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ビタミンと免疫†

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はじめに

多くのビタミンが種々の栄養素の代謝の中で酵素の働きを助ける補酵素として重要な役割を担っており，それらビタミンの欠乏は細胞内での栄養素代謝の破綻をもたらし，その結果として免疫能を含む細胞機能の低下を誘導する．さらに，ビタミン欠乏は単独で起こることは稀であり，一般にたん白・エネルギー栄養不良（PEM；Protein-energy malnutrition）に付随してみられる[1]（表1）．また，入院患者においても単一あるいは2，3のビタミン欠乏が混在していることも知られている[2]（表2）．個々のビタミン欠乏時にはほとんどの免疫能の低下することが知られている[3]（表3）．本稿ではビタミンを脂溶性ビタミン（ビタミンA，D，EおよびK）と水溶性ビタミン（ビタミンB群とC）に分け，宿主免疫能への影響についてこれまでの研究成果について要約した．

表1　たん白質・エネルギー栄養不良に伴うビタミン欠乏の発生頻度

ビタミン	重篤な栄養不良		中程度の栄養不良	
	調査数	欠乏（%）	調査数	欠乏（%）
ビタミンA	13/29	45	11/37	30
カロテン	32/33	97	25/32	78
葉　酸	5/33	15	7/36	19
ビタミンC	0/20	0	0/19	0
ビタミンB_1	12/28	43	18/27	67
ビタミンB_2	5/31	16	3/24	13
ビタミンB_6	12/34	35	8/42	19

表2　米国の入院患者にみられるビタミン欠乏の頻度

ビタミン	欠乏者の割合（%）
ビタミンA	13
ビタミンE	12
葉　酸	45
ビタミンC	12
ビタミンB_1	31
ビタミンB_2	12
ビタミンB_6	27
ナイアシン	29
パントテン酸	15
ビタミンB_{12}	10
ビオチン	1
2種の欠乏	38
3種の欠乏	14
4種の欠乏	6
5種の欠乏	10

I　脂溶性ビタミン

脂溶性ビタミンの中で免疫能との関連でこれまで多くの研究報告がみられるビタミンはAおよびEである．ビタミンDに関しては若干の報告はあるものの未だ十分ではない．さらに，ビタミンKに関しては免疫能に対する作用は低いと考えられている．

† Vitamins and Immunity

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表3　種々のビタミン欠乏と免疫能（抜粋）

ビタミン	免疫能の変化
ビタミンB_6	・ジフテリア毒素に対する抗体産生の低下 ・SRBCに対する抗体産生細胞数の低下 ・リンパ球混合培養反応の低下
パントテン酸	・サルモネラ菌に対する抗体価の低下 ・SRBCに対する抗体産生細胞数の低下
ビタミンB_1	・ラットにおけるヒト赤血球に対する抗体価の低下
ビタミンB_2	・ラットおよびブタにおけるヒト赤血球に対する抗体価の低下
ビオチン	・ジフテリアに対する二次抗体価の低下
ビタミンB_{12}	・TおよびB細胞数は正常 ・PHAに対するヒト末梢血リンパ球幼若化能の低下
ビタミンA	・ジフテリア毒素に対する抗体反応の低下 ・遅延型過敏反応の低下 ・末梢血T細胞数の低下とPHAに対する反応低下
ビタミンC	・ツ反に対する感受性発達を阻害 ・皮膚移植片の拒絶反応の低下 ・胸腺由来液性因子の産生低下

(1)ビタミンAおよびカロテノイド

一般にビタミンA欠乏状態ではT細胞の成熟の場である胸腺の萎縮がみられ，細胞性ならびに体液性免疫ともに抑制され，細菌に対する易感染性や発ガン物質投与によるガン発生の増加が認められている[4,5]．また，一方では高ビタミンA摂取により細胞性免疫や抗体産生の亢進することが見出されており，感染抵抗性の増大やマクロファージの殺腫瘍活性亢進を介した移植腫瘍に対する増殖抑制作用が報告されている[6,7]．例えば，マウスの皮膚ガン発生に対する高ビタミンA摂取の効果をみた実験では，発生腫瘍数や腫瘍重量が著明に低下することを認めている（表4）．この機序としてマクロファージ数の増加や殺腫瘍活性亢進との関連が示唆されている．特にB_{16}メラノーマ細胞に対するマクロファージの殺腫瘍活性が食餌中のビタミンA含量の増加に依存して高くなる傾向がみられている[8]．また，マクロファージ活性化因子（MAF）を用いてマクロファージを活性化したところ，基礎食群のマクロファージでは約35%の殺腫瘍活性の上昇を認めた．これに対し，高ビタミンA食群では食餌中のビタミンA含量が増えるに伴い，逆にマクロファージのMAFに対する反応性は低下する傾向がみられている．このことは高ビタミンA食摂取によりT細胞が活性化され，活性化されたT細胞から産生されたMAFによりマクロファージが既に活性化されていることを示唆している．さらに，ビタミンAとの*in vitro*培養によってもラット肺胞マクロファージ（AM）の殺腫瘍活性が亢進されること[9]や，遺伝的に胸腺が欠損したヌードマウスにおいても高ビタミンA食投与によりマクロファージ数の増加やオプソニン化羊赤血球（SRBC）に対する貪食能の有意な亢進を認めたこと[10]から，ビタミンAによるマクロファージ機能の亢進が活性化Tリンパ球から産生されるサイトカインの一つであるMAFによるものと，Tリンパ球を介さず，ビタミンAそのものが直接作用する場合があることが考えられる[11]．図1にマクロファージとNK細胞活性化の機序を要約した．換言すると，ビタミンAによるT細胞活性化を介して産生されるMAF，IL-2およびIFN-γなどのサイトカインによって二次的にマクロファージやNK細胞が活性化される系と，ビタミンAにより直接的に活性化される系とが存在する可能性が考えられる．しかし，ビタミンAの過剰摂取は皮膚の落屑，脱毛，筋肉痛や妊婦では胎児の奇形などの副作用をあらわすことが知られており[12]，その摂取には注意を要する．一方，ビタミンAの前駆物質として知られるβ-カロテンやその他のカロテノイドもまたビタミンAと同様に免疫賦活作用を有しており，しかもビタミンAとは異なり大量摂取によっても副作用はほとんど出現しないことが知られている[13]．カロテノイド摂取により，好中球やマクロファージなどの食細胞機能の亢進，NK細胞活性の上昇，細胞障害性Tリンパ球機能の亢進などがみられ，これらによって移植腫瘍細胞の増殖抑制や発ガン抑

制が誘導されることが報告されている[14].

表4　マウス皮膚癌発生に対するビタミンAパルミチン酸（RP）および13-cisレチノイン酸（13cRA）の影響

実験群	食餌中RP又は13cRA含量 (IU/kg diet)	マウス当りの腫瘍数	マウス当りの腫瘍重量 (g)
基礎食	3,500	15.6±2.7	1.373±0.20
RP	60,000	14.3±2.6	0.20±0.04
	200,000	8.2±2.0	0.131±0.03
	700,000	3.4±1.2	0.007±0.001
13cRA	200,000	13.0±1.9	0.312±0.07
	700,000	14.8±3.0	0.049±0.01

