検討している。葉酸欠乏（25 μg/日）期では，血漿葉酸が減少し，ホモシステインが増加した。葉酸添加期（99 μg/日）では，血漿葉酸は増加するが，ホモシステインの低下はみられていない。この結果，所要量200 μg/日では十分でないことを示している。

葉酸の過剰摂取については，食事のみから多量に摂取することは困難であり，多くの場合サプリメントの利用によるものと考えられる。400 μg/日のサプリメントによって，亜鉛の尿中への排泄量が低下することや糞便中の亜鉛量が増加することが報告されている[23-25]。今回所要量に見合った葉酸サプリメントの摂取量では，試験期間中に血清亜鉛量の変化はみられなかった。しかしながら，ビタミン B_1，B_{12}，C を増やした場合に血清亜鉛量の低下が認められた。葉酸のみでなく，これらのビタミンも腸管からの亜鉛の吸収や体内での亜鉛の代謝に影響している可能性がある。今後さらに検討する必要がある。

最後に，第六次改定日本人の栄養所要量—食事摂取基準—において，葉酸の所要量が200 μg/日とはじめて策定された。また日本人の食事摂取基準（2005年版）では，葉酸の推奨量が240 μg/日に改定された。今回使用した葉酸摂取量は200 μg/日であるが，生体利用率を考慮すると，368 μg あるいは340 μgDFEs/日となる。この結果では，男性ではやや不足している可能性があるが，女性では必要量を十分に満たしているものと考えられる。これまでの報告でも葉酸摂取量が200 μg/日で充足しているとする報告がある。わが国では，食品に葉酸が強化されていないため，葉酸の摂取量は食事から摂取可能な量として考える必要がある。今回の結果は，今後葉酸の食事摂取基準を策定するための基礎的な知見として重要である。

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文　献

1) 岩井和夫（1980）葉酸 6.1 序論：ビタミン学［II］，（ビタミン学会編），p. 363-8. 東京化学同人，東京.
2) 小橋昌裕（1996）葉酸：ビタミンの事典，（日本ビタミン学会編），p. 283-94. 朝倉書店，東京.
3) Daly S, Mills JL, Molloy AM, Conley M, Lee YJ, Kirke PN, Weir DG, Scott JM (1997) Minimum effective dose of folic acid for food fortification to prevent neural-tube defects. *Lancet* **350**：1666-9.
4) Czeizel AE, Dudas I (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* **327**：1832-5.
5) 渡辺敏明（2002）葉酸による先天異常の予防：ビタミン研究のブレークスルー—発見から最新の研究まで—，（日本ビタミン学会編），p. 265-70. 学進出版，吹田.
6) Ueland PM, Refsum H, Beresford SAA, Vollset SE (2000) The controversy over homocysteine and cardiovascular risk. *Am J Clin Nutr* **72**：324-32.
7) Schnyder G, Roffi M, Flammer Y, Pin R, Hess OM (2002) Effect of homocysteine-lowering therapy with folic acid, vitamin B_{12}, and vitamin B_6 on clinical outcome after percutaneous coronary intervention. *JAMA* **288**：973-9.
8) 渡辺敏明，大川恵子（2001）葉酸代謝と動脈硬化症．臨床検査 **45**，1117-22.
9) 厚生省（1999）葉酸：第六次改定日本人の栄養所要量—食事摂取基準—，p. 103-5. 厚生省保健医療局地域保健・健康増進栄養課生活習慣病対策室，東京都.
10) Institue of Medicine (2003) Folate. In: Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B_6, Folate, Vitamin B_{12}, Pantothenic Acid, Biotin, and Choline, p. 196-305. National Academy Press, Washington DC.
11) 厚生労働省（1999）葉酸：日本人の食事摂取基準，2005年版，p. 100-3. 厚生労働省健康局総務課生活習慣病対策室，東京都.
12) 平岡真実，安田和人（2000）女子大学生のビタミン B_{12}，葉酸栄養状態について—血清ビタミン B_{12}，葉酸濃度の分布範囲—．ビタミン **74**，271-80.
13) Melse-Boonstra A, de Bree A, Verhoef P, Bjorke-Monsen AL, Verschuren WMM (2002) Dietary monoglutamate and polyglutamate folate are associated with plasma folate concentrations in Dutch men and women aged 20-65 years. *J Nutr* **132**：1307-12.
14) Konings EJM, Roomans HHS, Dorant E, Goldbohm RA, Saris WHM, van den Brandt PA (2001) Folate intake of the Dutch population according to newly established liquid chromatography data for foods. *Am J Clin Nutr* **73**：765-76.
15) 健康・栄養情報研究会（2001）国民栄養の現状：平成13年度厚生労働省国民栄養調査結果．第一出版，東京.
16) Hiraoka M (2004) Folate intake, serum folate, serum total homocysteine levels and methylenetetrahydrofolate reductase C677T polymorphism in young Japanese women. *J Nutr Sci Vitaminol* **50**：238-45.
17) Selhub J, Jacques PF, Wilson PFW, Rush D, Rosenberg IH (1993) Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA* **270**：2693-8.
18) Higgins JR, Quinlivan EP, McPartlin J, Scott JM, Weir DG, Darling MRN (2000) The relationship between increased folate catabolism and the increased requirement for folate in pregnancy. *Br J Obstet Gynaecol* **107**：1149-54.
19) Milne DB, Johnson LK, Mahalko JR, Sandstead HH (1983) Folate status of adult males living in a metabolic unit: possible relationships with iron

nutriture. *Am J Clin Nutr* **37** : 768-73.
20) O'Keefe CA, Bailey LB, Thomas EA, Hofler SA, Davis BA, Cerda JJ, Gregory JF III (1995) Controlled dietary folate affects folate status in nonpregnant women. *J Nutr* **125** : 2717-25.
21) Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC (1987) Folate requirement and metabolism in nonpregnant women. *Am J Clin Nutr* **46** : 1016-28.
22) Jacob RA, Wu M-M, Henning SM, Swendseid ME (1994) Homocysteine increases as folate decreases in plasma of healthy men during short-term dietary folate and methyl group restriction. *J Nutr* **124** : 1072-80.
23) Milne DB, Canfield WK, Mahalko JR, Sandstead HH (1984) Effect of oral folic acid supplements on zinc, copper, and iron absorption and excretion. *Am J Clin Nutr* **39** : 535-9.
24) Butterworth CE, Jr, Tamura T (1989) Folic acid safety and toxicity: a brief review. *Am J Clin Nutr* **50** : 353-8.
25) Keating JN, Wada L, Stokstad EL, King JC (1987) Folic acid: effect on zinc absorption in humans and in the rat. *Am J Clin Nutr* **46** : 835-9.

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Original Paper

Dietary Requirement of Folate in Healthy Japanese Adults

Toshiaki Watanabe,*,[1] Misa Ohgushi,[1] Yukari Maekawa,[1] Mamoru Nishimuta,[2] Katsumi Shibata,[3] and Toru Fukui[1,4]

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Summary : In the 6th revision of the Japanese Recommended Dietary Allowance (RDA)—Dietary Reference Intakes (DRI)—the RDA of folate was stated to be 200 μg/day for an adult, all of which can be obtained from food. However, as evidence-based knowledge on this subject is insufficient, there is an obvious need for new data, collected by us in Japan, to be considered. To study the dietary requirement of folate, a group of healthy adults comprising 10 men and 10 women were fed a semi-purified diet following the Japanese RDA. In the women, the serum level and urinary excretion of folic acid were increased for the 8 days of the experiment. On the other hand, in the men, a decrease in the urinary excretion of folic acid was observed during the experimental period, but no change was noted in the serum level of folic acid. There was a sex difference in the dietary requirement of folic acid. These findings suggest that the 200 μg/day intake used in this study would be adequate for maintaining a constant serum level of folic acid in women, but that a deficiency of folic acid might occur in this situation in men.

Key words : folate, dietary requirement, urine, serum, dietary reference intakes

* Corresponding author (E-mail: watanabe@shse.u-hyogo.ac.jp)
[1] Laboratory of Dietary Environment Analysis, Department of Environment for Life and Living, School of Human Science and Environment, Himeji Institute of Technology University of Hyogo, 1-1-12 Shinzaike Honcho, Himeji 670-0092, Japan
[2] Division of Human Nutrition, The Incorporated Administrative Agency of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan
[3] School of Human Cultures, The University of Shiga Prefecture, 2500 Yasaka, Hikone, 522-8533, Japan
[4] Byotai Seiri Laboratory, 47-11 Kumanocho, Itabashi-ku, Tokyo 173-0025, Japan

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〈特集：栄養生化学に必要とされる食事摂取基準の知識〉

ビタミンの食事摂取基準の策定方法と策定に用いられた数値

柴田 克己、福渡　努、廣瀬 潤子

Process for establishing Japanese dietary reference intakes of vitamins and the reference values used

Katsumi Shibata, Tsutomu Fukuwatari and Junko Hirose

Summary The Dietary Reference Intakes were established for all of the 13 vitamins for a healthy Japanese population. Those Intakes for vitamins B_1, B_2, B_6, B_{12}, C, A, and niacin and folic acid were set as the Estimated Average Requirement (EAR), while, the Intakes of pantothenic acid, biotin, vitamins E, D, and K were set as the Adequate Intake, because the data that might have determined the ability to prevent a specific deficiency were not obtained for these vitamins.

Key words: Vitamin, Dietary reference intakes, Reference values, Decision method

Ⅰ．1歳～69歳の食事摂取基準の策定方法と策定に用いられた数値

1. 水溶性ビタミン

食事摂取基準で対象としている健康な人では、尿中への排泄量が、水溶性ビタミンの摂取量を反映していることは、すでに1945年にMelnick[1]らにより指摘されている。彼らは、アスコルビン酸、チアミン、リボフラビンの各尿中排泄量は各摂取量に応じて増大したこと、N^1-メチルニコチンアミド（ニコチンアミドの異化代謝産物の一つ）の尿中排泄量は摂取ニコチンアミド量に応じて増大したことを報告している。以来多くの研究者が、尿中の排泄量を利用してビタミン必要量の判定とビタミンの栄養状態を評価している。

ラットの実験のデータ[2]から作成したモデル図であるが、欠乏食投与後の血液中と尿中のビタミン含量の変化を図1に示した。

ところで、食事摂取基準を策定する目的は健康の維持・増進であり、具体的には、二つの項目に分けられる。一つは「欠乏症の予防」、二つ目は「生活習慣病の一次予防」である。現在の日本では、一つ目の目的である「欠乏症の予防」に関しては、克服することができたので、主目的が、「生活習慣病の一次予防」のための食事摂取基準へと変わりつつある。

滋賀県立大学人間文化学部生活文化学科
食生活専攻
〒522-8533 滋賀県彦根市八坂町2500

Laboratories of Food Science and Nutrition, Department of Life Style Studies, School of Human Cultures, The University of Shiga Prefecture, Hikone, Shiga 522-8533, Japan

