Feeding dried purple laver (nori) to vitamin B$_{12}$-deficient rats significantly improves vitamin B$_{12}$ status

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To clarify the bioavailability of vitamin B$_{12}$ in lyophilized purple laver (nori; Porphyra yezoensis), total vitamin B$_{12}$ and vitamin B$_{12}$ analogue contents in the laver were determined, and the effects of feeding the laver to vitamin B$_{12}$-deficient rats were investigated. The amount of total vitamin B$_{12}$ in the dried purple laver was estimated to be 54·5 and 58·6 (SE 5·3 and 7·5 respectively) mg/100 g dry weight by Lactobacillus bioassay and chemiluminescent assay with hog intrinsic factor respectively. The purple laver contained five types of biologically active vitamin B$_{12}$ compounds (cyano-, hydroxo-, sulfito-, adenosyl- and methylcobalamin), in which the vitamin B$_{12}$ coenzymes (adenosyl- and methylcobalamin) comprised about 60 % of the total vitamin B$_{12}$. When 9-week-old vitamin B$_{12}$-deficient rats, which excreted substantial amounts of methylmalonic acid (71·7(SE 20·2) mmol/d) in urine, were fed the diet supplemented with dried purple laver (10 mg/kg diet) for 20 d, urinary methylmalonic acid excretion (as an index of vitamin B$_{12}$ deficiency) became undetectable and hepatic vitamin B$_{12}$ (especially adenosylcobalamin) levels were significantly increased. These results indicate that vitamin B$_{12}$ in dried purple laver is bioavailable to rats.

Vitamin B$_{12}$ deficiency: Purple laver: Urinary methylmalonate excretion: Hepatic vitamin B$_{12}$ content

Various types of seaweed (arame, carragheen, dulse, hijiki, kelp, laver, wakame) are available as food items. Although seaweeds are known to be rich in vitamins and minerals as well as dietary fibres (Resources Council, Science and Technology Agency, 1984), the nutritional significance of seaweeds is not well understood. Dried purple laver (Porphyra sp.; nori), which appears to be the most widely eaten seaweed worldwide, has been reported to contain substantial amounts of vitamin B$_{12}$ (van den Berg et al. 1988), which is an essential nutrient for all animals and some other organisms, and is known to be synthesized in certain bacteria, but not in animals or plants (Schneider, 1987).

Several studies have indicated that most of the vitamin B$_{12}$ in seaweeds exists as vitamin B$_{12}$ analogues, so it may not be bioavailable to mammals (Herbert & Drivas, 1982; van den Berg et al. 1988; Dagnelie et al. 1991). Rauma et al. (1995) reported that some seaweeds can supply adequate amounts of bioavailable vitamin B$_{12}$ when consumed by strict vegetarians. Thus, it is still unclear whether the algal vitamin B$_{12}$ is available to mammals.

We determined the total vitamin B$_{12}$ and vitamin B$_{12}$ analogue contents of the dried purple laver (Porphyra yezoensis), and then investigated the effects on vitamin B$_{12}$ status of feeding the purple laver to vitamin B$_{12}$-deficient rats, to clarify the bioavailability of the vitamin B$_{12}$ from purple laver in mammals.

Materials and methods

Materials

Hydroxycobalamin (OH-B$_{12}$), cyanocobalamin (CN-B$_{12}$), 5'-deoxyadenosylcobalamin (ado-B$_{12}$) and methylcobalamin

Abbreviations: ado-B$_{12}$, 5'-deoxyadenosylcobalamin; CH$_{3}$B$_{12}$, methylcobalamin; CN-B$_{12}$, cyanocobalamin; OH-B$_{12}$, hydroxycobalamin; SO$_{3}$B$_{12}$, sulfitocobalamin.

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(CH$_3$-B$_{12}$) were obtained from Sigma (St Louis, MO, USA). Sulfitocobalamin (SO$_3$-B$_{12}$) was prepared from OH-B$_{12}$ and sodium sulfate by the method of Toraya (1983). A reversed-phase HPLC column (Wakosil-II SC18RS; 4.6 × 150 mm) was obtained from WAKO Pure Chemical Industries, Ltd, Osaka, Japan. Fresh purple laver, obtained from the Fisherman’s Association of Ishinoura in Akashi city, Hyogo prefecture, Japan, was immediately lyophilized using a freeze-dryer (FD-550; Tokyo Rikakikai Co. Ltd, Tokyo, Japan) and then powdered using a food mixer (National MK-50; National, Osaka, Japan).

