Different corrinoid specificities for cell growth and the cobalamin uptake system in *Euglena gracilis* z

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A variety of cobalamin analogues synthesized chemically or microbiologically were used to study the corrinoid specificities of cell growth and the cobalamin uptake system in *Euglena gracilis* z. Although *Euglena* could not utilize most of the analogues for cell growth, benzimidazolyl cobamide, cyanocobalamin-*O*⁵-phosphate, and cyanocobalamin-*b*-, *-d*- and *-e*-monocarboxylates had effects similar to that of cyanocobalamin on cell growth. It is suggested that *Euglena* cells have the ability to synthesize 'complete cobalamin' from the acid derivatives (amidation reaction) and/or the phosphate derivative (dephosphorylation reaction). Inhibition of uptake of radiolabelled cyanocobalamin caused by addition of various analogues indicates that both the α -lower axial ligand (the cobalt-coordinated nucleotide) and the (*b*)-propionamide side-chain of the cobalamin molecule are essential for the cobalamin uptake system in *Euglena*. These results indicate that there are different corrinoid specificities for cell growth and the cobalamin uptake system in *E. gracilis* z.

Introduction

Euglena gracilis z, a photosynthesizing unicellular protozoan, has characteristics of both animal and plant cells (Wolken, 1967). *Euglena* cells require cobalamin (Cbl) for growth and have the ability to take up and accumulate exogenous Cbl (Ross, 1952; Varma *et al.*, 1961). The kinetics of Cbl uptake in *Euglena* are biphasic, as in *Escherichia coli* (DiGirolamo & Bradbeer, 1971; Bradbeer & Woodrow, 1976) and the protozoan *Ochromonas malhamensis* (Bradbeer, 1971). *Euglena* pellicle (a cell-membrane complex) fragments isolated by sucrose density-gradient centrifugation contain Cbl-binding proteins which are involved in the Cbl uptake system (Watanabe *et al.*, 1988a).

In addition to Cbl-dependent enzymes (methionine synthase, methylmalonyl-CoA mutase and ribonucleotide reductase), *E. gracilis* z contains numerous non-enzymic Cbl-binding proteins, which are distributed in the cytosolic, mitochondrial, chloroplastic and microsomal fractions (Isegawa *et al.*, 1984). Some of these Cbl-binding proteins have been purified and characterized

(Watanabe *et al.*, 1987a, b; 1988a, b). Thus, *Euglena* cells have unique intracellular Cbl transfer and/or accumulation system(s), which differ from both mammalian and bacterial systems (Watanabe & Nakano, 1991).

In this study, we demonstrate the corrinoid specificities of *Euglena* cell growth and the Cbl uptake system, and discuss why there are differences between the two in this organism.

Methods

Organism and culture. *Euglena gracilis* z was normally cultured for 5 d at 27 °C with illumination (50 $\mu\text{E m}^{-2} \text{s}^{-1}$) in Koren-Hutner medium (CN-Cbl, 5 μg per litre of medium) (Koren & Hutner, 1967). For Cbl uptake experiments and Cbl analogue-feeding experiments, we used *Euglena* cells grown in Cbl-limited medium A (33.3 ng l⁻¹) or medium B (0.22 ng l⁻¹), to reduce their cellular Cbl contents.

Corrinoid compounds (see Table 1). CN-Cbl, OH-Cbl, CH₃-Cbl, AdoCbl and (CN)CN-Cbl were obtained from Sigma. [G-³H]CN-Cbl [9.2 Ci mmol⁻¹ (340 GBq mmol⁻¹)] was obtained from Du Pont-NEN. (2-AMP)CN-Cbl was kindly provided by Dr T. Kamikubo (Kobe Women's University, Japan).

(Bza)CN-Cba, (5-OH-Bza)CN-Cba, (Ade)CN-Cba and (*p*-Cre)CN-Cba were prepared microbiologically and purified as described previously (Stupperich *et al.*, 1986, 1988, 1990; Stupperich & Krautler, 1988).