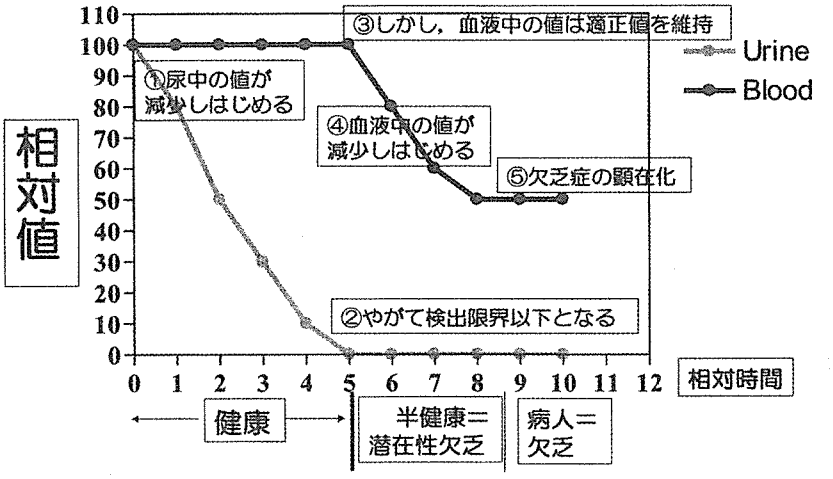

図1　欠乏食投与後の血液中と尿中のB群ビタミン量の変化

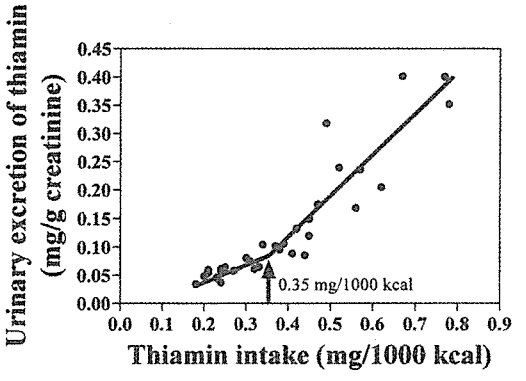

図2　チアミン摂取量と尿中へのチアミン排泄量との関係

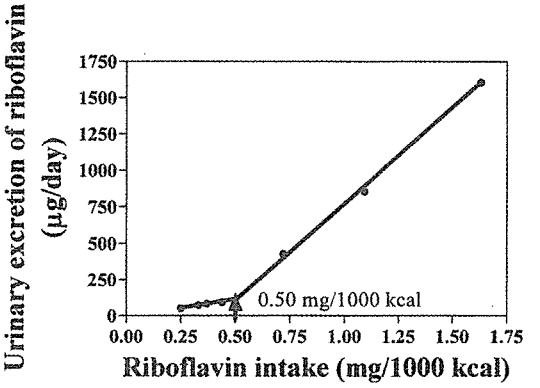

図3　リボフラビン摂取量と尿中へのリボフラビン排泄量との関係

1-1. ビタミンB_1（チアミン）

1) 背景

尿中へのチアミンの排泄形態はチアミンが主要な物であるが、チアミンの標識化合物の投与実験から、多くの異化代謝産物が存在することが知られている[3]。その中でも、主要な異化代謝産物として、4-methylthiazole-5-acetic acid[4]と3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid[5]などが報告されている。しかしながら、これら異化代謝産物の標準品が入手できないこと、定量方法も開発されていないことから、現在でも、チアミンの栄養評価は、尿中に排泄されるチアミンを利用している。

2) 尿中の値から推定平均必要量を策定

18カ国から報告された類似のデータのメタ分析から[6]、図2に示したように、必要量を満たすと、はじめて尿中への排泄量が実質的に増大してくる。この時の摂取量はチアミン（分子量=265.3）として、0.35 mg/1000 kcalである。チアミン塩酸塩（分子量=337.3）に換算すると0.45 mg/1000 kcalとなる。この値を推定平均必要量とした。推奨量は推定平均必要量×1.2としたので、0.54 mg/1000 kcalとなる。1日当たりの値にするには、対象年齢区分の推定エネルギー必要量を掛けて計算すればよい。

1-2. ビタミンB_2（リボフラビン）

1) 背景

リボフラビンの主要な異化代謝産物として、Ohkawaら[7]により、7α-hydroxyriboflavin、8α-hydroxyriboflavin、hydroxyethylflavinなどが報告されている。これら異化代謝産物の実用的な定

量方法も開発されている[7]が、標準品が入手できないこと及び排泄量がリボフラビンそのものに対して無視できることから、リボフラビンの栄養評価は、尿中に排泄されるリボフラビンを利用している。

2) 尿中の値から推定平均必要量を策定

リボフラビンもチアミンと同じく、必要量を満たすと、図3[8]に示したように、はじめて尿中への排泄量が実質的に増大してくる。この時の摂取量はリボフラビンとして、0.50 mg/1000 kcalである。この値を推定平均必要量とした。推奨量は推定平均必要量×1.2としたので、0.60 mg/1000 kcalとなる。1日当たりの値にするには、対象年齢区分の推定エネルギー必要量を掛けて計算すればよい。

1-3. ナイアシン

1) 背景

尿中へのナイアシンの排泄形態は、チアミンとリボフラビンと異なり、主要な摂取形態であるニコチンアミドではなく、異化代謝産物であるN^1-メチルニコチンアミド（MNA）、N^1-メチル-2-ピリドン-5-カルボキサミド（2-Py）とN^1-メチル-4-ピリドン-3-カルボキサミド（4-Py）である[9]。これらの異化代謝産物の排泄量比を表1に示した[9]。異化代謝産物の中で最も多いのは2-Pyであるが、市販品がないことから、MNAの排泄量から平均必要量が求められている。

表1　1日尿中に排泄されるMNA、2-Pyおよび4-Py量(日本人女性)[9]

化合物		値
ナイアシン摂取量	(μmol/日)	168.5±43.6 (78.1-293.4)
	(mg/日)	20.7±5.4 (9.6-36.1)
MNA	(μmol/日)	31.1±12.3 (11.9-66.9)
	(mg/日)	4.26±1.68 (1.63-9.17)
2-Py	(μmol/日)	59.8±26.5 (18.0-136.3)
	(mg/日)	9.10±4.03 (2.74-20.76)
4-Py	(μmol/日)	7.1±3.3 (2.4-15.8)
	(mg/日)	1.08±0.50 (0.36-2.40)

平均値±SD（n＝84）、かっこ内の数値は最低値と最大値

2) 尿中のMNAの値から推定平均必要量を策定

ナイアシン欠乏であるペラグラ発症の指標となるMNAの尿中排泄量は1.0 mg/日である（表2）[10-13]。これらの報告の再解析より（表3）、MNA尿中排泄量が1 mg/日となるナイアシン当量（NE）は、4.8 mg NE/1000 kcalである。この値を推定平均必要量とした。推奨量は推定平均必要量×1.2としたので、5.8 mg NE/1000 kcalとなる。1日当たりの値にするには、対象年齢区分の推定エネルギー必要量を掛けて計算すればよい。

3) トリプトファン-ナイアシン転換率

ヒトにおいても、肝臓でトリプトファンからNAD^+を経てニコチンアミドが合成される。その転換率は重量比で1/60程度である[10]。Fukuwatariら[14]も日本人女性を被検者として調べたが、概ねこの数値を支持するデータを報告しているので、60 mgのトリプトファンから1 mgのニコチンアミドが体内で合成されているものとした。

ナイアシン当量（mg）＝ニコチン酸(mg)＋ニコチンアミド（mg）＋1/60トリプト

表2　Goldsmithらの実験における尿中のMNA排泄量

摂取量	Days 2-13	Days 14-25	Days 26-41		
Trp, 180 mg	1.8 mg	1.6 mg	0.9 mg		
Niacin, 4.7 mg	(13.1 μmol)	(11.7 μmol)	(6.6 μmol)		
NE, 7.7 mg			皮膚炎，下痢，		
3.85 mg/1000kcal			舌炎		
摂取量	Day 2-13	Day 14-25	Day 26-41	Day 42-61	Day 62-95
Trp, 230 mg	1.9 mg	1.5 mg	1.4 mg	1.3 mg	1.1 mg
Niacin, 5.7 mg	(13.9 μmol)	(10.9 μmol)	(10.2 μmol)	(9.5 μmol)	(8.0 μmol)
NE, 9.5 mg					
4.75 mg/1000kcal					

Goldsmith GA, Sarett HP, Register UD, Gibbens J: Studies on niacin requirement inman. Ⅰ. Experimental pellagra in subjects on corn diets low in niacin and tryptophan. J. Clin. Invest., 31: 533-542, 1952.

表3　MNA排泄量が1 mg/日となるナイアシン当量摂取量

文献	被験者	MNA排泄量が1 mg/日となるNE摂取量
Goldsmithら(1952)	5名の女性、25-54歳	12.6±3.0 (23%) = 6.8 mg NE/1000 kcal
Goldsmithら(1955)	3名の女性、26-60歳	10.9±0.9 (8%) = 4.9 mg NE/1000 kcal
Horwittら(1956)	7名の男性、30-65歳	11.5±4.5 (39%) = 4.9 mg/1000 kcal
Jacobら(1989)	7名の男性、23-39歳	11.3±4.6 (41%) = 4.4 mg/1000 kcal

● Goldsimith GA, Sarett HP, Register UD: Gibbens J. Studies on niacin requirement in man 1. Experimental pellagra in subjects on corn diets low in niacin and tryptophan. J. Clin. Invest., 31, 533-542, 1952
● Goldsimth GA, Rosenthal HL, Bibbens J, Unglaub WG: Studies on niacin requirement in man. 2. Requirement on wheat and corn diets low in tryptophan. J. Nutr., 56, 371-386, 1955
● Horwitt MK, Harvey CG, Rothwell WS, Cutler JL, Haffron D: Tryptophan-niacin relationships in man: Studies with diets deficient in riboflavin and niacin, together with observations on the excretion of nitrogen and niacin metabolites. J. Nutr., 60, 1-43, 1956
● Jacob RA. Swendseid ME, McKee PW, Fu C. Clemens RC: Biochemical markers for assessment of niacin status in young men: Urinary and blood levels of niacin metabolites. J. Nutr. , 119, 591-598, 1989

ファン（mg）である。簡便法として、ナイアシン当量（mg）＝ニコチン酸（mg）＋ニコチンアミド（mg）＋1/6たんぱく質（g）の式を用いて計算してもよい。理由は食品たんぱく質のトリプトファン含量が重量比で1％程度であるからである。

1-4. ビタミンB_6（ピリドキシン）

1) 背景

血漿中に存在するビタミンB_6補酵素であるピリドキサルリン酸（PLP）は、体内組織のビタミンB_6貯蔵量を良く反映する[15]。血清中のPLP濃度が低下した若年女性において、脳波パターンに異常が見られたという報告がある[16]。

2) 血清中のピリドキサルリン酸から推定平均必要量を策定

神経障害の発生などのビタミンB_6欠乏に起因する障害が観察された報告を基に判断すると、血漿PLP濃度を30 nmol/Lに維持することができれば、これらの障害は観察されなくなる[17,18]。そこで、血漿PLP濃度を30 nmol/Lに維持できるビタミンB_6摂取量を推定平均必要量とすることにした。

一方において、ビタミンB_6の必要量は、たんぱく質摂取量が増加すると増し、血漿PLP濃度は、たんぱく質当たりのビタミンB_6摂取量と良く相関することが知られている[6]。この解析値から[18]、血清PLPを30 nmol/Lに50％の人が維持できるピリドキシン摂取量を指標として0.014 mg/gたんぱく質とした（図4）。生体利用率75％[19]を加味して、ビタミンB_6の推定平均必要量を0.019 mg/gたんぱく質とした。ビタミンB_6の推奨量は、EAR×1.2、つまり、0.023 mg/gたんぱく質とした。1日当たりの値は、対象年齢区分のたんぱく質の食事摂取基準の推奨量をかけて計算すればよい。

1-5. 葉酸

1) 背景

高ホモシステイン血症が、脳血管疾患や心疾患のリスクファクターとなる[20]。葉酸はホモシステインからメチオニンの変換に必要なビタミンである。このため、葉酸の摂取量が必要量を