**Animals and diets**

Forty male weanling Wistar rats (4 weeks old, 50 (SE 5·0) g), born to 14-week-old parents fed on a vitamin B$_{12}$-deficient diet for 8 weeks, were used. Parent rats aged 6 weeks were obtained from KIWA Laboratory Animals Co. Ltd, Wakayama, Japan. The vitamin B$_{12}$-deficient diet fed to the parents contained (g/kg): 400 soyabean protein (Fuji Oil Ltd, Osaka, Japan), 438 anhydrous glucose (Nacalai Tesque Ltd, Kyoto, Japan), 100 soyabean oil (Nacalai Tesque Ltd), 50 salt mixture, 5 DL-methionine (Nacalai Tesque Ltd), 100 vitamin mixture and 2 choline chloride (Nacalai Tesque Ltd), as described previously (Watanabe et al. 1991a). The 10 g purple laver powder contained 5-45 (50-53) µg vitamin B$_{12}$, which is identical to the amount of vitamin B$_{12}$ in the cyanocobalamin-supplemented diet.

At 6 weeks of age, the 9-week-old rats were housed in individual metabolic cages at 24°C in a room with a 12 h light–dark cycle. They were given free access to the three experimental diets and water for 20 d.

**Feeding experiments with purple laver**

The effects of feeding purple laver on growth and urinary methylmalonic acid levels in the vitamin B$_{12}$-deficient rats were studied using the diets shown in Table 1. Cellulose powder (10 g; Nacalai Tesque Ltd) was added to the original vitamin B$_{12}$-deprived diet and used as the vitamin B$_{12}$-deficient diet. The vitamin B$_{12}$-supplemented diet was identical to the vitamin B$_{12}$-deficient diet, except that 5·5 µg CN-B$_{12}$/kg diet was included. Freeze-dried purple laver powder (10 g, containing 5·45 (50·53) µg vitamin B$_{12}$) was added to the original vitamin B$_{12}$-deprived diet instead of cellulose powder, and used as a purple laver-supplemented diet. The vitamin B$_{12}$-deficient 9-week-old rats were given free access to the three experimental diets and water for 20 d.

**Urinary methylmalonic acid assay**

The urine of the vitamin B$_{12}$-deficient, vitamin B$_{12}$-supplemented and purple laver-supplemented rats was sampled for 24 h in individual metabolism cages at days 0, 10 and 20 during the experiments. Urinary methylmalonic acid was assayed by HPLC, as described previously (Toyoshima et al. 1994).

**Extraction and assay of vitamin B$_{12}$**

After food was withheld from rats overnight, the rats were killed by decapitation under diethyl ether anaesthesia. Livers were washed with a chilled 9 g NaCl/l solution, weighed, and stored at −80°C until analysed. A portion (1 g) of the liver was cut into small pieces using a razor blade and homogenized in 10 vol. acetate buffer (10 mM, pH 4·8). Total vitamin B$_{12}$ was extracted from the liver homogenate and from the dried purple laver powder (1 g) by boiling with KCN at acid pH (Frenkel et al. 1980). Acetate buffer (0·5 M, pH 4·8; 10 ml) and 20 mg KCN were added to the homogenate and the laver powder, and boiled for 30 min at 98°C in the dark. The solution was

**Table 1. Composition of the experimental diets (g/kg)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Vitamin B$_{12}$-deficient</th>
<th>Cyanocobalamin-supplemented</th>
<th>Purple laver-supplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defatted soyabean</td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Glucose, anhydrous</td>
<td>443</td>
<td>443</td>
<td>443</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Soyabean oil</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>10</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>Cyanocobalamin (µg/kg)</td>
<td>–</td>
<td>5·5</td>
<td>–</td>
</tr>
<tr>
<td>Purple laver powder</td>
<td>–</td>
<td>–</td>
<td>10</td>
</tr>
</tbody>
</table>

