Requirement for Vitamin B₁ for Growth of *Euglena gracilis*

By SHIGERU SHIGEOKA,¹* TOSHIO ONISHI,¹ YOSHIHISA NAKANO²
AND SHOZABURO KITAOKA²

¹ Department of Food and Nutrition, Kinki University, Higashiosaka, Osaka 577, Japan

² Department of Agricultural Chemistry, University of Osaka Prefecture, Sakai, Osaka 591, Japan

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Euglena gracilis Z showed an absolute requirement for vitamin B₁ for growth. Increase of cell number in vitamin B₁-deficient cultures occurred on the addition of vitamin B₁ and depended on the amount added. The phosphate esters of vitamin B₁ also supported growth. *E. gracilis* cells exhaustively took up vitamin B₁ within about 2 h of addition. Of the total amount of vitamin B₁ taken up, as much as 96% existed in the free form irrespective of the forms added. The addition of 4-amino-5-hydroxymethyl-2-methylpyrimidine, but not 5-(2-hydroxyethyl)-4-methylthiazole, to the vitamin B₁-deficient cells enhanced cell number at the same rate as the addition of vitamin B₁, indicating that *E. gracilis* Z is unable to synthesize the pyrimidine moiety of vitamin B₁.

INTRODUCTION

For the thiamin-pyrophosphate-dependent (TPP-dependent) decarboxylation of 2-oxo acids, *Euglena gracilis* Z contains an O₂-sensitive, NADP⁺-dependent pyruvate dehydrogenase distinct from the pyruvate dehydrogenase complex (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3) of other organisms (Inui *et al.*, 1984). In addition, the 2-oxoglutarate dehydrogenase complex (EC 1.2.4.2 + EC 2.3.1.61 + EC 1.6.4.3) requires TPP and MgCl₂ for maximum activity. However, we have reported recently that TPP-dependent 2-oxoglutarate decarboxylase occurs in mitochondria of *E. gracilis*, which lacks the 2-oxoglutarate dehydrogenase complex, and the complex activity reported by Inui *et al.* (1984) is due to the new decarboxylase coupling with NAD(P)⁺-dependent succinate semialdehyde dehydrogenase (EC 1.2.1.16) (Shigeoka *et al.*, 1986). Consequently, *E. gracilis* has an alternative route in the tricarboxylic acid cycle which involves two enzymes.

In this context the requirement for vitamin B₁ by *E. gracilis* Z must be elucidated. Cook (1968) reported that *Euglena* requires both vitamin B₁ and vitamin B₁₂ for normal growth. Vitamin B₁₂ depletion in *Euglena* causes cell enlargement accompanied by an arrest of cell division; replenishment of vitamin B₁₂ allows the cell to return to the normal state (Carell, 1969; Kempner, 1982). On the other hand, vitamin B₁ is not universally required among *Euglena* species: *E. gracilis* var. *bacillaris* requires vitamin B₁, but not *E. spirogyra* (Cramer & Myers, 1952; Leedale *et al.*, 1965). The extent and cause of the requirement for vitamin B₁ and/or its phosphate esters by *E. gracilis* Z is not known. Here we report that *E. gracilis* Z absolutely requires vitamin B₁ for growth, that 4-amino-5-hydroxymethyl-2-methylpyrimidine (OMP) but not 5-(2-hydroxyethyl)-4-methylthiazole (Th) can remedy vitamin B₁ deficiency, and that vitamin B₁ taken up by *E. gracilis* exists in the free form and not as its phosphate esters.

Abbreviations: CV, coefficient of variation; OMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; Th, 5-(2-hydroxyethyl)-4-methylthiazole; TMP, thiamin monophosphate; TPP, thiamin pyrophosphate; TTP, thiamin triphosphate.

METHODS

Organism and culture. *Euglena gracilis* Z was maintained aseptically in 150 ml Koren & Hutner (1967) medium, containing 100 µg vitamin B₁, which was prepared by dissolving 20 mg in 10 ml 70% (v/v) methanol. Cells were cultivated under illumination (3000 lx) at 27 °C with shaking (90 strokes min⁻¹) for 4 d, by which time the stationary phase was reached. These cells were termed vitamin B₁-sufficient.

