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5. Transformation reversed (100 to 4 percent) 1 hour after replacement of bPTH(1-34) (100 ng/ml) with additive-free medium and reoccurred (98 percent) 1 hour after readdition of the hormone 8, 12, or 24 hours later. Reversibility could be demonstrated through four such cycles over 60 hours (maximum tested to date); control transformation remained < 11 percent.
6. We prepared cultures as in Table 1, incubated them with bPTH(1-34) for 2½ minutes, extracted and then assayed their intracellular cAMP by methods of J. D. M. Albano, G. D. Barnes, D. M. Maudskey, B. L. Brown, R. W. Etkins [*Anal. Biochem.* **60**, 130 (1974)] and A. G. Gilman [*Proc. Natl. Acad. Sci. U.S.A.* **67**, 305 (1970)]: control, 4.65 pmole of cAMP per culture; PTH (1 ng/ml), 6.04 ($P < .001$ compared to control); and PTH (5 ng/ml), 7.37. Results are similar to those of Peck *et al.* (*9*).
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Ribonucleotide Reductase in Blue-Green Algae: Dependence on Adenosylcobalamin

Abstract. *Ten species of freshwater blue-green algae exhibit an adenosylcobalamin-dependent ribonucleotide reductase, thus explaining the requirement for cobalt by these organisms. The evidence suggests a phylogenetic affinity between the cyanophytes and bacteria, such as* Clostridium *and* Rhizobium, *and the euglenoid flagellates, which also use the cofactor-dependent reductase. In contrast, the ribonucleotide reductase reaction in the few green algae surveyed shows no dependence on cobalamins.*

The importance of vitamin B_{12} in algal nutrition has been widely documented (*1*). In contrast to a group such as the dinoflagellates, however, very few blue-green algae need an exogenous source of this vitamin. Only a small number of marine species have been shown by van Baalen to require vitamin B_{12} for growth (*2*). Nevertheless, the requirement for cobalt by these organisms is universal (*3*) and various corrinoids have been extracted from species such as *Anabaena cylindrica* (*4, 5*).

The nutritional requirement for vitamin B_{12}, either exogenous or synthesized by the organism, could be established by demonstrating a cobalamin-dependent metabolic reaction. Although several enzymatic reactions have been shown to require a coenzyme form of vitamin B_{12}, only three appear to be widespread. Methylcobalamin is used as a cofactor in methionine synthesis in some strains of *Escherichia coli* (*6*). This enzyme may also be of importance in mammalian cells (*7*). The methylmalonyl coenzyme A isomerase reaction uses adenosylcobalamin as cofactor. This enzyme has been isolated from various sources—from bacteria to sheep liver (*8*). However, it may be of limited importance in some cells, especially microorganisms. Ribonucleotide reductase also requires an adenosylcobalamin cofactor in some organisms. The reaction catalyzed by this enzyme is crucial to cell division, as it supplies the deoxyribonucleotide precursors for DNA synthesis. Two forms of the enzyme exist; as isolated from *E. coli* the enzyme contains two atoms of nonheme iron per molecule. It requires no B_{12} cofactors (*9*). A similar enzyme has been demonstrated in mammalian cells (*10, 11*). The adenosylcobalamin-dependent type of reductase was first demonstrated in *Lactobacillus leichmannii* (*12*). Although this type of reductase may be considered the more esoteric form of the enzyme, a survey of literature indicates that it is probably more common among prokaryotes than the iron-containing reductase. A ribonucleotide reductase that requires a cobalamin cofactor is found in *Clostridium* (*13*), *Rhizobium* (*14*), various denitrifying organisms (*15*), and *Bacillus megaterium* (*16*). In addition, the adenosylcobalamin-dependent reductase has been demonstrated in one class of algae, the euglenoid flagellates (*17*). In this report, we show the occurrence of this enzyme in various blue-green algae. Using the sensitive assay procedure of monitoring the exchange of ^3H from [5′-$^3H_2$]adenosylcobalamin to water during the enzymatic reaction (*13, 18*), we have demonstrated that the B_{12}-dependent type of enzyme is widespread among the cyanophytes.

Axenic cultures of blue-green algae were obtained from various sources and grown in media indicated in Table 1. Cell growth was monitored by optical density at 650 nm or direct counting in a Petroff-Hauser counting chamber. Cells were harvested in the logarithmic phase of growth in centrifugation at 7000*g* in a DuPont-Sorvall RC-5 refrigerated centrifuge. The cells were resuspended in a buffer containing 0.01*M* dimethylglutarate buffer (*p*H 7.2) and 0.001*M* mercaptoethanol. They were washed once and resuspended in the same buffer (1 g of cells, wet weight, per 5 ml of buffer). One gram of charcoal (neutral Norit A) was added per 10 ml of cell suspension. The suspension was then sonicated for 10 minutes with a Heat Systems Branson Sonifer. The sonicated extract was centrifuged at 27,000*g* for 20 minutes, and the supernatant was removed and assayed for ribonucleotide reductase activity as soon as possible. Protein was determined by the biuret (*19*) method, with bovine serum albumin as a standard.