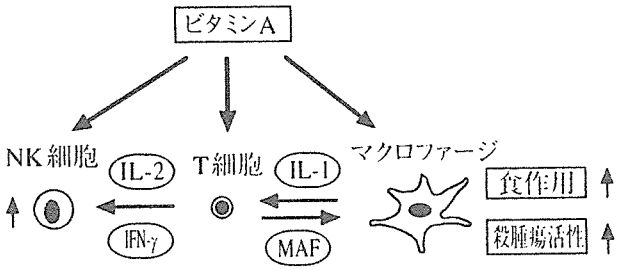

図1　ビタミンAによるマクロファージおよびNK細胞活性化の機序

(2)ビタミンE

ビタミンEは自然界にはトコフェロールと側鎖に二重結合を有するトコトリエノールとがあり，各々にクロマン環に結合したメチル基数の違いにより α-，β-，γ-および δ-の4つのタイプ，計8種の同族体が存在している．ビタミンEと言えば一般に α-トコフェロールのことを指し，抗酸化活性や体内含量が同族体の中では最も高いことが知られている．ビタミンE欠乏では免疫系だけでなく生殖機能を含め種々の組織・器官の機能と形態が損なわれる[15]．免疫系では抗体産生能，リンパ球増殖能，好中球の走化性や殺菌能など広範囲にわたる免疫低下を誘導することが知られている．但し，動物実験ではビタミンE欠乏時に肺胞マクロファージ貪食能が亢進するという報告もある[16]．一方，高ビタミンE食投与は体液性および細胞性免疫能を亢進し，生体防御能を高めることが知られている[17]．特に，細胞性免疫能の低下した高齢者では血中VLDLコレステロール当りのビタミンEレベルと末梢血リンパ球幼若化能との間に有意な正相関のあることが見出されている（図2）．さらに，ビタミンE補足により高齢者の低下した免疫能が改善されることが報告されている．この機序としてアラキドン酸から合成され，免疫抑制作用を有する PGE_2 産生をビタミンEが抑制することとの関連が示唆されている．その他，ビタミンEには骨髄で産生された未熟T細胞が分化・成熟する場である胸腺の機能を高める作用のあることも見出されている[18]．これらの作用の多くはビタミンEの抗酸化作用に帰するものであるが，同じく抗酸化作用を有する2-メルカプトエタノール（2-ME）投与ではビタミンE投与と同様の免疫能改善がみられないことから抗酸化作用それだけでは説明できないビタミンE固有の作用の存在が考えられている．

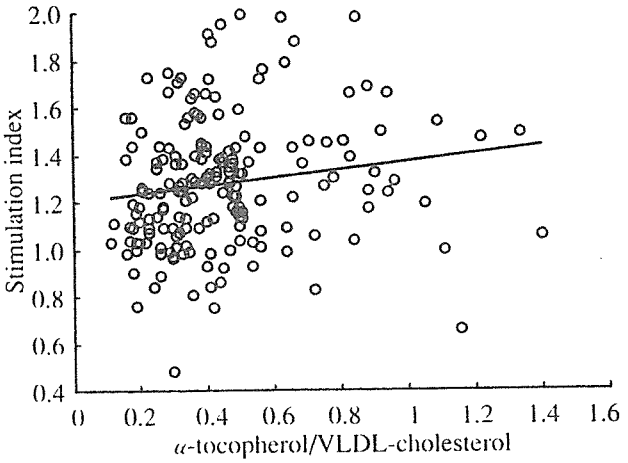

図2　末梢血リンパ球幼若化能と血中ビタミンE濃度との関係

(3)ビタミンD

ビタミンDはきのこや魚類に含まれるほかに，皮膚において紫外線によりプロビタミンDから合成される．このビタミンDが欠乏すると子どもではくる病，成人では骨軟化症を発症することが知られている．ビタミンDと免疫能との関連についての研究はこれまで比較的少なく，その中でくる病の子どもの好中球機能をみたものがある．ビタ

ミンD欠乏状態であるくる病においても好中球の殺菌能は保持されていたが，貪食活性は低下することが認められている[19]．また，動物実験ではビタミンD欠乏により抗体産生能の低下することが見出されており，宿主免疫能を保持するうえで十分なビタミンD摂取が必要である．ビタミンDの補足効果については，ビタミンAとの併用によりイースト菌に対する貪食能が相乗的に亢進されることも報告されている[20]．

II　水溶性ビタミン

(1)ビタミンB群

ビタミンB_1，B_2，B_6，B_{12}，葉酸およびパントテン酸などのビタミンB群と免疫能との関連についての研究は比較的少なく，しかもそのほとんどがこれら各ビタミンの欠乏時における免疫能について検討したものである．また，ヒトではこれらビタミン欠乏が単独で起こることはほとんどないことから，これまでの研究成果のほとんどが動物実験によるものである．いずれのビタミン欠乏においてもTおよびBリンパ球機能の低下が認められている．その中でも特にアミノ酸や核酸代謝に関与するビタミンB_6欠乏時にはTおよびBリンパ球数の減少をはじめとする顕著な免疫低下の生じることが知られている[21]．いずれの場合も欠乏しているビタミンを補足することによりそれら機能が回復することから，ビタミンB群に関しては免疫を保持するうえで少なくとも欠乏にならない程度の摂取が望まれる．また，ビタミンB群の補足による免疫変化に関してはビタミンB_1およびB_6補足により食細胞の走化性が亢進されたり[22]，ナイアシン補足によりヒト末梢血リンパ球やマウス脾臓リンパ球の増殖能が亢進されること[23]が知られている．

(2)ビタミンC

ビタミンCは多くの動物ではグルコースから酵素的に合成されるが，ヒトをはじめとする霊長類やモルモットでは合成酵素が欠損しているために，体外より食事として経口的にビタミンCを摂取する必要がある．ビタミンCの欠乏症状としては壊血病が知られている．ビタミンCが免疫の分野において脚光を浴びたのはノーベル賞を2度受賞したポーリング博士の「ビタミンC大量投与が風邪やガン発生を予防する」という発言に端を発する[24]．しかし，ビタミンCによる免疫賦活作用は図3に示すごとく，所要量（100mg/日）の10倍を越えるビタミンCを毎日連続して摂取している場合には末梢血リンパ球増殖能の亢進が誘導されるが，一旦，ビタミンC摂取を止めると1週間後には亢進していた免疫能が摂取前のレベルにまで戻り，さらに1日2gを越えるビタミンC摂取をしてもさらに高い免疫能の亢進は誘導されない[25]．これら結果はビタミンC補足によって風邪等の予防を図るうえでのビタミンCの量や摂取方法に対して示唆を与えるものである．また，ビタミンCは抗酸化ビタミンの中で唯一の水溶性ビタミンであり，その作用の一つとして同じく有力な抗酸化ビタミンであるビタミンEが活性酸素等により酸化された場合，その還元作用によりもとの抗酸化能を有するビタミンEに回復することが見出され，

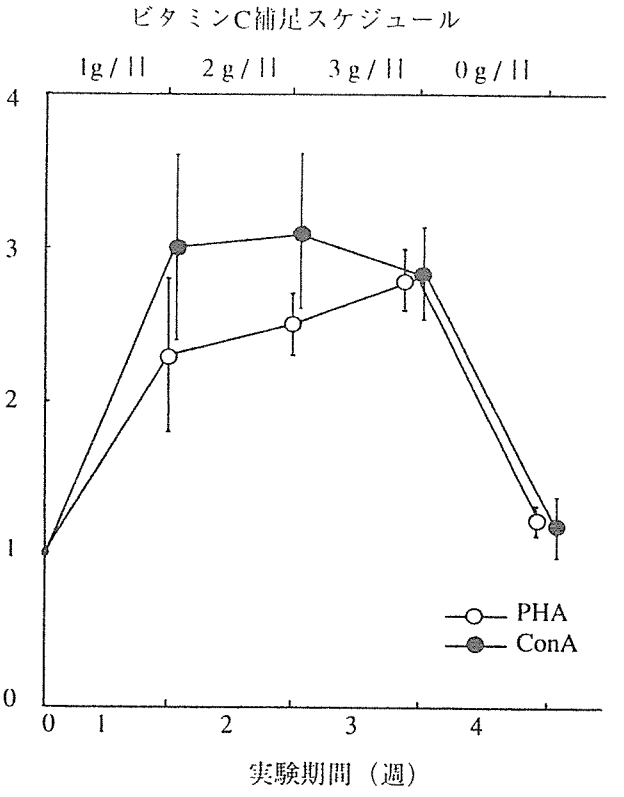

図3　末梢血リンパ球幼若化能に対するビタミンC大量投与の影響

注目されている[26]．このことは，ビタミンC補足による免疫賦活作用がビタミンC固有の作用だけではなく，ビタミンEとの共同作用によって免疫能が亢進される可能性を支持するものである．