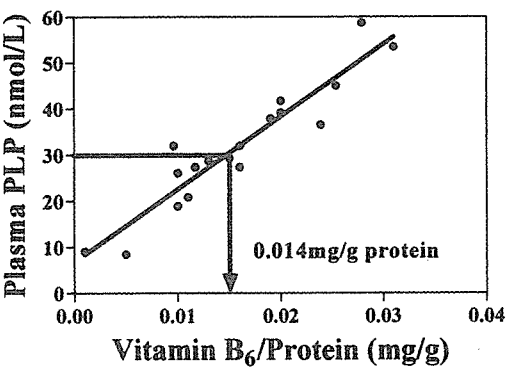

図4　ピリドキシン摂取量と血漿PLP濃度の関係

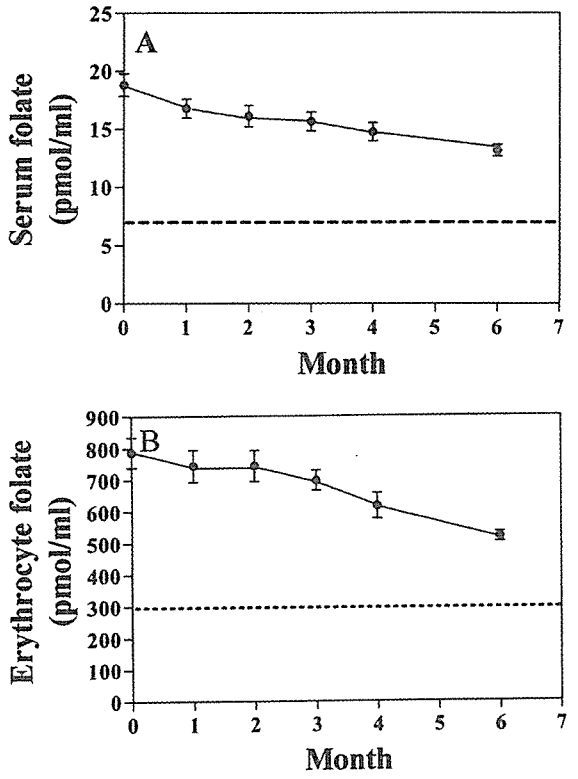

図5　約200μg/日の葉酸を含む食事を投与した時の血清中（A）と赤血球中(B)の葉酸濃度の変動

満たさないと、血漿中のホモシステイン濃度の上昇がみられる。

2) 血液中の葉酸値とホモシステイン値から推定平均必要量を策定

血清ホモシステイン値を14μmol/L未満[21]、血清葉酸値を7 nmol/L以上[22]、赤血球葉酸値を300 nmol/L以上[23]に50%の成人（18～49歳）が維持できる食事由来の葉酸摂取量から、推定平均必要量，200μg/日を求めた（図5）[24]。推奨量は、推定平均必要量×1.2としたので、240μg/日となる。

1-6. ビタミンB_{12}

1) 背景

ビタミンB_{12}が欠乏すると、赤血球のMCV（mean corpuscular volume＝平均血球体積）が大きくなり、MCH（mean corpuscular hemoglobin＝平均血球ヘモグロビン量）が高くなる。

2) 赤血球の性状維持と血清中のビタミンB_{12}濃度から推定平均必要量を策定

ビタミンB_{12}の必要量は、悪性貧血患者にさまざまな量のビタミンB_{12}を筋肉内注射し、彼らの血液学的性状および血清ビタミンB_{12}濃度を適正に維持するために必要な量を基にして算定した。20人の悪性貧血患者を対象とした研究によると、0.5μgから4.0μgまで投与量を変化させた結果、赤血球産生能は、投与量とともに増加し、およそ1.4μg/日で半数の患者が最大に到達した[25]。また、0.8～1.0μg/日の投与で患者の半数の血液学的性状および血清ビタミンB_{12}濃度が適正に保つことができ、1.7μg/日の投与では全患者の血液学的性状および血清ビタミンB_{12}濃度が適正に保つことができたとする報告[26,27]がある。一方、0.1μg/日、0.6～0.7μg/日では悪性貧血を治療するのに十分ではなかったとする報告[28]がある。これら一連の研究結果から、1.5μg/日程度がビタミンB_{12}の必要量と考えられる。ところで、悪性貧血患者では、胆汁中に含まれる多量のビタミンB_{12}を再吸収することができないため、その損失量（0.5μg/日）を差し引くことによって、正常な腸管吸収能力を有する健康な成人における必要量に換算することができる。すなわち、1.5－0.5＝1.0μg/日となる。

ビタミンB_{12}の吸収率は、食品中のビタミンB_{12}の含有量に左右される。つまり、1μg、5μg、25μgと摂取量を変えた場合の吸収率はそれぞれ、50%、20%、5%であったとの報告がある[29]。また、単一の食品を摂取させた場合の吸収率は、羊肉、卵、鶏肉、鱒肉で、それぞれ、65%、24～36%、60%、25～47%であったとの報告がある[30-33]。これらの報告から生体利用率を50%とした。したがって、推定平均必要量を2.0μg/日とした。推奨量はEAR×1.2から求め、2.4μg/日とした。

1-7. ビオチン

1) 背景

推定平均必要量を設定するに足る実験データはない。

2) 健康人の摂取量から目安量を策定

トータルダイエット調査による1日当たりのビオチン摂取量、45.1μgというデータを採用した[34]。この値を平滑化して、成人（18～69歳）の目安量を45μg/日とした。

1-8. パントテン酸

1) 背景

パントテン酸の欠乏症を実験的に再現できない[35]ため推定平均必要量を設定できない。そこで、食事調査の値[36]を用いた。

2) 健康人の摂取量から目安量を策定

Kimuraら[37]が調べた女子学生（34名、20～21歳）の結果では、平均値±SDが4.63±1.36 mg/日であった。また、平成13年国民栄養調査結果[36]における1歳～69歳のパントテン酸摂取量は、概ね4～6 mg/日の摂取量であった。この摂取量で欠乏が出たという報告はないので、パントテン酸の目安量を5 mg/日とした。

1-9. ビタミンC（アスコルビン酸）

1) 背景

ビタミンCの欠乏症は壊血病であり、その予防のためには、ビタミンCを10 mg/日程度摂取しておればよい[38]。しかしながら、さらに多く摂取すると抗酸化、心臓血管系の疾病予防が期待できることが明らかとなってきた。そこで、心臓血管系の疾病予防果を期待できる摂取量を設定した。

2) 血清中のアスコルビン酸値から推定平均必要量を策定

心臓血管系の疾病予防が期待できる血漿ビタミンC濃度は約50 μmol/L以上と報告されている[39-42]。ビタミンCの摂取量と血漿濃度を調べたメタ分析の報告から50%の人が50 μmol/Lの血漿濃度を維持する摂取量は約83 mg/日と算出されている[43]。そこで、成人（18～29歳）の推定平均必要量を85 mg/日とした。推奨量は、推定平均必要量×1.2から100 mg/日とした。

2. 脂溶性ビタミン

2-1. ビタミンA（レチノール）

1) 背景

ビタミンAの典型的な欠乏症として夜盲症がある。血漿レチノール濃度は肝臓のビタミンA貯蔵量が肝臓1 gあたり20 μgに低下するまで血漿レチノール濃度の低下は見られない[44]。

2) 肝臓内ビタミンA貯蔵量（20 μg/g）を維持できる摂取量から推定平均必要量を策定

成人が4か月にわたってビタミンAの摂取低下があった場合でもビタミンA欠乏症状に陥ることのない肝臓内ビタミンA貯蔵量は、肝臓1 g当たりで20 μgである[45]。この貯蔵量を維持するために必要なビタミンA摂取量を推定平均必要量とした。

そこで、このビタミンA蓄積量を維持するために必要なビタミンA摂取量を計算すると、体内のビタミンA消失率0.5%/日[45]、体重あたりの肝臓重量30 g/kg[45]、ビタミンA蓄積量の体全体と肝臓の比1.1、および摂取するビタミンAの蓄積効率を40%[46]と見積もることにより、0.005×20 μg/g×30 g/kg×1.1×2.5 (100/40)＝8.25 μg RAE/kg体重/日と算定される。

日本人成人男子の基準体重から求めると、530 μgRE/日となる。推奨量はEAR×1.4から750 μgRE/日となる。

3) レチノール当量（RE）

プロビタミンAとして体内でビタミンA作用を発揮するのは、β-カロテン、α-カロテン、クリプトキサンチンなどカロテノイドの一部である。

β-カロテンは中央開裂により2分子のビタミンA（レチナール）を生成する。他のプロビタミンAカロテノイドのプロビタミンA活性はβ-カロテンの約50%である。レチノールの吸収率は70～90%である[47]。食品素材によってβ-カロテンの吸収率は10%以下から60%まで大きく異なる[48]。通常の食事として摂取する野菜に由来するβ-カロテンの吸収率は、精製β-カロテンを油に溶かしたβ-カロテンサプリメントを摂取した場合と比べると1/7である[49]。果物やカボチャなどのようにβ-カロテンの吸収率の比較的高い（約50%[50]）食品の摂取頻度がわが国では8～11%であること[51]を考慮に加えると、日本人の食事由来のβ-カロテンの吸収率は、油に可溶化したβ-カロテンの1/6と推定される。したがって、β-カロテンからレチノールへの転換効率を50%と見積もり、食品由来β-カロテン12 μgのビタミンA活性は1 μgのレチノールと等価〔レチノール活性当量RAE（retinol activity equivalents）[18]〕であるとして換算するのが適当である。食品由来の場合、

1 μg RAE
＝1 μg レチノール
＝12 μg β-カロテン
＝24 μg α-カロテン

表4　健康な日本人を対象として血漿中α-トコフェロール濃度と摂取量（平均値±SD）を測定した場合

参考文献	性別	人数（人）	年齢（歳）	血漿濃度（μmol/L）	摂取量（mg/日）	国民栄養調査[1] 年齢階級（歳）	国民栄養調査[1] 摂取量（mg/日）
A)	男性	42	31～58	25.4±5.6	11.1±4.9	30～49	9.1±4.3
	女性	44	24～67	31.8±10.5	9.5±3.9	30～49	8.2±3.7
B)	女性	150	21～22	32.0±10.5	7.0±2.4[2]	18～29	8.2±4.0
C)	女性	10	21.6±0.8	22.2±2.2	7.1±2.0[3]		
		11	21.2±0.8	26.3±4.2	6.2±2.4[3]		
		10	21.0±0.7	28.5±3.6	5.6±2.0[3]		

1参考値として、平成13年国民栄養調査における類似した年齢階級における摂取量を示した。
2トコフェロール含量。
3トコフェロール、トコフェロール摂取量(mg/kg)と平均体重（kg）から算出した。
A) Sasaki S, Ushio F, Amano K, et al.: Serum biomarker-based validation of a self-administrered diet history questionnaire for Japanese subjects. J. Nutr. Sci. Vitaminol., 46, 285-296, 2000
B) Hiraoka N: Nutritional status of vitamin A, E, C, B1, B2, B6, nicotinic acid, B12, folate, and beta-carotene in young women. J. Nutr. Sci. Vitaminol., 47, 20-27, 2001
C) Maruyama C, Imamura K. Oshima S, et al.: Effects of tomato juice consumption on plasma and oxidative modification. J. Nutr. Sci. Vitaminol., 47, 213-221, 2001

