Vitamin B-12 content and bioavailability of spirulina and nori in rats

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*The availability of vitamin B-12 from two different seafood products, nori (*Porphyra tenera*) and spirulina (*Spirulina *sp.), was evaluated in rats. Male weanling Wistar rats (n = 30) were fed a vitamin B-12 deficient diet for six weeks, followed by a four-week repletion period in which the rats were supplemented with equal doses of vitamin B-12, either supplied as pure cyanocobalamin, or as dried spirulina or nori. No difference in body weight gain, relative liver, or relative kidney weight could be demonstrated between the groups in either the depletion or repletion period. Low serum, liver, and kidney cobalamin contents were measured after the depletion period using an Intrinsic Factor-based radioassay. However, no hematological abnormalities could be demonstrated in the B-12 depleted rats. After repletion, cobalamin contents of serum and kidney were significantly lower, and liver cobalamin content was higher, for both the nori- and spirulina-fed rats than for the cyanocobalamin-supplemented controls. These data illustrate that cobalamins from algae are indeed absorbed by the rat. However, the distribution pattern over liver and kidneys indicates that at least part of the cobalamins, measured by a specific radioassay, may actually be analogues.*

Keywords: vitamin B-12; bioavailability; vitamin B-12 analogues; algae

Introduction

Vegetarian and macrobiotic foods are associated frequently with a "healthy" lifestyle. Alongside the many beneficial effects, for example on the blood lipid profile, there may be some nutritional "risks" accompanying a vegetarian or macrobiotic diet.*[1-3] Both types of diet are almost devoid of foods of animal origin.[2] One of the vitamins which may be deficient is vitamin B-12, which is considered to be present in animal products only. However, vitamin B-12 activity has been demonstrated in some vegetable food products, such as algae and fermented products.[4-6] Spirulina, a bluish green alga, is marketed widely as a vitamin B12-containing health food. However, most of the vitamin B-12 activity measured in this product has been found to be present as biologically inactive vitamin B-12 analogues.[7] These analogues are mainly corrinoids, cobalt-containing compounds with a similar corrin nucleus as the biologically active cobalamins, but lacking the 5,6-dimethylbenzimidazole group in the β-position to the Co^{3+} atom centered in the corrin nucleus.[8] With some methods for quantitation of vitamin B-12 in food products, such as the microbiological assay with *Lactobacillus leichmannii* or radioassays using non-purified Intrinsic Factor (IF), both the active cobalamins and the inactive corrinoids are measured. We recently reported data on the vitamin B-12 content of some algae, seaweed and fermented soy products, measured both by microbiological assay (*L. leichmannii*) and by a "pure IF"-based radioassay.[4] Remarkably, a high (true) vitamin B-12 content was found for nori, roasted leaves prepared from *Porphyra tenera*. In this report, we present results of a comparative study to test the capability of nori and spirulina to replete vitamin B-12 plasma and tissue stores of vitamin B-12 depleted rats.

Materials and methods

Animals and diets: study design

Thirty male 4-week-old Wistar rats (Wi; Winkelmann GmbH, Borchen, FRG), weighing 75–90 g, were housed in groups of five each in stainless steel wire-mesh floored cages. Animals were kept in a well-

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ventilated room at 22 ± 2° C, a relative humidity of 50 ± 10%, and a light-dark cycle of 12 hours. Purified diet and tap water were provided ad libitum.

After an acclimatization period of 1 week in which all rats were fed a vitamin B-12 deficient diet, rats were matched for weight, divided into six groups of five each, and caged individually (groups A–F). The experimental protocol is presented schematically in *Table 1*. Five groups (A, C–F) were maintained on the B-12 deficient diet for another 6 weeks, while one control group (B) was fed the same diet supplemented with cyanocobalamin (100 μg/kg). After this 6-week depletion period, the rats were fed either a vitamin B-12 "repletion" diet containing pure cyanocobalamin (100 μg/kg; groups A and B) or a purified diet supplemented with spirulina (*Spirulina* sp.) or nori (*P. tenera*) as the source of vitamin B-12 (groups D and E, respectively). The composition of the purified diets is given in *Table 2*. A soy protein isolate was used as protein source because of its low cobalamin content (< 2 μg/kg) as compared to casein. The amount of spirulina and nori added provided the same dose of vitamin B-12 as the control diet (100 μg/kg). The diets were corrected for the protein content of the algae added to the diets to provide an isonitrogenous and isoenergetic diet. The protein contents of nori, spirulina, and the soy protein isolate were 39.2, 65.0, and 87.3%, respectively.