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Characterization of Corrinoid Compounds from a Japanese Black Tea (Batabata-cha) Fermented by Bacteria

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Characterization of Corrinoid Compounds from a Japanese Black Tea (Batabata-cha) Fermented by Bacteria

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A Japanese fermented black tea (Batabata-cha) contained a considerable amount of vitamin B_{12} (456 ± 39 ng per 100 g dry tea leaves and 2.0 ± 0.3 ng per 100 mL of tea drink). A corrinoid compound was partially purified and characterized from the tea leaves. The patterns of the purified compound by the silica gel 60 thin-layer chromatography and C18 reversed phased high-performance liquid chromatography were identical to those of authentic vitamin B_{12}. When 20 week old vitamin B_{12} deficient rats, which excreted substantial amounts (about 250 mg/day) of methylmalonic acid in urine as an index of vitamin B_{12} deficiency, were fed the tea drink (50 mL/day, 1 ng of vitamin B_{12}) for 6 weeks, urinary methylmalonic acid excretion (169 ± 29 mg/day) of the tea drink-supplemented 26 week old rats decreased significantly relative to that (250 ± 32 mg/day) of the deficient rats. The results indicate that the vitamin B_{12} found in the fermented black tea is bioavailable in mammals.

KEYWORDS: Vitamin B_{12}; cobalamin; corrinoid; tea; vitamin B_{12} deficient rat; methylmalonic acid; hepatic vitamin B_{12}

INTRODUCTION

In manufacturing processes of black teas fermented by bacteria, such as Pu'erh tea, tea leaves are heat-treated with steam or roasting and then fermented with certain naturally occurring bacteria (*1*). Thus, they are completely different from the types of self-oxidized black teas (Keemun tea and Darjeeling tea, etc.). These black teas fermented by bacteria, which are found in some Asian countries, may contain various vitamins and/or biofactors synthesized by the concomitant bacteria.

Vitamin B_{12} (B_{12}) is synthesized only in certain bacteria (*2*). Usual dietary sources of B_{12} are known to be animal products but not plant products (*3*). If the fermented black teas contain considerable amounts of B_{12}, the black tea would contribute to human B_{12} needs, especially for vegetarians.

Here, we describe the partial purification and characterization of a corrinoid compound from Japanese fermented black tea (Batabata-cha) leaves and also investigated the effect of feeding the tea drink on the B_{12} status of B_{12} deficient rats.

MATERIALS AND METHODS

Materials. Cyano-B_{12} was obtained from Wako Pure Chemical Industries (Osaka, Japan). A B_{12} assay medium for *Lactobacillus delbrueckii* subsp. *lactis* (formerly *Lactobacillus leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin-layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). All other reagents used were of the highest purity commercially available. Japanese fermented black tea (Batabata-cha) leaves and the tea drink were provided by Asahi, Ltd. (Toyama-city, Japan).

A UV-1600 UV−vis spectrophotometer (Shimadzu, Kyoto, Japan) was used for measuring the turbidity of the *L. delbrueckii* test culture in the microbiological method. A fully automated ACS 180 chemiluminescence B_{12} analyzer (Chiron Diagnostics, East Walpole, MA) was used for the B_{12} assay.

Extraction of Corrinoid Compounds for the Determination of B_{12} Content of the Tea Leaves. The dried tea leaves (5 g) were powdered by a food mill and then suspended in 50 mL of 0.25 mol/L acetate buffer, pH 4.8, containing 0.2% (w/v) KCN as cyanation for stabilization. The total corrinoids were extracted from the suspension by boiling for 60 min at 98 °C in the dark. The suspension was centrifuged for 10 min at 5000*g*, and the supernatant was used for the B_{12} assay.

Concentration of the Fermented Black Tea Drink. For the determination of B_{12} in the tea drink, B_{12} was concentrated with a Sep-Pak Vacc 20 cm^3 (5 g) C18 cartridge (Waters Corp., Milford, MA). After the C18 cartridge was washed with 75% ethanol and equilibrated with distilled water, an aliquot (50 mL) of the drink was put on the cartridge. B_{12} was eluted with 50 mL of 25% ethanol, and the eluate was evaporated to dryness under reduced pressure, dissolved in 1.0 mL of distilled water, and used for the B_{12} assay.

Assay of B_{12}. B_{12} was assayed by the microbiological method with *L. delbrueckii* subsp. *lactis* ATCC 7830 and a B_{12} assay medium (Nissui) and by the chemiluminescence B_{12} analyzer with intrinsic factor (IF) as described previously (*4*). The above B_{12} extract and the concentrated drink were directly applied to the chemiluminescence

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analyzer. They were diluted with distilled water to a B_{12} concentration range of 0.01−0.1 μg/L and used as samples for the microbiological method.

Purification of Corrinoid Compounds from the Fermented Black Tea. About 0.5 kg of the dried tea leaves was powdered by a model MX-X51-H food mill (National, Osaka, Japan), and suspended in 2 L of 0.25 mol/L acetate buffer, pH 4.8. KCN was added to the suspension at the final concentration of 10 mmol/L. Total B_{12} was extracted from the suspension under the same conditions described above. The boiled suspension was centrifuged at 10 000*g* for 10 min. The supernatant was used for purification of corrinoid compounds. Amberlite XAD-4 resin (500 g) washed with 5 L of methanol and equilibrated with distilled water was added to the supernatant fraction and stirred for 3 h at room temperature in the dark. The resin suspension was passed through a glass funnel (Buchner type) with a type 25G1 glass filter (Iwaki, Tokyo, Japan), and the resin was washed with 5 L of distilled water. One liter of 80% methanol solution was added to the washed resin, and the suspension was stirred for 3 h at room temperature in the dark. The resin suspension was passed through the glass funnel. The 80% methanol eluant (about 1 L) containing corrinoid compounds was pooled and evaporated to a final volume of 20 mL under reduced pressure. The solution was put on a 24 mm × 70 mm column of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan), which was washed with 75% ethanol solution and equilibrated with distilled water. A corrinoid compound was eluted with 100 mL of a linear gradient (0−25%) of ethanol. The B_{12} active fractions assayed by the microbiological method were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of 70% 2-propanol solution containing 2.8% NH_4OH. The solution was put on a silica gel 60 TLC sheet and developed with 2-propanol/28% NH_4OH/water (7:1:2) as a solvent in the dark at room temperature. The dried TLC sheet was cut into small pieces (0.5 cm × 1.0 cm) with scissors. Corrinoid was extracted from the pieces in 70% 2-propanol solution containing 2.8% NH_4OH several times, and the extract was evaporated to dryness under reduced pressure, dissolved in 1.0 mL of distilled water, and used as samples for the microbiological B_{12} assay. The B_{12} active fractions assayed by the microbiological method were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was further purified by high-performance liquid chromatography (HPLC) using a Shimadzu HPLC apparatus consisting of a LC-6A pump, SPD-6A spectrophotometer, CTO-6A column oven, and C-R6A Chromatopac. The sample (100 μL) was put on a 150 mm × 4.6 mm i.d., 5 μm, Wakosil-II 5C18RS reversed phase HPLC column equilibrated with 20% methanol solution containing 1% acetic acid at 35 °C. The flow rate was 1 mL/min. The corrinoid compound was isocratically eluted with the same solution, monitored by measuring absorbance at 278 nm, and collected in 1 mL fractions. The B_{12} active fractions assayed by the microbiological method were collected, concentrated, and used as a purified corrinoid compound.

Analytical TLC and HPLC of Corrinoid Compound Purified from the Black Tea. The concentrated solution (10 μL) of corrinoid compound purified from the tea leaves and authentic cyano-B_{12} was spotted on the silica gel 60 TLC sheets and developed with solvents I [2-propanol/NH_4OH (28%)/water (7:1:2)] and II [1-butanol/2-propanol/water (10:7:10)] in the dark at room temperature. The TLC sheets were dried, and R_f values of the pink-colored spot of cyano-B_{12} were determined.

