Vitamin B\textsubscript{6} (B\textsubscript{6}) deficiency affects homocysteine metabolism, and this leads to hyperhomocysteinemia. In this study, we examined i) the effects of B\textsubscript{6}-deficiency and graduated levels of dietary methionine on homocysteine metabolism, and ii) the effects of fortified folate on homocysteine metabolism. In experiment 1, Wistar male rats were fed a control or a B\textsubscript{6}-deficient diet supplemented with \textit{L}-methionine at a level of 3, 6, or 9 g/kg of diet for 5 weeks. The resulting plasma homocysteine levels in the B\textsubscript{6}-deficient groups increased in relation to the increase in dietary methionine level. Next, in experiment 2, rats were fed a control, B\textsubscript{6}-deficient, or folate enriched (10 mg\textit{p}-tert-butylmethylglycine acid/kg) B\textsubscript{6}-deficient diet containing \textit{L}-methionine at 9 g/kg for 5 weeks. Although the B\textsubscript{6}-deficient diet induced hyperhomocysteinemia, folate fortification ameliorated the plasma homocysteine concentration. Overall, our results indicate that folate fortification ameliorates the hyperhomocysteinemia induced by B\textsubscript{6} deficiency and supplemental \textit{L}-methionine intake.

**Key words:** vitamin B\textsubscript{6} deficiency; hyperhomocysteinemia; \textit{pyridoxal 5-phosphate}; folate fortification; \textit{L}-methionine supplementation

Homocysteine (Hcy) is a metabolic intermediate of methionine, an essential amino acid (Fig. 1). Epidemiologically, an elevated plasma concentration of Hcy is an independent risk factor for cardiovascular disease and atherosclerosis,\textsuperscript{1-3} and hence it is important to reduce Hcy formation or to increase its removal via metabolism. Homocysteine has two metabolic pathways. In the trans-sulfuration pathway, Hcy is metabolized to cysteine by cystathionine \(\beta\)-synthase (EC 4.2.1.22; CBS) and cystathionine \(\gamma\)-lyase (EC 4.4.1.1; CGL). These two enzymes require \textit{pyridoxal 5-phosphate} (PLP) as coenzyme. The second pathway is called the remethylation pathway. In it, folate and vitamin B\textsubscript{12} (B\textsubscript{12}) or betaine is required. Thus three B-group vitamins are involved in the metabolism of Hcy, and deficiencies in the respective vitamins have been reported to cause hyperhomocysteinemia.\textsuperscript{4,8} In addition, dietary methionine content is reported to be another parameter in hyperhomocysteinemia.\textsuperscript{9} We have reported that vitamin B\textsubscript{6} (B\textsubscript{6}) deficiency in a 70% casein diet did not always cause severe hyperhomocysteinemia in rats.\textsuperscript{4,5} Thus the way methionine levels affect Hcy levels, under B\textsubscript{6}-deficient conditions is an issue of concern. The involvement of the three B-group vitamins in Hcy metabolism led us to speculate that additional dietary folate should contribute toward improving hyperhomocysteinemia under B\textsubscript{6}-deficient dietary conditions.

To test this hypothesis, we planned two experiments. In experiment 1, the effect of B\textsubscript{6}-deficiency with a graduated level of dietary methionine on methionine metabolism, including Hcy, was examined. In experiment 2, the effect of supplemental folate on hyperhomocysteinemia caused by B\textsubscript{6}-deficiency was examined. In the latter experiment, we also undertook a pilot study of the way folate relieves a deficiency of B\textsubscript{6} in cooperatively operating metabolic pathways.

**Materials and Methods**

\textbf{Animals.} This study was approved by the Committee for Animal Research and Welfare of Gifu University on the Proper Use of Laboratory Animals. Male 4-week-old Wistar rats weighing 80–100 g were obtained from Japan SLC (Hamamatsu, Japan). The rats were housed individually in stainless-steel wire-bottomed cages in an animal room at 23 ± 1°C on an inverted 12h light-dark cycle (lights on at 06:00) and 12h dark-light cycle (lights off at 18:00) for 5 weeks. To provide energy and a complete diet, the rats received a standard laboratory diet (MF, Oriental Yeast, Tokyo, Japan) that was supplemented with 1% methionine, 1% casein, 1% L-cystine, 0.5% choline chloride, 0.1% sucrose, 0.5% vitamin mixture (Shionogi, Osaka, Japan), and 0.1% calcium chloride. The rats were also offered distilled water ad libitum.

