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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 lyophylized 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) μg/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} coezymes (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) μmol/d) in urine, were fed the diet supplemented with dried purple laver (10 μg/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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Table 1. Composition of the experimental diets (g/kg)*

Ingredient	Diet (g/kg diet)		
	Vitamin B_{12}-deficient	Cyanocobalamin-supplemented	Purple laver-supplemented
Defatted soyabean	400	400	400
Glucose, anhydrous	443	443	443
DL-Methionine	5	5	5
Soyabean oil	100	100	100
Salt mixture	50	50	50
Vitamin mixture	5	5	5
Choline Chloride	2	2	2
Cellulose powder	10	10	–
Cyanocobalamin (μg/kg)	–	5·5	–
Purple laver powder	–	–	10

* 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.* 1991*a*). The 10 g purple laver powder contained 5·45 (SED 0·53) μg vitamin B_{12}, which is identical to the amount of vitamin B_{12} in the cyanocobalamin-supplemented diet.

(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 5C18RS; 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}-deprived 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), 5 vitamin B_{12}-free vitamin mixture and 2 choline chloride (Nacalai Tesque Ltd), as described previously (Watanabe *et al.* 1991*a*). The vitamin B_{12}-supplemented diet (control) was identical to the vitamin B_{12}-deprived diet, except that 5 μg CN-B_{12}/kg diet was included. The 3-week-old weanling rats were housed in individual metabolism cages at 24°C in a room with a 12 h light–dark cycle. They were given free access to the vitamin B_{12}-deprived and control diets and tap water for 6 weeks. The animals used in these studies were maintained in accordance with the guidelines of the National Research Council (1985). The body weights of the 9-week-old rats fed with the vitamin B_{12}-deficient diet were less than 35 % of those of the control rats. The 9-week-old rats fed the vitamin B_{12}-deficient diet excreted 71·7 (SE 20·2) μmol methylmalonic acid/d in urine (an index of vitamin B_{12} deficiency). Severely vitamin B_{12}-deficient rats (14 weeks old) have been reported to excrete 214·3 (SE 115·2) μmol methylmalonic acid/d in urine (Watanabe *et al.* 1991*a*). These results indicated that the 9-week-old rats fed on the vitamin B_{12}-deprived diet developed a moderate vitamin B_{12} deficiency.

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 (SE 0·53) 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

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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.* (1991*b*). 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 μl) was put onto a reversed-phase HPLC column (Wakosil-II 5C18RS, 4·6 × 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 $P < 0{\cdot}05$.

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) μg/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 μg) 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) μg respectively) reported by Watanabe *et al.* (1999*b*). 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 alga was grown. These vitamin B_{12} contents of the dried purple laver were markedly higher than those of other seaweeds (kelp, 0·1 μg, hijiki 0 μg, wakame, 0·6 μg; 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 most of 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 radiodilution assay with hog intrinsic factor (Herbert & Drivas, 1982; van den Berg *et al.* 1988). Our recent study (Watanabe *et al.* 1999*a*) 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.* 1999*b*).

Table 2. Vitamin B_{12} analogue contents (μg/100 g dry weight) of the purple laver (*Porphyra yezoensis*)

(Mean values with their standard errors for four samples)

	Mean	SE
Total vitamin B_{12} analogues	55·1	2·3
OH-B_{12}	2·9	0·3
SO_3-B_{12}	7·6	0·8
CN-B_{12}	7·8	0·9
ado-B_{12}	10·3	1·1
CH_3-B_{12}	24·8	1·8

OH-B_{12}, hydroxycobalamin; SO_3-B_{12}, sulfitocobalamin; CN-B_{12}, cyanocobalamin; ado-B_{12}, 5′-deoxyadenosylcobalamin; CH_3-B_{12}, methylcobalamin.

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 ($P < 0{\cdot}05$; 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.

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Table 3. Effects of feeding the dried purple laver-supplemented diet on the body weight and urinary methylmalonic acid excretion of 9-week-old vitamin B_{12}-deficient rats*

(Mean values with their standard errors for four rats)

	Body weight (g)		Urinary methylmalonic acid (μmol/g body wt)	
Dietary group	Mean	SE	Mean	SE
Day 0				
Vitamin B_{12}-deficient	113·2[a]	12·3	1·22[a]	0·15
CN-B_{12}-supplemented	108·3[a]	8·5	1·32[a]	0·15
Purple laver-supplemented	105·4[a]	15·1	1·41[a]	0·12
Day 10				
Vitamin B_{12}-deficient	145·3[b]	15·4	4·31[b]	4·22
CN-B_{12}-supplemented	151·7[b]	8·8	ND	
Purple laver-supplemented	153·1[b]	11·4	0·41[c]	0·14
Day 20				
Vitamin B_{12}-deficient	173·2[c]	20·4	3·21[b]	3·21
CN-B_{12}-supplemented	191·4[c]	13·4	ND	
Purple laver-supplemented	201·8[c]	14·8	ND	

CN-B_{12}, cyanocobalamin; ND, not detected.
[a,b,c]Mean values within a column with different superscript letters were significantly different ($P < 0·05$).
* For details of diets, animals and procedures, see Tables 1 and 2 and p. 700.