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Abbreviation: Cbl, cobalamin.

CN-Cbl(*b*-OH), CN-Cbl(*d*-OH) and CN-Cbl(*e*-OH) were prepared as described by Yamada & Hogenkamp (1972) and Toraya *et al.* (1979). The CN-Cbl(*b*-OH), (*d*-OH) and (*e*-OH), which were prepared and designated, have been reported to be CN-Cbl(*d*-OH), (*e*-OH) and (*b*-OH), respectively, as determined by ^{13}C -NMR analysis (Anton *et al.*, 1980). The revised designations of the acid derivatives were used in the experiments described in this paper. CN-Cbl(13-epi) (Bonnett *et al.*, 1971*a*), CN-Cbl(*c*-lactam) (Bonnett *et al.*, 1957) and CN-Cbl(5'-*O*-P) (Friedmann, 1971) were prepared by the methods described in the references cited. CN-Cbl(5'-*O*-Suc) and CN-Cbl[2'-*O*,5'-*O*-(Suc)₂] were prepared as described by Toraya *et al.* (1975*a*).

Each of the CN-Cbl derivatives was further purified by HPLC using a Shimadzu HPLC apparatus (LC-7A pump, SPD-7AV spectrophotometer) and a Hitachi chromato-data processor (D-2500). The sample was put on a reversed-phase HPLC column (Wakosil 5C18-200; 4.6 × 150 mm) and isocratically eluted with water/methanol/acetic acid (79:20:1, by vol.) at a flow rate of 1.0 ml min⁻¹. Corrinoids were monitored by measuring the absorbance at 361 nm. Each of the CN-Cbl derivatives gave a single peak in HPLC. The purity of the Cbl analogues was further checked as follows. CN-Cbl(*b*-OH), (*d*-OH) and (*e*-OH) were checked by TLC on precoated silica gel plates with 2-butanol/acetic acid/water (127:1:50, by vol.) or 2-propanol/NH₄OH/water (7:1:2, by vol.) as the solvent (Toraya *et al.*, 1979). CN-Cbl(13-epi) was characterized by paper chromatography with 1-butanol/ethanol/water (50:15:35, by vol.) or 1-butanol/2-propanol/water (37:36:37, by vol.) as the solvent (Tkachuck *et al.*, 1974). CN-Cbl(*c*-lactam) was checked by paper chromatography with 2-butanol containing aqueous ammonia solution as the solvent (Bonnett *et al.*, 1957). The phosphate derivative was checked by TLC on silica gel with 2-butanol/acetic acid/water (127:1:50, by vol.) or 1-butanol/2-propanol/water (10:7:10, by vol.) (Renz, 1971). The succinate derivatives were checked by paper chromatography with 2-butanol/water/acetic acid (70:30:1, by vol.) (Toraya *et al.*, 1975*a*).

The concentrations of the Cbl analogues prepared chemically were determined spectrophotometrically as follows. The concentrations of CN-Cbl(*b*-OH), CN-Cbl(*d*-OH), CN-Cbl(*e*-OH), CN-Cbl(5'-*O*-P), CN-Cbl(5'-*O*-Suc) and CN-Cbl[2'-*O*,5'-*O*-(Suc)₂] were determined on the basis of $\epsilon_{361} = 28.06 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for CN-Cbl (Friedrich, 1964). That of CN-Cbl(13-epi) was done on the basis of $\epsilon_{367} = 20.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for (CN)CN-Cbl(13-epi) (Bonnett *et al.*, 1971*b*). That of CN-Cbl(*c*-lactam) was done on the basis of $\epsilon_{359} = 22.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Bonnett *et al.*, 1957).