\textbf{Experimental design.} The rats were randomly divided into eight groups (12 rats per group). The control group received a control diet without methionine supplementation. The B\textsubscript{6}-deficient group received a B\textsubscript{6}-deficient diet without methionine supplementation. In experiment 1, the rats were fed a control, B\textsubscript{6}-deficient, or folate enriched diet containing \textit{L}-methionine (3, 6, or 9 g/kg of diet) for 5 weeks. In experiment 2, the rats were fed a control or folate enriched diet containing \textit{L}-methionine (9 g/kg of diet) for 5 weeks. The rats were housed individually in stainless-steel wire-bottomed cages in an animal room at 23 ± 1°C on an inverted 12h light-dark cycle (lights on at 06:00) and 12h dark-light cycle (lights off at 18:00) for 5 weeks. To provide energy and a complete diet, the rats received a standard laboratory diet (MF, Oriental Yeast, Tokyo, Japan) that was supplemented with 1% methionine, 1% casein, 1% L-cystine, 0.5% choline chloride, 0.1% sucrose, 0.5% vitamin mixture (Shionogi, Osaka, Japan), and 0.1% calcium chloride. The rats were also offered distilled water ad libitum.

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The other reagents were purchased from Nacalai (Tokyo). Methanol and acetonitrile (HPLC grade) were from Kanto (Tokyo). Phosphate (PMP) were from Wako Pure Chemical Industries (Osaka).

0900 h on the final morning of the feeding period, the rats were anesthetized with diethyl ether, and blood was collected via the abdominal aorta with a heparinized syringe. The livers were immediately excised, rinsed in ice-cold 0.25M sucrose, blotted on paper towels, weighed, quickly frozen in liquid nitrogen, and stored at −80 °C until analyzed. In experiment 2, the rats were randomly divided into six groups (n = 6 each) that were fed for 5 d.

**Dietary methionine levels did not affect the plasma Hcy concentration.** In contrast, the plasma Hcy levels in the D9 group (Fig. 2) were significantly higher than in the other groups (Table 2). Both liver PLP and liver PMP levels were significantly lower in the D9 group than in the other groups (Table 2).

**Results**

**Experiment 1**

**Growth parameters**

Final body weight and total food intake did not differ among the six experimental groups. However, the whole liver weight in the D9 group was significantly higher than in the other groups (Table 2).

**Vitamin B<sub>6</sub> status**

Plasma PLP was significantly lower in the three B<sub>6</sub>-deficient diet groups. The reduction in B<sub>6</sub> in the liver was not as severe as in the plasma.

**Methionine metabolites in the plasma and liver**

Plasma Hcy showed a significant increase only with the D9 group (Fig. 2). In the control groups, increased dietary methionine levels did not affect the plasma Hcy concentration. In contrast, the plasma Hcy levels in the B<sub>6</sub>-deficient groups increased as the dietary methionine level increased, and a significant increase in Hcy, was observed for the D9 group. The hepatic SAM concentration was not affected by the dietary methionine level or B<sub>6</sub> deficiency, but the hepatic SAH concentration in the D9 group showed a significant increase (Table 2).

### Table 1. Compositions of the Experimental Diets (g/kg diet)

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>Control&lt;sup&gt;1&lt;/sup&gt;</th>
<th>B&lt;sub&gt;6&lt;/sub&gt;-Deficient&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Folate-enriched B&lt;sub&gt;6&lt;/sub&gt;-deficient&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td></td>
</tr>
<tr>
<td>Ingredients</td>
<td>C3</td>
<td>C6</td>
<td>C9</td>
</tr>
<tr>
<td>Vitamin-free casein</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Sucrose</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Gelatinized cornstarch</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>AIN-76 vitamin mixture</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;-free vitamin mixture</td>
<td>—</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>AIN-76 mineral mixture</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>l-Methionine</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PL: Ex. 305 nm; Em. 525 nm.</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>1</sup>Control diets supplemented with 3 (C3), 6 (C6), 9 (C9) g of l-methionine per kg of diet.

<sup>2</sup>Vitamin B<sub>6</sub>-deficient diets supplemented with 3 (D3), 6 (D6), 9 (D9) g of l-methionine per kg of diet.

<sup>3</sup>Folate-enriched B<sub>6</sub>-deficient diet supplemented with 9 (F9) g of l-methionine per kg of diet.