The TLC sheets were also cut into small pieces (0.5 cm × 1.0 cm) with scissors. Corrinoids were extracted from the pieces in 80% (v/v) methanol several times, evaporated to dryness under reduced pressure, dissolved in 1.0 mL of distilled water, and used as samples for the microbiological B_{12} assay.

In the case of HPLC, the concentrated solutions (10 μL) of the purified corrinoid compound and authentic cyano-B_{12} were analyzed with the same reversed phase HPLC column as used for purification. The corrinoids were isocratically eluted with 20% methanol solution containing 1% acetic acid at 35 °C and monitored by measuring absorbance at 278 nm. The retention times of corrinoids were determined at a flow rate of 1 mL/min. The eluate from the HPLC column was collected, evaporated to dryness, dissolved in 1.0 mL of distilled water, and used as samples for the microbiological B_{12} assay.

Animals and Experimental Diets. Fifteen male Wister rats (20 weeks old), born to 14 week old parents fed on a B_{12} deficient diet for 8 weeks, were used. The B_{12} deficient diet fed to the parents contained (g/kg diet): 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.), and 5 B_{12}-free vitamin mixture and 2 choline chloride (Nacalai Tesque Ltd.), as described previously (*5*). 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 16 g/day of the B_{12} deficient diet and distilled water for 17 weeks. In the feeding experiments, the 20 week old B_{12} deficient rats (four rats/group) were given free access to 16 g/day of the B_{12} deficient diet and 50 mL of either distilled water, authentic cyano-B_{12} solution (1 ng of B_{12} per 50 mL), or the tea drink (1 ng of B_{12} per 50 mL) for 6 weeks. All experimental procedures involving laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

Urinary Methylmalonic Acid Assay. The urine of the B_{12} deficient, cyano-B_{12}-supplemented, and tea drink-supplemented rats was sampled for 24 h in individual methabolic cages at weeks 0, 1, 2, and 6 during the experiments. Urinary methylmalonic acid was assayed by HPLC as described previously (*6*).

Extraction of B_{12} from Rat Liver. After food was withheld from the 26 week old rats overnight, the rats were killed by decapitation under diethyl ether anesthesia. Livers were washed with a chilled 9 g/L NaCl solution, weighed, and stored at −80 °C until analyzed. A portion (1 g) of the liver was cut into small pieces using a razor blade and homogenized in 10 times its volume of 10 mmol/L acetate buffer, pH 4.8. B_{12} was extracted from the liver homogenate by boiling with KCN at acidic pH as described above and assayed by the microbiological assay.

Statistics. Statistical analysis was performed using GB-STAT5.4 (Dynamic Microsystems, Inc., Silver Spring, MD). One way and two way repeated measure analysis of variance (ANOVA) were used for assay of hepatic B_{12} and urinary methylmalonic acid in the animal feeding test, respectively. When ANOVA results were significant, a posthoc two-tailed Student's *t*-test also was performed and considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

B_{12} Content Determination. The B_{12} contents were determined by two methods, the IF-chemiluminescence and the microbiological methods. The B_{12} contents of the dry tea leaves were 456 ± 39 ng and 368 ± 56 ng per 100 g dry weight by the IF-chemiluminescence method and the microbiological method, respectively. In the case of B_{12}, contents of the tea drink used for the feeding experiments were 2.0 ± 0.3 ng and 2.0 ± 0.8 ng per 100 mL by those two methods, respectively. Although B_{12} contents were considerably lower in the black tea drink than in cow's milk (0.3 μg/100 g) (*7*), which greatly contributes to the B_{12} intake of U.S. adult women (*8*), this is the first report on the occurrence of B_{12} in tea leaves and their drinks.

Characterization of the Corrinoid Compound from the Black Tea. A corrinoid compound was purified from the tea leaves. The purified corrinoid compound and authentic cyano-B_{12} were analyzed by TLC and HPLC. The R_f values for the purified compound were 0.64 and 0.22 on silica gel 60 TLC in solvents I and II, respectively. These values were identical to those for authentic cyano-B_{12}. The retention time of authentic B_{12} by reversed phase HPLC was 9.4 min; it was also identical to that of the purified compound. These results strongly suggest that the compound purified from the fermented tea leaves is true B_{12} but not corrinoid compounds inactive for humans. UV−vis spectroscopy and NMR spectroscopy could not be determined because a substantial amount of the purified compound was not obtained.

Feeding Test of the Tea Drink with the B_{12} Status of B_{12} Deficient Rats. To evaluate whether the B_{12} found in the tea

Table 1. Effects of Feeding the Fermented Black Tea Drink on Urinary Methylmalonic Acid of B_{12} Deficient Rats[a]

	urinary methylmalonic acid (mg/day)		
groups	control	CN-B_{12} supplement	tea drink supplement
week 0	246 ± 51[a]	253 ± 41[a]	251 ± 35[a]
week 1	246 ± 32[a]	200 ± 25[a]	181 ± 66[a]
week 2	251 ± 104[a]	218 ± 35[a]	151 ± 50[b]
week 6	250 ± 32[a]	201 ± 28[a]	169 ± 29[b]

[a] The 20 week old B_{12} deficient rats (four rats/group) were given free access to 50 mL of either distilled water, the cyano-B_{12} solution (1 ng/50 mL), or the tea drink (1 ng of B_{12}/50 mL) per day. [ab]The mean values with different superscript letters are significantly different; $P < 0.05$.

Table 2. Hepatic B_{12} Contents of the 26 Week Old B_{12} Deficient Rats Fed the CN-B_{12} Solution and Tea Drink[a]

groups	B_{12} contents (pg/g wet tissue)
control	746 ± 97[a]
CN-B_{12} supplement	768 ± 129[a]
tea drink supplement	1473 ± 252[b]

[a] The 20 week old B_{12} deficient rats (four rats/group) were given free access to 50 mL of either distilled water, the cyano-B_{12} solution (1 ng/50 mL), or the tea drink (1 ng of B_{12}/50 mL) per day. [ab]The mean values within a column with different superscript letters are significantly different; $P < 0.01$.

leaves is absorbed in the mammalian intestine and accumulated in the liver, feeding experiments of the tea drink to 20 week old B_{12} deficient rats were conducted. There was no significant difference in the intakes of the diet and drink (water, cyano-B_{12} solution, or the tea drink) among the rat groups during the experimental time course. When the 20 week old B_{12} deficient rats, which excreted substantial amounts of methylmalonic acid (about 250 mg/day) in urine (as an index of B_{12} deficiency), were given the tea drink (1 ng of B_{12} per day) for 6 weeks, urinary methylmalonic acid excretion of the tea drink-supplemented 26 week old rats decreased significantly relative to the B_{12} deficient (control) rats, but that of the cyano-B_{12}-supplemented rats did not (**Table 1**).

Although the rate of growth (61.6 ± 18.2 g) of the B_{12} deficient rats given the tea drink had a tendency to be greater than that of the control (28.2 ± 7.4 g) and cyano-B_{12}-supplemented (20.6 ± 15.8 g) rats during the experiment, there was no significant difference in body weight among the rats fed the three experimental drinks after 6 weeks.

The hepatic B_{12} contents were about 2-fold greater in the tea drink-supplemented rats than in both control and cyano-B_{12}-supplemented rats (**Table 2**); there was no significant difference in the hepatic B_{12} contents between control and cyano-B_{12}-supplemented rats. Although the methylmalonic aciduria of the B_{12} deficient rats could not be completely recovered by the 6 week feeding of the tea drink, the significant increase in the hepatic B_{12} content of the tea drink-supplemented rats indicated that the feeding of the tea drink considerably improved B_{12} statue in the B_{12} deficient rats.

Our previous study (*9*) has indicated that urinary levels of methylmalonic acid became undetectable in the B_{12} deficinet rats fed a cyano-B_{12} (about 100 ng/day)-supplemented diet for 10 days. In this study, however, the cyano-B_{12} (1 ng/day)-supplemented 26 week old rats did not show both significant recovery of methylmalonic aciduria and increase in hepatic B_{12} content. Even the 26 week old B_{12} deficient rats given the tea drink did not completely recover from methymalonic aciduria. The results may be due to a lesser B_{12} content (1 ng/day) of the administered authentic B_{12} and tea drink. We did not use any concentrated or purified compound from the tea leaves because of evaluation for bioavailability of B_{12} found in the tea drink commercially available for humans.