Cbl analogue-feeding experiments. Each of the Cbl analogues (5 µg per litre of medium) was added aseptically to a culture (50 ml) of *Euglena* cells (about 0.5×10^6 cells ml⁻¹) which had been grown for 5 d in the Cbl-limited medium B. Cells that had been fed each Cbl analogue were further cultured for 6 d under the same conditions. A sample (1.0 ml) of culture was taken every 24 h after addition of the Cbl analogue and cell numbers were counted in a haemocytometer.

Assay of Cbl uptake activity in living cells of *Euglena*. *Euglena* cells (about 10^7 cells per ml of medium) grown for 5 d in 50 ml of the Cbl-limited medium A were collected aseptically by centrifugation at 2000 *g* for 5 min, washed with glutamate buffer, pH 4.0, containing 56 mM-glucose, 18 mM-sodium glutamate, 2.6 mM-histidine/HCl and 2.2 mM-KH₂PO₄, and suspended in 50 ml of the same buffer. The suspended cells were pre-incubated with shaking at 27 °C for 5 h under the same irradiance as used for growth. The assay mixture (1.0 ml) for Cbl uptake activity contained 10 nM-[G-³H]CN-Cbl, glutamate buffer, pH 4.0, and 10^5 *Euglena* cells. In the case of experiments to study inhibition of uptake, 100 nM each of the Cbl analogues was added to the mixture. The mixture, after incubation for 2 h at 4 °C (to study the initial phase or energy-independent process) or 27 °C (to study total Cbl uptake = energy-independent and -dependent processes) in the dark, was immediately filtered through a membrane filter (cellulose acetate

type, 0.45 µm pore size, 25 mm diameter) and washed three times with 5 ml 0.9% (w/v) NaCl. The radioactivity on the dried membrane filter was counted with a liquid scintillation counter (Aloka LSC 903). The Cbl uptake in the secondary phase (energy-dependent process) was calculated by subtracting the radioactivity in the initial phase (at 4 °C) from that in the initial and secondary phases (at 27 °C).

Corrinoid extraction and assay with HPLC. Corrinoids were extracted from cells by the KCN-boiling method as described previously (Watanabe *et al.*, 1991). The extract was desalted and concentrated by Amberlite XAD-4 column chromatography as described by Stupperich *et al.* (1986). The 80% methanol eluent was evaporated to dryness and dissolved in 500 µl of water. Corrinoids in the concentrated solution were partially purified by HPLC (Wakosil 5C18-200 HPLC column) with 0.1% (v/v) acetic acid as a mobile phase; corrinoids were eluted with 80% (v/v) acetonitrile/0.1% (v/v) acetic acid and monitored by measuring absorbance at 551 nm. Corrinoid fractions were pooled, evaporated to dryness and dissolved in 200 µl of water. Corrinoids in the final solution were assayed by the same HPLC system with water/methanol/acetic acid (79:20:1, by vol.) as a mobile phase.

Materials. The Wakosil 5C18-200 HPLC column (4.6 × 150 mm) was obtained from Wako Chemicals, Japan. Cellulose acetate membrane filters were obtained from Advantec, Japan. Amberlite XAD-4 was obtained from Japan Organo Co.

Results

Structural formulae of Cbl analogues

A variety of Cbl analogues was synthesized chemically or microbiologically to study the corrinoid specificities of cell growth and the Cbl uptake system in *E. gracilis* z. The structural formulae of Cbl and partial structures of Cbl analogues used in this study are shown in Fig. 1, and their abbreviations are given in Table 1.

