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Metabolism Clinical and Experimental 55 (2006) 899–906

Metabolism
Clinical and Experimental

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Distinct effects of folate and choline deficiency on plasma kinetics of methionine and homocysteine in rats

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Received 5 October 2005; accepted 21 February 2006

Abstract

Both folate and betaine, a choline metabolite, play essential roles in the remethylation of homocysteine to methionine. We have studied the effects of folate and choline deficiency on the plasma kinetics of methionine, especially remethylation of homocysteine to methionine, by means of stable isotope methodology. After a bolus intravenous administration of [$^2H_7$]methionine (5 mg/kg body weight) into the rats fed with folate-, choline-, folate + choline–deficient or control diets, the plasma concentrations of [$^2H_7$]methionine, demethylated [$^2H_4$]homocysteine, and remethylated [$^2H_4$]methionine were determined simultaneously with endogenous methionine and homocysteine by gas chromatography-mass spectrometry–selected ion monitoring. The total plasma clearance of [$^2H_7$]methionine was not significantly different among groups, suggesting that the formation of [$^2H_4$]homocysteine from [$^2H_7$]methionine was not influenced by deficiencies of folate and choline. The area under concentration-time curve of [$^2H_4$]homocysteine significantly increased in the folate- and folate + choline–deficient group as compared with the control, but not in the choline-deficient group. The time profile of plasma concentrations of [$^2H_4$]methionine in the folate-deficient group was the same as the control group, whereas the appearance of [$^2H_4$]methionine in plasma was delayed in the choline- and folate + choline–deficient group. These results suggested plasma levels of remethylated methionine were influenced by choline deficiency rather than folate deficiency.

1. Introduction

Homocysteine, a sulfur amino acid, is an intermediate metabolite of methionine. Homocysteine in plasma is predominantly bound with itself, cysteine, and protein sulfhydryl groups to form homocystine, homocysteine-cysteine, and protein-bound homocysteine, respectively. Total homocysteine refers to all the forms of homocysteine. Human plasma concentrations of total homocysteine normally range from 5 to 15 μmol/L, and patients with plasma concentration of total homocysteine of more than 15 μmol/L are considered to have hyperhomocysteinemia [1]. Hyperhomocysteinemia is recognized as an independent risk factor for cardiovascular disease [2,3]. In the absence of renal impairment, hyperhomocysteinemia is caused either by genetic defects in the enzymes involved in homocysteine metabolism or nutritional deficiencies in vitamin cofactors [4].

Homocysteine is a product of *S*-adenosylmethionine–dependent methylation reactions. Intracellular homocysteine is then metabolized by transsulfuration or remethylation [5]. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5′ -phosphate–containing enzyme, cystathionine-β-synthase (EC 4.2.1.22). Remethylation can occur by transfer of a methyl group from 5-methyltetrahydrofolate (5-methyl-THF) catalyzed by methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase, EC 2.1.1.13) or from betaine, trimethylglycine, catalyzed by betaine-homocysteine methyltransferase (EC 2.1.1.5). The reaction with 5-methyl-THF is vitamin B_{12}–dependent, whereas the reaction with betaine is vitamin B_{12}–independent. Recent studies in humans show that deficiencies of folate and vitamin B_6 play an important causative role in hyperhomocysteinemia [6].

Betaine-homocysteine methyltransferase is contained in the liver and kidney, whereas methionine synthase and cystathionine-β-synthase are widely distributed among tissues [7-9]. Finkelstein [10] reported that the k_m values for

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doi:10.1016/j.metabol.2006.02.017

both methyltransferases were at least 1 order of magnitude smaller than that for cystathionine-β-synthase and that remethylation would be favored relative to transsulfuration in rat liver. It is also known that both remethylation pathways seemed to contribute equally by in vitro assay using rat liver [11]. Schalinske and Steele [12] examined the folate-dependent remethylation in the rat liver using a radioisotope tracer. However, the regulation of remethylation by both pathways in vivo is not fully understood.

Various dietary modifications have been used for establishing the hyperhomocysteinemic rat model [13-16]. Plasma total homocysteine markedly increased during folate deficiency [17,18]. Miller et al. [19] showed that plasma total homocysteine in rat was elevated to greater extent in folate deficiency rather than in vitamin B_6 deficiency, whereas post–methionine-loading plasma homocysteine was greater in vitamin B_6 deficiency. Although betaine, a choline metabolite, may also play essential roles in the remethylation of homocysteine, it is not known how important choline deficiency might be in causing hyperhomocysteinemia. Moreover, the relative contributions of dietary folate and choline on plasma levels of remethylated methionine are still unclear.