Our preliminary experiments indicated that considerable amounts of the coenzyme B_{12} (adenosylcobaramin and methylcobalamin) were found in the tea leaves and that the B_{12} found in the tea drink existed as a free form (without binding to a macromolecular compound). These results suggest that the B_{12} found in the tea drink would be assimilated more easily in the B_{12} deficinet rats than authentic cyano-B_{12}.

The results presented here indicate that the B_{12} found in the Japanese black tea (Batabata-cha) fermented by bacteria is bioavailable in mammals. Although only 1−2 L of the fermented tea drink (20−40 ng of B_{12}) is not sufficient to satisfy the recommended dietary allowance (2.4 μg/day) for human adults, intakes of various B_{12}-containing plant foods [purple and green lavers (*10*), Chlorella tablets (*11*), and the fermented tea extract] would contribute to prevention of B_{12} deficiency for vegetarians.

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Euglena gracilis Zの生育に伴うビタミンB_{12}依存性メチオニン合成酵素活性の変動

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Time Course of Changes in the Activity of Vitamin B_{12}-Dependent Methionine Synthase during Cell Growth of *Euglena gracilis* Z

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要　旨

ビタミンB_{12}給与および欠乏*Euglena gracilis* Zの生育に伴うビタミンB_{12}依存性メチオニン合成酵素活性の変動を検討した。本酵素活性はビタミンB_{12}給与*Euglena*細胞で生育2日目の対数増殖初期で最大(2.1nmol/min/10^6cells)となり，その後顕著に減少した。一方，ビタミンB_{12}欠乏*Euglena*細胞では生育期間を通して非常に低い活性(0.01-0.04nmol/min/10^6cells)を示した。本酵素はビタミンB_{12}給与細胞の対数増殖初期にのみ高い活性が認められたため，対数増殖初期のビタミンB_{12}給与細胞を用いて本酵素の細胞内局在性の解明を試みた。その結果，ほとんどの本酵素活性が細胞質画分に回収されたが，単離・精製したミトコンドリアと葉緑体にも本酵素活性が検出され，これらオルガネラにおいても本酵素が存在し，メチオニン代謝に関与する可能性が示唆された。

Abstract

To verify physiological roles of vitamin B_{12} (or cobalamin)-dependent methionine synthase in the vitamin B_{12}-requiring protozoan *Euglena gracilis* Z, the time course of changes in the enzyme activity during *Euglena* cell growth was studied. The enzyme activity of the vitamin B_{12}-sufficient *Euglena* cells reached a maximum (2.1nmol/min/10^6cells) at the early logarithmic growth phase and significantly decreased thereafter. While that of the vitamin B_{12}-deficient cells was significantly low (0.01-0.04nmol/min/10^6cells) during cell growth. Preliminary experiments indicated that most of the enzyme activity found in a homogenate of the cells grown for 2 days in the vitamin B_{12}-sufficient medium was recovered in the cytosolic fraction. The enzyme activity, however, was found in the percoll-purified mitochondria and chloroplasts, suggesting that the enzyme can function in methionine metabolism in the organelle.

Keywords : cobalamin, *Euglena gracilis* Z, methionine synthase, subcellular distribution, vitamin B_{12}

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1　目的

Euglena gracilis Zは紡錘形の形をした原生生物で，長さ約50μm，幅約10μmの大きさをしており，動物と植物の両方の特徴を有している[1]。*E. gracilis* Zは生育にビタミンB_{12}（B_{12}）を要求し[2]，細胞内にB_{12}を活発に取込み・蓄積する[2]ためB_{12}の生理機能解明のための研究材料として適している。

E. gracilis ZにはB_{12}依存性酵素としてN^5-メチルテトラヒドロ葉酸とホモシステインからメチオニンの生成を触媒するメチオニン合成酵素（EC 2.2.2.13)[3]，アミノ酸や奇数鎖脂肪酸の異化経路で*R*-メチルマロニル-CoAからスクシニル-CoAの異性化反応を触媒するメチルマロニルCoAムターゼ（EC 5.4.99.2)[4]，DNA合成に関与するリボヌクレオチドリダクターゼ（EC 1.17.4.2)[5]が存在している。

B_{12}依存性メチオニン合成酵素は生物界に広く分布し，哺乳動物の各種細胞では細胞質に局在する酵素として知られている[6]。一方*E. gracilis* Zにおいて本酵素は細胞質，ミトコンドリア，葉緑体の各オルガネラに分布することが報告[3]されているが，*E. gracilis* Zの本酵素は非常に不安定なため詳細な酵素化学的性質や生理機能についての知見はない。そこで，*E. gracilis* ZにおけるB_{12}依存性メチオン合成酵素の生理機能を解明する目的で生育に伴う本酵素活性の変動やB_{12}欠乏の影響について検討した。

2　実験方法

2－1　培養法

E. gracilis ZはKH培地[7]（B_{12}を5 μg/L含む）で光照射下（30 μE/m²/s）27℃で5日間振盪培養した。B_{12}欠乏細胞の調製は，KH培地で5日間前培養した*E. gracilis* Zの培養液1.0mLを無菌的にB_{12}を除去したKH培地150mLに接種し，同条件で5日間培養した。

2－2　細胞数の測定法

E. gracilis Zの細胞数は血球計算盤を用いて測定した。

2－3　粗酵素液の調製法

数日間生育させた*E. gracilis* Zの培養液（1～10mL）を無菌的にサンプリングした後，3,000 x*g* 10分間の遠心分離により*Euglena*細胞を集めた。細胞は蒸留水で2回洗浄後，10%（w/v）ショ糖を含む10mMリン酸カリウム緩衝液（pH7.0）に懸濁後，超音波処理により破砕した。細胞破砕液は10,000x*g* 30分間遠心分離し，その上清を粗酵素液として実験に用いた。全ての操作は2～4℃で行った。

2－4　ビタミンB_{12}依存性メチオニン合成酵素活性の測定法

本酵素活性の測定はHuangらの方法[8]を改良して行った。また，Banerjeeら[9]の半嫌気的酵素活性測定法に準じて酵素反応液を調製した。酵素反応液の組成は，100mMリン酸カリウム緩衝液（pH7.0），152 μM*S*-アデノシルメチオニン（シグマ社製），50mMヒドロキソB_{12}（シグマ社製），25mMアスコルビン酸，25mMジチオスレイトール，500 μM*L*-ホモシステイン（シグマ社製），25 μMN^5-メチルテトラヒドロ葉酸（シグマ社製），粗酵素液とし全容量を1.0mLとした。酵素反応液は，N^5-メチルテトラヒドロ葉酸を含まない反応液を予め調製し，37℃ 5分間保温し，本酵素を還元的に活性化させた。

その後，基質N^5-メチルテトラヒドロ葉酸を加え，37℃10分間酵素反応を行った。酵素反応終了後，直ちに100℃ 2分間の加熱処理により酵素反応を停止させた。酵素反応液は氷中で5分間冷却した後，遠心分離により変性タンパク質を除去した。この遠心分離上清液をメンブレンフィルター（Millex-LH，0.45 μm，ミリポア社製）でろ過し，ろ過液10 μLを高速液体クロマトグラフィー（HPLC）の試料とした。

HPLC分析システムは島津社製SCL-10Avpシステムコントローラー，LC-10ADvp HPLCポンプ，DGU-12Aデガッサー，CTO-10Avpカラムオーブン，C-R6Aクロマトパック，分光蛍光光度計RF-5000を用いた。HPLCの分析条件はTSK-GEL ODS-120A（4.6×250mm，東ソー社製）カラムを用い，カラム温度30℃，移動相7.0%（v/v）アセトニトリルを含む33mMリン酸カリウム緩衝液（pH3.0）を用い，流速0.5mL/minで分析した。酵素反応により生成したテトラヒドロ葉酸を励起波長290nm，吸収波長356nmで測定した。また，本酵素活性は対照の反応液（予め100℃ 5分間加熱処理した粗酵素液を用いて上述の反応液を調製し，直ちに100℃ 2分間の加熱処理を行った後，N^5-メチルテトラヒドロ葉酸を添加した反応液）中のテトラヒドロ葉酸量を差引き求めた。