Effect of Cbl analogues on *Euglena* cell growth

To clarify the corrinoid specificity of cell growth of *E. gracilis* z, each of the Cbl analogues (5 µg per litre of medium) was added aseptically to a 5 d Cbl (0.22 ng l⁻¹)-limited culture (containing about 0.5×10^6 cells ml⁻¹). Addition of (Bza)CN-Cba or CN-Cbl(5'-*O*-P) increased significantly the growth of *Euglena*, and the culture reached a maximum density of about 20×10^6 cells ml⁻¹ at day 6. An identical growth curve was obtained for the CN-Cbl-supplemented culture, while in 6 d cultures supplemented with CN-Cbl(5'-*O*-Suc), (5-OH-Bza)CN-Cba, (Ade)CN-Cba, (*p*-Cre)CN-Cba or CN-Cbl[2'-*O*,5'-*O*-(Suc)₂] growth increased by about 47, 33, 30, 12 and 5%, respectively, over that of the CN-Cbl-supplemented culture (Fig. 2*a*, *c*). Growth in the CN-Cbl(*b*-OH)-, CN-Cbl(*d*-OH)- and CN-Cbl(*e*-OH)-supplemented cultures increased to similar levels at day 6 as in the CN-Cbl-supplemented culture, but the rates of cell growth in the CN-Cbl-acid-derivative- and CN-Cbl-supplemented cultures were different during the period

Table 1. Corrinoid compounds

Corrinoid	Abbreviation	Structure*
5'-Deoxyadenosylcobalamin	AdoCbl	1
Methylcobalamin	CH ₃ -Cbl	2
Hydroxocobalamin	OH-Cbl	3
Cyanocobalamin	CN-Cbl	4
Cyanocobalamin- <i>O</i> ⁵ -phosphate	CN-Cbl(5'- <i>O</i> -P)	5
Cyanocobalamin- <i>O</i> ⁵ -succinate	CN-Cbl(5'- <i>O</i> -Suc)	6
Cyanocobalamin- <i>O</i> ² , <i>O</i> ⁵ -disuccinate	CN-Cbl[2'- <i>O</i> ,5'- <i>O</i> -(Suc) ₂]	7
2-Amino-2-methylpropanolyl cyanocobalamin	(2-AMP)CN-Cbl	8
Cyanocobalamin(<i>c</i> -lactam)	CN-Cbl(<i>c</i> -lactam)	9
Cyano-13-epicobalamin	CN-Cbl(13-epi)	10
Cyanocobalamin- <i>b</i> -monocarboxylate	CN-Cbl(<i>b</i> -OH)	11
Cyanocobalamin- <i>d</i> -monocarboxylate	CN-Cbl(<i>d</i> -OH)	12
Cyanocobalamin- <i>e</i> -monocarboxylate	CN-Cbl(<i>e</i> -OH)	13
Benzimidazolyl cobamide	(Bza)CN-Cba	14
5-Hydroxybenzimidazolyl cobamide	(5-OH-Bza)CN-Cba	15
7-Adenyl cobamide	(Ade)CN-Cba	16
<i>p</i> -Cresolyl cobamide	(<i>p</i> -Cre)CN-Cba	17
Dicyanocobinamide	(CN)CN-Cbi	Lacks the nucleotide (1- α -ribofuranosyl- 5,6-dimethylbenzimidazol- 3'-phosphate)

* Numbers 1-17 correspond to the structures in Fig. 1.

studied (Fig. 2*b*). Neither CN-Cbl(13-epi), CN-Cbl(*c*-lactam), (2-AMP)CN-Cbl nor (CN)CN-Cbi could stimulate *Euglena* cell growth at all (Fig. 2*b*, *d*).

Intracellular form of the Cbl analogues taken up by *Euglena* cells

For the Cbl analogues that had the same biological activity as CN-Cbl (as shown in Fig. 2*a*, *b*, *c*), the intracellular form of the analogue after uptake by *Euglena* cells was determined by HPLC (Table 2). In the (Bza)CN-Cba-supplemented cells, most of the intracellular corrinoids were recovered in the (Bza)CN-Cba fraction, but not in CN-Cbl. While in the CN-Cbl(5'-*O*-P)-, CN-Cbl(*b*-OH)-, CN-Cbl(*d*-OH)- and CN-Cbl(*e*-OH)-supplemented cells, each of the Cbl analogues accumulated by the cells was converted to CN-Cbl (Table 2). The results suggest that *Euglena* cells have the ability to dephosphorylate CN-Cbl(5'-*O*-P) and amidate CN-Cbl(*b*-OH), (*d*-OH) and (*e*-OH) to form CN-Cbl.