The vitamin B-12 supplemented diets were fed for a period of 4 weeks. One group (C) was kept on the vitamin B-12 deficient diet for the whole study period. Animals from group F were sacrificed after the 6-week depletion period to check whether vitamin B-12 stores were indeed depleted. During the depletion period (week 4), blood samples were collected from all rats via the tip of the tail. At week 10, rats were anesthetized with ether and bled to death by puncture of the aorta abdominalis. Part of the blood was used for hematological determinations; serum was prepared after blood clotting by centrifuging for 15 min at 1700*g*. Liver and kidney were dissected out and, after weighing, immediately frozen in liquid nitrogen. Homogenates were prepared in a solution containing 50 mg/L potassium cyanide (KCN) using a Potter-Elvehjem tissue grinder. All serum samples and organ homogenates were stored at −20° C until analysis.

Table 1 Experimental protocol

Group	Depletion period (6 week)	Repletion period (4 week)
A (− B12/+ B12)	− Vit. B-12	+ Vit. B-12
B (+ B12/+ B12)	+ Vit. B-12	+ Vit. B-12
C (− B12/− B12)	− Vit. B-12	− Vit. B-12
D (− B12/Nori)	− Vit. B-12	Nori
E (− B12/Spir)	− Vit. B-12	Spirulina
F (− B12/—)[a]	− Vit. B-12	

[a] Animals of group F were sacrificed immediately after the depletion period.

Table 2 Composition of experimental diets (in g/kg)

Ingredients	Vit. B-12 deficient	Vit. B-12 repletion	Nori	Spirulina
Soy protein isolate	200	200	147	47
DL-Methionine	2	2	2	2
Wheat starch	329	329	296	302
Sucrose	329	329	296	302
Cellulose (Dicacel 10)	5	5	5	5
Soybean oil	5	5	5	5
Vitamin B-mixture[a]				
Vitamin B without B-12	2	—	2	2
Vitamin B with B-12	—	2	—	—
Vitamin ADEK preparation[a]	3	3	3	3
Mineral mixture[a]	35	35	35	35
Nori (Akwarius)	—	—	119	—
Spirulina (M. Rohrer)	—	—	—	207

[a] For composition of vitamin and mineral mixtures, see ref. 19.

Analysis

Hematological variables (hemoglobin, hematocrit, erythrocyte, and differential leukocyte counts in peripheral blood smears) were measured by conventional methods. Serum and tissue vitamin B-12 contents* were measured using an in-house radioassay, based on the assay principle originally described by Lau.[9] Purified porcine IF (Sigma Chemical Co., St. Louis, MO, USA) was used as the binder. Purity was checked by binding studies with cobinamide (cross-reactivity: 0.002%). The cyanocobalamin standard solution was calibrated spectrophotometrically (absorbance at 368 nm in 0.5 M NaOH/0.077 M KCN; E 1 cm, 368 nm = 30.800). Standard range in the assay: 2–300 pg/tube. The serum assay was performed in 0.05 M borate buffer pH 9.3, containing 0.77 mM KCN (0.005%). Endogenous serum-binding proteins were denaturated by heating the samples for 20 min in a boiling water bath. Tissue homogenates were prepared by autoclaving in 0.1 M sodium acetate buffer pH 4.6, containing 50 mg/L M KCN (15 min/120° C). The tissue assay was performed in 0.044 M L-glutamic acid buffer pH 4.1. In both assays, cyano[^{57}Co]cobalamin (Amersham, UK) was used as the tracer (15 pg/tube). The within-assay coefficient of variation (CV) was 3–5%, the between-assay CV 3–7%. The vitamin B-12 content[1] of the batches of spirulina and nori used for preparation of the diets was determined using the same assay protocol as for liver and kidney homogenates. Total vitamin B-12 (corrinoid) content was measured using a radioassay with porcine R-protein (Sigma) as the binder, but using the same assay protocol.

* In the Results and Discussion sections, the vitamin B-12 content measured with the "pure IF" assay will be referred to as cobalamins, the contents measured using the R-binder assay as corrinoids.