2－5　タンパク質定量法

タンパク量はオボアルブミンを標準タンパク質としてバイオ(ラ)ッド社製プロテインアッセイ試薬を用いて定量した。

2－6　細胞分画法

生育2日目の培養液を3,000x*g*10分間の遠心分離により*Euglena*の細胞を集めた。細胞は蒸留水で2回洗浄後，0.33Mマンニトールを含む25mMグリシルグリシン-KOH緩衝液（pH7.4）に懸濁後，乳鉢・乳棒を用いて細胞を海砂と共に温和に摩砕することで細胞破砕液を調製した。細胞破砕液をガーゼでろ過し，3,000x*g*10分間の遠心分離上清画分を細胞抽出液とした。

細胞抽出液を5,000x*g*10分間遠心分離し，沈殿画分は0.33Mマンニトールを含む25mMグリシルグリシン-KOH緩衝液（pH7.4）で懸濁し，粗葉緑体とした。

上清画分はさらに11,000x*g*10分間遠心分離し，沈殿画分は0.25Mショ糖を含む25mMグリシルグリシン-KOH緩衝液（pH7.4）で懸濁し粗ミトコンドリアとした。また，上清画分は細胞質画分として実験に用いた。全ての操作は，2－4℃で行った。

粗ミトコンドリアおよび葉緑体はさらにWatanabeら[10]の方法でパーコール精製を行い無傷のミトコンドリアと葉緑体を調製した。酵素活性測定に際して各画分を超音波破砕し，粗酵素液として使用した。

3　結果と考察

B_{12}給与および欠乏*Euglena*細胞の生育に伴うB_{12}依存性メチオニン合成酵素活性の変動をFig. 1に示す。B_{12}給与細胞では生育2日目で対数増殖初期となり，生育4日目には定常期に達した。一方，B_{12}欠乏細胞では実験期間を通じて顕著な細胞増殖は確認できなかった。

B_{12}依存性メチオニン合成酵素活性はB_{12}給与細胞で生育2日目の対数増殖初期で最大（2.1nmol/min/10^6cells）となり，その後顕著に減少した。この結果は，対数増殖期の活発な細胞分裂に備えタンパク質合成に必須のメチオニンを供給するために本酵素が機能しているものと思われる。

一方，B_{12}欠乏細胞では生育期間を通して非常に低い酵素活性（0.01-0.04nmol/min/10^6cells）を示した。この結果は，B_{12}欠乏により不安定なアポ酵素が増加するためと考えられ，大腸菌[11]や哺乳動物[12]で同様の結果が報告されている。

Isegawaら[4]は生育6日目のB_{12}欠乏*Euglena*細胞を用いて本酵素の細胞内局在性を検討し，本酵素が細胞質，葉緑体，ミトコンドリアにそれぞれ68.9%（14.5pmol/min/mg protein），18.4%（7.6 pmol/min/mg protein），9.5%（6.6pmol/min/mg protein）の割合で存在していることを報告している。しかし，今回の実験結果から本酵素はB_{12}給与細胞の対数増殖初期にのみ高い活性が検出されたため，対数増殖初期のB_{12}給与細胞を用いて本酵素の細胞内局在性を検討する必要がある。

B_{12}欠乏*Euglena*細胞を用いたトリプシン消化法によるオルガネラの分画法[4]が確立されているが，B_{12}給与細胞の細胞膜複合体は強固なため本

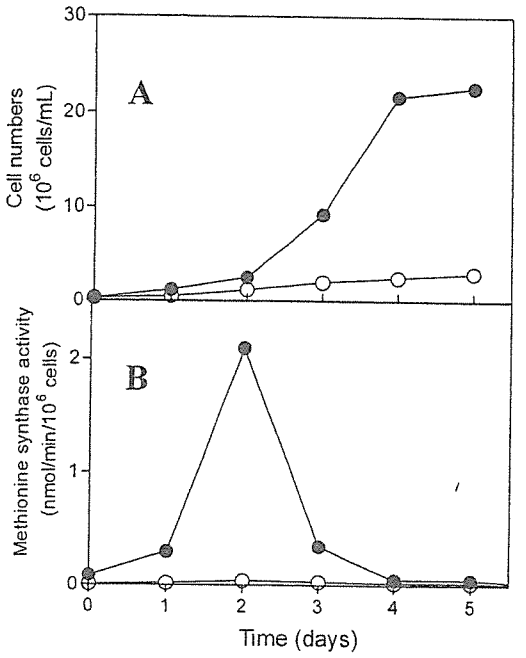

Fig. 1 Changes in the activity of vitamin B_{12}-dependent methionine synthase during cell growth of *E. gracilis* Z.
(A) cell growth, (B) methionine synthase activity. (●) B_{12}-sufficient cells, (○) B_{12}-defficient cells.

法を用いることができないので*Euglena*細胞を海砂で摩砕することで細胞を破砕し，分画遠心法によりミトコンドリアと葉緑体を分離した。その結果，本酵素活性の約99%が細胞質画分（5.0nmol /min/mg protein）に回収されたが，各オルガネラのマーカー酵素のクロスコンタミネーションによる補正を行っていないので正確な本酵素の細胞内分布とは言えず，今後さらにB_{12}給与細胞の細胞分画方法を含めて再検討する必要がある。また，パーコール精製した無傷のミトコンドリア（19.2nmol/min/mg protein）や葉緑体（1.2nmol /min/mg protein）に本酵素活性が検出されたことから，これらオルガネラにおいても本酵素が存在し，メチオニン代謝に機能していることが推測された。

B_{12}依存性メチオニン合成酵素はヒトを含めた哺乳動物の細胞で細胞質にのみ局在することが報告[6]されているが，今回の実験結果からミトコンドリアにも本酵素が存在する可能性が示唆され，哺乳動物での本酵素の細胞内局在性を再検討する必要があると思われる。

また，*E. gracilis* Zの各オルガネラに存在する本酵素の生理機能や代謝調節機構について生化学的および分子生物学的手法を用いて今後解明する予定である。

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Purification and characterization of methylmalonyl-CoA mutase from a photosynthetic coccolithophorid alga, *Pleurochrysis carterae*

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Abstract

Low activity (about 4 mU/mg protein) of 5′ -deoxyadenosylcobalamin-dependent methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) was found in a cell homogenate of a photosynthetic coccolithophorid alga, *Pleurochrysis carterae*. Most of the enzyme occurred as the apo-enzyme, which was labile during purification. The holo-enzyme, which was converted from the apo-enzyme by incubation with 10 μM 5′ -deoxyadenosylcobalamin at 4 °C in the dark, was purified to homogeneity and partially characterized. An apparent molecular mass for the enzyme of 150 ± 5 kDa was calculated by Superdex 200 pg gel filtration. SDS-polyacrylamide gel electrophoresis of the purified enzyme gave a single protein band with an apparent molecular mass of 80 ± 5 kDa, indicating that the *P. carterae* enzyme occurs as a homodimer. Some properties of methylmalonyl-CoA mutase from *P. carterae* were studied.

Keywords: Cobalamin; Coccolithophorid; 5′ -Deoxyadenosylcobalamin; Food supplement; Marine alga; Methylmalonyl-CoA mutase; *Pleurochrysis carterae*; Vitamin B_{12}

1. Introduction

The photosynthetic coccolithophorid alga, *Pleurochrysis carterae* (formerly *Hymenomonas carterae*) is a unicellular marine calcareous phototroph and produces $CaCO_3$ structures (calcified scales around the cells) (Takenaka et al., 1996). This marine alga absolutely requires vitamin B_{12} (cobalamin, Cbl) for growth (Provasoli and Pintner, 1953). Considerable amounts of Cbl (30–40 pg Cbl/10^5 cells) were taken up and accumulated by the algal cells grown in Cbl (10 μg/l)-supplemented medium. Most Cbl accumulated by the cells was converted to methylcobalamin (MeCbl; 46.1%) and 5′ -deoxyadenosylcobalamin (AdoCbl; 26.0%), which function as coenzyme of methionine synthase (EC 2.1.1.13) and methylmalonyl-CoA mutase (MCM; EC 5.4.99.2), respectively (Miyamoto et al., 2002b).

