# RIBONUCLEOTIDE REDUCTASE ACTIVITY IN GREEN ALGAE

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SUMMARY. A ribonucleoside diphosphate reductase is demonstrated in the algae, Scenedesmus obliquus and Chlorella pyrenoidosa. In synchronized cultures an activity maximum at the 12th hour of the cell cycle coincides with maximum DNA production. Induction of reductase activity is prevented by cycloheximide. The enzyme requires dithiols for reduction of CDP in vitro; it is not significantly stimulated by iron or magnesium ions nor dependent upon deoxyadenosylcobalamin. ATP stimulates the reaction but dATP or dTTP act as inhibitors. The ribonucleotide reductase of green algae differs from the $B_{12}$-requiring enzyme characterized in Euglena gracilis.

## INTRODUCTION

The enzymatic conversion of ribonucleoside 5'-phosphates to 2'-deoxyribonucleotides, catalysed by ribonucleotide reductases (1,2) has rarely been studied in plant cells. Ribonucleotide reductase activity could be demonstrated in germinating wheat, in root tips of the broad bean (Vicia faba), and in growing soybean callus tissue (3,4,5) but the study of these enzymes is difficult due to their low amounts. A more detailed knowledge of plant enzymes in comparison with bacterial and animal ribonucleotide reductases is necessary because of their important function in cell proliferation and because of the uncertain distribution of deoxyadenosylcobalamin (coenzyme $B_{12}$) requirement for ribonucleotide reduction in eucaryotes. Thus, the algae Euglena gracilis and Astasia longa (6,7,8), as well as the fungus Pithomyces chartarum (9) possess $B_{12}$-dependent reductases, while deoxyade-

nosylcobalamin is without effect on ribonucleotide reduction in yeast or the higher plants mentioned above.

Microalgae offer themselves as a source of ribonucleotide reductases because cell proliferation leads to formation of 8 or 16 daughter cells, requiring a high supply of DNA precursors, and because synchronous growth is easily achieved (1o). Although many aspects of nucleic acid metabolism have been investigated in various algal species, study of the reductive pathway to deoxyribonucleotides has obviously been neglected. We here describe some characteristics of ribonucleotide reduction in the green algae, Scenedesmus obliquus and Chlorella pyrenoidosa.

## MATERIALS AND METHODS

Synchronised cultures of S.obliquus (strain D3) and C.pyrenoidosa (strain 211-8b) were derived from stock cultures maintained in the collection of Dr.H.Senger, Botany Department, Marburg. Cells were grown in a light thermostat in inorganic, aerated (3 % $CO_2$) media at 28°C essentially as described (1o); synchrony was induced in a periodic light-dark regime of 14:1o hours. Algae were harvested at the desired time by centrifugation at 2,5oo rpm. The cells (75o - 1ooo µl packed cell volume) were suspended in 1.5 ml of potassium phosphate buffer (o.o5 M, pH 7.5) containing 5 mM mercaptoethanol; the mixture was frozen in liquid nitrogen and ruptured in a high-frequency disintegrator (Micro-Dismembrator, Braun-Instrumente, Melsungen, Germany) using a Teflon capsule and steel balls. The homogenate was diluted with buffer (2 ml) and centrifuged for 3o min at 3o,ooo x g. Unless otherwise stated, the green supernatant was directly used for enzyme assays.

Nucleotides and other chemicals were of highest purity available, deoxyadenosylcobalamin was a gift of Dr.H.P.C.Hogenkamp, Iowa City, U.S.A. Solutions of the radioactive substrate, CDP (Amersham; specific activity, 23 Ci/mmole) had to be freed from ethanol prior to use by evaporation in vacuo.

Ribonucleotide reductase assays contained, in a total volume of o.3 ml: [5-$^3$H]cytidine diphosphate, 1o µCi; 1o mM dithiothreitol; 3.5 mM ATP; 5 mM $Mg^{++}$; o.o6 mM $Fe^{++}/Fe^{+++}$; and o.2 ml of an enzyme extract; final pH, 7.2. After incubation for 1 hr at 3o°C the reaction was terminated by addition of 1 ml perchloric acid, nucleotides were hydrolysed to the monophosphates and separated on Dowex 5o ($H^+$) columns (11). The eluted fractions corresponding to CMP and dCMP were pooled, and radioactivity was determined in a liquid scintillation counter. Although under the above conditions the substrate (CDP) concentration was not saturating and specific enzyme ac-