* Defatted soyabean was obtained from Fuji Oil Ltd, Osaka, Japan. Glucose, soyabean oil, choline chloride, DL-methionine and cellulose powder were purchased from Nacalai Tesque Ltd, Kyoto, Japan. Salt and vitamin mixtures were prepared as described previously (Watanabe et al. 1991a). The 10 g purple laver powder contained 5·45 (SE 0·53) µg vitamin B$_{12}$, which is identical to the amount of vitamin B$_{12}$ in the cyanocobalamin-supplemented diet.
centrifuged at 10 000 \( g \) for 10 min. The vitamin B\(_{12} \) remaining in the precipitate was re-extracted under the same conditions. The combined supernatant fractions were diluted with distilled water and used as a sample for the microbiological assay of vitamin B\(_{12} \). In the assay of total vitamin B\(_{12} \) in the dried purple laver the amount of vitamin B\(_{12} \) was also determined by an automated chemiluminescent vitamin B\(_{12} \) assay system ACS-180 with hog intrinsic factor (Chiron Diagnostics, East Walpole, CA, USA) as described previously (Watanabe et al. 1998).

Vitamin B\(_{12} \) analogues were extracted from the liver homogenate and the laver powder (1 g) by the method reported by Watanabe et al. (1991b). Ethanol was added to the vitamin B\(_{12} \) extract (4:1, v/v) vigorously shaken, heated at 98°C for 30 min, and then cooled in an ice bath. The solution was centrifuged at 5000 \( g \) for 10 min and the vitamin B\(_{12} \) remaining in the precipitate was re-extracted under the same conditions. The combined supernatant fractions were evaporated to dry and the residue was dissolved in a small amount of distilled water. The solution was used as a sample for HPLC. All procedures were performed in the dark. A sample of the extract (200 \( \mu l \)) was put onto a reversed-phase HPLC column (Wakosil-II SC18RS, 4.6 x 150 mm), equilibrated at 40°C with 40 mM-tartaric acid–sodium phosphate buffer, pH 3.0, containing 25% (v/v) methanol. The flow rate was 1 ml/ min. Vitamin B\(_{12} \) analogues were eluted with 30 ml eluent using a linear gradient (25–75% (v/v) methanol in the same buffer. The retention times of OH-B\(_{12} \), CN-B\(_{12} \), SO\(_3\)-B\(_{12} \), ado-B\(_{12} \) and CH\(_3\)-B\(_{12} \) were 9.0, 12.0, 13.5, 18.0 and 22.0 min respectively. Fractions (1 ml) were collected from the HPLC column, allowed to evaporate to dryness and dissolved in 1 ml distilled water. The solution was used for the microbiological assay of vitamin B\(_{12} \).

Vitamin B\(_{12} \) was assayed with *Lactobacillus leichmannii* ATCC 7830 and a vitamin B\(_{12} \) assay medium (Nissui, Tokyo, Japan) according to the manufacturer’s instructions.

**Statistics**

Statistical analysis was performed using GB-STAT™ 5.4 (Dynamic Microsystems, Inc., Silver Spring, MD, USA). One-way and two-way repeated-measures ANOVA were used with *post-hoc* two-tailed Dunnett’s test for assay of the vitamin B\(_{12} \) in the dried purple laver and rat liver, and the purple laver feeding experiments respectively. Differences were considered significant at \( P < 0.05 \).