The use of gas chromatography–mass spectrometry (GC-MS) and stable isotope–labeled compounds as tracers has found a broad application in pharmacokinetic studies [20-23]. Storch et al. [24,25] used [1-^{13}C, methyl-$^2H_3$]methionine as a tracer to investigate the basic kinetics of methionine. Flux rates under the steady-state condition were calculated using the infusion rate and the isotopic enrichments. However, the method cannot provide exact information about the remethylation pathway [26]. Martinez et al. [27] examined the remethylation of homocysteine in vitamin B_6–deficient rats by a bolus injection of [$^2H_3$]serine. We have initiated studies to assess the methionine-homocysteine kinetics after a single administration of a multiple stable isotope–labeled methionine (Fig. 1). [3,3,4,4,*S*-Methyl-$^2H_7$]methionine ([$^2H_7$]methionine) was chosen for a tracer. [$^2H_7$]Methionine is metabolized to [3,3,4,4-$^2H_4$]homocysteine ([$^2H_4$]homocysteine) with the loss of [$^2H_3$]methyl group. [$^2H_4$]Homocysteine formed is remethylated to form [3,3,4,4-$^2H_4$]methionine ([$^2H_4$]methionine) by accepting an unlabeled methyl group from either 5-methyl-THF or betaine. Because endogenous methionine is interconverted with homocysteine, it is not possible to differentiate in the circulating blood between methionine remethylated and methionine administered. With a deuterium label in the methyl position, therefore, the obvious advantage of administered [$^2H_7$]methionine is that the tracer made it possible to recognize directly the elimination of methionine. Moreover, it has become feasible to investigate the extent of homocysteine remethylation after [$^2H_7$]methionine dosing because [$^2H_7$]methionine, [$^2H_4$]methionine, and nonlabeled methionine were distinguishable from each other by GC-MS. We have already reported the synthesis of [$^2H_7$]methionine [28] and the determination of plasma levels of methionine and total homocysteine by GC-MS [29]. In the previous article, a stable isotope method was used to evaluate the pharmacokinetics of methionine and to estimate the extent of remethylation in normal rats [30]. The purpose of the present study was to assess how deficiencies of folate and choline affect to the plasma kinetics of [$^2H_7$]methionine in rats.

2. Methods

2.1. Chemicals and reagents

DL-[3,3,4,4-$^2H_4$]Methionine (DL-[$^2H_4$]methionine; >99% atom ^2H) and DL-[3,3,3′,3′,4,4,4′,4′-$^2H_8$]homocystine (DL-[$^2H_8$]homocystine; 97.9% atom ^2H) were purchased from CDN isotopes (Quebec, Canada). Optically pure L-[3,3,4,4,*S*-methyl-$^2H_7$]methionine ([$^2H_7$]methionine) was synthesized in our laboratory as described previously [28]. The isotopic purity and the enantiomeric purity were 99.3% atom ^2H and >99.8% enantiomeric excess, respectively. Isobutyl chlorocarbonate and dithiothreitol were obtained from Wako (Osaka, Japan). A strong cation-exchange solid-phase extraction column BondElut SCX (H^+ form; size, 1 mL/100 mg) was purchased from Varian (Harbor City, OH). All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

