatori N. Glutathione-degrading capacities of liver and kidney in different species. Biochem Pharmacol 1990;40: 1131–5.
[18] Kashiwamata S, Greenberg DM. Studies on cystathionine synthase of rat liver. Properties of the highly purified enzyme. Biochim Biophys Acta 1970;212:488–500.
[19] Matsuo Y, Greenberg DM. A crystalline enzyme that cleaves homoserine and cystathionine. I. Isolation procedure and some physicochemical properties. J Biol Chem 1957;230:545–60.
[20] Bagley PJ, Hirschberger LL, Stipanuk MH. Evaluation and modification of an assay procedure for cysteine dioxygenase activity: high-performance liquid chromatography method for measurement of cysteine sulfinate and demonstration of physiological relevance of cysteine dioxygenase activity in cysteine catabolism. Anal Biochem 1995;227:40–8.
[21] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagent. J Biol Chem 1952;193:265–75.
[22] Kaplowitz N, Aw TY, Ookhtens M. The regulation of hepatic glutathione. Annu Rev Pharmacol Toxicol 1985;25:715–44.
[23] Lu SC. Regulation of hepatic glutathione synthesis. Semin Liver Dis 1998;18:331–43.
[24] Meister A, Anderson ME. Glutathione. Annu Rev Biochem 1983;52: 711–60.
[25] Lu SC, Ge JL, Kuhlenkamp J, Kaplowitz N. Insulin and glucocorticoid dependence of hepatic gamma-glutamylcysteine synthetase and glutathione synthesis in the rat. Studies in cultured hepatocytes and *in vivo*. J Clin Invest 1992;90:524–32.
[26] Beatty PW, Reed DJ. Involvement of the cystathionine pathway in the biosynthesis of glutathione by isolated rat hepatocytes. Arch Biochem Biophys 1980;204:80–7.
[27] Finkelstein JD, Martin JJ. Homocysteine. Int J Biochem Cell Biol 2000;32:385–9.
[28] Wilcken DE, Wilcken B, Dudman NP, Tyrrell PA. Homocystinuria: the effects of betaine in the treatment of patients not responsive to pyridoxine. N Engl J Med 1983;309:448–53.
[29] Barak AJ, Beckenhauer HC, Tuma DJ. Use of *S*-adenosylmethionine as an index of methionine recycling in rat liver slices. Anal Biochem 1982;127:372–5.
[30] Xue GP, Snoswell AM. Comparative studies on the methionine synthesis in sheep and rat tissues. Comp Biochem Physiol B 1985; 80:489–94.
[31] Guidotti GG, Gazzola GC. Amino acid transporters: systematic approach and principles of control in mammalian amino acid transport. New York: Plenum Press; 1992. p. 3–29.
[32] Peters-Regehr T, Bode JG, Kubitz R, Hussinger D. Organic osmolyte transport in quiescent and activated rat hepatic stellate cells (Ito cells). Hepatology 1999;29:173–80.
[33] Weik C, Warskulat U, Bode J, Peters-Regehr T, Hussinger D. Compatible organic osmolytes in rat liver sinusoidal endothelial cells. Hepatology 1998;27:569–75.
[34] Kilberg MS, Christensen HN, Handlogten ME. Cysteine as a system-specific substrate for transport system ASC in rat hepatocytes. Biochem Biophys Res Commun 1979;88:744–51.
[35] Mailliard ME, Kilberg MS. Sodium-dependent neutral amino acid transport by human liver plasma membrane vesicles. J Biol Chem 1990;265:14321–6.
[36] Saiki H, Chan ET, Wong E, Yamamuro W, Ookhtens M, Kaplowitz N. Zonal distribution of cysteine uptake in the perfused rat liver. J Biol Chem 1992;267:192–6.
[37] Christensen HN, Oxender DL, Liang M, Vatz KA. The use of *N*-methylation to direct route of mediated transport of amino acids. J Biol Chem 1965;240:3609–16.
[38] Petronini PG, De Angelis E, Borghetti AF, Wheeler KP. Osmotically inducible uptake of betaine via amino acid transport system A in SA-3T3 cells. Biochem J 1994;300:45–50.

[39] Abeles RH, Walsh CT. Acetylenic enzyme inactivators. Inactivation of γ-cystathionase, *in vitro* and *in vivo* by propargylglycine. J Am Chem Soc 1973;95:6124–5.
[40] Kim SK, Kim YC. Effect of propargylglycine on synthesis of glutathione in mice. Nutr Res 2001;21:1373–81.
[41] Finkelstein JD, Kyle WE, Harris BJ. Methionine metabolism in mammals: regulatory effects of *S*-adenosylhomocysteine. Arch Biochem Biophys 1974;165:774–9.
[42] Finkelstein JD, Kyle WE, Martin JL, Pick AM. Activation of cystathionine synthase by adenosylmethionine and adenosylethionine. Biochem Biophys Res Commun 1975;66:81–7.
[43] Finkelstein JD, Martin JJ, Harris BJ. Effect of dietary cystine on methionine metabolism in rat liver. J Nutr 1986;116:985–90.
[44] Kwon YH, Stipanuk MH. Cysteine regulates expression of cysteine dioxygenase and γ-glutamylcysteine synthetase in cultured rat hepatocytes. Am J Physiol Endocrinol Metab 2001; 280:E804–15.
[45] Richman PG, Meister A. Regulation of γ-glutamylcysteine synthetase by nonallosteric feedback inhibition by glutathione. J Biol Chem 1975;250:1422–6.
[46] Huang CS, Chang LS, Anderson ME, Meister A. Catalytic and regulatory properties of the heavy subunit of rat kidney γ-glutamylcysteine synthetase. J Biol Chem 1993;268:19675–80.
[47] Borroz KI, Buetler TM, Eaton DL. Modulation of gamma-glutamylcysteine synthetase large subunit mRNA expression by butylated hydroxyanisole. Toxicol Appl Pharmacol 1994;126:150–5.
[48] Warskulat U, Wettstein M, Hussinger D. Osmoregulated taurine transport in H4IIE hepatoma cells and perfused rat liver. Biochem J 1997;321:683–90.
[49] Stipanuk MH, Coloso RM, Garcia RA, Banks MF. Cysteine concentration regulates cysteine metabolism to glutathione, sulfate and taurine in rat hepatocytes. J Nutr 1992;122:420–7.
[50] Bella DL, Hirschberger LL, Hosokawa Y, Stipanuk MH. Mechanisms involved in the regulation of key enzymes of cysteine metabolism in rat liver *in vivo*. Am J Physiol 1999;276:E326–35.