<sup>4</sup>The content of folic acid in this vitamin mixture was 100 mg/100 g, 5 times higher than the AIN-76 vitamin mixture.

### Materials

- S-Adenosylhomocysteine (SAH), S-adenosylmethionine (SAM), and cysteine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO).
- 4-Fluoro-7-sulfobenzofurazan ammonium (SAM), and cysteine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO).
- 4-Fluoro-7-sulfobenzofurazan ammonium (SAH), and cysteine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO).
- Methanol and acetonitrile (HPLC grade) were from Wako Pure Chemical Industries (Osaka, Japan).

### Analysis of vitamin B<sub>6</sub> in plasma and liver

Plasma PLP, liver PLP, PMP, and pyridoxal (PL) levels were determined by isocratic HPLC with fluorescence detection (PLP: Ex. 320 nm; Em. 420 nm, PMP and PL: Ex. 305 nm; Em. 390 nm) by the method of Tsuge.10)

### Measurement of plasma Hcy and cysteine

Plasma total Hcy was measured by the method of Yamaguchi et al.,13 which uses an isocratic HPLC system equipped with a fluorescence detector (Ex. 380 nm; Em. 420 nm). In experiment 2, plasma cysteine was measured by the same method.
Table 2. Body Weight Gain, Total Food Intake, Liver Weight, Plasma and Liver Vitamin B6 Status, and Contents of S-Adenosylmethionine and S-Adenosylhomosysteme in the Livers of the Rats Fed the Experimental Diets (Exp. 1)

<table>
<thead>
<tr>
<th>t-Methionine supplemented (g/kg diet)</th>
<th>Two-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( P_{\text{M6}} )</td>
</tr>
<tr>
<td>Control diet</td>
<td>64.2 ± 5.8</td>
</tr>
<tr>
<td>B6-deficient diet</td>
<td>78.5 ± 6.2</td>
</tr>
<tr>
<td>Total food intake (g)</td>
<td>386 ± 10</td>
</tr>
<tr>
<td>Liver weight (g/100 g B.W.)</td>
<td>2.49 ± 0.08(^a)</td>
</tr>
<tr>
<td>Plasma PLP (nmol/g)</td>
<td>114.4 ± 37.2(^a)</td>
</tr>
<tr>
<td>B6-deficient diet</td>
<td>6.1 ± 1.4(^b)</td>
</tr>
<tr>
<td>Liver PPL (nmol/g)</td>
<td>13.7 ± 1.5(^a)</td>
</tr>
<tr>
<td>B6-deficient diet</td>
<td>8.0 ± 0.4(^b)</td>
</tr>
<tr>
<td>Liver PMP (nmol/g)</td>
<td>36.2 ± 3.0(^e)</td>
</tr>
<tr>
<td>B6-deficient diet</td>
<td>22.8 ± 2.9(^ab)</td>
</tr>
<tr>
<td>Liver SAM (nmol/g)</td>
<td>28.3 ± 6.4</td>
</tr>
<tr>
<td>B6-deficient diet</td>
<td>32.7 ± 4.2</td>
</tr>
<tr>
<td>Liver SAH (nmol/g)</td>
<td>50.7 ± 5.2(^ab)</td>
</tr>
<tr>
<td>B6-deficient diet</td>
<td>48.3 ± 3.0(^b)</td>
</tr>
</tbody>
</table>

Each value is mean ± SE (n = 6).
Means not sharing the same superscript are significantly different at \( p < 0.05 \) as determined by one-way ANOVA followed by Tukey’s multiple comparison test.

**Fig. 2.** Concentrations of Homocysteine in the Plasma of the Rats Fed the Experimental Diets (Exp. 1).
Each value is the mean ± SE (n = 6). For the experimental groups, see the legend to Table 1. Means not sharing the same superscript are significantly different at \( p < 0.05 \) as determined by one-way ANOVA followed by Tukey’s multiple comparison test.

**Experiment 2**

**Growth parameters**

Final body weight and total food intake did not differ among the three experimental groups. The liver weight in the D9 group was significantly higher than in the C9 group (Table 3).

**Vitamin B6 status**

Plasma PLP significantly decreased with B6 deprivation (Table 3), and the concentrations of PLP and total B6 in the livers of the B6-deficient rats were also significantly lower than those of the control rats (Fig. 3). Plasma and liver PLP levels were not affected by folate fortification, but liver PMP, PL, and total B6 were significantly higher in the folate enriched group.