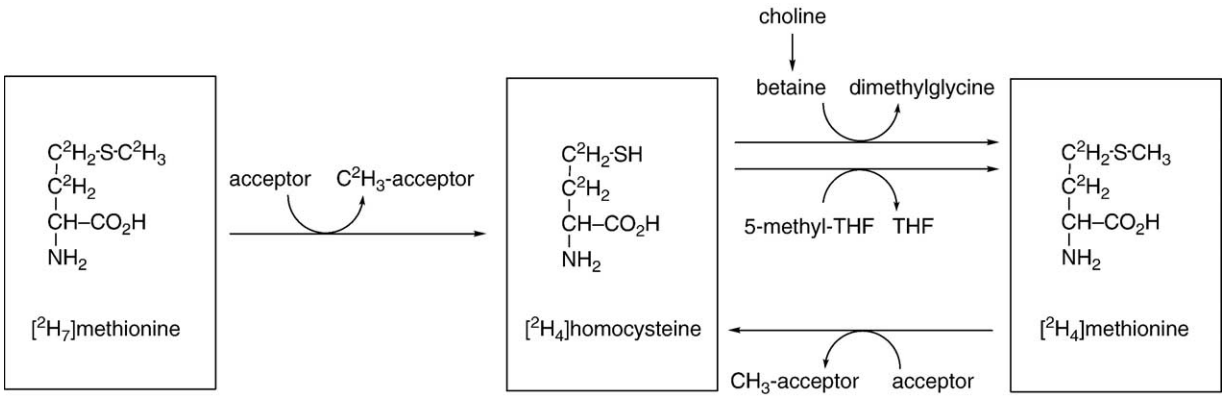

Fig. 1. Metabolic scheme for the conversion of [$^2H_7$]methionine to [$^2H_4$]methionine through [$^2H_4$]homocysteine.

2.2. Animals and dietary treatment

The experimental protocols were approved by the institutional animal care committee of the Tokyo University of Pharmacy and Life Science. Male Sprague-Dawley rats aged 4 weeks were obtained from the Tokyo Laboratory Animal Center (Tokyo, Japan) and housed in stainless-steel cages in an air-conditioned room maintained at 23°C ± 1°C and 55% ± 5% humidity with a 12-h dark-light cycle. On arrival, the rats were acclimated for 7 days, during which time they were allowed free access to water and food (CE-2, Clea Japan, Tokyo, Japan). The rats then were allocated randomly to 4 groups (n = 6 per group). In the control and folate-deficient groups, the rats were fed ad libitum for 3 weeks with AIN-93G diet (Oriental Yeast, Tokyo, Japan) containing 1% succinylsulfathiazole with 8 mg folate/kg diet (control) or without folate (folate-deficient). Addition of succinylsulfathiazole was necessary to reduce folate provided from intestinal bacteria [31]. A pilot study comparing AIN-93G diet with and without succinylsulfathiazole indicated no differences in plasma levels of methionine and homocysteine, and the plasma kinetics of [$^2H_7$]methionine. In the folate + choline–deficient group, the rats were fed ad libitum for 1 week with AIN-93G diet containing 1% succinylsulfathiazole without folate and then for 2 weeks with AIN-93G diet containing 1% succinylsulfathiazole without folate and choline. In the choline-deficient group, the rats were fed ad libitum for 1 week with AIN-93G diet, and then for 2 weeks AIN-93G diet without choline. Body weights were recorded weekly.

2.3. Dose experiment

After an overnight fast, each rat aged 8 weeks was anesthetized with pentobarbital (50 mg/kg body weight, IP). On the day of the experiment the body weights of the rats were 285 ± 19 g. [$^2H_7$]Methionine was dissolved in saline (12.5 mg/mL) and administered (5 mg/kg body weight) into the femoral vein. Heparinized blood samples (0.6 mL) were obtained from the jugular vein at 5 minutes before and 5, 10, 30, 60, 90 and 120 minutes after dosing. Plasma was separated and stored at −20°C until analysis.

2.4. Gas chromatography-mass spectrometry–selected ion monitoring

Gas chromatography-mass spectrometry–selected ion monitoring (GC-MS-SIM) was made with a Shimadzu (Kyoto, Japan) QP-1000EX quadrupole gas chromatograph–mass spectrometer equipped with a data processing system. The GC-MS analyses were performed under the conditions described previously [29]. Briefly, the GC column was a fused-silica capillary column SPB-1 (Supelco, Bellefonte, PA), and the mass spectrometer was operated in chemical ionization mode with isobutane as the reagent gas. Selected ion monitoring was performed on the molecular-related ions at *m*/*z* 285, 282, and 278 for the *N*(*O*,*S*)-isobutyloxycarbonyl ethyl ester derivatives of [$^2H_7$]methionine, [$^2H_4$]methionine, and methionine, respectively, and on the molecular-related ions at *m*/*z* 368 and 364 for the *N*(*O*,*S*)-isobutyloxycarbonyl ethyl ester derivatives of [$^2H_4$]homocysteine and homocysteine, respectively.

