Effect of Vitamin B₁₂-Deficiency on the Activity of Hepatic Cystathionine β -Synthase in Rats

Tadashi DOI,¹ Tetsunori KAWATA,² Naoto TADANO,¹
Takeshi IJIMA,¹ and Akio MAEKAWA¹

¹ *Department of Agricultural Chemistry, Tokyo University of Agriculture,
Setagaya-ku, Tokyo 156, Japan*

² *Faculty of Education, Okayama University, Okayama 700, Japan*

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Summary The effect of vitamin B₁₂(B₁₂)-deficiency on the activities of hepatic methionine synthase, homocysteine methyltransferase, and cystathionine β -synthase was investigated in rats. The rats bred from B₁₂-deficient dams were fed the B₁₂-deficient diets for 150 days after weaning. Growth retardation of the B₁₂-deficient rats was already observed on day 30 and continued through 150 days. But dietary supplementation of 0.5% DL-methionine slightly improved the growth retardation. Urinary excretion of methylmalonic acid increased to about 15 mg/mg creatinine and hepatic B₁₂ concentration declined to about 2 ng/g liver after a 150-day feeding of the B₁₂-deficient diets. Hepatic methionine synthase activity in rats fed the B₁₂-deficient diets supplemented with or without methionine decreased to about 5% of B₁₂-supplemented controls. Hepatic betaine-homocysteine methyltransferase activity showed no significant change caused by B₁₂-deficiency. Hepatic cystathionine β -synthase activity in rats fed the B₁₂-deficient diets supplemented with or without methionine decreased to about 61% and 27% of their B₁₂-supplemented controls, respectively, but the decrease was partially improved by methionine supplementation. In conclusion, the rats bred from B₁₂-deficient dams showed a severe B₁₂-deficiency after a 150-day feeding of the B₁₂-deficient diets. The decrease of hepatic cystathionine β -synthase activity was supposed to be due to the adaptation by the defect of methionine resynthesis.

Key Words vitamin B₁₂-deficient rats, methionine synthase, betaine-homocysteine methyltransferase, cystathionine β -synthase

In mammals, vitamin B₁₂ (B₁₂) is a coenzyme for two enzymes. Methylmalonyl-CoA mutase [EC 5.4.99.2] requires adenosylcobalamin as a coenzyme and it catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA.

¹ 土居 忠, ² 河田哲典, ¹ 只野尚登, ¹ 飯島健志, ¹ 前川昭男

Abbreviations used: B₁₂, vitamin B₁₂; CN-B₁₂, cyanocobalamin.

Accordingly the increased urinary excretion of methylmalonic acid occurs in B₁₂-deficient rats (1) and this is a useful indicator of the B₁₂-deficiency (2). Methionine synthase [EC 2.1.1.13] requires methylcobalamin as a coenzyme and it catalyzes the transfer of methyl group of 5-methyltetrahydrofolate to homocysteine, which forms methionine and tetrahydrofolate. It is known that B₁₂ can spare the dietary requirement of animals for labile methyl group such as choline and methionine. Rats can grow on a methionine-free, homocysteine-supplemented diet, if the diet contains ample amount of folic acid and B₁₂ (3).

Methionine is resynthesized from homocysteine, *via* S-adenosylmethionine and S-adenosylhomocysteine. Two reactions are available to methylate homocysteine. The one is catalyzed by B₁₂-dependent methionine synthase. The other is catalyzed by betaine-homocysteine methyltransferase [EC 2.1.1.5], which is independent of B₁₂. Furthermore, homocysteine reacts with serine to form cystathionine, which is catalyzed by cystathionine β -synthase [EC 4.2.1.22]. Homocysteine which flowed out from the cycle of methionine synthesis can no longer serve as a methionine precursor (4). This step seems to play a regulatory role in methionine metabolism (5).