Preparation of vitamin B₁-limited and -deficient cells. Vitamin B₁-limited cells were obtained by transferring vitamin B₁-sufficient cells (12×10^6) to basal medium (150 ml) without vitamin B₁ and culturing them for 5 d. Cultivation for obtaining vitamin B₁-deficient cells was done in the same manner as above by using the vitamin B₁-limited cells (5×10^6). Cell number (counted with a haemocytometer) was about 10^6 ml⁻¹ after 4–5 d.

Determination of vitamin B₁ and its phosphate esters. Cells were harvested, suspended in 5 ml 5% (w/v) trichloroacetic acid, and disrupted by sonication (10 kHz, 2 min). The sonicate was centrifuged at 10000 g for 15 min, and the supernatant washed three times with 2 vols diethyl ether. The aqueous phase was then used for the assay of vitamin B₁ and its phosphate esters. The concentrations of these compounds remaining in the medium were assayed directly as described below.

To 0.8 ml of a sample were added 0.1 ml 0.3 M-cyanogen bromide and 0.1 ml 1 M-sodium hydroxide. The mixture was agitated thoroughly to convert vitamin B₁ and its phosphate esters into the corresponding thiochromes. Separation of thiochromes was done by HPLC according to Ishii *et al.* (1979) with modifications. The reaction mixture (20 µl) was immediately applied onto an HPLC apparatus (Shimadzu LC-5A) equipped with a stainless steel column (4.6 × 250 mm) packed with LiChrosorb-NH₂ (10 µm, Merck). The mobile phase was acetonitrile/90 mM-potassium phosphate buffer (pH 7.5) (60:40, v/v). The flow rate was 2.0 ml min⁻¹ and the column oven was maintained at room temperature. The eluate was monitored fluorometrically by excitation at 365 nm and emission at 430 nm. Thiochromes of vitamin B₁, thiamin monophosphate (TMP), TPP and thiamin triphosphate (TTP) had retention times of 1.38, 3.56, 6.70 and 10.34 min, respectively. The relation of peak area to the quantity of each compound injected was linear from 1 to 100 pmol. The recoveries of vitamin B₁, TMP and TPP added to the harvested cells as internal standards were 100.1, 102.4 and 100.1%, respectively.

Chemicals. Vitamin B₁, TMP and TPP were purchased from Sigma. OMP and Th were kindly supplied by Dr Suzuki, Shimane University. TPP was a gift from Dr Okada, Takeda Industry Co. Ltd, Japan.

RESULTS

Growth under vitamin B₁ sufficiency, limitation and deficiency. Fig. 1 shows the growth curves of vitamin B₁-sufficient, -limited and -deficient *E. gracilis* Z cultures. Both vitamin B₁-limited and vitamin B₁-sufficient cells reached stationary phase in about 4 d, but the maximum growth of the former was about 40% of that of the latter. Transfer of the vitamin B₁-limited cells into 27 nM-vitamin B₁ (Fig. 3), the optimal amount of vitamin B₁ required for maximal growth was number was about 10^6 ml⁻¹ at stationary phase.

Cellular contents of vitamin B₁ and its phosphate esters. Table 1 shows the contents of vitamin B₁ and its phosphate esters in *Euglena* cells in stationary phase after growth under the three conditions. The vitamin B₁-deficient cells contained little vitamin B₁ and only traces of TMP and TPP. TTP was not detected during the growth of *E. gracilis* under any conditions.