2.5. Analysis of methionine and homocysteine

Plasma concentrations of [$^2H_7$]methionine, [$^2H_4$]methionine, methionine, [$^2H_4$]homocysteine, and homocysteine were determined by the double-isotope dilution method as described previously [29,30]. Plasma sample was divided into 2 sets (0.1 mL each). To the sample in one set only was added DL-[$^2H_4$]methionine (10 nmol) and DL-[$^2H_8$]homocystine (2 nmol) as analytical standards. These 2 sets of plasma sample were then subjected to the GC-MS-SIM measurements after the reduction of the disulfide bond with dithiothreitol, the precipitation of proteins with trichloroacetic acid, the purification by cation exchange chromatography using BondElut SCX cartridge, and the derivatization with isobutyl chlorocarbonate in water-ethanol-pyridine.

2.6. Analysis of vitamins

Blood concentration of 5-methyltetrahydrofolate (5-methyl-THF) was determined by using high-performance liquid chromatography with fluorescence detection developed by Luo et al. [32]. Plasma concentration of betaine was determined by using high-performance liquid chromatography with ultraviolet detection developed by Laryea et al. [33].

2.7. Data analysis

Pharmacokinetic parameters were calculated by model-independent analysis using a macro program MOMENT(EXCEL) ([34]; http://www.pharm.kyoto-u.ac.jp/byoyaku/Kinetics/download.html) running on Microsoft Excel. The half-life ($t_{1/2}$) of the terminal elimination phase of the plasma concentration-time curve was estimated using a regression equation. The area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal method. Total plasma clearance (CL) was calculated using the equation CL = dose/AUC.

2.8. Statistical analysis

All data were expressed as means ± SD. Data were analyzed by 1- or 2-way analysis of variance, and then differences among means were analyzed using Tukey-Kramer multiple comparison tests. All analyses were performed using StatView version 5 (SAS Institute, Cary, NC). Results were considered statistically significant at $P < .05$.

3. Results

Moderate deficiencies of folate and choline were sought to resemble the likely degree of deficiencies that might be encountered in a free-living population. There was no difference in the body weights among rats fed with the various dietary treatments. They did not exhibit any

Table 1
Pharmacokinetic parameters for [$^2H_7$]methionine, [$^2H_4$]homocysteine, and [$^2H_4$]methionine

	Control	Deficient		
		Folate	Choline	Folate + choline
[$^2H_7$]methionine				
$t_{1/2}$ (min)	34.8 ± 4.4	37.3 ± 1.7	36.0 ± 10.2	36.5 ± 4.0
$AUC_{0\text{-}120}$ (min nmol/mL)	1037 ± 215	897 ± 125	845 ± 108	936 ± 52
$AUC_{0\text{-}\infty}$ (min nmol/mL)	1103 ± 238	948 ± 138	887 ± 119	977 ± 61
CL (mL · min^{-1} · kg^{-1})	28.9 ± 5.0	32.3 ± 4.0	33.8 ± 4.4	31.7 ± 1.9
[$^2H_4$]homocysteine				
$AUC_{0\text{-}120}$ (min nmol/mL)	66 ± 16	141 ± 17*,‡	75 ± 9	240 ± 60*,‡,†
[$^2H_4$]methionine				
$AUC_{0\text{-}120}$ (min nmol/mL)	145 ± 7	139 ± 20	142 ± 11	113 ± 8*,‡,†

Each value represents the mean ± SD (n = 6).
$^*P < .05$ vs control.
$^†P < .05$ vs folate-deficient.
$^‡P < .05$ vs choline-deficient.

abnormal behavior. After dietary treatment, blood concentrations of 5-methyl-THF in the folate-deficient group (459 ± 160 pmol/mL; $P < .05$ vs control) and folate + choline–deficient group (453 ± 111 pmol/mL; $P < .05$ vs control) were about 70% lower than those in the control group (1492 ± 106 pmol/mL). With the mild choline depletion at 2-week periods, the plasma concentrations of betaine were not different between the choline-deficient group (112.9 ± 13.5 nmol/mL) and the control group (123.4 ± 7.4 nmol/mL), but were significantly lower in the folate + choline–deficient group (93.6 ± 19.2 nmol/mL) than the control group ($P < .05$). No significant difference in plasma methionine concentrations was found among all groups (control, 64.3 ± 7.5 nmol/mL; folate deficient, 58.7 ± 5.4 nmol/mL; choline deficient, 73.4 ± 12.4 nmol/mL; folate + choline deficient, 65.8 ± 5.6 nmol/mL). As expected, the total homocysteine levels in the folate-deficient group (27.4 ± 5.2 nmol/mL; $P < .05$ vs control) and the folate + choline–deficient group (34.4 ± 9.8 nmol/mL; $P < .05$ vs control) were markedly and significantly greater than the controls (11.3 ± 1.4 nmol/mL). In contrast, the total homocysteine levels in the choline-deficient group (10.8 ± 1.0 nmol/mL) were within the reference range.