＝24μg β-クリプトキサンチン
として換算できる。

なお、サプリメントとして摂取する油溶化β-カロテンは2μgで1μgのレチノールに相当する[44]。

2-2. ビタミンE

1) 背景

ビタミンEの欠乏症の一つに溶血性貧血がある。そこで、必要量は、血中α-トコフェロール値と過酸化水素による赤血球溶血試験結果を参考にして求めた。その結果、血中α-トコフェロール値が6～12μmol/Lの範囲にある場合には、過酸化水素による溶血反応が上昇するが[52]、14μmol/Lあれば過酸化水素による溶血反応を防止できる[53]ことを認めている。このことから、米国/カナダの食事摂取基準[18]では50%のヒトに過酸化水素による溶血を防止できる血中α-トコフェロール濃度として、12μmol/Lが採用され、この濃度を維持できる摂取量として12 mg/日という数値を推定平均必要量として策定している。しかしながら、日本人を対象としたα-トコフェロール摂取量と血中α-トコフェロール濃度を測定した報告は、異なっていた（表4）。

2) 血中α-トコフェロール値と過酸化水素による赤血球溶血試験結果を参考にして目安量を策定

表4に示したように、日本人の平均摂取量は5.6～11.1mg/日であるにもかかわらず、血漿中の濃度の平均値は22μmol/L以上に保たれていた。そして、これらの値は、平成13年国民栄養調査[35]に近いものであった。つまり、日本人では、米国/カナダの食事摂取基準で採用された報告よりも低いα-トコフェロール摂取量で、過酸化水素による溶血を防止できる血漿中のα-トコフェロール濃度が達成されていた。言い換えれば、現在の日本人の摂取量（中央値）であれば、血漿中のα-トコフェロール濃度は12μmol/L以上に保つことができるものと判断した。したがって、平成13年国民栄養調査[35]における性・年齢階級別の摂取量中央値を目安量とした。

2-3. ビタミンD

1) 背景

欠乏症として、小児に現れるくる病がある。成人では、骨折と骨粗鬆症がある。

2) 血清中の25-ヒドロキシビタミンD値を参考にして目安量を策定

小児ではくる病の発症リスクが低いと考えられる血漿中25-ヒドロキシビタミンD濃度を維持できるビタミンD摂取量を目安量とした。血漿中25-ヒドロキシビタミンD濃度は、皮膚で産生

されたビタミンD量あるいは食物から摂取されたビタミンD量を正確に反映して変動する[54]。したがって、ビタミンD摂取量と血漿中25-ヒドロキシビタミンD濃度との関係に関する観察疫学的な報告はあるが、一貫性に乏しい。したがって、現在の日本人の摂取量中央値を用いて[35]、1～17歳までは策定した。

成人では、骨折と骨粗鬆症のリスク低減を指標として、血漿中副甲状腺ホルモン濃度が上昇しない血漿中25-ヒドロキシビタミンD濃度を維持できるビタミンD摂取量を目安量とした。血中25-ヒドロキシビタミンD濃度の平均値が25 nmol/L以上50 nmol/L未満に保つに必要な摂取量は、30～49歳では4.1μg/日、50～69歳では6.3μg/日である[55-60]。したがって、(4.1＋6.3)÷2＝5.2μg/日となる。この値、5μg/日を成人の目安量とした。

2-4. ビタミンK（フィロキノン）

1) 背景

ビタミンKと総称する場合、フィロキノン、メナキノン-4およびメナキノン-7をいい、血液凝固因子としてのこれらの生物活性はほぼ同等と考えられている。ビタミンKの主な生理作用は、プロトロンビンやその他の血液凝固因子を活性化し、血液の凝固を促進することにある[61]。また、骨に存在するたんぱく質オステオカルシンを活性化し、骨形成を促すことも重要な生理作用である[62]。さらに、ビタミンK依存性蛋白質MGP（Matrix Gla Protein）の活性化を介して動脈の石灰化を防止することも重要な生理作用の一つである[63]。しかし、人でビタミンK欠乏症が明確に認められているのは、現在のところ血液凝固に関してのみである。そこで、正常な血液凝固能を維持するに必要なビタミンK摂取量を基準としてビタミンKの食事摂取基準を策定した。

2) 正常な血液凝固能を維持するに必要な量から目安量を策定

血中フィロキノン濃度の低下や血中非カルボキシル化プロトロンビンの上昇が起こらないビタミンK摂取量を求め、これを目安量とする。潜在的欠乏状態を回避できる摂取量として80μg/日（成人、体重72 kg, アメリカ/カナダの食事摂取基準[18]）を採用した。体重比の0.75乗で外挿することによって日本人成人の目安量を算出する。成人男子では75μg/日となる。

3) ビタミンK量

ビタミンK同族体の相対的な生理活性に関する科学的根拠は極めて乏しい。今回は、五訂日本食品標準成分表との整合性を重視してフィロキノンとメナキノン-4の合計量をビタミンK量として食事摂取基準を策定した。ただし、納豆などの発酵食品には栄養上無視できない量のメナキノン-7が含まれているので、このような食品についてはメナキノン-7の分子量（649）から換算し求めたメナキノン-4（分子量444.65）相当量を加算してビタミンK量とする。

表5　母乳中のビタミン含量

ビタミン名	採用値
ビタミンB_1	0.15 mg/L
ビタミンB_2	0.40 mg/L
ナイアシン	2.0 mg/L
ビタミンB_6	0.25 mg/L
葉酸	54 μg/L
ビタミンB_{12}	0.2 μg/L
パントテン酸	5.0 mg/L
ビオチン	5.2 μg/L
ビタミンC	50 mg/L
ビタミンA	0.352 mg/L
ビタミンE	3.5 mg/L
ビタミンD	3 μg/L
ビタミンK	5.17 μg/L

II. 乳児（0～5か月と6～11か月）の食事摂取基準の策定方法と策定に用いられた数値

1. 0～5か月

乳児（0～5か月）は、母乳を適当量摂取している限り、健常に発育する。したがって、食事摂取基準は目安量（AI）とした。目安量は母乳中のビタミン含量×泌乳量から計算した。表5に採用された値をまとめて示した。

2. 6～11か月

乳児（6～11か月）の食事摂取基準は目安量（AI）とした。基本的に、①乳児（0～5か月）からの外挿、乳児（0～5か月）の目安量×{(6～11か月の体重)/(0～5か月の体重)}$^{0.75}$と②成人（18～29歳）からの外挿、成人（18～29歳）

のデータ×{(6～11か月の体重)/(成人(18～29歳)の体重)}$^{0.75}$×1.3、の二つの値の平均値として策定した。

Ⅲ. 70歳以上の食事摂取基準の策定方法と策定に用いられた数値

身体活動は15～29歳をピークにして、それ以降の年齢では漸減する。しかし、加齢に伴う消化吸収率の低下などを考慮して、基本的に15～29歳の値と同じとした。

Ⅳ. 妊婦の食事摂取基準の策定方法と策定に用いられた数値

ビタミンの代謝特性を考慮して付加量を策定した。

Ⅴ. 授乳婦の食事摂取基準の策定方法と策定に用いられた数値

基本的に母乳中のビタミン含量と1日当たりの泌乳量から策定した。泌乳量は授乳量と同じであるとし、0.78 Lを採用した[64]。

文献

1) Melnick D, Hochberg M, Oser BL: Physiological availability of the vitamins. I. The human technic. J. Nutr., 30: 67-79, 1945

2) Shibata K, Matsuo H: Effect of gradually increasing levels of nicotinamide in a niacin-free and tryptophan-limited diet on the blood NAD levels and the urinary excretion of nicotinamide metabolites in rats. Agric. Biol. Chem., 53: 1333-1336, 1989

3) Tanphaichitr V: Thiamine, edited by Rucker RB, Suttie JW, McCormick DB, Machlin LJ: "Handbook of Vitamins Third edition" Marcel Dekker, Inc. New York, USA, 275-316, (2001)

4) Matsuo T, Suzuoki Z: The occurrence of 4-methylthiazole-5-acetic acid as a thiamine metabolites in rabbit, dog, man and rat. J. Biochem., 65: 953-960, 1969

5) Amos WH, Neal RA: Isolation and identification of 3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid (thiamine acetic acid) and 2-methyl-4-amino-5-formylaminomethylpyrimidine as metabolites of thiamine in the rat. J. Biol. Chem., 245: 5643-5648, 1970

6) World Health Organization Technical Report Series No. 362. FAO Nutrition Meeting Report Series No. 41. Requirements of Vitamin A, Thiamine, Riboflavin and Niacin. Reports of a Joint FAO/WHO Expert Group. Rome, Italy, 6-17 September 1965. pp.30-38, Published by FAO and WHO. World Health Organization, Geneva. (1967)

7) Ohkawa H, Ohishi N, Yagi K: New metabolites of riboflavin appear in human urine. J. Biol. Chem., 258: 5623-5628, 1983

8) World Health Organization Technical Report Series No. 362. FAO Nutrition Meeting Report Series No. 41. Requirements of Vitamin A, Thiamine, Riboflavin and Niacin. Reports of a Joint FAO/WHO Expert Group. Rome, Italy, 6-17 September 1965. pp.39-44 Published by FAO and WHO. World Health Organization, Geneva. (1967)

9) Shibata K, Matsuo H: Correlation between niacin equivalent intake and urinary excretion of its metabolites, N^1-methylnicotinamide, N^1-methyl-2-pyridone-5-carboxamide, and N^1-methyl-4-pyridone-3-carboxamide, in humans consuming a self-selected food. Am. J. Clin. Nutr., 50: 114-119, 1989

10) Horwitt MK, Harper AE, Henderson LM: Niacin-tryptophan relationships for evaluating niacin equivalent. Am. J.Clin. Nutr., 34: 423-427, 1981

11) Goldsimith GA, Sarett HP, Register UD, Gibbens J: Studies on niacin requirement in man. Ⅰ. Experimental pellagra in subjects on corn diets low in niacin and tryptophan. J. Clin. Invest., 31: 533-542, 1952

12) Goldsimth GA, Rosenthal HL, Bibbens J, Unglaub WG: Studies on niacin requirement in man. Ⅱ. Requirement on wheat and corn diets low in tryptophan. J. Nutr., 56: 371-386, 1955

13) Horwitt, MK, Harvey, CC, Rothwell WS, Cutler JL, Haffron D: Tryptophan-niacin relationships in man: Studies with diets deficient in riboflavin and niacin, together with observations on the excretion of nitrogen and niacin metabolites. J. Nutr., 60: 1-43, 1956

14) Fukuwatari T, Ohta M, Kimura N, Sasaki R, Shibata K: Conversion ratio of tryptophan to niacin in Japanese women fed on a purified diet conforming to the Japanese Dietary Reference Intakes. J. Nutr. Sci. Vitaminol., 50: 385-391, 2004

15) Lui A, Lumeng L, Aronoff GR, et al.: Relationship between body store of vitamin B_6 and plasma pyridoxal-P clearance: Metabolic balance studies in humans. J. Lab. Clin. Med., 106: 491-497, 1985

16) Kretsch MJ, Sauberlich HE, Newbrun E: Electroencephalographic changes and periodontal status during short-term vitamin B_6 depletion of young, nonpregnant women. Am. J. Clin. Nutr., 53: 1266-1274, 1991

17) Leklem JE: Vitamin B_6: A status report. J. Nutr., 120: 1503-157, 1990

18) Institute of Medicine. The B vitamins and choline: overview and methods. In: Institute of Medicine. Dietary

Reference Intakes: For thiamine, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic Acid, biotin, and choline. Washington, D.C., National Academy Press, 150-195, (1998)

19) Tarr JB, Tamura T, Stokstad EL: Availability of vitamin B6 and pantothenate in an average American diet in man. Am. J. Clin. Nutr., 34: 1328-1337, 1981

20) Gerhard GT, Duell PB: Homocysteine and atherosclerosis. Curr. Poin. Lipidol., 10: 417-428, 1999

21) Herbert V: Making sense of laboratories tests of folate status: Folate requirements to sustain normality. Am. J. Hem., 26: 199-207, 1987

22) Herbert V: Experimental nutritional folate deficiency in man. Trans. Assoc. Am. Physicians., 75: 307-320, 1962