Table 3 Mean food intake (g/day) and food conversion efficiency (g/g) for the various diet groups (5 rats/group). The corresponding standard deviations are given in parentheses

Group	Vit. B-12 Depl/Repl	Food intake Week 6	Food intake Week 9	Food conversion efficiency Week 6	Food conversion efficiency Week 9
A	(- B12/+ B12)	20.1 (1.4)	20.5 (1.6)	0.16 (0.02)	0.12 (0.02)
B	(+ B12/+ B12)	22.5 (1.6)	19.7 (2.0)	0.15 (0.02)	0.12 (0.02)
C	(- B12/- B12)	18.3 (1.4)	19.6 (1.0)	0.16 (0.02)	0.12 (0.02)
D	(- B12/Nori)	20.0 (1.7)	22.0 (1.9)	0.18 (0.04)	0.12 (0.02)
E	(- B12/Spir)	20.0 (1.4)	21.9 (1.7)	0.16 (0.02)	0.12 (0.02)

Statistical analysis

All data were analyzed by computer by one-way analysis of variance (ANOVA). Weekly body weight gain and food consumption were also analyzed by ANOVA follwed by Dunnett's test for multiple comparisons with the control group. For comparison of serum and organ cobalamin and corrinoid contents, the standard errors of difference (SED) were calculated. Corresponding t values can be calculated by dividing the difference between two means by the corresponding SED. Probability levels (P values) < 0.05 were considered significant.

Results

Weight gain, food, and vitamin B-12 intake

Mean growth curves for rats from the various groups are shown in *Figure 1*. Animals from group C, kept on the vitamin B-12 deficient diet throughout the whole experimental period, tended to have lower body weights. However, no significant differences in weight gain could be demonstrated between the experimental groups B–E as compared to the control group (A), both in the depletion and repletion period. Relative liver and kidney weights (data not shown) were also not significantly different between groups.

Mean food consumption and food conversion efficiency (FCE) are summarized in *Table 3*. No significant differences among groups were apparent.

The cobalamin content of the nori used for preparation of the diets was 840 μg/kg, and for spirulina 484 μg/kg; the corrinoid content was 990 μg/kg and 1460 μg/kg, respectively. In the actual diets, the cobalamin content measured (IF assay) was 88.5 μg/kg (nori) and 100.0 μg/kg (spirulina), and the corrinoid content (R-binder assay) 104.9 and 301.7 μg/kg, respectively. The cobalamin content of the control diet (as added cyanocobalamin) was 85.1 μg/kg, the vitamin B-12 deficient diet contained < 0.2 μg/kg.

From the cobalamin contents of the various diets and the mean food intake, the mean cobalamin intake (μg/day) during the repletion period was calculated as 1.71 ± 0.11 (group A), 1.61 ± 0.10 (group B), 0.04 ± 0.002 (group C), 1.85 ± 0.11 (group D), and 2.01 ± 0.14 (group E). For the corrinoid intake calculated, intakes were: 1.84 ± 0.11 (group A), 1.75 ± 0.11 (group B), 0.04 ± 0.002 (group C), 2.22 ± 0.13 (group D), and 6.08 ± 0.44 (group E).

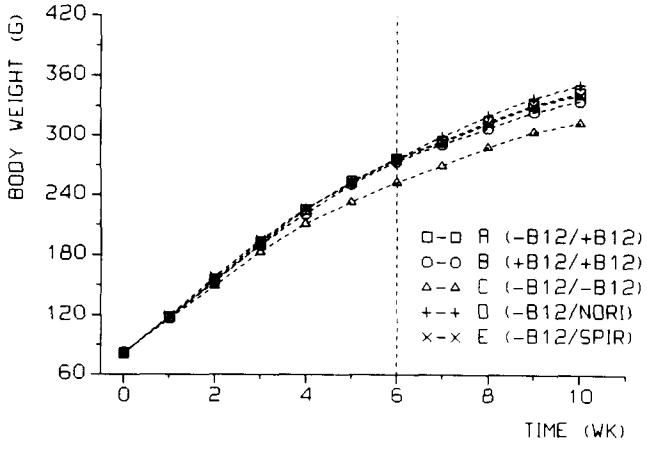

Figure 1 Mean body weight during the depletion (week 0–5) and repletion (week 6–10) periods