After a bolus intravenous administration of [$^2H_7$]methionine (5 mg/kg body weight) to rats fed with the various dietary treatments, plasma concentrations of [$^2H_7$]methionine, [$^2H_4$]homocysteine, and [$^2H_4$]methionine were followed at various times simultaneously with endogenous methionine and homocysteine for 120 minutes. The results are presented in Figs. 2-6. The calculated pharmacokinetic parameters were presented in Table 1. Fig. 2 shows the plasma concentration vs time profiles for [$^2H_7$]methionine. [$^2H_7$]Methionine disappeared biexponentially from the plasma, and no significant difference among all groups was observed at any of the time points after the administration. Consequently, there was no difference in the pharmacokinetic

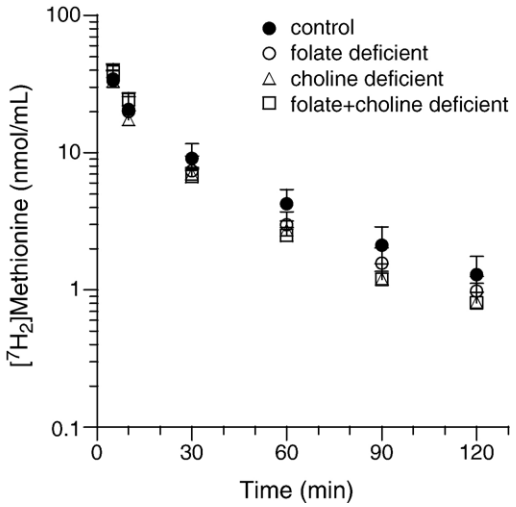

Fig. 2. Semilogarithmic plots of plasma concentrations of [$^2H_7$]methionine vs time profiles after a bolus intravenous administration of [$^2H_7$]methionine (5 mg/kg body weight) to vitamin-deficient rats. Each value represents the mean ± SD (n = 6).

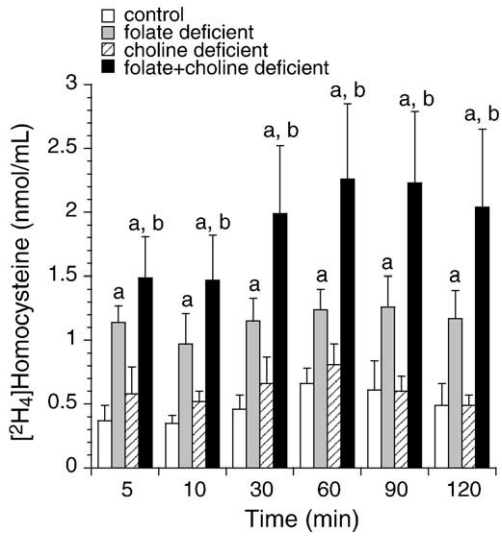

Fig. 3. Plasma concentrations of total [$^2H_4$]homocysteine after a bolus intravenous administration of [$^2H_7$]methionine (5 mg/kg body weight) to vitamin-deficient rats. Each value represents the mean ± SD (n = 6). $^aP < .05$ compared with control group; $^bP < .05$ compared with folate-deficient group.