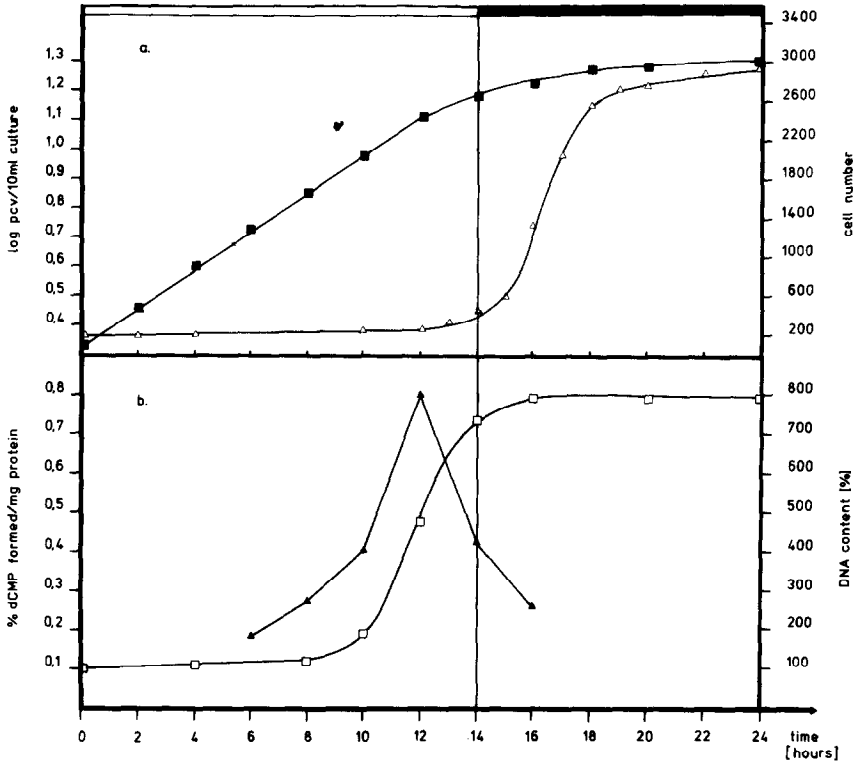


Figure 1. a. Development of cell volume (pcv, logarithmic) ( ■ ) and cell number ( △ ) in a synchronous culture of Scenedesmus obliquus, growing in light-dark regime (14:1o hrs). b. Ribonucleotide reductase activity ( ▲ ) and relative increase of DNA content ( □ ) (12) in the same culture.

tivities could not be obtained, relative values of product formation (dCMP, based upon unreacted CMP) were reproducible to better than ± 1o %.

## RESULTS AND DISCUSSION

The green algae used in this study can be cultured with a very good degree of cell synchrony (1o), as is evident from the time course of cell volume and cell number during the 24-hr cell cycle shown in Figure 1, a. Ribonucleotide reductase activity in extracts of Scenedesmus obliquus was measured between the 6th and 16th hour and is seen to rise sharply from low levels

Table 1. Ribonucleotide reductase activity in algae extracts

| Source and assay conditions | Relative activity (%) |
|---|---|
| *Chlorella pyrenoidosa* | |
| Standard culture, standard assay* | 1oo |
| culture + cycloheximide (1o μM) | 18 |
| *Scenedesmus obliquus* | |
| Standard assay* | 1oo |
| - [$^3$H]CDP, + [$^3$H]CMP or [$^3$H]CTP | < 1o |
| - dithiothreitol | 44 |
| - dithiothreitol, + NADPH (5 mM) | 75 |
| - $Fe^{++}/Fe^{+++}$ | 1o5 |
| - $Mg^{++}$ | 97 |
| - ATP | 67 |
| - ATP, - $Mg^{++}$ | 38 |
| + hydroxyurea (12 mM) | 61 |
| + dATP (3.5 mM), - ATP | 8 |
| + dTTP (3.5 mM), - ATP | 15 |

* Absolute values for standard assay (see Materials and Methods): 4.7 pmoles dCMP/mg protein with *Chlorella* extract, 2.o pmoles dCMP/mg protein with *Scenedesmus* extract; approximately 5 % product formed from labeled ribonucleotide substrate.

to a maximum at the 12th hour, followed by a decline (Fig.1,b). This activity peak coincides precisely with the maximum rate of DNA synthesis during the S phase (12). In extracts of the closely related species, *Chlorella pyrenoidosa*, high reductase activity is also present at the 12th hour. The enzymes are clearly responsible for nuclear DNA synthesis since production of chloroplast DNA occurs during the whole cell cycle in these algae. Cycloheximide, when added to the culture at the 9th hour, reduced the 12th hr-ribonucleotide reductase activity by more than 8o % (Table 1), indicating that the cell cycle-dependent increase is the result of *de novo* enzyme synthesis.