23) Selhub J, Jacques PF, Wilson PWF, et al.: Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. J. Am. Med. Assoc., 270: 2693-2698, 1993

24) Milne DB, Johnson LK. Mahalko JR, Sandstead HH: Folate status of adult males living in a metabolic unit: possible relationships with iron nutriture. Am. J. Clin. Nutr., 37: 768-773, 1983

25) Darby WJ, Bridgforth EB, LE Brocquy J, et al.: Vitamin B_{12} requirement of adult man. Am. J. Med., 25: 726-732, 1958

26) Bastrup-Madsen P, Helleberg-Rasmussen I, Norregaard S, et al.: Long term therapy of pernicious anaemia with the depot cobalamin preparation betolvex. Scand. J Haemato., 31: 57-62, 1983

27) Lindenbaum J, Savage DG, Stabler SP, et al.: Diagnosis of cobalamin deficiency: Ⅱ. Relative sensitivities of serum cobalamin, methylmalonic acid, and total homocysteine concentrations. Am. J. Hematol., 34: 99-107, 1990

28) Sullivan LW, Herbert V: Studies on the minimum daily requirement for vitamin B_{12}. Hematopoietic responses to 0.1 microgram of cyanocobalamin or coenzyme B_{12} and comparison of their relative potency. N. Engl. J. Med., 272: 340-346, 1965

29) Adams JF, Ross SK, Mervyn L, et al.: Absorption of cyanocobalamin, coenzyme B_{12}, methylcobalamin, and hydroxocobalamin at different dose levels. Scand. J. Gastroenterol., 6: 249-252, 1971

30) Heyssel RM, Bozian RC, Darby WJ, et al.: Vitamin B_{12} turnover in man. The assimilation of vitamin B_{12} from natural foodstuff by man and estimates of minimal daily dietary requirements. Am. J. Clin. Nutr., 18: 176-184, 1966

31) Doscherholmen A, McMahon J, Ripley D: Vitamin B_{12} absorption from eggs. Proc. Soc. Exp. Biol. Med., 149: 987-990, 1975

32) Doscherholmen A, McMahon J, Ripley D: Vitamin B_{12} assimilation from chicken meat. Am. J. Clin. Nutr., 31: 825-830, 1978

33) Doscherholmen A, McMahon J, Economon P: Vitamin B_{12} absorption from fish. Proc. Soc. Exp. Biol. Med., 167: 480-484, 1981

34) 齋藤由紀, 牛尾房雄: トータルダイエット調査による東京都民のビオチン、ビタミンB_6、ナイアシンの一日摂取量の推定. 栄養学雑誌, 62: 165-169, 2004

35) Fry P C, Fox HM., Tao HG: Metabolic response to a pantothenic acid deficient diet in humans. J. Nutr. Sci. Vitaminol., 22: 339-346, 1976

36) 厚生労働省　国民栄養の現状　平成13年国民栄養調査結果, 東京, (2003)

37) Kimura N, Fukuwatari T, Sasaki R, Hayakawa F, Shibata K: Vitamin intakes in Japanese college women students. J. Nutr. Sci. Vitaminol., 49: 149-155, 2003

38) Hodges RE, Hood J, Canham JE, et al.: Clinical manifestations of ascorbic acid deficiency in man. Am. J. Clin. Nutr., 24: 432-443, 1971

39) Byun J, Mueller DM, Fabjan JS, et al.: Nitrogen dioxide radical generated by the myeloperoxidase-hydrogen peroxide-nitrite system promotes lipid peroxidation of low density lipoprotein. FEBS Lett., 455: 243-246, 1999

40) Frei B: Vitamin C as antiatherogen: Mechanism of action. In: Packer L, Fuchs J. Eds. Vitamin C in health and disease. Marcel Dekker Inc. New York. pp. 163-182, (1997)

41) Gey KF: Vitamins E plus C and interacting conutrients required for optimal health. A critical and constructive review of epidemiology and supplementation data regarding cardiovascular disease and cancer. Biofactors, 7: 113-174, 1998

42) Yokoyama T, Date C, Koubo Y, et al.: Serum vitamin C concentration was inversely associated with subsequent 20-year incidence of stroke in a Japanese rural community. Stroke, 31: 2287-2294, 2000

43) Brubacher D, Moser U, Jordan P: Vitamin C concentrations in plasma as a function of intake: meta-analysis. Int. J .Vitam. Nutr. Res., 70: 226-237, 2000

44) Sauberlich HE, Hodges RE, Wallace DL, et al.: Vitamin A metabolism and requirements in the human studied with the use of labeled retinol. Vitam. Horm., 32: 251-275, 1974

45) Olson JA: Recommended dietary intakes (RDI) of vitamin A in humans. Am. J. Clin. Nutr., 45: 704-716, 1987

46) Haskell MJ, Handelman GJ, Peerson JM, et al.: Assessment of vitamin A status by the deuterated-retinol-dilution technique and comparison with hepatic vitamin A concentration in Bangladeshi surgical patients. Am. J. Clin. Nutr., 66: 67-74, 1997
47) Sivakumar B, Reddy V: Absorption of labeled vitamin A in children during infection. Br. J. Nutr., 27: 299-304, 1972
48) Castenmiller JJ, West CE: Bioavailability and bioconversion of carotenoids. Ann. Rev. Nutr., 18: 19-38, 1998
49) Van her Hof KH, Brouwer IA, West CE, et al.: Bioavailability of lutein from vegetables is five times higher than that of beta-carotene. Am. J. Clin. Nutr., 70: 261-268, 1999
50) de Pee S, West CE, Permaesih D, et al.: Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and beta-carotene in schoolchildren in Indonesia. Am. J. Clin. Nutr., 68: 1058-1067, 1988
51) Kobayashi M, Sasaki S, Tsugane S: Validity of a self-administered food frequency questionnaire used in the 5-year follow-up survey of the JPHC study cohort I to assess carotenoids and vitamin C intake: comparison with dietary records and blood level. J. Epidemiol., 13: S82-S91, 2003
52) Horwitt MK, et al.: Erythrocyte survival time and reticulocyte levels after tocopherol depletion in man. Am. J. Clin. Nutr., 12: 99-106, 1963
53) Farrel PM, et al.: The occurrence and effects of human vitamin E deficiency. A study in patients with cystic fibrosis. J. Clin. Invest., 60: 233-244, 1977
54) Brustad M, Alsaker E, Engelsen O, et al.: Vitamin D status of middle-aged women at 65-71 degrees N in relation to dietary intake and exposure to ultraviolet radiation. Public Health Nutr., 7: 327-335, 2004
55) Nakamura K, Nashimoto M, Tsuchiya Y. et al.: Vitamin D insufficiency in Japanese female college students: a preliminary report. Int. J. Vit. Nutr. Res., 71: 302-305, 2001
56) Nakamura K, Nashimoto M, Matsuyama S, et al.: Low serum concentrations of 25-hydroxtvitamin D in young adult Japanese women: a cross sectional study. Nutrition, 17: 921-925, 2001
57) Nakamura K, Nashimoto M, Hori Y, et al.: Serum 25-hydroxyvitamin D levels in active women of middle and advanced age in a rural community in Japan. Nutrition, 15: 870-873, 1999
58) Nakamura K, Nashimoto M,Yamamoto M: Summer/winter differences in the serum 25-hydroxyvitamin D3 and parathyroid hormone levels of Japanese women. Int. J. Biometeorol., 44: 186-189, 2000
59) Nakamura K, Nashimoto M, Hori Y, et al.: Serum 25-hydroxyvitamin D concentrations and related dietary factors in peri- and postmenopausal Japanese women. Am. J. Clin. Nutr., 71: 1161-1165, 2000
60) Nakamura K, Nashimoto M,Yamamoto: Are the serum 25-hydroxyvitamin D concentrations in winter associated with forearm bone mineral density in healthy elderly Japanese women? Int. J. Vit. Nutr. Res., 71: 25-29, 2001
61) Suttie JW: Synthesis of vitamin K-dependent proteins. FASEB J., 7: 445-452, 1993
62) Price PA: Role of vitamin K-dependent proteins in bone metabolism. Ann. Rev. Nutr., 8: 565-583, 1988
63) Ferland G: The vitamin K-dependent proteins: An update. Nutr. Rev., 56: 223-30, 1998
64) 鈴木久美子, 佐々木晶子, 新澤佳代ら: 離乳前乳児の哺乳量に関する研究. 栄養学雑誌, 62: 369-372, 2004

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Note

Characterization of a Corrinoid Compound in the Edible (Blue-Green) Alga, Suizenji-nori

Fumio WATANABE,[1,†] Emi MIYAMOTO,[2] Tomoyuki FUJITA,[3] Yuri TANIOKA,[4] and Yoshihisa NAKANO[4]

[1]*School of Agricultural, Biological and Environmental Sciences, Tottori University, Tottori 680-8553, Japan*
[2]*Department of Health and Nutrition, Nagasaki International University, Sasebo 859-3298, Japan*
[3]*Graduate School of Agriculture, Shinshu University, Kami-ina 399-4598, Japan*
[4]*Graduate School of Life and Environmental Science, Osaka Prefecture University, Sakai 599-8531, Japan*

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The edible blue-green alga (cyanobacterium), Suizenji-nori, contained 143.8 ± 22.4 μg of vitamin B_{12} per 100 g dry weight of the alga (mean ± SE, $n = 4$). A corrinoid compound was purified from the dried Suizenji-nori, and partially characterized. The silica gel 60 TLC and reversed-phase HPLC patterns of the purified corrinoid compound were not identical to those of true vitamin B_{12}, but to those of pseudovitamin B_{12} which is inactive for humans.

Key words: *Aphanothece sacrum*; blue-green alga; pseudovitamin B_{12}; Suizenji-nori; vitamin B_{12}

The usual dietary sources of vitamin B_{12} (B_{12}) are animal food products (meat, milk, eggs and shellfish), but not plant food products.[1)] Substantial amounts of B_{12}, however, can be found in various edible algae.[2,3)] Our previous studies have demonstrated that true B_{12} was the predominant cobamide of some eukaryotic algae,[4–7)] although pseudo-B_{12}, an inactive corrinoid for humans, predominates in the prokaryotic, blue-green alga (cyanobacterium), *spirulina*.[8)] It is still unclear whether other edible blue-green algae contain true B_{12} or an inactive corrinoid compound.

Suizenji-nori (*Aphanothece sacrum*) is an edible blue-green alga indigenous to Japan. The dried alga is used as an ordinary food item after being soaked in water as well as a nutritional supplementary food. The nutrition labeling of the algal product shows that the dried algal cells contain substantial amounts of B_{12}. In this study, we purified and characterized a corrinoid compound from suizenji-nori and demonstrate the bioavailability of the algal corrinoids in humans.