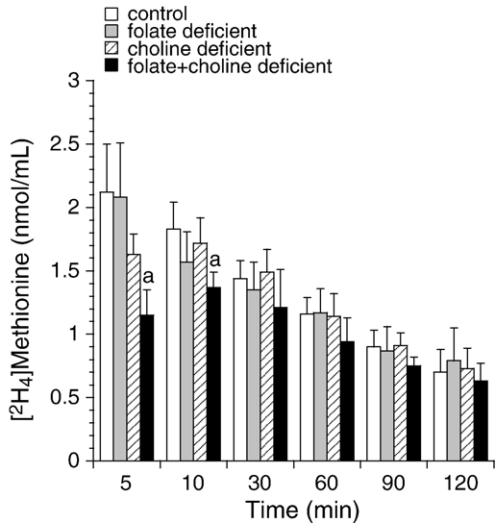

Fig. 4. Plasma concentrations of [$^2H_4$]methionine after a bolus intravenous administration of [$^2H_7$]methionine (5 mg/kg body weight) to vitamin-deficient rats. Each value represents the mean ± SD (n = 6). $^aP < .05$ compared with control.

parameters of [$^2H_7$]methionine ($t_{1/2}$, AUC, and CL) in the deficient groups as compared with the control.

The appearance of total [$^2H_4$]homocysteine in plasma was very rapid (Fig. 3). Plasma concentrations of total [$^2H_4$]homocysteine in the folate-deficient group and the folate + choline–deficient group were significantly higher compared with the control group at any time. The $AUC_{0\text{-}120}$ values of total [$^2H_4$]homocysteine in the folate-deficient group and the folate + choline–deficient group significantly increased in comparison with those in the control group (Table 1). The rise was significantly greater in the folate + choline–deficient group than in the folate-deficient group.

The appearance of [$^2H_4$]methionine in plasma was rapid in the control group, such that the time to achieve maximum

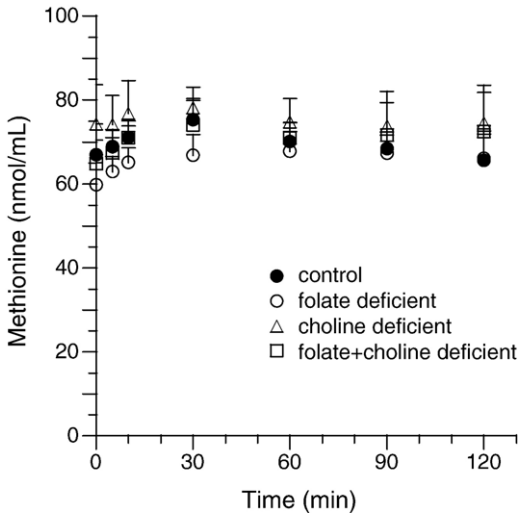

Fig. 5. Plasma concentrations of endogenous methionine after a bolus intravenous administration of [$^2H_7$]methionine (5 mg/kg body weight) to vitamin-deficient rats. Each value represents the mean ± SD (n = 6).

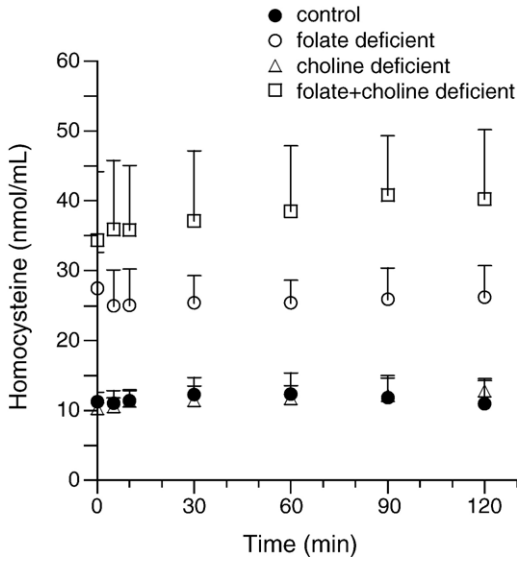

Fig. 6. Plasma concentrations of endogenous total homocysteine after a bolus intravenous administration of [$^2H_7$]methionine (5 mg/kg body weight) to vitamin-deficient rats. Each value represents the mean ± SD (n = 6).

plasma concentration (t_{max}) was observed by the first time point determined (5 minutes) as shown in Fig. 4. In the folate-deficient group, the time profile of [$^2H_4$]methionine was not significantly different from that in the control group. In the choline-deficient group and the folate + choline–deficient group, however, the appearance of [$^2H_4$]methionine in plasma was delayed (t_{max}, 10 minutes). The plasma concentrations of [$^2H_4$]methionine at 5 minutes in the choline-deficient group (1.63 ± 0.16 nmol/mL) and in the folate + choline–deficient group (1.15 ± 0.20 nmol/mL; $P < .05$ vs control) were lower than in the control group (2.12 ± 0.38 nmol/mL). Furthermore, the concentration of [$^2H_4$]methionine at 10 minutes in the folate + choline–deficient group (1.37 ± 0.12 nmol/mL) was significantly lower than in the control group (1.83 ± 0.21 nmol/mL). The $AUC_{0\text{-}120}$ values of [$^2H_4$]methionine in the folate + choline–deficient group significantly decreased in comparison with those in the control group (Table 1).