**Results and discussion**

Total vitamin B\(_{12} \) content of the dried purple laver was estimated to be 54.5 (SE: 5.3) and 58.6 (SE: 7.5) \( \mu g \)/100 g dry weight by the *Lactobacillus* vitamin B\(_{12} \) bioassay and chemiluminescent vitamin B\(_{12} \) assay with hog intrinsic factor respectively. These values were slightly lower than the value (83.6 \( \mu g \)) described in the Standard Table of Food Composition (Resources Council, Science and Technology Agency, 1995), but were higher than the values (32.36 (SE 1.61) and 25.07 (SE 0.54) \( \mu g \) respectively) reported by Watanabe et al. (1999b). The differences in vitamin B\(_{12} \) content of the dried purple laver may have been due to different strains and growing conditions, or it might simply have reflected different degrees of vitamin B\(_{12} \) concentration in different areas where the algae was grown. These vitamin B\(_{12} \) contents of the dried purple laver were markedly higher than those of other seaweeds (kelp, 0.1 \( \mu g \), hijiki 0 \( \mu g \), wakame, 0.6 \( \mu g \); Resources Council, Science and Technology Agency, 1995); similar results have been reported by van den Berg et al. (1988). Yamada et al. (1997) reported that the vitamin B\(_{12} \) in some seaweeds (wakame (*Undaria pinnatifida*) and akaba-gin-nansou (*Rhodoglossum pulcherum*), may be cobamide-like vitamin B\(_{12} \) analogues, which are inactive in mammals. Several studies have also reported that spirulina tablets (*Spirulina sp.*) contain substantial amounts of corrinoid-like vitamin B\(_{12} \) analogues, which are assayable by the *L. leichmannii* assay, but not by a radiodiution assay with hog intrinsic factor (Herbert & Drivas, 1982; van den Berg et al. 1988). Our recent study (Watanabe et al. 1999a) demonstrated the presence of pseudo-vitamin B\(_{12} \), an inactive vitamin B\(_{12} \) analogue, in the predominant cobamide of spirulina tablets. The purple laver would not contain such inactive vitamin B\(_{12} \) analogues, because there was no significant difference between the amounts of vitamin B\(_{12} \) determined by the microbiological assay and the chemiluminescent vitamin B\(_{12} \) (with hog intrinsic factor) assay (data not shown). Identical results have been obtained previously (Watanabe et al. 1999b).

The purple laver contained five types of biologically active vitamin B\(_{12} \) compounds (OH-B\(_{12} \), SO\(_3\)-B\(_{12} \), CN-B\(_{12} \), ado-B\(_{12} \) and CH\(_3\)-B\(_{12} \)), in which the vitamin B\(_{12} \) coenzymes (ado-B\(_{12} \) and CH\(_3\)-B\(_{12} \)) predominated (about 60% of total vitamin B\(_{12} \); Table 2). Yamada et al. (1997) have also reported that CH\(_3\)-B\(_{12} \) is predominantly found in a purple laver (*Porphyra suborbiculata*).

To establish the bioavailability of the dried purple laver in mammals, the feeding experiments of the purple laver-supplemented diet to 9-week-old vitamin B\(_{12} \)-deficient rats was conducted. The urinary methylmalonic acid excretion as an index of vitamin B\(_{12} \) deficiency significantly increased in the rats fed the vitamin B\(_{12} \)-deficient diet (\( P < 0.05 \); Table 3), suggesting that the rats fed a vitamin B\(_{12} \)-deficient diet for 20 d further develop a severe vitamin B\(_{12} \) deficiency. However, in the rats fed the CN-B\(_{12}\) and the purple laver-supplemented diets, methylmalonic acid became undetectable after 10 and 20 d respectively; the level of methylmalonic acid excretion in the rats supplemented with purple laver for 10 d was not significantly different from that in the CN-B\(_{12}\)-supplemented rats.

<table>
<thead>
<tr>
<th>Table 2. Vitamin B(_{12} ) analogue contents (( \mu g/100 ) g dry weight) of the purple laver (<em>Porphyra yezoensis</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mean values with their standard errors for four samples)</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
</tr>
<tr>
<td>Total vitamin B(_{12} ) analogues</td>
</tr>
<tr>
<td>OH-B(_{12} )</td>
</tr>
<tr>
<td>SO(<em>3)-B(</em>{12} )</td>
</tr>
<tr>
<td>CN-B(_{12} )</td>
</tr>
<tr>
<td>ado-B(_{12} )</td>
</tr>
<tr>
<td>CH(<em>3)-B(</em>{12} )</td>
</tr>
</tbody>
</table>

OH-B\(_{12} \), hydroxycobalamin; SO\(_3\)-B\(_{12} \), sulfotocobalamin; CN-B\(_{12} \), cyanocobalamin; ado-B\(_{12} \), S\(^-\)deoxyadenosylcobalamin; CH\(_3\)-B\(_{12} \), methylcobalamin.
Although the rate of growth of the vitamin B12-deficient rats given CN-B12 or the purple laver had a tendency to be greater than that of rats not receiving CN-B12 during the experiment, there was no significant difference in body weight among the rats fed the three experimental diets after 20 d (Table 3).