Effect of Cbl analogues on the activities of the *Euglena* Cbl uptake system

To clarify the corrinoid specificity of the Cbl uptake system of living *Euglena* cells, inhibition of Cbl uptake was studied using Cbl analogues (Table 3). Cbl uptake in *Euglena* has been reported to be a biphasic process which consists of Cbl-binding to a cell-surface receptor (energy-independent process; initial phase) and active transport (energy-dependent process; secondary phase) (Varma *et*

Table 2. Determination of the intracellular form of corrinoids taken up by *Euglena* cells

Each of the analogues indicated (5 μ g per litre of medium) was added aseptically to a culture (1 litre) of *Euglena* cells (about 0.5×10^6 cells ml⁻¹) which had been grown for 5 d in a Cbl-limited (0.22 ng l⁻¹) medium. The cells were cultured for a further 6 d and then collected by centrifuging at 2000 *g* for 10 min and washed three times with distilled water. The corrinoids taken up by the cells were extracted using the KCN-boiling method, and then partially purified and analysed by HPLC as described in the text. Retention times of authentic CN-Cbl, (Bza)CN-Cba, CN-Cbl(5'-*O*-P), CN-Cbl(*b*-OH), CN-Cbl(*d*-OH) and CN-Cbl(*e*-OH) were 12.8, 9.8, 21.5, 29.3, 17.5 and 23.5 min, respectively, in water/methanol/acetic acid (79:20:1, by vol.) as a mobile phase. The amount of intracellular Cbl in the CN-Cbl-supplemented cells (control) was estimated to be 2.96 nmol by the HPLC analysis; the amounts in the analogue-supplemented cells are expressed as a percentage of this control. The data represent mean values from three experiments. ND, Not detectable.

Addition to medium	Intracellular corrinoids (% of control)	
	Main	Minor
CN-Cbl (control)	CN-Cbl (100)	ND
(Bza)CN-Cba	(Bza)CN-Cba (89.2)	ND
CN-Cbl(5'- <i>O</i> -P)	CN-Cbl (98.5)	CN-Cbl(5'- <i>O</i> -P) (<0.5)
CN-Cbl(<i>b</i> -OH)	CN-Cbl (66.8)	CN-Cbl(<i>b</i> -OH) (<1.8)
CN-Cbl(<i>d</i> -OH)	CN-Cbl (97.8)	CN-Cbl(<i>d</i> -OH) (<2.2)
CN-Cbl(<i>e</i> -OH)	CN-Cbl (98.3)	CN-Cbl(<i>e</i> -OH) (<2.5)

al., 1961; Sarhan *et al.*, 1980). [G -³H]CN-Cbl uptake in the initial phase (Cbl-binding to a cell-surface receptor) was inhibited about 41, 61 and 65% by addition of 100 nM-CN-Cbl[2'-*O*,5'-*O*-(Suc)₂], -CN-Cbl(*b*-OH) and

Fig. 1. Structural formulae of Cbl and partial structures of Cbl analogues. The partial structures of Cbl analogues show only those portions of the molecule that differ from CN-Cbl. Table 1 lists the actual compounds used for experiments. Numbers (1–17) of the structures correspond to those in Table 1.