Plasma levels of endogenous methionine (Fig. 5) and total homocysteine (Fig. 6) were almost constant for 120 minutes after an administration of [$^2H_7$]methionine in all groups.

4. Discussion

The present study was carried out to determine the effects of folate, choline, and folate + choline deficiency on the pharmacokinetic behavior of [$^2H_7$]methionine in rats. Our stable isotope approach offered unique advantages because it allowed determining plasma concentrations of the exogenously administered methionine ([$^2H_7$]methionine), the remethylated methionine ([$^2H_4$]methionine), and the demethylated homocysteine ([$^2H_4$]homocysteine) simultaneously with endogenous (unlabeled) methionine and homocysteine. No significant difference on the CL of

[$^2H_7$]methionine was observed among all groups (Table 1), indicating that the effect of folate or choline deficiency on the elimination of [$^2H_7$]methionine in plasma was almost negligible in rats. These results implied that the formation of [$^2H_4$]homocysteine from [$^2H_7$]methionine was not also influenced by deficiencies of folate and choline.

Administered methionine is taken up by cells and is metabolized to homocysteine via *S*-adenosylmethionine, the major intracellular methyl donor [35]. Intracellular homocysteine is either converted to cystathionine or remethylated to methionine. Remethylation can occur by transfer of a methyl group from 5-methyl-THF catalyzed by methionine synthase or from betaine catalyzed by betaine-homocysteine methyltransferase. Intracellular homocysteine is exported into plasma when the rate of homocysteine formation exceeds the metabolic capacity. Plasma homocysteine can be derived only from the intracellular homocysteine formed by transmethylation of methionine. In the present study, plasma concentration of total [$^2H_4$]homocysteine in the folate-deficient group was significantly higher compared with the control group at any of time points after the administration of [$^2H_7$]methionine (Fig. 3). The AUC values of total [$^2H_4$]homocysteine in folate-deficient group were about twice of those in control group (Table 1). These data indicated that homocysteine remethylation was inhibited by the folate deficiency and excess homocysteine accumulated in cells was exported to plasma.

Rats have a very high activity of hepatic choline oxidase, which oxidizes choline to betaine [36]. Rats fed with the choline-deficient diet for 2 weeks have diminished hepatic concentration of choline (<50% of control) and betaine (30% of control) [37]. In the present study, the plasma level of endogenous total homocysteine in 2-week choline-deficient group was within the reference range. Moreover, a small elevated plasma total [$^2H_4$]homocysteine level was observed in the choline-deficient group after administration of [$^2H_7$]methionine (Fig. 3), but the difference in AUC values of [$^2H_4$]homocysteine was insignificant between the choline-deficient and the control group (Table 1). These data indicated that the effects of choline deficiency on an elevated plasma level of homocysteine were less pronounced than those of folate deficiency.

Little information is available on the fate of plasma homocysteine. Urinary excretion is small relative to the amount of homocysteine formed because only a small fraction of homocysteine is excreted in human urine (about 6 μmol/d) [38]. Bostom et al. [39] also reported that the urinary excretion of homocysteine was negligible in healthy rats. Guttormsen et al. [40] reported a pharmacokinetics in healthy humans after an oral administration of homocysteine. The elimination half-life of homocysteine was about 4 hours, and only 2% was excreted unchanged in the urine. They suggested that plasma homocysteine was taken up by cells and metabolized, mainly remethylated to methionine.

There has been a strong presumption that failure of remethylation in the folate-deficient rats would result in a decreased plasma level of remethylated methionine. However, plasma concentrations of the remethylated methionine, [$^2H_4$]methionine, in the folate-deficient group were the same as the control rats at any time (Fig. 4). On the other hand, delay of appearance of [$^2H_4$]methionine in plasma was observed in the choline-deficient group and the concentration at 5 minutes was significantly lower as compared with the control group. These data indicated that plasma levels of remethylated methionine were influenced by choline deficiency rather than folate deficiency.