After 2 g of the dried suizenji-nori had been suspended in 40 ml of distilled water and homogenized with a UD-200 ultrasonic disruptor (Tomy, Tokyo, Japan), total B_{12} was extracted from the suspension while boiling in the acidic pH range and assayed by the microbiological method with *Lactobacillus delbrueckii* ATCC 7830 as described in the Japanese Standard Tables of Food Composition.[9)]

About 600 g of the dried suizenji-nori was added to 40 liters of a 50 mM acetate buffer at pH 4.8. Total B_{12} was extracted from the suspension by boiling with KCN in the acidic pH range;[9)] KCN was added to the suspension to a final concentration of 10 mM. The suspension was boiled for 30 min at 98 °C in a draught chamber. The boiled suspension was left for several hours up to 30 °C, and then centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant fraction (about 20 liters) was put into a column (7 × 100 cm) of Amberlite XAD-4 resin (Japan Organo Co., Tokyo, Japan), which had been washed with 10 liters of methanol and then equilibrated with distilled water, at room temperature in the dark. The column was washed with 10 liters of distilled water, and eluted with 10 liters of 80% (v/v) ethanol. The eluate was pooled, evaporated to dryness under reduced pressure, and dissolved in 100 ml of distilled water. The solution was loaded into a column (2.4 × 30 cm) of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan), which had been washed with a 75% (v/v) ethanol solution and then equilibrated with distilled water, and eluted with 400 ml of a linear gradient (0–30% v/v) of ethanol. The B_{12}-active fractions were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The solution was further purified by the Cosmosil column (2.4 × 15 cm) chromatography under the same conditions, except for eluting with 200 ml of a linear gradient (0–25% v/v) of ethanol. The B_{12}-active fractions were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was put on to a silica gel 60 TLC sheet (Merck, Darmstadt, Germany) and developed with 2-propanol/

† To whom correspondence should be addressed. Tel/Fax: +81-857-31-5412; E-mail: watanabe@muses.tottori-u.ac.jp

NH_4OH (28%)/water (7:1:2 v/v) as a solvent in the dark at room temperature. The red-colored spot on the TLC sheet was dried, collected, extracted with an 80% (v/v) methanol solution, evaporated to dryness under reduced pressure, and dissolved in 50 μl of distilled water. The solution was further purified by TLC under the same conditions. The concentrated solution was purified by HPLC with Shimadzu (Kyoto, Japan) apparatus (two LC-10ADvp pumps, a DGV-12A degasser, SCL-10Avp system controller, SPD-10Avvp ultraviolet-visible detector, CTO-10Avp column oven, 100-μl sample loop, and C-R6A chromatopac integrator). The sample (50 μl) was loaded into a reversed-phase HPLC column (Wakosil-II 5C18RS, ϕ4.6 × 150 mm; 5-μm particle size; Wako Pure Chemical Industries, Osaka, Japan) which had been equilibrated with a 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C. The flow rate was 1 ml/min. The corrinoid compound was isocratically eluted with the same solution, monitored by measuring the absorbance at 361 nm, and collected in 1-ml fractions. The final red-colored fraction was collected, evaporated to dryness under reduced pressure, dissolved in 20 μl of distilled water, and used as the purified corrinoid compound.

The B_{12} concentration in the edible (blue-green) alga, suizenji-nori, was determined by the microbiological method. The dried alga contained 143.8 ± 22.4 μg of B_{12} per 100 g dry weight of the alga (mean ± SE, n = 4). A similar B_{12} concentration (94 μg) has been shown in the nutrition labeling of the algal product.

To evaluate whether the B_{12} activity detected in the suizenji-nori extract was derived from true B_{12} nor not, a corrinoid compound was purified and partially characterized. The final purified preparation gave a single red-colored spot by silica gel 60 TLC and a single peak by reversed-phase HPLC, indicating that the corrinoid compound had been purified to homogeneity.

The ultraviolet-visible spectrum of the compound purified from the suizenji-nori showed a typical absorption of cobalt-containing corrinoid (Fig. 1); λ_{max} nm (absorbance) was at 548.0 (0.350), 518.0 (0.334), and 360.0 (1.366). The purified corrinoid compound, authentic B_{12}, and cyanocobamides (pseudo-B_{12} and 5-hydroxybenzimidazolyl, and benzimidazolyl cyanocobamides; all kindly provided by Dr. E. Stupperich, Ulm University, Germany) were compared by silica gel 60 TLC and reversed-phase HPLC (Table 1). The R_f values (0.14 and 0.48 in solvents I and II, respectively, by TLC) for the purified compound were identical to those for authentic pseudo-B_{12}, whose retention time (6.7 min by HPLC) was also identical to that of the purified compound. The authentic pseudo-B_{12} (identified by Dr. Stupperich) has been independently analyzed with ^{1}H-NMR spectroscopy by ourselves (unpublished data); the spectrum of the authentic compound was identical to that of the cited reference.[10] The authentic pseudo-B_{12} also gave identical patterns of TLC and HPLC to those of the main *Spirulina* corrinoid compound which has been identified as pseudo-B_{12}.[8] The TLC and HPLC patterns of the compound purified from suizenji-nori, pseudo-B_{12} purified from *Spirulina* tablets, and authentic pseudo-B_{12} are shown in Fig. 2. These results indicate that the red-colored compound purified from suizenji-nori was not true B_{12}, but pseudo-B_{12} that is inactive for humans. No further detailed information on the purified compound is available because only a small amount of the purified sample was obtained (for the NMR study).

These results indicate that suizenji-nori is not suitable for use as a B_{12} source, especially for vegetarians.

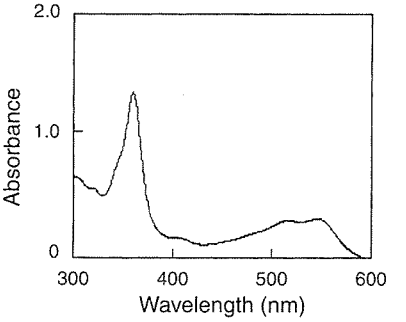

Fig. 1. Ultraviolet-Visible Spectrum of the Purified Compound from the Suizenji-nori.

A portion of the purified preparation was dissolved in 0.1 ml of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature, super-micro quartz cuvettes (0.1 ml, d = 1 cm) being used.

Table 1. R_f Values and Retention Times for the Purified Compound from Suizenji-nori, Authentic B_{12}, and Cyanocobamides on TLC and HPLC

Concentrated solutions (2 μl each) of the compound purified from suizenji-nori and cyanocobamides were spotted on silica gel 60 TLC sheets and developed with 1-butanol/2-propanol/water (10:7:10 v/v) and 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as solvents I and II, respectively, in the dark at room temperature.

In the case of HPLC, diluted solutions (10 μl each) of the purified compound and the cyanocobamides were analyzed in a reversed-phase HPLC column (Wakosil-II 5C18RS) under the same conditions as those described in the text.

	TLC (R_f value)		HPLC (retention time, min)
	Solvent I	Solvent II	
Purified compound	0.14	0.48	6.7
Vitamin B_{12}	0.24	0.61	7.9
Benzimidazolyl cyanocobamide	0.18	0.57	6.3
5-Hydroxybenzimidazolyl cyanocobamide	0.20	0.49	6.1
Pseudovitamin B_{12}	0.14	0.48	6.7

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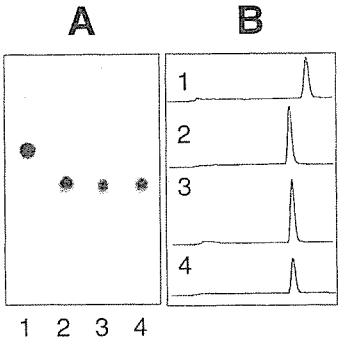

Fig. 2. TLC and HPLC Patterns of the Compound Purified from the Suizenji-nori, Pseudo-B_{12} Purified from *Spirulina* Tablets, and Authentic Pseudo-B_{12}.

In the TLC analysis (A), concentrated solutions (2 μl) of authentic B_{12} (1), authentic pseudo-B_{12} (2), pseudo-B_{12} purified from *Spirulina* tablets (3), and the purified compound from suizenji-nori (4) were spotted on silica gel 60 TLC sheets and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) in the dark at room temperature. In the case of HPLC (B), diluted solutions (10 μl) of authentic B_{12} (1), authentic pseudo-B_{12} (2), pseudo-B_{12} purified from *Spirulina* tablets (3), and the purified compound from suizenji-nori (4) were analyzed in a reversed-phase HPLC column (Wakosil-II 5C18RS) under the same conditions as those described in the text.

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References

1) Ball, G. F. M., Vitamin B_{12}. In "Bioavailability and Analysis of Vitamins in Foods," Chapman & Hall, London, pp. 497–515 (1998).
2) Watanabe, F., Takenaka, S., Katsura, H., Mazumder, S. A. M. S. H., Abe, K., Tamura, Y., and Nakano, Y., Dried green and purple lavers (nori) contain substantial amounts of biologically active vitamin B_{12} but less of dietary iodine relative to other edible seaweeds. *J. Agric. Food Chem.*, **47**, 2341–2343 (1999).
3) Watanabe, F., Takenaka, S., Kittaka-Katsura, H., Ebara, S., and Miyamoto, E., Characterization and bioavailability of vitamin B_{12}-compounds from edible alage. *J. Nutr. Sci. Vitaminol.*, **48**, 325–331 (2002).
4) Watanabe, F., Takenaka, S., Katsura, H., Miyamoto, E., Abe, K., Tamura, Y., Nakatsuka, T., and Nakano, Y., Characterization of a vitamin B_{12} compound in the edible purple laver, *Porphyra yezoensis*. *Biosci. Biotechnol. Biochem.*, **64**, 2712–2715 (2000).
5) Watanabe, F., Katsura, H., Miyamoto, E., Takenaka, S., Abe, K., Yamasaki, Y., and Nakano, Y., Characterization of vitamin B_{12} in an edible green laver (*Entromopha prolifera*). *Appl. Biol. Sci.*, **5**, 99–107 (1999).
6) Kittaka-Katsura, H., Fujita, T., Watanabe, F., and Nakano, Y., Purification and characterization of a corrinoid-compound from *chlorella* tablets as an algal health food. *J. Agric. Food Chem.*, **50**, 4994–4997 (2002).
7) Miyamoto, E., Watanabe, F., Ebara, S., Takenaka, S., Takenaka, H., Yamaguchi, Y., Tanaka, N., Inui, H., and Nakano, Y., Characterization of a vitamin B_{12} compound from unicellular coccolithophorid alga (*Pleurochrysis carterae*). *J. Agric. Food Chem.*, **49**, 3486–3489 (2001).
8) Watanabe, F., Katsura, H., Takenaka, S., Fujita, T., Abe, K., Tamura, Y., Nakatsuka, T., and Nakano, Y., Pseudovitamin B_{12} is the predominant cobamide of an algal health food, spirulina tablets. *J. Agric. Food Chem.*, **47**, 4736–4741 (1999).
9) "Standard Tables of Food Composition in Japan—Vitamin K, B_6 and B_{12}," Resource Council, Science and Technology Agency, Tokyo, pp. 16–56 (1995).
10) Hoffmann, B., Obrhuber, M., Stupperich, E., Bothe, H., Buckel, W., Konrat, R., and Kräuter, R., Native corrinoids from *Clostridium cochlearium* are adeninyl-cobamides: spectroscopic analysis and identification of pseudovitamin B_{12} and factor A. *J. Bacteriol.*, **182**, 4773–4782 (2000).

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Purification and Characterization of a Corrinoid-Compound in an Edible Cyanobacterium *Aphanizomenon flos-aquae* as a Nutritional Supplementary Food

EMI MIYAMOTO,*,† YURI TANIOKA,‡ TOMOYUKI NAKAO,‡ FLORIN BARLA,‡ HIROSHI INUI,‡ TOMOYUKI FUJITA,§ FUMIO WATANABE,|| AND YOSHIHISA NAKANO‡

Department of Health and Nutrition, Nagasaki International University, Sasebo 859-3298, Japan, Department of Applied Biological Chemistry, Osaka Prefecture University, Sakai 599-5831, Japan, Graduate School of Agriculture, Shinsyu University, Kami-ina 399-4598, Japan, and School of Agricultural, Biological and Environmental Sciences, Tottori University, Tottori 680-8553, Japan

The vitamin B_{12} concentration of the dried cells of *Aphanizomenon flos-aquae* was determined by both microbiological method with *Lactobacillus delbrueckeii* ATCC7830 and chemiluminescence method with intrinsic factor. The *Aphanizomenon* cells contained 616.3 ± 30.3 μg ($n = 4$) of vitamin B_{12} per 100 g of the dried cells by the microbiological method. The values determined with the chemiluminescence method, however, were only about 5.3% of the values determined by the microbiological method. A corrinoid-compound was purified from the dried cells and characterized. The purified corrinoid-compound was identified as pseudovitamin B_{12} (an inactive corrinoid-compound for humans) by silica gel 60 TLC, C18 reversed-phase HPLC, ultraviolet−visible spectroscopy, and ^{1}H NMR spectroscopy. The results suggest that the *Aphanizomenon* cells are not suitable for use as a vitamin B_{12} source, especially in vegans.