On the other hand, the effect of methionine supplementation on the deranged metabolism of folate in B₁₂-deficiency is well known. Methionine supplementation to B₁₂-deficient animals decreases urinary excretion of formiminoglutamic acid (6) as well as restores liver folate concentration and the ratio of the forms of folate coenzyme to normal or subnormal (7, 8). The interrelationship between these two vitamins and the restorative effect of methionine on the folate metabolism is often explained by the "methylfolate trap" hypothesis. Scott and Weir proposed that the "methylfolate trap" is an essential physiological response to the insufficient synthesis of methionine and results in an appropriate response to B₁₂-deficiency (9). However, the information related to methionine metabolism in dietary B₁₂-deficient rats is insufficient.

In previous study, we observed that the activities of hepatic methionine synthase and methylmalonyl-CoA mutase markedly decreased in the rats fed the B₁₂-deficient diet for 150 days (10). We suggested that the rats were useful for the study of metabolic changes in B₁₂-deficiency as an experimental animal model. In this study, in order to provide fundamental information about the effect of B₁₂-deficiency on methionine metabolism, we examined the activities of hepatic homocysteine methyltransferases and cystathionine β -synthase in B₁₂-deficient rats.

METHODS

Animals and diets. Male Wistar weanling rats (about 30 g) were used in all experiments. They were born from dams which were fed the B₁₂-deficient diets during pregnancy and lactation. Newborn rats produced in this way were weaned on day 23 after birth, and divided into four dietary treatment groups as follows: -B₁₂-Met (fed the basal B₁₂-deficient diet), +B₁₂-Met (fed the basal B₁₂-

deficient diet and administered B₁₂), -B₁₂ + Met (fed the basal B₁₂-deficient diet supplemented with 0.5% DL-methionine) and +B₁₂ + Met (fed the basal B₁₂-deficient diet supplemented with 0.5% DL-methionine and administered B₁₂). Rats were individually housed in stainless steel screen-bottom cages under room conditions of constant temperature ($22 \pm 2^\circ\text{C}$) and humidity ($60 \pm 5\%$), and a 12-h cycle of light (7 a.m.-7 p.m.) and dark (7 p.m.-7 a.m.).

The basal soybean protein diet contained the following: (in %) soybean protein*¹ 18.0; glucose anhydrous*² 66.6; lard*³ 10.0; salt mixture*⁴ 5.0; vitamin mixture*⁴ 0.25; choline chloride*⁵ 0.15. When DL-methionine*⁵ was supplemented, the amount of glucose anhydrous was reduced. Each group was fed its respective diet for 150 days. Diets and water were given *ad libitum*. B₁₂-supplemented rats received 1 μg of CN-B₁₂ per day by oral administration.

Urinary methylmalonic acid. A few days before the animals were killed, they were transferred to metabolism cages and urine was collected for 24 h. The amount of methylmalonic acid was determined by the method of Giorgio and Plaut (12).

Vitamin B₁₂ concentration in liver. B₁₂ concentration in liver was determined by microbiological assay with *Lactobacillus leichmannii* (ATCC 4797). Extraction procedures of B₁₂ from rat liver were followed as previously described by Hayashi *et al.* (13).

Preparation of enzyme solution. Rats were anesthetized with ether, and blood was collected by cardiocentesis. Liver was rapidly removed after perfusion with cold saline and homogenized in a Teflon pestle-glass homogenizer using 4 volumes of 40 mM potassium phosphate buffer, pH 7.5, per gram of liver. The homogenate was centrifuged at $105,000 \times g$ for 60 min at 4°C . The supernatant fluid was collected and used for determination of methionine synthase, betaine-homocysteine methyltransferase, and cystathionine β -synthase activities.

Methionine synthase activity. Measurement of methionine synthase activity was carried out according to the method described by Matsuno *et al.* (14), except we used NADH instead of FMNH₂. The formed [¹⁴C]methionine was separated from the reaction mixture by a Dowex 1 $\times$ 8 (Cl⁻-form) column. An aliquot containing labeled methionine was counted in a liquid scintillation counter.