The assay mixture contained the following: 0.12*M* 3,3-dimethylglutarate buffer, *p*H 7.2; 1.2 m*M* ethylenediaminetetraacetic acid; 26 m*M* dithiothreitol; 2 m*M* adenosine triphosphate (P-L Biochemicals); 0.01 m*M* [5′-$^3H_2$]adenosylcobalamin (specific activity, 15 μc/μmole (*20*); and 1 to 5 mg of protein of crude algal extract in a final volume of 0.5 ml. Coenzyme was added in dim light and the reaction initiated by addition of

extract. The reaction vessels were incubated in the dark at 37°C and the reaction was terminated by freezing the vessels in liquid nitrogen. Water was removed by sublimation and a 0.1-ml sample added to 10 ml of PCS scintillation fluid (Amersham/Searle); the samples were counted in a Searle Mark II liquid scintillation system. The results are presented in Table 1.

All the blue-green algae listed in Table 1 except the marine species *Agmenellum quadruplicatum* are freshwater organisms that do not require an exogenous source of vitamin B_{12}. All except the marine organism exhibit adenosylcobalamin-dependent ribonucleotide reductase activity. The green alga *Chlorella* has no activity in this system. An extract of *Euglena gracilis* Z is active as previously reported (*17*). Although all the orders of blue-green algae are not represented in Table 1, the occurrence of a B_{12}-dependent reductase is probably quite wide, at least among freshwater forms. The results also indicate that these organisms are capable of synthesizing cobalamins.

According to the theories of Oparin (*21*), the coenzyme-requiring form of an enzyme may be more primitive than one that does not utilize a cofactor. The B_{12}-dependent reductase is found among the more primitive type of bacteria, the obligate anaerobes. The fossil evidence indicates that cyanophytes are also an ancient group of organisms (*22*). Thus the occurrence of a cofactor-requiring reductase may be further proof of their early evolution. The lack of activity in the few green algae [*Chlorella* and *Chlamydomonas* (*5*)] that were tested is surprising as the Chlorophyceae supposedly evolved from a blue-green algal ancestor. It may be that the green algae are more closely related to the higher plants that contain no vitamin B_{12} (*23*) and only remotely related to extant blue-green algae. The occurrence of the adenosylcobalamin-dependent reductase in blue-green algae and the Euglenophyceae may indicate a closer phylogenetic affinity between these two groups than between the cyanophytes and the green algae. However, a final resolution of the phylogeny based on cobalamin enzymology will depend on a more complete survey of the green algae.

The fact that no activity was found in *A. quadruplicatum* merits further investigation. A negative result in the exchange assay suggests that this organism uses another reaction for reduction of ribonucleotides, presumably the iron-containing enzyme. Cells of *A. quadruplicatum* in the logarithmic phase were harvested and assayed by use of ^{3}H-labeled cytidine diphosphate. This preparation was able to reduce cytidine diphosphate to deoxycytidine diphosphate in an assay system described by Fuchs and Warner (*24*). Adenosylcobalamin was not required for the reaction. Since the vitamin B_{12} requirements of *A. quadruplicatum* are similar to those of *E. coli* auxotrophs, it is probable that the vitamin functions in methionine synthesis in this organism as it does in the chrysophyte *Ochromonas malhamensis* (*25*).

The ribonucleotide reductase reaction is a vital step in DNA synthesis in all cells since few are able to make exclusive use of the nucleotide salvage pathway (*26*). Characterization of the ribonucleotide reductase in these and other algae may yield information on the phylogenetic relationships among these organisms. In addition, it is known that the ribonucleotide reductase reaction is repressed by a number of small molecules, especially nucleotides [see (*27*) for review]. Although no highly specific, in vivo inhibitor for the adenosylcobalamin-dependent reductase is available, it is conceivable that the growth of the cyanophytes could be controlled by substances that would modify this reaction. Presumably such an inhibitor would not affect other organisms in the ecosystem which use the iron-containing enzyme for ribonucleotide reduction.

Table 1. The source of the organisms, growth media used, and the specific activity of ribonucleotide reductase are given here.

Organism	Source*	Medium	Specific activity† (count/min) per milligram of protein
Coccochloris peniocystis	ICC 1548	Bg 11 (*28*)	240
Synechococcus sp.	ATCC 27146	Cg 10 (*29*)	230
Anacystis nidulans	ICC 625	Cg 10	345
Agmenellum quadruplicatum	PR-6‡	ASP-2 (*30*)	0
Nostoc commune	ICC 584	Bg 11	2910
Anabaena flos-aquae	ICC 1444	Bg 11	474
Oscillatoria prolifera	ICC 1270	Bg 11	390
Scytonema hofmanni	ICC 1581	Bg 11	740
Fremyella diplosiphon	ICC 481	Bg 11	620
Plectonema boryanum	ICC 581	Bg 11	360
Phormidium autumnale	ICC 1580	Bg 11	425
Euglena gracilis Z (dialyzed preparation)	ICC 753	*E. gracilis* medium (*31*)	5662
Chlorella nocturna	ICC 1804	*E. gracilis* medium	0
Chlorella vulgaris	ICC 29	*E. gracilis* medium	0

*Sources: ICC, Indiana Culture Collection, Department of Botany, Indiana University, Bloomington 47401; ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852. †Specific activity is reported as the radioactivity transferred to the water in a 0.5-ml reaction mixture per milligram of algal extract protein after 30 minutes of incubation. ‡The B_{12}-requiring marine organism, gift of C. van Baalen, University of Texas Marine Science Institute, Port Aransas 78373.

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