**Modification of methionine metabolism by folate fortification**

Although the B6-deficient diet induced hyperhomocysteinemia in the D9 group, folate fortification (F9 group) ameliorated this condition (Fig. 4A). In contrast, the plasma cysteine concentration was lower in the D9 group, and improved in the F9 group (Fig. 4B). Similarly to plasma Hcy, the hepatic SAH concentration increased significantly in the D9 group and was significantly lower in the F9 group (Table 3). There was no significant difference in the amount of SAM in the liver for these groups.

**Discussion**

Vitamin B6 (PLP) is a cofactor of CBS, which catalyzes the conversion of Hcy to cysteine. Smolin et al. have reported that B6 deficiency reduced CBS activity and induced hyperhomocysteinemia.13 Referring to earlier experiments, She et al. reported that CBS activity was significantly decreased by B6 deficiency.14
found that 9 g/kg of methionine supplementation caused severe hyperhomocysteinemia in B6-deficient rats. According to recent reports,17,18 plasma homocysteine levels increase in response to the methionine intake level, and a dietary methionine loading of about 2% is likely to trigger severe hyperhomocysteinemia in rats.17,19 In the present study, B6-deficiency was regarded as the main cause of severe hyperhomocysteinemia, since methionine supplementation alone did not cause hyperhomocysteinemia (Fig. 2). Thus B6-deficiency and methionine supplementation functioned synergistically to cause an accumulation of Hcy in the plasma. Although CBS activity was decreased by B6 deficiency, it held at a sufficient level to metabolize homocysteine with moderate methionine supplementation. However, with high methionine supplementation, Hcy formation should outstrip Hcy metabolism by both the trans-sulfuration and the re-methylation pathway, leading to increased plasma homocysteine levels and accumulation.

We also observed SAH accumulation in the livers of the B6-deficient rats (Table 2), similarly to the results obtained in previous studies.4,20 Isa et al. found that the accumulation of SAH was due to the increased SAH synthetic activity of SAH hydrolase, due to Hcy accumulation caused by B6 deficiency.3

Amelioration of hyperhomocysteinemia by folate fortification has also been observed in murine and human studies,21,22 but with only a small effect, but there have been no reports to the effect that folate fortification can reduce plasma Hcy concentrations elevated by B6 deficiency. Thus this study is the first to indicate that hyperhomocysteinemia caused by B6 deficiency can be improved by folate fortification. In this study, folate fortification suppressed the increase in the plasma Hcy concentration (Fig. 4A). In other studied, plasma Hcy in rats was increased by B12-deficiency,7 methionine supplementation,24 or choline-deficiency,23 and was reduced by folate fortification. Since 5-methyltetrahydrofolate, a substrate for methionine synthase, imparts a methyl group to Hcy,24 an increased supply of folate might promote the re-methylation of Hcy by increasing the levels of 5-methyltetrahydrofolate. Considering that the formation of 5-methyltetrahydrofolate is catalyzed mainly by methylenetetrahydrofolate reductase (EC 1.5.1.20) and that its polymorphism relates to increased plasma Hcy,25 5-methyltetrahydrofolate may be a limiting substance in the re-methylation pathway. Hence we speculate that 5-methyltetrahydrofolate is more efficient than folate. In any case, it should be clarified how folate fortification increases 5-methyltetrahydrofolate levels in the liver.

The plasma cysteine concentration decreased in the D9 group, and it appeared to be influenced by CBS activity, which had been reduced by B6-deficiency (Fig. 4B). Improvement in plasma cysteine level, in the F9 group might have resulted from a slight increase in liver B6 due to folate fortification, although we have no idea how folate fortification affected liver B6 levels.

Currently no report has indicated that hyperhomocysteinemia induced by B6-deficiency is ameliorated by folic acid supplementation. Since both B6 and folate are involved in Hcy metabolism, the plasma Hcy level must be affected by the nutritional levels of both vitamins. This suggests that Hcy metabolism is affected not only...
by the relevant vitamins (e.g., B₆) but also by related vitamins (e.g., folate). Further studies are needed to explore the involvement of these vitamins in regulation of the plasma Hcy concentration.

In summary, we found that supplementation of a B₆-deficient diet with methionine induced a significant elevation in the plasma homocysteine concentration. We also found evidence that fortification by folate effectively ameliorates the hyperhomocysteinemia caused by B₆-deficient and high dietary methionine conditions.

Acknowledgment

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References