Betaine-homocysteine methyltransferase activity. The determination of betaine-homocysteine methyltransferase activity was carried out according to the procedure of Finkelstein and Mudd (15). The products, labeled methionine and dimethylglycine, were separated by a Dowex 1 $\times$ 8 (OH⁻-form) column. An aliquot of eluates were measured by liquid scintillation counter. The substrate, [methyl-¹⁴C]betaine was prepared and purified as follows. The reaction mixture contained 100 μmol of Tris-HCl buffer, pH 8.0, 100 μCi of [methyl-¹⁴C]choline chloride

*¹ "Ajipron S₂" (crude protein content 85.4%) produced by Ajinomoto Co. *² Produced by Aito Co. *³ Produced by Nihon Yurou Yakuhin Co. *⁴ Formulated according to reference (11). B₁₂ was omitted from vitamin mixture. All chemicals were purchased from Wako Pure Chemicals Industries Co. *⁵ Produced by Tokyo Kasei Industries Co.

(52.0 mCi/mmol), 20 unit of choline oxidase (Toyo Jozo Co.), in a total volume of 5.0 ml. After the reaction was carried out at 37°C for 90 min, the formed [methyl- ^{14}C]betaine was purified with an Amberlite CG-50 column and paper chromatographies as described by Speed and Richardson (16).

Cystathionine β -synthase activity. Cystathionine β -synthase activity was determined by the method of Suda *et al.* (17). The formed ^{14}C -labeled cystathionine was separated from the reaction mixture using a Dowex 50W $\times$ 4 (H^+ -form) column.

Determination of protein. Protein was determined by the method of Lowry *et al.* with bovine serum albumin as standard (18).

RESULTS

Growth of rats

Growth curves of rats are shown in Fig. 1. Growth of rats was markedly depressed by B_{12} -deficiency but slightly improved by dietary methionine supplementation. Final body weights of $-\text{B}_{12}-\text{Met}$ and $-\text{B}_{12}+\text{Met}$ groups were 57% and 67% of those of B_{12} -supplemented rats ($+\text{B}_{12}\pm\text{Met}$), respectively.

Urinary excretion of methylmalonic acid

Urinary methylmalonic acid excretion by rats fed on B_{12} -deficient diets for 150 days was about 15 mg/mg creatinine and not influenced by dietary methionine supplementation. In the B_{12} -supplemented group, urinary methylmalonic acid was not detected. The amount of urinary methylmalonic acid is presented in Table 1.

Fig. 1. Growth curves of the vitamin B_{12} -supplemented and -deficient rats with or without dietary methionine. Each point presents mean $\pm$ SD of five rats. Symbols: ○, $+\text{B}_{12}-\text{Met}$; □, $+\text{B}_{12}+\text{Met}$; ●, $-\text{B}_{12}-\text{Met}$; ■, $-\text{B}_{12}+\text{Met}$.

Table 1. Urinary excretion of methylmalonic acid and hepatic vitamin B₁₂ concentration in the vitamin B₁₂-supplemented and -deficient rats with or without dietary methionine supplementation.

Dietary treatment	Methylmalonic acid ^a (mg/mg creatinine)	Vitamin B ₁₂ ^a (ng/g liver)
+ B ₁₂ - Met	N.D. ^b	93.00 ± 8.01
- B ₁₂ - Met	15.72 ± 1.18	2.39 ± 0.99*
+ B ₁₂ + Met	N.D. ^b	97.23 ± 15.34
- B ₁₂ + Met	15.46 ± 0.75	2.12 ± 0.97*

^a Data presented as means ± SD of five rats fed the experimental diets for 150 days. ^b Not detected. * Significantly different from means of the vitamin B₁₂-supplemented controls; $p < 0.01$.