-(CN)CN-Cbi, respectively, while other Cbl analogues completely inhibited the uptake activity. Although CN-Cbl[2'-O,5'-O-(Suc)₂], CN-Cbl(*b*-OH) and (CN)CN-Cbi did not affect Cbl uptake in the secondary phase (active transport), this phase of uptake was inhibited by about 47, 80, 83 and 90% by (*p*-Cre)CN-Cba, CN-Cbl(*d*-OH), CN-Cbl(5'-O-P) and CN-Cbl(5'-O-Suc), respectively, and other analogues inhibited

uptake completely. The results indicate that the cell-surface Cbl-binding reaction (initial phase) has much lower corrinoid specificity in the Cbl uptake system than does the active transport reaction (secondary phase), and that both the α -lower axial ligand (the cobalt-coordinated nucleotide) and the (*b*)-propionamide side-chain of the Cbl molecule are essential for the uptake system in *E. gracilis* z.

Fig. 2. Effect of Cbl analogues on growth of *Euglena*. Each of the Cbl analogues ($5 \mu\text{g}$ per litre of medium) was added aseptically to cells which had been grown for 5 d in a Cbl-limited (0.22 ng l^{-1}) medium. The cells were cultured for a further 6 d after addition of analogues. Cell numbers were counted in a haemocytometer every 24 h. The data represent mean values from three experiments. (a) Cbl analogues with different base moieties in the α -lower axial ligand: $\bullet$, CN-Cbl; $\blacktriangle$, (5-OH-Bza)CN-Cba; $\blacksquare$, (p-Cre)CN-Cba; $\triangle$, (Bza)CN-Cba; $\square$, (Ade)CN-Cba; $\circ$, no analogue added. (b) Cbl analogues with modified corrin ring side-chains: $\bullet$, CN-Cbl; $\blacktriangle$, CN-Cbl(b-OH); $\triangle$, CN-Cbl(d-OH); ∇ , CN-Cbl(e-OH); $\blacksquare$, CN-Cbl(c-lactam); $\square$, CN-Cbl(13-epi); $\circ$, no analogue added. (c) Cbl analogues with a modified ribose moiety in the α -lower axial ligand: $\bullet$, CN-Cbl; $\triangle$, CN-Cbl(5'-O-Suc); $\blacksquare$, CN-Cbl[2'-O,5'-O-(Suc) $_2$]; $\circ$, no analogue added. (d) Cbl analogues with a modified alkanolamine moiety and without the α -lower axial ligand: $\bullet$, CN-Cbl; $\blacktriangle$, (2-AMP)CN-Cbl; $\triangle$, (CN)CN-Cbi; $\circ$, no analogue added.

Discussion

To clarify the corrinoid specificity of *Euglena* cell growth and the Cbl uptake system, a variety of Cbl analogues synthesized chemically or microbiologically was used (Fig. 1 and Table 1).

Table 3. Effect of Cbl analogues on the *Euglena* Cbl uptake system

Euglena cells grown for 5 d in a Cbl-limited (33.3 ng l^{-1}) medium were used for assay of Cbl uptake activity as described in the text. The assay mixture (1.0 ml) contained 10 nM [$G\text{-}^3\text{H}$]CN-Cbl, glutamate buffer, pH 4.0, and 10^5 cells of *E. gracilis* z. The Cbl analogues were added to the mixture to a final concentration of 100 nM . The mixture was incubated for 2 h at 4°C or 27°C in the dark. Cbl uptake in the secondary phase (energy-dependent) was calculated by subtracting the radioactivity accumulated in the initial phase (energy-independent, at 4°C) from that at 27°C . The data represent means $\pm$ SD ($n = 5$).