Requirements for vitamin B₁ and its phosphate esters for growth. When vitamin B₁ (60 nM) was fed to vitamin B₁-deficient cells, cell division commenced and cell number reached the same level as with vitamin B₁-sufficient cells in 2 d (Fig. 2). The proliferation of vitamin B₁-deficient cells was dependent on the amount of vitamin B₁ added. Thus the added vitamin B₁ affected the doubling time during the exponential phase and the total cell number once the stationary phase had been attained. Since the maximum increase of cell number was obtained by the addition of 27 nM-vitamin B₁ (Fig. 3), the optimal amount of vitamin B₁ required for maximal growth was calculated as 7.7×10^{-16} g per cell. The addition of TMP or TPP to the vitamin B₁-deficient cells also allowed cell division to begin. Proportionality between the increase of cell number and the amount of TMP and TPP supplied was observed up to 334 nM and 1050 nM, respectively. The requirements for TMP and TPP for optimal growth were 1.1×10^{-14} and 5.9×10^{-14} g per cell, respectively.

Requirement for OMP and Th. Supply of OMP to vitamin B₁-deficient cells grown for 4 d caused a rapid enhancement of cell growth at the same rate as observed with vitamin B₁,

Fig. 1. Growth curves of vitamin B₁-sufficient (●), -limited (■) and -deficient (▲) *E. gracilis* Z. Each point represents the mean of four assays (CV% ≤ 5).

Fig. 2

Fig. 3

Fig. 2. Effects of vitamin B₁, OMP and Th on growth of vitamin B₁-deficient *E. gracilis* Z. Each compound was added at 60 nM to vitamin B₁-deficient cells grown for 4 d. OMP was also added to the cells prefed Th for 2 d. Each experimental point represents the mean of four assays (CV% ≤ 5). ▲, No vitamin B₁ added (deficient cells); ○, + vitamin B₁; ■, + OMP; △, + Th; □, prefed Th + OMP.

Fig. 3. Requirement for vitamin B₁ for *Euglena* growth. Vitamin B₁-deficient cultures received vitamin B₁ at various concentrations as shown on the abscissa. The number on the ordinate shows the increase of cell number after 48 h compared to vitamin B₁-deficient cultures. Each point represents the mean of four assays (CV% ≤ 5).

replenishment (Fig. 2). The addition of Th to the deficient cells did not affect cell number, whereas the addition of OMP to cells prefed Th for 2 d allowed cell division to occur.

Uptake of vitamin B₁ and its phosphate esters and conversion by *E. gracilis*. When 1 μM-vitamin B₁ was added to vitamin B₁-deficient cultures, the cellular content of vitamin B₁ increased linearly to reach a peak after 2 h and then gradually decreased (Fig. 4). The vitamin B₁ was actively taken up by *E. gracilis* for 2 h and no leakage from the cells to the medium was found thereafter. The cellular content of TPP increased on the addition of vitamin B₁, reaching a stationary level after about 4 h. The cellular content of TMP did not change during vitamin B₁ uptake.

When 150 nmol TMP or TPP was fed to vitamin B₁-deficient cells, which were suspended in 150 ml 20 mM-Tris/HCl buffer, pH 7.5, these compounds accumulated in the cells in the form of free vitamin B₁, but not in the ester forms (Fig. 5). The cellular content of vitamin B₁ increased linearly up to 48 h after addition of TMP or TPP and then reached a stationary level. The contents of TMP and TPP changed little. The addition of 1 μM-OMP also caused an increase in intracellular vitamin B₁ content.

Fig. 4. Changes in the levels of vitamin B₁ and its phosphate esters in vitamin B₁-supplemented cells and medium. Vitamin B₁ (1 μ M) was added to vitamin B₁-deficient *E. gracilis* Z grown for 4 d. Each point represents the mean of five assays (CV% $\leq$ 8). ●, Vitamin B₁ in cells; ■, TMP in cells; ▲, TPP in cells; ○, vitamin B₁ in medium; □, cell number.

Fig. 5. Effect of addition of TMP or TPP on contents of vitamin B₁ and its phosphate esters in cells and medium. Vitamin B₁-deficient cells grown for 4 d were aseptically resuspended to 10^6 cells ml⁻¹ in 20 mM-Tris/HCl buffer, pH 7.5. TMP or TPP was added to 1 μ M to the deficient cultures. Each point represents the mean of four assays (CV% $\leq$ 8). ●, Vitamin B₁ in cells; ■, TMP in cells; ▲, TPP in cells; □, TMP in medium; △, TPP in medium.