Although the rate of growth of the vitamin B_{12}-deficient rats given CN-B_{12} or the purple laver had a tendency to be greater than that of rats not receiving CN-B_{12} 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 B_{12} 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 B_{12} 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 B_{12} taken up by hepatic cells of the vitamin B_{12}-deficient rats would be immediately converted to ado-B_{12}, 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-B_{12}- and the purple laver-supplemented diets for 20 d was it possible to recover completely from methylmalonic aciduria, recovery from growth retardation was not complete, because considerably longer-term feeding of the vitamin B_{12}- or the purple laver-supplemented diets would be necessary for complete recovery from the severe growth retardation.

Total vitamin B_{12} and vitamin B_{12} compounds were assayed in the livers of rats fed the vitamin B_{12}-deficient diet (control), the CN-B_{12}-supplemented diet and the purple laver-supplemented diet for 20 d (Table 4). The hepatic total vitamin B_{12} levels of the CN-B_{12}- and purple laver-supplemented rats were about 2·8-fold and 1·9-fold greater respectively than the control. The increased total vitamin B_{12} level in the purple laver-supplemented rats was about 50 % of that in the CN-B_{12}-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 B_{12} from the dried purple laver during intestinal digestion.

Although the hepatic levels of OH-B_{12}, SO_3-B_{12} and CH_3-B_{12} in the CN-B_{12}-supplemented and the purple laver-supplemented rats were not significantly different from those of the control, the ado-B_{12} level increased significantly in both dietary groups of rats ($P < 0·05$). The hepatic CN-B_{12} level was 3·7-fold greater in the CN-B_{12}-supplemeted rats than in the control and the purple laver-supplemented rats, showing that about 50 % of the vitamin B_{12} taken up by the liver is accumulated as CN-B_{12}

Table 4. Hepatic vitamin B_{12} contents (μg/kg liver) of rats fed the vitamin B_{12}-deficient diet, the cyanocobalamin (CN-B_{12})-supplemented diet and the purple laver-supplemented diet*

(Mean values with their standard errors for four rats)

	Vitamin B_{12}-deficient		CN-B_{12}-supplemented		Purple laver-supplemented	
Dietary group	Mean	SE	Mean	SE	Mean	SE
Total vitamin B_{12} content	73·8[a]	14·2	224·2[b]	5·8	158·2[a,b]	22·3
Total vitamin B_{12} analogues	72·1[a]	21·1	223·1[b]	10·2	132·7[a]	18·6
OH-B_{12}	2·2[a]	0·4	3·4[a]	0·2	6·8[a]	3·2
SO_3-B_{12}	7·0[a]	1·1	8·6[a]	1·6	7·7[a]	1·2
CN-B_{12}	33·2[a]	0·7	131·0[b]	9·8	30·0[a]	12·2
ado-B_{12}	23·2[a]	2·2	72·1[b]	4·3	80·1[b]	12·1
CH_3-B_{12}	7·8[a]	2·4	12·2[a]	5·1	14·8[a]	4·2

OH-B_{12}, hydroxycobalamin; SO_3-B_{12}, sulfitocobalamin; ado-B_{12}, 5′-deoxyadenosylcobalamin; CH_3-B_{12}, methylcobalamin.
[a,b]Mean values within a row with different superscript letters were significantly different ($P < 0·05$).
* For details of diets, animals and procedures, see Tables 1 and 2 and p. 700.

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in the CN-B_{12}-supplemented rats. Although the increased hepatic vitamin B_{12} level in the purple laver-supplemented rats was about 50 % of that in the CN-B_{12}-supplemented rats, there was no significant difference between them in the levels of hepatic vitamin B_{12} coenzyme. These results indicate that the feeding of the dried purple laver significantly improved the vitamin B_{12} status of vitamin B_{12}-deficient rats.

van den Berg *et al.* (1988) reported that feeding nori was ineffective in vitamin B_{12}-deficient children, and Rauma *et al.* (1995) also demonstrated that vegans with high seaweed intakes have decreasing serum vitamin B_{12} levels with time. Recently, Yamada *et al.* (1999) reported that methylmalonic acid excretion in human female volunteers given dried *Porphyra tenera* (asakusa-nori) increased, and that air drying seemed to produce vitamin B_{12} analogues that are not only inactive, but also appear to be inhibitory to the efficient use of biologically active vitamin B_{12}. It is likely that the lyophilized purple laver used in the present study differs from the air-dried purple laver with regard to biologically active vitamin B_{12}:vitamin B_{12} analogues. These observations suggest that lyophilization is an effective drying method without loss of the biologically active vitamin B_{12}. The results presented here indicate that vitamin B_{12} in the lyophilized purple laver is bioavailable to rats, indicating that vitamin B_{12} compounds found in the dried purple laver are active in rats. Although our results strongly suggest that the biologically active vitamin B_{12} compounds from the lyophilized purple laver are also active in man, the bioavailability of the algal vitamin B_{12} compounds in man remains to be determined in detail, because rat metabolism is not necessarily similar to human metabolism.

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