For further characterisation of the reductase, *Scenedesmus* cells

harvested at the 12th hour were used. The algal enzyme is a soluble protein because total activity is found in a 1oo,ooox g supernatant of cell extracts. It is most likely a ribonucleoside diphosphate reductase (EC 1.17.4.1); this follows from the observation that labeled cytidine diphosphate (CDP) yields much better reduction than do the mono- or triphosphates (CMP, CTP) (Table 1). However, because ATP is added as an effector and the cell extract contains both kinase and phosphatase activities, the substrate phosphorylation level was confirmed independently: The time dependence of CDP, CMP, and CTP concentrations during an incubation with CDP as substrate (followed chromatographically) shows an initial increase of monophosphate leveling off after 3o min; in contrast, deoxyribonucleotide (product) formation is most rapid at an earlier time when the initial CDP concentration is high. Deoxyadenosylcobalamin (25 μM) did not stimulate the reaction, and exposure to light had no effect. Both these specificities were also confirmed by partial purification of the Scenedesmus reductase on $P^3$-(6-aminohex-1-yl)dATP-Sepharose as an affinity adsorbent (13), after which the enzyme solution was free from low-molecular weight compounds and was only dependent upon CDP but not upon added coenzyme $B_{12}$.

Dithiols such as dithiothreitol serve as reductants in vitro (Table 1); reduced lipoate is much less efficient. The dependence of enzyme activity upon added dithiothreitol has a maximum (at 5-1o mM concentration) similar to the dithiol requirement of ribonucleotide reduction observed in mammalian tissues and in Escherichia coli. The physiological reductant in the green algae is as yet unknown; however, addition of NADPH to crude extracts also supports ribonucleotide reduction, suggesting the existence of a thioredoxin-like hydrogen donor system. Whether

mercaptoethanol alone is a reductant remains to be established after further purification.

The metal requirement of ribonucleotide reduction in the algae extracts differs from that of most other organisms in that magnesium and iron ions which are stimulatory for many bacterial and eucaryotic reductases have little, if any effect. Addition of hydroxyurea, however, is inhibitory as is typical of the iron-containing enzymes. This observation may explain the inhibition of DNA synthesis by hydroxyurea in Chlorella (14). The endogenous iron content of dried algae (o.14-o.19 mg/g) would yield an iron concentration as low as o.o3 μM in the reductase assay. Therefore, if the algal enzyme is an iron-containing protein the metal must be very tightly bound.

Nucleotides act as allosteric effectors of all ribonucleotide reductases known so far, and the plant enzymes make no exception. ATP, at an optimum concentration of 3.5 mM, stimulates the Scenedesmus reductase but dTTP and dATP are strong inhibitors (Table 1). The latter compound is also inhibitory for the E.coli, yeast, wheat, and mammalian enzymes while the action of dTTP is stimulatory in some organisms.

Ribonucleotide reduction in green algae obviously differs from that of the Euglenophyta. Euglena gracilis is well known as a $B_{12}$-requiring organism, and a deoxyadenosylcobalamin-dependent ribonucleoside triphosphate reductase (EC 1.17.4.2) has been purified from this algae (6,8). In contrast to higher plants, Scenedesmus obliquus also contains considerable quantities of vitamin $B_{12}$ (15) but its diphosphate reductase does not depend upon $B_{12}$ coenzyme. Judging from our present data the enzyme is similar to the ones found in wheat or broad bean. This dissimi-

larity between Scenedesmus or Chlorella and Euglena is in accord with the proposed phylogenetic place of the latter on a branch outside the main line of plant evolution.

The results described above bear resemblance to the induction of ribonucleotide reductase in synchronous cultures of yeast, Saccharomyces cerevisiae (16). However in Scenedesmus the rise and fall of enzyme activity during the S phase and its correlation with DNA synthesis are even more distinct despite the much longer generation time. It appears justified to expect the same key role of ribonucleotide reduction for cell division in all eucaryotic cells.

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