Cobalamin content in serum, liver, and kidney

In the 4th week of the depletion period, serum cobalamin levels were measured in tail blood samples from all animals. In the groups on the vitamin B-12 deficient diet (groups A, C–F), serum levels were below 20 ng/L (15 pmol/L), for the group B (+ vitamin B-12) mean serum content was 1620 ± 93 ng/L (1200 ± 69 pmol/L). The serum, liver, and kidney cobalamin and corrinoid contents measured after the repletion period (week 10) are summarized in *Table 4*. The cobalamin content in liver and kidney tissue of the rats of group F after the depletion period (week 6) was 25 ± 5 and 128 ± 22 μg/kg wet tissue, respectively, indicating that depletion of stores had indeed occurred. Both for serum and tissues, corrinoid contents were not significantly different from the cobalamin levels measured. However, the distribution between liver and kidney cobalamin, but also corrinoids, was significantly different between groups D and E and the control group. For groups D and E, the liver/kidney ratio was 0.14 and 0.18, respectively, while for group A this ratio was 0.04. Total organ cobalamin contents (organ content × organ weight) are presented in *Figure 2*.

Table 4 Mean serum (S; ng/L), liver (L; μg/kg), and kidney (K; μg/kg) cobalamin and corrinoid contents after vitamin B-12 repletion (5 rats/group)

Group	A	B	C	D	E	
Parameter	−B12/+B12	+B12/+B12	−B12/−B12	−B12/Nori	−B12/Spir	SED[a]
S-cobalamins	1216[a]	1276[a]	< 20[b]	904[c]	908[c]	52
S-corrinoids	1024[a]	1158[a]	21[b]	870[c]	767[c]	52
L-cobalamins	127[a]	188[b]	23[c]	229[d]	234[d]	14
L-corrinoids	126[a]	172[b]	23[c]	220[d]	223[d]	16
K-cobalamins	2949[a]	4615[b]	137[c]	1601[d]	1288[d]	195
K-corrinoids	2803[a]	4281[b]	129[c]	1558[d]	1279[d]	180

[a] SED: standard error of difference between groups. Means not sharing a common superscript are significantly different at $P < 0.05$.

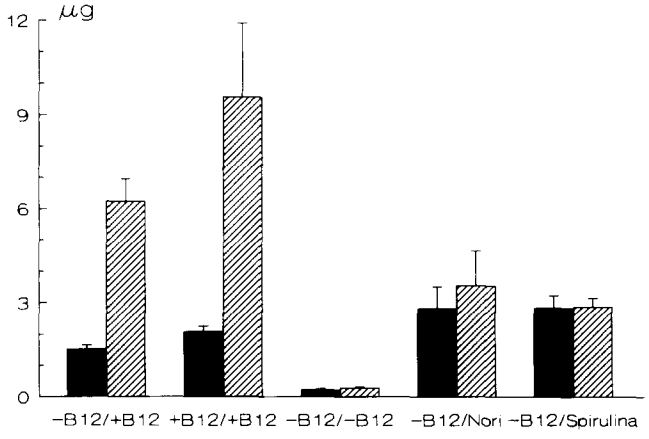

Figure 2 Mean (SD) total liver and kidney cobalamin contents (liver: solid bars; kidney: hatched bars)

Hematological findings

The hematological data are summarized in *Table 5*. None of the parameters differed significantly among the groups. Also, leukocyte differentiation in a peripheral blood smear did not differ between the vitamin B-12 depletion and repletion groups (data not shown).

Discussion

From the data presented in *Table 4*, we may conclude that the cobalamins present in nori and spirulina are indeed absorbed by the rat. Apparent availability was calculated as the mean serum or organ cobalamin content measured for group D (nori) or group E (spirulina) expressed as percentage of the content in the control group (A), after correction for the difference in cobalamin intake between the groups. Based on serum cobalamin levels, the apparent availability of cobalamins in nori is about 70%; for spirulina this figure is about 65%. Departing from cobalamin accumulation in the kidney, these figures can be calculated to be 53% and 40%, respectively. Remarkably, liver cobalamin contents of rats fed the nori or spirulina diets were higher than for rats supplied with pure cyanocobalamin resulting in apparent availabilities > 100%. This difference in organ distribution between the experimental

Table 5 Mean values for some relevant hematological parameters after the first 6 weeks for the vitamin B-12 deplete (group A, C, D, E) and replete rats (group B); (5 rats/group)

Group	RBC (10^{*2}/L)	Hb (nM)	PCV (L/L)	MCV (fL)	MCHC (mM)	MCH (mM)
+ Vit. B-12 (group B)	6.5	9.0	0.465	71.6	19.4	1.39
− Vit. B-12 (groups A,C,D,E)	6.6	9.0	0.460	70.3	19.6	1.38

groups D and E and the control group, but also the similar response for the two seafood products tested with respect to serum and tissue contents, need some further comment.