Vitamin B12 deficiency causes multiple metabolic disorders (Weidemann et al. 1970; Williams & Spray, 1971; Fehling et al. 1978; Brass & Stabler, 1988), which appear to lead to severe growth retardation in rats. Toyoshima et al. (1994) have demonstrated that an unusual accumulation of methylmalonic acid caused by vitamin B12 deficiency disrupts normal cellular metabolism (especially metabolic inhibition of the Krebs cycle) in rat liver. To prevent the accumulation of the toxic methylmalonic acid, the vitamin B12 taken up by hepatic cells of the vitamin B12-deficient rats would be immediately converted to ado-B12, which functions as the coenzyme of methylmalonyl-CoA mutase, catalysing the isomerization of L-methylmalonyl-CoA to succinyl-CoA. Although these observations suggest that by feeding CN-B12- and the purple laver-supplemented diets for 20 d it was possible to recover completely from methylmalonic aciduria, recovery from growth retardation was not complete, because considerably longer-term feeding of the vitamin B12- or the purple laver-supplemented diets would be necessary for complete recovery from the severe growth retardation.

Total vitamin B12 and vitamin B12 compounds were assayed in the livers of rats fed the vitamin B12-deficient diet (control), the CN-B12-supplemented diet and the purple laver-supplemented diet for 20 d (Table 4). The hepatic total vitamin B12 level of the CN-B12-supplemented and purple laver-supplemented rats were about 2.8-fold and 1.9-fold greater respectively than the control. The increased total vitamin B12 level in the purple laver-supplemented rats was about 50% of that in the CN-B12-supplemented rats. These results suggest that the slightly delayed recovery from methylmalonic aciduria in the purple laver-supplemented rats was due to the incomplete release of free vitamin B12 from the dried purple laver during intestinal digestion. Although the hepatic levels of OH-B12, SO3-B12 and CH3-B12 in the CN-B12-supplemented and the purple laver-supplemented rats were not significantly different from those of the control, the ado-B12 level increased significantly in both dietary groups of rats (P < 0.05). The hepatic CN-B12 level was 3.7-fold greater in the CN-B12-supplemented rats than in the control and the purple laver-supplemented rats, showing that about 50% of the vitamin B12 taken up by the liver is accumulated as CN-B12.
in the CN-B₁₂-supplemented rats. Although the increased hepatic vitamin B₁₂ level in the purple laver-supplemented rats was about 50% of that in the CN-B₁₂-supplemented rats, there was no significant difference between them in the levels of hepatic vitamin B₁₂ coenzyme. These results indicate that the feeding of the dried purple laver significantly improved the vitamin B₁₂ status of vitamin B₁₂-deficient rats.

van den Berg et al. (1988) reported that feeding nori was ineffective in vitamin B₁₂-deficient children, and Rauma et al. (1995) also demonstrated that vegans with high seaweed intakes have decreasing serum vitamin B₁₂ levels with time. Recently, Yamada et al. (1999) reported that methylmalonic acid excretion in human female volunteers biologically active vitamin B₁₂: vitamin B₁₂ analogues. The study differs from the air-dried purple laver with regard to likely that the lyophilized purple laver used in the present study is strongly suggest that the biologically active vitamin B₁₂. The results presented here indicate that vitamin B₁₂ in the lyophilized purple laver is bioavailable to rats, indicating that vitamin B₁₂ compounds found in the dried purple laver are active in rats. Although our results strongly suggest that the biologically active vitamin B₁₂ compounds from the lyophilized purple laver are also active in man, the bioavailability of the algal vitamin B₁₂ compounds in man remains to be determined in detail, because rat metabolism is not necessarily similar to human metabolism.

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