KEYWORDS: ***Aphanizomenon flos-aquae*; cyanobacteria; nutritional supplementary food; pseudovitamin B_{12}; vitamin B_{12}**

INTRODUCTION

Strict vegetarians (vegans) have a greater risk of developing vitamin B_{12} deficiency relative to non-vegetarians because natural food sources of vitamin B_{12} are not plant food products, but animal food products (*1*). They must consume vitamin B_{12}-fortified foods or vitamin B_{12}-containing dietary supplements to prevent vitamin B_{12} deficiency. Plant foods, edible algae, and/or blue-green algae (cyanobacteria), however, contain substantial amounts of B_{12} (*2*, *3*). Our previous studies have demonstrated that true vitamin B_{12} is the predominant cobamide of many species of eukaryotic algae (*4*−*7*), although pseudovitamin B_{12}, an inactive corrinoid for humans, predominated in a cyanobacterium *Spirulina* (*8*). Substantial amounts of cyanobacteria, *Spirulina* (3000 t/year), *Nostoc* (600 t/year), and *Aphanizomenon* (500 t/year), are produced worldwide to meet the high demands of both food and pharmaceutical industries (*9*). It is still unclear whether the other edible cyanobacteria, which are used as nutritional supplementary foods, contain true vitamin B_{12} or the inactive corrinoid-compound.

* Author to whom correspondence should be addressed [telephone/fax +81-956-20-5530; e-mail miyamo@niu.ac.jp].
† Nagasaki International University.
‡ Osaka Prefecture University.
§ Shinsyu University.
|| Tottori University.

Aphanizomenon flos-aquae, a fresh water cyanobacterium, grow naturally in Upper Klamath Lake, OR. The bacterial cells contain various nutrients (polyunsaturated fatty acids, protein, carotenoids, vitamins, minerals, and so on) and also have therapeutic effects (*9*−*13*). Kay (*13*) has described that the bacterial cells contain some corrinoid-compounds that can be utilized as vitamin B_{12} in humans.

Thus, the dried *Aphanizomenon* cells (commercially available in a capsule form) are used as a vitamin B_{12}-rich nutritional supplementary food. The bacterial cells can contribute to human vitamin B_{12} needs, especially for vegans. There is, however, little information available on chemical properties of the corrinoid-compound in the *Aphanizomenon* cells.

In the present paper, we determine vitamin B_{12} concentration of the dried *Aphanizomenon* cells, which are used as a nutritional supplementary food by both microbiological method with *Lactobacillus delbrueckii* ATCC7830 and chemiluminescence method with intrinsic factor. We also describe the purification and characterization of corrinoid-compound from the bacterial cells to clarify whether the bacterial corrinoid-compound is true vitamin B_{12} or not.

MATERIALS AND METHODS

Materials. Vitamin B_{12} (cyanocobalamin) was obtained from Sigma (St. Louis, MO). Silica gel 60 TLC aluminum sheets were obtained

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Table 1. Vitamin B_{12} Concentrations of the Three Microalgae Commercially Available for Human Nutritional Supplementary Food (or Health Food)

	vitamin B_{12} concentration (μg/100 g of dry weight)			
	claim on bottle[a]	microbiological assay	chemiluminescence assay	refs
Chlorella	20–150	201.3–285.7	200.9–211.6	7
Spirulina	100–250	127.2–244.3	6.2–17.4	8
Aphanizomenon	800	616.3 ± 30.3[b]	32.3[c]	this study

[a] Determined by microbiological assay. [b] Values obtained represent mean ± SEM (n = 4). [c] Values obtained represent mean values (n = 2).

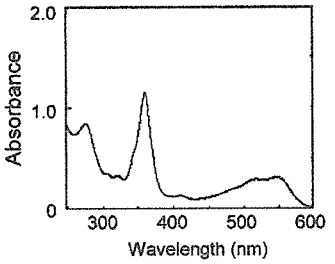

Figure 1. Ultraviolet–visible spectrum of the purified compound from the dried *Aphanizomenon* cells. A portion of the purified preparation was dissolved in 3.0 mL of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature, quartz cuvettes (3.0 mL, d = 1 cm) being used.

Table 2. R_f Values and Retention Times of the Purified Corrinoid-Compound from the Dried *Aphanizomenon* Cells, Authentic Vitamin B_{12}, and Pseudovitamin B_{12} on TLC and HPLC[a]

	TLC (R_f values)		
	solvent I	solvent II	reversed-phase HPLC (retention time, min)
purified compound	0.10	0.46	6.4
vitamin B_{12}	0.12	0.58	8.7
pseudovitamin B_{12}	0.10	0.46	6.4

[a] Concentrated solutions (2 μL) of the compound purified from the dried cells, vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were spotted on silica gel 60 TLC sheets and developed with 1-butanol/2-propanol/water (10:7:10 v/v) and 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as solvents I and II, respectively, in the dark at room temperature. In the care of HPLC, concentrated solutions (2 μL) of the purified compound from the dried cells, vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were analyzed in a reversed-phase HPLC column (Wakosil-II 5C18RS) under the same conditions described in the text.

from Merck (Darmstadt, Germany). A B_{12} assay medium for *Lactobacillus delbrueckii* (formerly *Lactobacillus leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Pseudovitamin B_{12} was kindly provided by Dr. E. Stupperich, Ulm University, Germany. A reversed-phase high-performance liquid chromatography (HPLC) column (Wakosil-II 5C18RS, ϕ4.6 × 150 mm; particle size, 5 μm) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The dried *Aphanizomenon* cells as a nutritional supplementary food were purchased from market in Japan.

Extraction of Corrinoid-Compound from the Dried *Aphanizomenon* Cells. One gram of the dried cells was added to 10 mL of 0.1 mol/L acetate buffer, pH 4.8. Corrinoid-compound was extracted from the cell suspension by the method of boiling with KCN at acidic pH; specifically 0.05% (w/v) of KCN was added to the cell suspension, which was boiled for 30 min at 98 °C in the dark. The extraction procedures were done in a Dalton (Tokyo, Japan) draft chamber. The boiled cell suspension was centrifuged at 10 000g for 10 min. The supernatant was used for vitamin B_{12} assay.

Assay of Total Vitamin B_{12}. The bacterial corrinoid-compound was assayed as vitamin B_{12} by the microbiological method with *L. delbrueckii* subsp. *lactis* ATCC 7830 and by the fully automated chemiluminescence B_{12} analyzer ACS 180 (Chiron Diagnostics, East Walpole, MA) according to the manufacturer's instructions as described previously (*14*). The extracts were diluted with distilled water up to a vitamin B_{12} concentration range of 10–100 ng/L and used as samples for the microbiological method. The turbidity (%T) of the test culture of *L. delbrueckii* ATCC7830 grown at 37 °C for 16–21 h was measured at 660 nm with the UV-1600 UV–visible spectrophotometer according to the manufacturer's recommended method.

Purification of a Corrinoid-Compound from the Dried *Aphanizomenon* Cells. About 550 g of the dried *Aphanizomenon* cells was added to 5.5 L of 0.1 mol/L acetate buffer, pH 4.8. Corrinoid-compound was extracted from the suspension by boiling with KCN at acidic pH; KCN was added to the suspension at the final concentration of 10 mmol/L. The suspension was boiled for 30 min at 98 °C in the dark. The extraction procedures were done in the Dalton draught chamber. The boiled suspension was centrifuged at 10 000g for 10 min. Corrinoid-compound remaining in the precipitate fraction was re-extracted under the same conditions. The combined supernatant fractions (about 8 L) were put on a column (5 × 100 cm) of Amberlite XAD-4 resin (Japan Organo Co., Tokyo, Japan), which had been washed with 5 L of methanol and then equilibrated with distilled water. The column was washed with 5 L of distilled water and then eluted with 5 L of 80% (v/v) methanol solution in the dark. The eluate containing a corrinoid-compound was evaporated to dryness under reduced pressure, and dissolved in 60 mL of distilled water. Each 20 mL of the concentrated solution was put on a column (24 × 180 mm) of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan), which had been washed with 75% (v/v) ethanol solution and then equilibrated with distilled water. The column was eluted with a stepwise gradient [0%, 10%, 20%, 30%, and 80% (v/v)] of ethanol. The 10% (v/v) ethanol fraction containing a corrinoid-compound was evaporated to dryness under reduced pressure, and dissolved with a small amount of distilled water. The concentrated solution was put on a silica gel 60 TLC sheet (Merck, Darmstadt, Germany) and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as a solvent in the dark at room temperature. Red-colored spots on the dried TLC sheet were collected, extracted with 80% (v/v) methanol solution, evaporated to dryness under reduced pressure, and dissolved in 50 μL of distilled water. The concentrated solution was put on a silica gel 60 TLC sheet and developed with 1-butanol/2-propanol/water (10:7:10 v/v) as a solvent in the dark at room temperature. Red-colored spots on the dried TLC sheet were collected, extracted with 80% (v/v) methanol solution, evaporated to dryness under reduced pressure, and dissolved in 100 μL of distilled water. The concentrated solution was purified by HPLC using Shimadzu HPLC apparatus (two LC-10ADvp pumps, DGV-12A degasser, SCL-10Avp system controller, SPD-10Avp ultraviolet–visible detector, CTO-10Avp column oven, 100 μL sample loop, C-R6A Chromatopac integrator). The sample (50 μL) was put on a reversed-phase HPLC column (Wakosil-II 5C18RS) equilibrated with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C. The flow rate was 1 mL/min. The corrinoid-compound was isocratically eluted with the same solution and monitored by measuring absorbance at 361 nm. The fractions (1 mL) were collected from the reverse phase HPLC column with a Bio-Rad Laboratories fraction collector (model 2110). The final red-colored fractions were collected, evaporated to dryness under reduced pressure, dissolved in 100 μL of distilled water, and used as a purified corrinoid-compound.

Analytical TLC and HPLC. The concentrated solutions (2 μL) of the corrinoid-compound purified from the *Aphanizomenon* cells, vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were spotted on the silica gel 60 TLC sheets and developed with 1-butanol/2-propanol/water (10:7:10 v/v), solvent I, and 2-propanol/NH_4OH (28%)/water (7:1:2 v/v), solvent II, in the dark at room temperature. After TLC sheets were dried, R_f values of the red-colored spots of these corrinoid-compounds were determined.