Corrinoid	Cbl uptake activity (pmol per 10^{-6} cells)	
	Initial phase (Cbl-binding to cell-surface receptor)	Secondary phase (active transport)
None	0.75 ± 0.02	0.76 ± 0.03
CN-Cbl	0	0
OH-Cbl	0	0
CH $_3$ -Cbl	0	0
AdoCbl	0	0
CN-Cbl(5'-O-P)	0	0.13 ± 0.01
CN-Cbl(5'-O-Suc)	0.02 ± 0.01	0.07 ± 0.01
CN-Cbl[2'-O,5'-O-(Suc) $_2$]	0.44 ± 0.02	0.74 ± 0.03
CN-Cbl(13-epi)	0	0
CN-Cbl(c-lactam)	0.01 ± 0.01	0.03 ± 0.01
CN-Cbl(b-OH)	0.29 ± 0.01	0.78 ± 0.02
CN-Cbl(d-OH)	0.03 ± 0.01	0.14 ± 0.01
CN-Cbl(e-OH)	0	0.03 ± 0.01
(2-AMP)CN-Cbl	0	0
(Bza)CN-Cba	0	0
(5-OH-Bza)CN-Cba	0	0
(Ade)CN-Cba	0	0
(p-Cre)CN-Cba	0	0.40 ± 0.02
(CN)CN-Cbi	0.26 ± 0.02	0.72 ± 0.03

Regarding the base specificity in the cobalt-coordinated α -lower axial ligand, radioactively labelled CN-Cbl uptake inhibition experiments (Table 3) show that *Euglena* cells have the ability to take up (Bza)-, (5-OH-Bza)- and (Ade)-CN-Cba, as well as CN-Cbl. *Euglena* cells in the (Bza)CN-Cba- and CN-Cbl-supplemented cultures increased in number at identical rates through the experimental period (Fig. 2a). When total corrinoids were extracted from the (Bza)CN-Cba-supplemented cells and analysed by HPLC, most of the corrinoids extracted were recovered in the (Bza)CN-Cba fraction (Table 2), of which the synthetic coenzyme form, (Bza)AdoCba, has been reported to be active in AdoCbl-dependent methylmalonyl-CoA mutases (Abeles & Lee, 1961; Lengyel *et al.*, 1960). This strongly suggests that the (Bza)CN-Cba taken up by *Euglena* cells was converted to the coenzyme forms that function as the prosthetic groups of the three Cbl-dependent enzymes known in *E. gracilis* z (methionine synthase, methylmalonyl-CoA mutase and ribonucleotide reductase). The (5-OH-Bza)- and (Ade)-CN-Cba-supplemented cultures

gave about 30% of the cell growth increase seen in the CN-Cbl-supplemented cultures (Fig. 2a). This lower increase in Cba-supplemented cultures could be due to difficulty in the formation of the Cbl-dependent enzyme-Cba complex because (Ade)AdoCba has been reported to have much lower affinity for some AdoCbl-dependent enzymes than AdoCbl itself (Lengyel *et al.*, 1960; Kung & Stadmann, 1971). The (*p*-Cre)CN-Cba-supplemented *Euglena* cultures grew at only about 10% of the rate of CN-Cbl-supplemented cultures. This seems to be because (*p*-Cre)CN-Cba has a considerably lower affinity for the *Euglena* uptake system (Table 3). (CN)CN-Cbi did not affect the activities of the Cbl uptake system (Table 3) nor did it support an increase in growth of (CN)CN-Cbi-supplemented cultures (Fig. 2d). The results indicate that *Euglena* cells are not able to take up 'incomplete' corrinoids like (CN)CN-Cbi, because a suitable α -lower axial ligand (presence of the cobalt-coordinated nucleotide) of the Cbl molecule is essential for the *Euglena* Cbl uptake system.

Radioactive CN-Cbl uptake inhibition experiments with OH-Cbl, CH₃-Cbl or AdoCbl (Table 3) indicate that the β -upper axial ligand of Cbl molecule is not essential for the *Euglena* uptake system.