Table 2. Activity of hepatic methionine synthase in the vitamin B₁₂-supplemented and -deficient rats with or without dietary methionine supplementation.

Dietary treatment	Methionine synthase activity ^a (pmol/min/mg protein)
+ B ₁₂ - Met	170.1 ± 24.9
- B ₁₂ - Met	8.1 ± 1.0*
+ B ₁₂ + Met	161.7 ± 44.8
- B ₁₂ + Met	7.7 ± 0.8*

^a Data presented as means ± SD of five rats fed the experimental diets for 150 days. * Significantly different from means of the vitamin B₁₂-supplemented controls; $p < 0.01$.

Hepatic vitamin B₁₂ concentration

The concentration of B₁₂ in liver is shown in Table 1. Hepatic B₁₂ concentration in rats fed on B₁₂-deficient diets declined to about 2 ng/g of wet tissues. The effect of dietary methionine supplementation on the hepatic B₁₂ concentration was not observed.

Hepatic methionine synthase activity

Methionine synthase activity in liver is shown in Table 2. Methionine synthase activity in the livers of B₁₂-deficient rats decreased to about 5% of the B₁₂-supplemented control. The supplementation of methionine did not influence the decrease in methionine synthase activity.

Hepatic betaine-homocysteine methyltransferase activity

Table 3 shows the activity of betaine-homocysteine methyltransferase in the liver. The effect of B₁₂-deficiency on the activity was not observed. But the activity showed a tendency to increase by dietary methionine supplementation.

Table 3. Activity of hepatic betaine-homocysteine methyltransferase in the vitamin B₁₂-supplemented and -deficient rats with or without dietary methionine supplementation.

Dietary treatment	Betaine-homocysteine methyltransferase activity ^a (nmol/min/mg protein)
+ B ₁₂ - Met	1.38 ± 0.17
- B ₁₂ - Met	1.51 ± 0.11
+ B ₁₂ + Met	1.95 ± 0.38*
- B ₁₂ + Met	1.82 ± 0.32

^a Data presented as means ± SD of five rats fed the experimental diets for 150 days.

* Significantly different from means of + B₁₂ - Met group; $p < 0.05$.

Table 4. Activity of hepatic cystathionine β -synthase in the vitamin B₁₂-supplemented and -deficient rats with or without dietary methionine supplementation.

Dietary treatment	Cystathionine β -synthase activity ^a (nmol/min/mg protein)
+ B ₁₂ - Met	1.40 ± 0.12
- B ₁₂ - Met	0.38 ± 0.13*
+ B ₁₂ + Met	1.65 ± 0.24
- B ₁₂ + Met	1.00 ± 0.18*.*

^a Data presented as means ± SD of five rats fed the experimental diets for 150 days.

* Significantly different from means of the vitamin B₁₂-supplemented controls; $p < 0.01$.

. Significantly different from means of - B₁₂ - Met group; $p < 0.01$.

Hepatic cystathionine β -synthase activity

As shown in Table 4, the hepatic cystathionine β -synthase activity in - B₁₂ - Met and - B₁₂ + Met groups were 27% and 61%, respectively, of the B₁₂-supplemented control (+ B₁₂ ± Met). The activity of B₁₂-deficient rats was restored by the supplementation of methionine.

DISCUSSION

Growth rate of B₁₂-deficient rats is generally known to be lower than that of B₁₂-supplemented rats. Fehling *et al.* (19) reported that body weight of rats after 48-week feeding on the B₁₂-deficient diets is 67% of the B₁₂-supplemented rats. Brink *et al.* (20) also reported that the body weight was 77% of the B₁₂-supplemented rats at 37 weeks. In this study the growth retardation was already observed after 30-day feeding of the B₁₂-deficient diet. Final body weights of the - B₁₂ - Met and - B₁₂ + Met groups after 150 days were 57% and 67% of B₁₂-supplemented control groups (+ B₁₂ ± Met), respectively. In addition to elevated methylmalonic acid

excretion into urine, the hepatic B₁₂ concentration and methionine synthase activity severely decreased after 150 days of feeding on the B₁₂-deficient diets. From these results obvious B₁₂-deficiency was confirmed in the rats bred from B₁₂-deficient dams and then fed on the B₁₂-deficient diets for 150 days.