In the case of HPLC, the concentrated solutions (2 μL) of the purified corrinoid-compound, vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were analyzed with the reversed-phase HPLC column (Wakosil-II

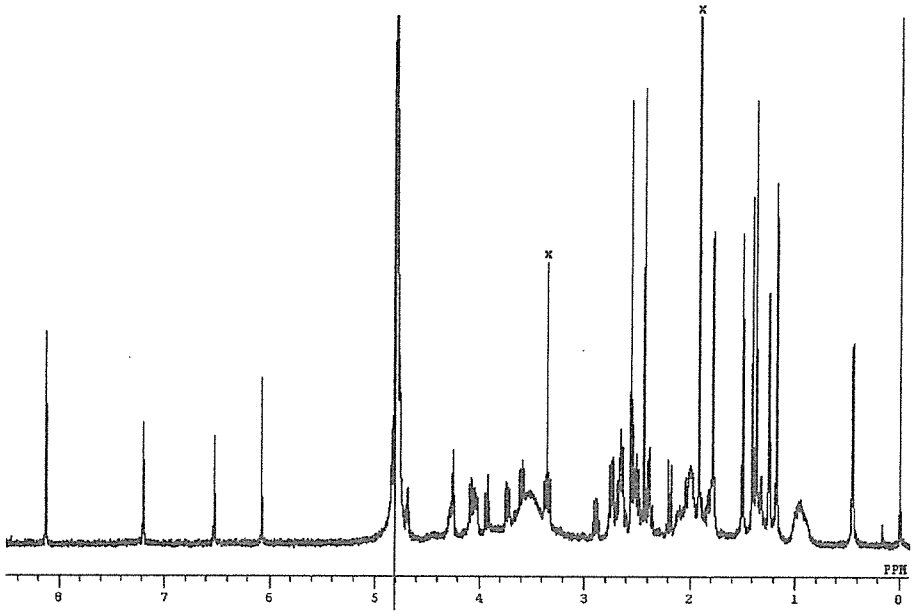

Figure 2. ^{1}H NMR spectrum of the corrinoid purified from dried *Aphanizomenon* cells (500 MHz, D_2O).

5C18RS). They were isocratically eluted with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C, and monitored by measuring absorbance at 361 nm. The flow rate was 1 mL/min.

Ultraviolet–Visible Spectrum. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature. Quartz cuvettes (d = 1 cm) were used. A portion of the purified corrinoid-compound was dissolved in 3 mL of distilled water.

^{1}H NMR Spectrum. ^{1}H NMR spectrum was obtained in D_2O with a JEOL JNM α-500 spectrometer. Chemical shifts are given on a δ (ppm) scale with 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP) as an internal standard. ^{1}H NMR spectral data of the purified corrinoid-compound: δ_H 8.13 (B2, s), 7.20 (B8, s), 6.53 (R1, d, J = 3.4 Hz), 6.08 (C10, s), 4.69 (R3, dt, J = 4.3, 8.5 Hz), 4.29 (Pr2, m), 4.26 (R2, t-like, J = 3.7 Hz), 4.08 (C3, m), 4.08 (C19, m), 4.03 (R4, m), 3.93 (R5a, dd, J = 2.1, 13.1 Hz), 3.74 (R5b, dd, J = 3.7, 13.1 Hz), 3.60 (Pr1a, br d, J = 14.3 Hz), 3.37 (C8, m), 3.35 (C13, m), 2.89 (Pr1b, dd, J = 10.1, 14.3 Hz), 2.56 (C53, s), 2.44 (C35, s), 1.79 (C25, s), 1.50 (C47, s), 1.41 (C54, s), 1.37 (C36, s), 1.25 (Pr3, d, J = 6.1 Hz), 1.18 (C46, s), 0.45 (C20, s). The assignment of these signals was carried out in comparison with those of authentic vitamin B_{12}.

RESULTS AND DISCUSSION

Vitamin B_{12} Concentration of the Dried *Aphanizomenon* Cells. Total vitamin B_{12} concentration of the dried *Aphanizomenon* cells was determined by both microbiological and chemiluminescence methods. Using the microbiological assay, the *Aphanizomenon* cells contained significantly higher amounts of vitamin B_{12} (616.3 ± 30.3 μg, n = 4) relative to the other edible microalgae (*Chlorella* and *Spirulina*) previously characterized (*8*, *14*) (**Table 1**). The values determined with the microbiological assay were, however, 20.5-fold greater than the values determined with the chemiluminescence assay in the *Aphanizomenon* cells. The similar result has been obtained in the *Spirulina* cells that contain pseudovitamin B_{12} (*8*). In the *Chlorella* cells containing true vitamin B_{12}, the values determined with the microbiological assay are similar to the values determined by the chemiluminescence assay (*14*).

Purification and Characterization of a Corrinoid-Compound from the Dried *Aphanizomenon* Cells. To evaluate whether the vitamin B_{12} activity detected in the *Aphanizomenon* cells by the microbiological assay method is derived from true vitamin B_{12} or not, a corrinoid-compound was purified and characterized.

The final purified preparation gave a single red-colored spot on the silica gel 60 TLC and a single peak by the C18 reversed-phase HPLC, indicating that the corrinoid-compound was purified to homogeneity. The ultraviolet–visible spectrum of the purified corrinoid-compound showed a typical absorption spectrum of cobalt-containing corrinoid (**Figure 1**); λ_{max} nm (absorbance) values were at 548.0 (0.317), 518.0 (0.296), 360.0 (1.153), and 277.5 (0.846).

The purified corrinoid-compound, authentic vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were analyzed by the silica gel 60 TLC and reversed-phase HPLC (**Table 2**). The R_f values (0.10 and 0.46 in solvents I and II, respectively, TLC) for the purified compound were identical to the values for authentic pseudovitamin B_{12}, whose retention time (6.4 min) by HPLC was also identical to that of the purified compound.

In the ^{1}H NMR spectrum of the corrinoid purified from the dried *Aphanizomenon* cells (**Figure 2**), the typical signals due to corrin skeleton and adenyl and ribose moieties were observed (see Material and Methods). These spectral data were identical to those of pseudovitamin B_{12} isolated from *Spirulina* tablet (*7*).

These results indicate that the red-colored compound purified from the dried *Aphanizomenon* cells is not true vitamin B_{12}, but pseudovitamin B_{12} inactive for humans.

Although only one corrinoid-compound (true vitamin B_{12}) has been purified from the *Chlorella* cells, the *Spirulina* cells contain two corrinoid-compounds (main, pseudovitamin B_{12}; and minor, true vitamin B_{12}). Our unpublished work demonstrated that the true vitamin B_{12} found in the *Spirulina* cells was derived from some vitamin B_{12}-synthesizing bacteria concomitant with the *Spirulina* cells grown under the open culture system. *Eschricha coli* 215-bioautography of the *Aphanizomenon* extract indicated that the bacterial cells contained substantial amounts of pseudovitamin B_{12} alone (data not shown). Peudovitamin B_{12} has been reported to reveal moderate affinity to the intrinsic

factor (most specific vitamin B_{12}-binding protein) (*15*) used in the chemiluminescence vitamin B_{12} assay method. These observations suggest that these cyanobacteria have the ability to synthesize pseudovitamin B_{12} *de novo*.

Some preclinical studies suggest that the *Aphanizomenon* cells have therapeutic properties such as macrophage-activation (*16*), antioxidant (*17*), immunological (*18*), and anti-inflammatory (*12*, *17*) activities. Although the taking of the *Aphanizomenon* cells may give some health promotion effects for humans, the results presented here strongly suggest that the bacterial cells are not suitable for use as a vitamin B_{12} source, especially in vegans.

LITERATURE CITED

(1) Millet, P.; Guilland, J. C.; Fuchs, F.; Klepping, J. Nutrient intake and vitamin status of health French vegetarians and nonvegetarians. *Am. J. Clin. Nutr.* **1989**, *50*, 718−727.

(2) Herbert, V.; Drivas, G. Spirulina and vitamin B_{12}. *J. Am. Nutr. Assoc.* **1982**, *248*, 3096−3097.

(3) Dagnelie, P. C.; van Sttaveren, W. A.; van den Berg, H. Vitamin B_{12} from algae appears not be bioavailable. *Am. J. Clin. Nutr.* **1991**, *53*, 695−697.

(4) Watanabe, F.; Katsura, H.; Miyamoto, E.; Takenaka, S.; Abe, K.; Yamasaki, Y.; Nakano, Y. Characterization of a vitamin B_{12} compound in an edible green laver (*Entromopha prdifera*). *Appl. Biol. Sci.* **1995**, *5*, 99−107.

(5) Watanabe, F.; Takenaka, S.; Katsura, H.; Miyamoto, E.; Abe, K.; Tamura, Y.; Nakatsuka, T.; Nakano, Y. Characterization of a vitamin B_{12} compound in the edible purple laver, *Porphyra yezoensis*. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 2712−2715.

(6) Miyamoto, E.; Watanabe, F.; Ebara, S.; Takenaka, S.; Takenaka, H.; Yamaguchi, Y.; Tanaka, N.; Inui, H.; Nakano, Y. Characterization of a vitamin B_{12} compound from unicellular coccolithophorid alga (*Pleurochrysis carterae*). *J. Agric. Food Chem.* **2001**, *49*, 3486−3489.

(7) Kittaka-Katsura, H.; Fujita, T.; Watanabe, F.; Nakano, Y. Purification and characterization of a corrinoid-compound from chlorella tablets as an algal health food. *J. Agric. Food Chem.* **2002**, *50*, 4994−4997.

(8) Watanabe, F.; Katsura, H.; Takenaka, S.; Fujita, T.; Abe, K.; Tamura, Y.; Nakatsuka, T.; Nakano, Y. Pseudovitamin B_{12} is the predominate cobamide of an algal health food, spirulina tablets. *J. Agric. Food Chem.* **1999**, *47*, 4736−4741.

(9) Pulz, O.; Gross, W. Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 635−648.

(10) Jensen, G. S.; Ginsberg, D. I.; Huerta, P.; Citton, M.; Drapeau, C. Consumption of *Aphanizomenon flos-aquae* has rapid effect on the circulation and function of immune cells in humans. *J. Am. Nutr. Assoc.* **2000**, *2*, 50−58.

(11) Kushak, R. I.; Drapeau, C.; Van Cott, E. M.; Winter, H. H. Favorable effect of blue-green algae *Aphanizomenon flos-aquae* on rat plasma lipids. *J. Am. Nutr. Assoc.* **2000**, *2*, 59−65.

(12) Drapeau, C. Anti-inflammatory and antidepressant properties on the blue-green algae *Aphanizomenon flos-aquae*. Abstracts from the 25th Annual Scientific Congerence of the American Holistic Medical Association (May 15−18, 2002, Tronto, Canada). *Complementary Health Practice Review* **2003**, *8*, 161−162.

(13) Kay, R. A. Microalgae as food and supplement. *Crit. Rev. Food Sci. Nutr.* **1991**, *30*, 555−573.

(14) Watanabe, F.; Takenaka, S.; Abe, K.; Tamura, Y.; Nakano, Y. Comparison of a microbiological assay and a fully automated chemiluminescent system for the determination of vitamin B_{12} in food. *J. Agric. Food Chem.* **1998**, *46*, 1433−1436.

(15) Stupperich, E.; Nexo, E. Effect of the cobalt-N coordination on the cobamide recognition by the human vitamin B12 binding proteins intrinsic factor, transcobalamin, and haptocorrin. *Eur. J. Biochem.* **1991**, *199*, 299−303.

(16) Pugh, N.; Pasco, D. S. Characterization of human monocyte activation by a water soluble preparation of *Aphanizomenon flos-aquae*. *Phytomedicine* **2001**, *8*, 445−453.

(17) Benedetti, S.; Benvenuti, F.; Pagliarani, S.; Francogli, S.; Scoglio, S.; Canestrari, F. Antioxidant properties of a novel phycocyanin extract from the blue-green alga *Aphanizomenon flos-aquae*. *Life Sci.* **2004**, *75*, 2353−2362.

(18) Pugh, N.; Ross, S. A.; ElSohly, H. N.; ElSohly, M. A.; Pasco, D. S. Isolation of three high molecular weight polysaccharide preparations with potent immunostimulatory activity form *Spirulina platensis*, *Aphanizomenon flos-aquae* and *Chlorella pyrenoidosa*. *Planta Med.* **2001**, *67*, 737−742.

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