(2-AMP)CN-Cbl, which contains 2-amino-2-methylpropanol instead of 1-amino-2-methylpropanol as an alkanolamine group, has been reported to inhibit cell growth of *E. coli* and *O. malhamensis* (Friedrich *et al.*, 1964). This analogue had no effect on *Euglena* cell growth (Fig. 1d). The results in Table 3 suggest that (2-AMP)CN-Cbl can be taken up by *Euglena* cells. Inhibition of the Cbl-dependent enzymes by the analogue, which has been reported to completely inhibit activity of an AdoCbl-dependent diol dehydrase (Kung & Stadmann, 1971), may explain why this analogue does not support a growth increase. The present results were different from our previous studies, which suggested that (2-AMP)CN-Cbl did not affect the activity of some *Euglena* Cbl-binding proteins (Watanabe *et al.*, 1987a, b; 1988a, b). We are, however, unable to explain why such differences in the analogue-binding behaviour occurred.

Although it has been reported that CN-Cbl(5'-O-P), (5'-O-Suc) and [2'-O,5'-O-(Suc)₂] do not inhibit labelled CN-Cbl uptake by *E. coli* (White *et al.*, 1973; Toraya *et al.*, 1975a), the results in Table 3 indicate that *Euglena* cells have the ability to take up CN-Cbl(5'-O-P) and (5'-O-Suc), but not [2'-O,5'-O-(Suc)₂], which had no effect on *Euglena* cell growth. The increase in growth of CN-Cbl(5'-O-P)-supplemented cultures was the same as that of CN-Cbl-supplemented cultures (control), but CN-Cbl(5'-O-Suc)-supplemented cultures grew at only about 50% of the rate of the control (Fig. 2c). It became clear that the CN-Cbl(5'-O-P) taken up by the cells was dephosphorylated to CN-Cbl (Table 2). *E. gracilis* z has

been reported to contain a non-specific phosphatase which is involved in vitamin B₁ (in particular thiamin pyrophosphate) uptake and is located in the cell surface region (Shigeoka *et al.*, 1987; Nakano *et al.*, 1987). The non-specific phosphatase may function in the dephosphorylation of CN-Cbl(5'-O-P) as well as thiamin pyrophosphate prior to uptake of the compounds. An enzymic system for dephosphorylation of CN-Cbl(5'-O-P) has been studied in some bacteria (Schneider & Friedmann, 1972). The lower growth rate of the CN-Cbl(5'-O-Suc)-supplemented cultures in comparison with CN-Cbl(5'-O-P)-supplemented cultures may be because the conversion of CN-Cbl(5'-O-Suc) to CN-Cbl is a rate-limiting step for cell growth.

It has been reported that both CN-Cbl(13-epi) and -(c-lactam) inhibit *E. coli* growth (Bonnett *et al.*, 1957; 1971a) and that AdoCbl(13-epi) also is inactive in some AdoCbl-dependent enzymes (Renz, 1971; Toraya *et al.*, 1975b). The results in Table 3 indicate that *Euglena* cells have the ability to take up both CN-Cbl(13-epi) and -(c-lactam), but neither analogue affected cell growth (Fig. 2b). The results in Table 3 also show that *Euglena* cells are capable of taking up CN-Cbl(*d*-OH) and (*e*-OH), but not (*b*-OH), indicating that the (*b*)-propionamide side chain of the Cbl molecule is essential for the *Euglena* Cbl uptake system. CN-Cbl(*b*-OH)-supplemented *Euglena* cultures, however, grew as effectively as CN-Cbl(*d*-OH)- or CN-Cbl(*e*-OH)-supplemented cultures. Each of the CN-Cbl acid derivatives accumulated by the cells was converted to CN-Cbl (Table 2). These results suggest that the enzyme involved in the conversion of each of the acid derivatives to CN-Cbl (CN-Cbl monocarboxylic acid amide synthase) occurs in *E. gracilis* z, probably in the cell surface region. A Cbl-producing bacterium, *Pseudomonas denitrificans*, has been reported to contain the enzyme cobyric acid *a,c*-diamide synthase, which is involved in Cbl biosynthesis (Debussche *et al.*, 1990).

The results presented here also provide useful information for the microbiological assay of Cbl, because *E. gracilis* z has been widely used for the bioassay of Cbl (Ross, 1952).

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