In the rat, liver and kidney are the main stores for the cobalamins. In particular, the kidney plays a role in the regulation of cobalamin uptake and release.[10] Only protein-bound cobalamins can be taken up by the cells. In rats, transcobalamin II (TC-II) is the principal transport protein.[11] TC-II has a high affinity for cobalamins but can also bind some B-12 analogues. In man, TC-II is also essential to delivery of cobalamins to the target tissues, but about 75% of plasma cobalamins are bound to so-called R-binders, especially the glycoprotein TC-I.[11] These R-binders can bind both corrinoids and cobalamins, but have a higher affinity for most corrinoids than for cobalamins. It has, therefore, been speculated that the main function of R-binders is the removal of potentially toxic vitamin B-12 analogues from the circulation.[13] The R-binder-bound B-12 analogues are cleared rapidly from the circulation by hepatocytes and retained in the liver and/or excreted in the feces.[12]

As with the differential analysis, no significant differences were obtained between the IF and R-binder assays. It seems that no corrinoids had accumulated in either tissue and analogues apparently do not interfere with cobalamin absorption. The fact that in rats, contrary to the situation in other mammals such as man and rabbit,[12,14,15] corrinoids apparently do not accumulate may be explained by the difference in binding proteins between species. However, we cannot exclude

completely the presence of vitamin B-12 analogues. Kondo et al.[14] isolated cobalamin analogues from rabbit kidney that showed growth-promoting activity for *Euglena gracilis* and *L. leichmannii* as well as binding affinity for R-protein and TC-II, but also for IF. So, in principle, cobalamin analogues with equal affinity for R-binder and IF might have been present.

Also the different distribution pattern found in this study for the algae-fed rats as compared to the control rats (i.e., a higher accumulation in the liver than in the kidney, is suggestive of the presence of cobalamin analogues).[12]

Of course, other compounds present in these seafood products may play a role as well, as we used crude preparations of both nori and spirulina rather than cobalamin-containing isolates. The only provision made was for the energy, vitamin B-12, and protein contents of the various diets.

Another, more functional criterion in evaluating whether cobalamins from nori and spirulina are indeed (bio)available is to use these products therapeutically (i.e., for correcting vitamin B-12 deficiency). The 6-week depletion period used in our study was sufficiently long to deplete plasma and tissue cobalamin stores, but not to induce a functional B-12 deficiency. Even in rats fed the deficient diet for the complete 10-week study period (group C), no significant effect on growth rate or on hematological indices was found. This is not very surprising since other studies have shown that rats are well protected from developing a diet-induced vitamin B-12 deficiency due to coprophagy, viz. the ingestion of vitamin B-12 produced by intestinal bacteria.[8] Brink et al.[16] reported a slight growth retardation already after 3 weeks, but hematological signs, such as macrocytosis, were observed only after 33 weeks. Differences with respect to type and time of deficiency symptoms may be related to differences in composition of the diet, experimental conditions, and rat strain used. Methyl malonate excretion seems to be an early symptom of functional vitamin B-12 deficiency, although it may be quite variable among rats and may be affected by other dietary factors, especially dietary fiber.[17] Unfortunately, no 24-hour urine samples were collected in our study.

That cobalamins from algae are indeed unavailable to man is also indicated by a recent, small-scale intervention study in vitamin B-12 deficient macrobiotically fed children.[18] Inclusion of nori and/or spirulina in these children's diet resulted in a further deterioration of mean corpuscular volume (MCV) despite an increase in vitamin B-12 plasma levels. This would also suggest the presence of compounds interfering with vitamin B-12 metabolism and/or DNA synthesis or of biologically inactive cobalamin analogues responsive to the porcine IF-based assay. Further studies are therefore needed to identify the nature of cobalamins and B-12 analogues present in algae, as well as dose-response studies in vitamin B-12 deficient rats using functional parameters of the vitamin B-12 status and cobalamin-containing isolates, rather than crude algal preparations.

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