The AdoCbl-dependent MCM that catalyzes isomerization of (*R*)-methylmalonyl-CoA to succinyl-CoA has been purified and characterized from mammalian tissues (Fenton et al., 1982), an intestinal nematode (Han et al., 1984), and aerobic (Miyamoto et al., 2002a, 2003) and anaerobic bacteria (Francalanci et al., 1986). To elucidate the mechanisms for the enzyme reaction, the anaerobic bacterium *Propionibacterium shermanii* MCM has been studied extensively (Mancia and Evans, 1998; Mancia et al., 1999). However, little information is available on the enzymological properties of MCM in marine algae.

Here, we describe the purification and characterization of MCM from the photosynthetic coccolithophorid alga *P. carterae* from the viewpoint of comparative biochemistry.

2. Materials and methods

2.1. Culture and organism

P. carterae was provided by Prof. M. Okazaki (Tokyo Gakugei University, Japan). Algal cells were cultured for 10 days at 26 °C in a modified Eppley medium (containing 20 μg cyanocobalamin/l; Miyamoto et al., 2002b) prepared with synthetic seawater. The culture (1.5 l) was bubbled with air and illuminated at 40 μmol photon/m^2/s.

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2.2. Enzyme assay

Methylmalonyl-CoA mutase was assayed by a modification of the HPLC method described by Gaire et al. (1999). Briefly, the assay mixture (0.15 ml) for total MCM activity contained 100 mM potassium phosphate buffer, pH 7.5, 30 μM AdoCbl (Sigma, St. Louis, MO, USA), 0.15 mM (*R,S*)-methylmalonyl-CoA (Sigma), and enzyme. AdoCbl was replaced by the same volume of distilled water for measuring holo-MCM activity. Apo-MCM activity was calculated by subtracting the holo-MCM activity from total MCM activity in the sample. The components except for (*R,S*)-methylmalonyl-CoA were mixed in microcentrifuge tubes in the dark and the temperature was equilibrated by incubation in a heating bucket (e-Heating Bucket, Taitec, Saitama, Japan) maintained at 30 °C. The reaction mixture was preincubated for 5 min, started by the addition of (*R,S*)-methylmalonyl-CoA, and left for 5 min. The enzyme reaction was stopped by the addition of 50 μl 10% (w/v) trichloroacetic acid. The reaction mixture was filtered through a 0.45-μm membrane filter (Millex Syringe Driven Filter Unit, LH-type, Millipore, USA). Aliquots (20 μl) of the filtrate were analyzed by HPLC using a Shimadzu HPLC apparatus (two LC-10ADvp pumps, DGV-12A degasser, SCL-10Avp system controller, SPD-10Avvp UV–VIS detector, CTO-10Avp column oven, 100 μl sample loop, C-R6A chromatopac integrator). The sample (20 μl) was put on a reversed-phase HPLC column (Cosmosil 5C18-AR-II, ∅ 3.0 × 150 mm) equilibrated with 50% (v/v) solvent A (100 mM acetic acid in 100 mM potassium phosphate buffer, pH 7.0) and 50% (v/v) solvent B [18% (v/v) methanol in solvent A]. (*R,S*)-Methylmalonyl-CoA and succinyl-CoA were eluted with a linear gradient of methanol [50–100% (v/v) solvent B] for 7.0 min at 40 °C and assayed by measurement of absorbance at 254 nm. The flow rate was 1.0 ml/min. MCM activity was calculated from the amount of succinyl-CoA formed. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of succinyl-CoA at the rate of 1 μmol/min.

2.3. Purification of P. carterae MCM

Purification was performed at 0–4 °C with a BioLogic HR chromatography system (Bio-Rad Laboratories, Hercules, CA, USA) in a dark room. The stored *P. carterae* cells (about 15 g wet mass) were suspended in 30 ml, 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose. The cells were disrupted by sonic oscillation (10 kHz, 10 s × 6) and centrifuged at 10,000 × *g* for 10 min. AdoCbl was added to the supernatant fraction at a final concentration of 10 μM and left overnight in the dark at 4 °C to form the holo-enzyme. The treated solution was put on a column (2.4 × 20 cm) of TSKgel QAE-Toyopearl HW55C equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose, and eluted with 300 ml of a linear gradient (0–0.5 M) of KCl in the same buffer. The active fractions were collected and concentrated in a Centriprep-30 centrifugal concentrator (Millipore, Bedford, MA, USA) to a final volume of 1.0 ml. The concentrated solution (1.0 ml) was put on a HiLoad 16/60 Superdex 200 pg column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose and 200 mM KCl, and eluted with the same buffer. The active fractions were combined and dialyzed overnight against 1 l of 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose. The dialyzed solution was concentrated in a Centricon-30 microconcentrator (Millipore) to a final volume of 1.0 ml. The concentrated solution (1.0 ml) was put on a UNO Q-1 column (Bio-Rad) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose, and eluted with 40 ml of a linear gradient (0–0.5 M) of KCl in the same buffer. The active fractions were combined and desalted in Centricon-30 microconcentrators. The solution was put on a Mono Q column HR 5/5 (Amersham Pharmacia Biotech) and eluted with 40 ml of a linear gradient (0–0.5 M) of KCl in the same buffer. The peak fraction of the enzyme activity was concentrated to a final volume of 0.1 ml as above and stored at −80 °C.

2.4. Polyacrylamide gel electrophoresis in the presence or absence of SDS

Polyacrylamide gel electrophoresis (PAGE) was carried out on 5–20% precast Ready Gels J (Bio-Rad, Tokyo, Japan). Purified enzyme (1 μg protein) was electrophoresed in the presence or absence of SDS at constant current (12 mA/gel) with bromophenol blue as a migration marker. After electrophoresis on the slab gel, proteins in the gel were stained with Coomassie brilliant blue R-250 and destained in acetic acid solution according to the manufacturer's instructions. Standard proteins (phosphorylase *b* from rabbit muscle, 97.4 kDa; albumin from bovine serum, 66.2 kDa; ovalubmin, 45.0 kDa; carbonic anhydrase from bovine erythrocyte, 31.0 kDa; trypsin-inhibitor from soybeans, 21.5 kDa; lysozyme from egg whites, 14.4 kDa) in a Bio-Rad kit were used for the calibration of the molecular mass of the subunit of *P. carterae* MCM.

2.5. Gel filtration experiments

The molecular mass of *P. carterae* MCM was determined with a HiLoad 16/60 Superdex 200 pg gel filtration column (Amersham Pharmacia Biotech) using the BioLogic HR chromatography system (Bio-Rad). The column was equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose and 200 mM KCl, and eluted with the same buffer. The molecular mass of the MCM was calibrated with blue dextran (average 2000 kDa), horse spleen apoferritin (480 kDa), yeast alcohol dehydrogenase

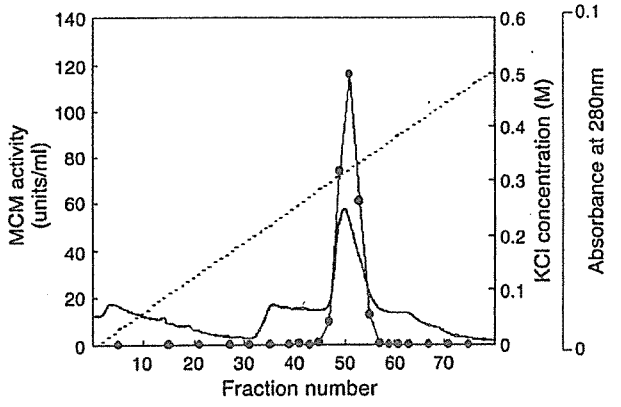

Fig. 1. Elution patterns of *Pleurochrysis carterae* methylmalonyl-CoA mutase activity during Mono-Q column chromatography.(−) absorbance at 280 nm; (●) MCM activity; and (---) KCl concentration. The data are typical elution patterns of the enzyme activity during Mono-Q HR 5/5 column chromatography from four independent experiments.