Table 1. Content of vitamin B₁ and its phosphate esters in vitamin B₁-sufficient, -limited and -deficient *Euglena gracilis* Z

Concentrations are expressed as pmol per 10^6 cells (mean $\pm$ SD, $n = 5$). Values in parentheses represent the content as a percentage of the total vitamin B₁.

Cells	B ₁	TMP	TPP	TTP
B ₁ -sufficient	74.5 $\pm$ 3.7 (70.0)	2.9 $\pm$ 0.1 (2.7)	29.1 $\pm$ 1.1 (27.3)	ND
B ₁ -limited	12.1 $\pm$ 0.3 (91.0)	trace	1.2 $\pm$ 0.1 (9.0)	ND
B ₁ -deficient	5.5 $\pm$ 0.2	trace	trace	ND

ND, Not detected.

DISCUSSION

For the decarboxylation of 2-oxo acids, *E. gracilis* Z contains unique TPP-dependent pyruvate dehydrogenase (Inui *et al.*, 1984) and 2-oxoglutarate decarboxylase (Shigeoka *et al.*, 1986). However, the requirement for vitamin B₁ by *E. gracilis* has not been established. The results presented here clearly demonstrate that vitamin B₁ is required absolutely for the growth of *E. gracilis* Z.

When fed to vitamin B₁-deficient cells, 96% of the total vitamin B₁ accumulated as the free form to attain a concentration of 1 mM. Vitamin B₁ uptake is mediated by an active transport system and accumulates as the free form in various micro-organisms (Iwashima *et al.*, 1973; Henderson & Zerely, 1978) and in the small intestine (Komai *et al.*, 1974) and isolated hepatocytes (Chen, 1978; Yoshioka, 1984) of rats. The cellular content of vitamin B₁ at 2 h gradually decreased to reach about half of the maximum level in 8 h. The fact that this decline was not offset by an increase of TPP suggests that some metabolism of vitamin B₁ may occur in *E. gracilis* Z. Further support for this possibility is given by the presence of 74.5 pmol vitamin B₁ per 10⁶ cells in the sufficient cells after 5 d growth (Table 1). This corresponds to 0.3 µg (ml medium)⁻¹, compared to the initial content of 0.67 µg ml⁻¹. The content of TPP at 4 h corresponded to about 30 pmol TPP per 10⁶ cells, suggesting that vitamin B₁-deficient cells promptly synthesize TPP to supply the coenzymes for 2-oxoglutarate decarboxylase (Shigeoka *et al.*, 1986), pyruvate dehydrogenase (Inui *et al.*, 1984) and other enzymes.

Although vitamin B₁-deficient cells grew upon addition of TMP or TPP, the requirements for optimal growth were about 12- and 39-fold higher, respectively, than that for vitamin B₁. Since free vitamin B₁ predominated in the cells after addition of TMP or TPP, this suggests that the esters were subjected to hydrolytic cleavage prior to incorporation into the cells as the vitamin B₁ form. This system resembles that in yeasts in which exogenous vitamin B₁ phosphate esters were converted to free vitamin B₁ by phosphatases located on the cell membrane (Nishimura *et al.*, 1982).

Vitamin B₁-requiring micro-organisms are divided into three types: those that utilize vitamin B₁ or its pyrimidine portion, those that utilize either vitamin B₁ or its thiazole portion, and those that utilize vitamin B₁ alone (Brown, 1972). The supply of OMP to vitamin B₁-deficient cells of *E. gracilis* Z caused rapid cell growth, whereas Th had no effect indicating that this organism requires either vitamin B₁ or its pyrimidine portion for growth. In addition, the increase in cellular vitamin B₁ content after the addition of OMP suggested that *E. gracilis* Z possesses an enzyme system which synthesizes vitamin B₁ from the exogenous pyrimidine moiety and endogenous thiazole moiety.

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