A hypothesis is proposed to explain the metabolic basis for observed change under various conditions (Fig. 7). In rats, methionine synthase is widely distributed in all tissues, whereas betaine-homocysteine methyltransferase is only contained in liver [7-9]. The remethylation catalyzed by methionine synthase is important for the maintenance of tissue methionine, which may be used for the synthesis of either proteins or *S*-adenosylmethionine. The remethylated methionine, [$^2H_4$]methionine, is retained within the cell of

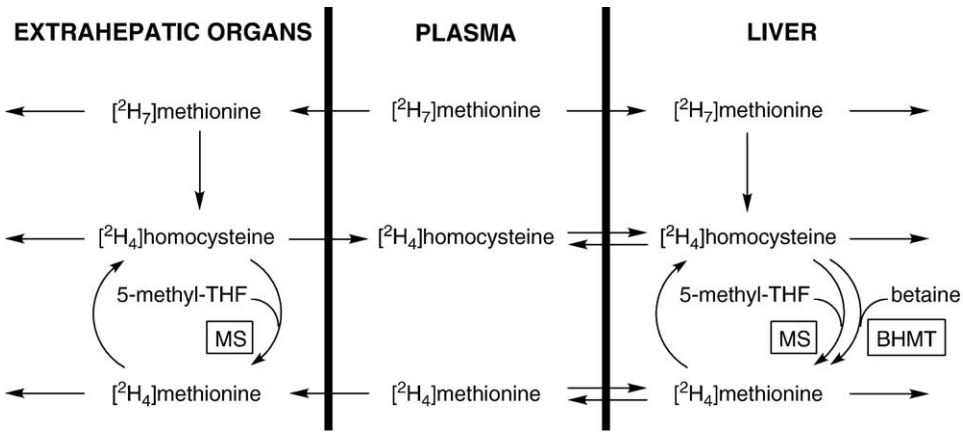

Fig. 7. Contribution to plasma levels of [$^2H_4$]homocysteine and [$^2H_4$]methionine by 5-methyl-THF and betaine. MS indicates methionine synthase; BHMT, betaine-homocysteine methyltransferase.

its origin, except liver. In folate deficiency, remethylation by methionine synthase is inhibited and excess [$^2H_4$]homocysteine is exported from the cells into plasma. [$^2H_4$]Homocysteine in plasma is taken up by liver and salvaged to [$^2H_4$]methionine through remethylation by methionine synthase or betaine-homocysteine methyltransferase. The excess [$^2H_4$]methionine formed in liver, mainly catalyzed by betaine-homocysteine methyltransferase, is exported to plasma. Betaine-dependent pathway may be a primary means to provide the remethylated methionine in plasma because plasma levels of remethylated methionine, [$^2H_4$]methionine, were influenced by the choline deficiency rather than the folate deficiency in this study. Further studies of the methionine/homocysteine kinetics in the liver are needed to reinforce the hypotheses.

With the mild choline depletion at the 2-week period, the plasma concentration of betaine was not different between the choline-deficient group and the control group. It may be that betaine was also synthesized via the cytidine diphosphocholine pathway. Phosphatidylcholine can be synthesized de novo from phosphatidylethanolamine and *S*-adenosylmethionine, which is partly dependent of a supply of folate [41]. In the folate + choline–deficient group, the de novo synthesis of choline derived from folate probably also decreased, thereby a significant reduction of plasma betaine was observed. Moreover, the AUC of [$^2H_4$]methionine in the folate + choline–deficient group was also decreased in comparison with those in the choline-deficient group because salvage to [$^2H_4$]methionine by the betaine pathway might be further depressed because of insufficient betaine. In the folate + choline–deficient group, remethylation by the betaine pathway did not only decrease, but [$^2H_4$]homocysteine export from the cells might also increase. Hence, the AUC of [$^2H_4$]homocysteine in folate + choline–deficient group was more increased than the folate-deficient group.

In summary, the present stable isotope methodology has made possible to evaluate the plasma kinetics of methionine, especially remethylation of homocysteine to methionine. The results showed that plasma levels of remethylated methionine were influenced by choline deficiency rather than folate deficiency.

Acknowledgment

This work was supported in part by grants from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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