(150 kDa), bovine serum albumin (66.0 kDa), and horse heart cytochrome *c* (12.4 kDa). Blue dextran and the proteins were monitored by measuring the absorbance at 280 nm.

2.6. Properties of MCM

2.6.1. Optimum temperature and pH

The optimum temperature of the enzyme was determined by incubating at temperatures ranging from 5 to 50 °C for 5 min at pH 7.5. In the case of the thermal stability of the enzyme, the enzyme activity was assayed at 30 °C for 5 min at pH 7.5 after the purified enzyme was preincubated at temperatures ranging from 5 to 50 °C for 10 min at pH 7.0.

The optimum pH of the enzyme was determined by incubation at various pH values ranging from 5.0 to 9.5 in 10 mM Tris–acetate buffer for 5 min at 30 °C.

2.6.2. Effect of SH-inhibitors

Enzyme activity was assayed at 30 °C for 5 min at pH 7.5 using the purified enzyme treated with 3 mM of SH-inhibitors [$HgCl_2$, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, 5,5′ -dithiobis-(2-nitrobenzoic acid), and iodoacetoamide] at 30 °C for 10 min at pH 7.0.

2.6.3. Effect of monovalent and divalent cations

Enzyme activity was assayed at 30 °C for 5 min at pH 7.5 in the presence of each monovalent and divalent cation (NaCl, KCl, NH_4Cl, $CoCl_2$, $MnCl_2$, $FeCl_2$ and $MgCl_2$ all at 3 mM).

2.6.4. Effect of substrate concentrations

In the case of determination of K_m values for methylmalonyl-CoA and succinyl-CoA (reverse reaction), the enzyme activity was assayed at various concentrations of (*R,S*)-methylmalonyl-CoA or succinyl-CoA under the standard assay conditions.

2.7. Protein assay

Protein was assayed by the use of Bio-Rad protein assay kit, with ovalbumin as a standard according to the manufacturer's instructions.

3. Results and discussion

3.1. MCM activity in a cell homogenate of P. carterae

MCM activity was considerably lower in a cell homogenate of *P. carterae* (about 4 mU/mg protein) grown for 10 days (stationary phase) than in those of the other organisms (about 25–50 mU/mg protein) previously reported (Kellermeyer et al., 1964; Han et al., 1984; Miyamoto et al., 2002a). Low specific activity (2.6 mU/mg protein) of this enzyme is also found in a cell homogenate of *Euglena gracilis* Z (a fresh water-Cbl-requiring alga) grown photoautotrophically; it is significantly increased in those cells adapted to propionate-supplemented medium (Watanabe et al., 1996).

Most (about 95%) of the *P. carterae* enzyme occurred as the apo-enzyme. Our preliminary experiments indicated that the enzyme activity, when assayed in the presence of AdoCbl, was completely lost through several column chromatographies. As a consequence of the lability of the apo-enzyme, the enzyme protein could not be purified to homogeneity. The holo-enzyme, which was converted from the apo- enzyme by incubation with 10 μM AdoCbl at 4 °C overnight in the dark, was considerably more stable relative to the apo-enzyme. Thus, the holo-enzyme was purified to homogeneity.

3.2. Purification of P. carterae MCM

Fig. 1 shows the elution profile of *P. carterae* MCM during Mono-Q HR 5/5 column chromatography (the final purification step). The enzyme activity gave a single peak at 0.35 M KCl and most of the protein was recovered in this fraction. The purification procedures for MCM from a homogenate of *P. carterae* are summarized in Table 1.

Table 1
Purification of methylmalonyl-CoA mutase from *P. carterae*

Step	Total protein (mg)	Total activity (units/min)	Specific activity (units/mg protein)	Yield (%)
Crude homogenate	871.9	3.54	0.004	100.0
TSKgel QAE-Toyopearl 550	84.6	2.33	0.03	65.9
HiLoad 16/60 Superdex 200 pg	2.72	0.51	0.19	14.5
UNO Q-1	0.30	0.17	0.58	4.9
Mono-Q	0.003	0.03	11.94	0.87

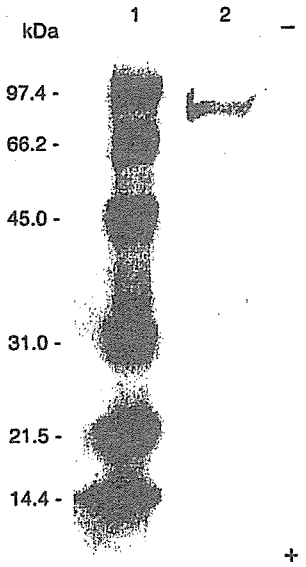

Fig. 2. PAGE of methylmalonyl-CoA mutase from the final purification step. (1) Molecular mass standard proteins; (2) the purified enzyme in the presence of SDS. Typical migration patterns of the purified enzyme after polyacrylamide slab gel electrophoresis in the presence of SDS from three independent experiments.

MCM was purified about 2985-fold over the homogenate with a yield of 0.87%. PAGE of the purified enzyme in the absence of SDS using a precast gel showed a single protein band (data not shown).

3.3. Molecular mass

An apparent molecular mass of the enzyme was calculated to be 150 ± 5 kDa by Superdex 200 pg gel filtration. SDS-PAGE of the purified enzyme gave a single protein band with an apparent molecular mass of 80 ± 5 kDa, indicating that the *P. carterae* enzyme is composed of two identical subunits (Fig. 2).

P. carterae MCM has a similar subunit structure to the mammalian (Fenton et al., 1982) and worm (Han et al., 1984) enzymes, while the *P. shermanii* (Francalanci et al., 1986) and *Methylobacterium extorquens* NR-1 (Miyamoto et al., 2002a) enzymes consist of two non-identical subunits with molecular masses of 79–85 and 67–70 kDa, respectively.

3.4. Some properties of MCM

The optimum temperature for MCM activity was 30 °C. The enzyme, when incubated at various temperatures for 10 min at pH 7.5, was stable up to 10 °C; activity was completely lost at 50 °C. The optimum pH of the enzyme was 7.5.

The apparent K_m values of the enzyme were 0.59 mM for (*R,S*)-methylmalonyl-CoA and 5.5 mM for succinyl-CoA

The purified enzyme activity was inhibited completely by the addition of 3 mM $HgCl_2$, but not by the addition of commonly used SH-inhibitors, iodoacetamide, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid and 5,5′ -dithiobis-(2-nitrobenzoic acid).

MCM activities of *P. shermanii* (Kellermeyer et al., 1964), *M. extorquens* NR-1 (Miyamoto et al., 2002a) and intestinal worm (Han et al., 1984) are not affected by the SH-inhibitors, but those of *Sinorhizobium meliloti* (Miyamoto et al., 2003) and mammals (Fenton et al., 1982) are considerably inhibited.

Although the *S. meliloti* MCM (Miyamoto et al., 2003) is activated by the addition of some monovalent cations (NH_4^+, K^+ and so on), the addition of monovalent (Na^+, K^+ and NH_4^+) and divalent cations (Co^{2+}, Mn^{2+}, Fe^{2+} and Mg^{2+}) at 3 mM did not affect the enzyme activity of *P. carterae*. The identical result has been reported in MCM of other organisms (Fenton et al., 1982; Kellermeyer et al., 1964; Miyamoto et al., 2002a).

Due to the low specific activity of the enzyme in the cell homogenate and lability of the enzyme during purification, we could not obtain enough enzyme to study further detailed enzyme properties. However, these results presented here indicate for the first time that the marine alga *P. carterae* contains an AdoCbl-dependent homodimeric MCM.

Acknowledgements

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