In spite of the decrease of methionine synthase activity, hepatic betaine-homocysteine methyltransferase activity was not affected by B₁₂-deficiency. However, methionine supplementation elevated slightly the activity of betaine-homocysteine methyltransferase in B₁₂-supplemented rats (Table 3).

Finkelstein and co-workers reported that the hepatic betaine-homocysteine methyltransferase activity in rat was increased by feeding of a high protein diet and an intraperitoneal injection of methionine, while methionine synthase activity was decreased (21). Namely, methionine synthase more likely contributes to the maintenance of a methionine pool, while betaine-homocysteine methyltransferase may function as a means both for the catabolism of choline and for the removal of homocysteine (4). However, betaine-homocysteine methyltransferase may conserve methionine under some nutritional conditions (22-24). Smith *et al.* (25) reported that a decrease of hepatic choline concentration and an increase of hepatic lipid content occurred in B₁₂-deficient sheep and that methionine supplementation prevented these changes. Further investigation is needed to clarify the relationship between methionine and choline (or betaine) metabolism in B₁₂-deficient rats (25).

Methionine is also metabolized through a single, irreversible out-flow step of cystathionine β -synthase (4). It seems to be one of the regulatory factors in methionine catabolism whether homocysteine flows to the remethylation reaction or to the cystathionine synthesis. The high methionine diet increases hepatic cystathionine β -synthase activity (5). In this study hepatic cystathionine β -synthase activity of B₁₂-deficient rats was lower than that of the B₁₂-supplemented control, and this can be restored to subnormal by supplementation of methionine (Table 4). *S*-adenosylmethionine stimulates the activity of partially purified cystathionine β -synthase (26). And it is reported that in an *in vitro* system which contained three homocysteine-metabolizing enzymes, substrates, and other metabolites at concentrations which approximated to the *in vivo* conditions in rat liver, the conversion ratio of homocysteine to cystathionine has been raised by increasing the concentration of *S*-adenosylmethionine (24). We observed that hepatic *S*-adenosylmethionine level was decreased by B₁₂-deficiency (unpublished data). The decrease of hepatic cystathionine β -synthase activity in B₁₂-deficient rats may be a metabolic response for the decreased resynthesis of methionine. However, it is reported that the hepatic cystathionine β -synthase is affected by protein content of diets, fasting, age of animals, and hormones such as hydrocortisone and glucagon (27). In this study the total food intakes of rats after 150-day feeding of +B₁₂-Met, -B₁₂-Met, +B₁₂+Met, and -B₁₂+Met diets were 2,337, 1,542, 2,602, and 1,819 g, respectively. Pair-feeding experiments will be required to estimate to what extent food intakes or growth retardation may influence the hepatic cystathionine β -synthase activity in our system.

The reduced activity of cystathionine β -synthase results in accumulation of homocysteine (4, 28). Homocystinuria has been observed in patients with defective B₁₂ metabolism (29, 30). The accumulation of homocysteine was also observed in the plasma of nitrous oxide-exposed fruit bats (31). Moreover, the levels of non-protein sulfhydryl compounds have decreased in rats fed B₁₂-deficient diets (32, 33). We also found that the hepatic glutathione level in B₁₂-deficient rats decreased to about 78% of B₁₂-supplemented control (34). It is known that hepatic glutathione is able to be a reservoir of cysteine (35, 36). Hepatic glutathione level may reflect the sulfur amino acid pool. This information supports the above argument of the decrease in cystathionine β -synthase activity in B₁₂-deficient rats.

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