Ribonucleotide Reductase Activity in Vitamin B\textsubscript{12}-Deficient \textit{Euglena gracilis}

Edgar F. CARELL and John W. SEEGER, Jr.

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, U.S.A.

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The ribonucleotide reductase activities in vitamin B\textsubscript{12}-sufficient and -deficient cells of \textit{Euglena gracilis} were measured. We found that as the cells progress into vitamin B\textsubscript{12} deficiency the enzyme activity increases, reaching a maximum value of 20-fold in advanced deficiency. No significant differences in the activities were found to result as a consequence of different growth conditions. We propose that the increased activity in vitamin B\textsubscript{12}-deficient cells is due to an increase in enzyme protein.

Ribonucleotide reductase catalyses the conversion of the ribonucleotides into the corresponding deoxyribonucleotides. This enzyme system is considered to play a key role in regulating DNA synthesis and subsequent cell division (Elford et al., 1970; Cory & Whitford, 1972; Gleason & Wood, 1976; Lewis et al., 1977).

With regard to substrate requirement, there are two known types of ribonucleotide reductase. Ribonucleoside diphosphate reductase (EC 1.17.4.1) catalyses the conversion of ribonucleoside diphosphates and ribonucleoside triphosphate reductase (EC 1.17.4.2) reduces ribonucleoside triphosphates. These enzymes may be further classified on the basis of their cofactor requirements. Under this scheme, three types of ribonucleotide reductase have been identified. The most extensively studied type reduces the ribonucleoside diphosphates, is inhibited by hydroxyurea, is a non-haem-iron-containing protein, is found in \textit{Escherichia coli} and appears to be characteristic of mammals and some higher plants (cf. Follman, 1974; Reichard, 1978). The enzyme found in \textit{Lactobacillus leichmannii} (Abrams, 1965; Blakley et al., 1965; Beck & Hardy, 1965) and \textit{Pithomyces chartarum} (Stutzerberger, 1974) requires 5'-deoxyadenosylcobalamin as a cofactor and reduces the ribonucleoside triphosphates. The third type is characteristic of \textit{Bacillus megaterium} (Yau & Wachsmann, 1973) and \textit{Rhizobium meliloti} (Cowles & Evans, 1968). This enzyme requires deoxyadenosylcobalamin, but reduces the ribonucleoside diphosphates.

Ribonucleotide reductase of \textit{Euglena gracilis} has been shown to require vitamin B\textsubscript{12}, in the form of 5'-deoxyadenosylcobalamin, as a cofactor and to utilize ribonucleoside triphosphates as substrate (Gleason & Hogenkamp, 1970, 1972; Hamilton, 1974).

Previous studies, carried out on the pool sizes of the deoxyribonucleoside triphosphates (dNTP) in vitamin B\textsubscript{12}-deficient and -replenished \textit{Euglena}, have shown the pools to be undetectable in vitamin B\textsubscript{12} deficient cells, but that they appear rapidly on replenishment with the vitamin. The pools reach a maximum size, which is about 6 times that of normal exponentially growing cells, but decrease and become undetectable as the cells divide. Addition of cycloheximide to vitamin B\textsubscript{12}-replenished cells did not inhibit the accumulation of the dNTP, suggesting that vitamin B\textsubscript{12}-deficient cells may accumulate ribonucleotide reductase apoenzyme, which lacks the vitamin B\textsubscript{12} coenzyme for activity (Carell & Goetz, 1976; Goetz & Carell, 1978).

A number of studies utilizing inhibitors of DNA synthesis such as hydroxyurea (Murphree et al., 1969), fluorouracil (Lowdon & Vitolis, 1973) and fluorodeoxyuridine and methotrexate (Elford et al., 1977) have resulted in elevated ribonucleotide reductase activities.

In the present paper we show that vitamin B\textsubscript{12}-deficient \textit{Euglena gracilis} displays manifolds increases in the activity of this enzyme. Our findings suggest that the increase is due to an increase in enzyme protein rather than to enzyme activation.

**Experimental**

**Growth conditions**

\textit{Euglena gracilis} Klebs strain Z (Pringsheim) and the aplastidic mutant Y-ZHL (Egan & Carell, 1972) were grown in a pH 3.6 medium as previously described (Carell, 1969). These cells were also grown in a newly developed medium, similar to Hutner's modified medium (Price & Vallee, 1962), but with K\textsubscript{2}HPO\textsubscript{4} as buffer and titrated to pH 6.8 with aq. NH\textsubscript{3}. Growth measurements in terms of cell number, production of vitamin B\textsubscript{12} deficiency and criteria of the deficiency condition have been described (Carell, 1969).

**Chemicals and reagents**

Bovine serum albumin, 3,3-dimethylglutaric acid, ATP and dATP were from Sigma Chemical Co., St.
Louis, MO, U.S.A.; diphenylamine, 2-chloroaceta-
mine and 2-mercaptoethanol were from Eastman
dithiothreitol was from United States Biochemical
Corp., Cleveland, OH, U.S.A.; all other chemicals
were from Fisher Scientific Co., Fair Lawn, NJ,
U.S.A.

Preparation of cell-free extracts
Partially purified extracts were prepared by the
method of Gleason & Hogenkamp (1970), modified
as follows. Harvested and washed cells were
resuspended at $1 \times 10^8$/ml for vitamin B$_{12}$-sufficient
and $5 \times 10^7$ cells/ml for vitamin B$_{12}$-deficient
cultures. After the addition of neutral Norit-A char-
coal, the resulting cell slurries were stored at $-80^\circ$C
before sonication. The cells were disrupted by sonic
oscillation for 10 min at 1 min intervals with a
Bronwell–Blackstone 20 KC Biosonik ultrasonic
probe. The lysate was then centrifuged twice, the
supernatant was dialysed, the nucleic acids were
precipitated and centrifuged off, and the super-
natant was dialysed as described by Gleason &
Hogenkamp (1970). In our preliminary studies we
were unable to detect significant and consistent
values for the activity in the partially purified extracts
prepared from vitamin B$_{12}$-sufficient cells. However,
no activity was found in the sediments produced
after centrifugation of lysates of either the vitamin
B$_{12}$-sufficient or the vitamin B$_{12}$-deficient cells. Also,
mixing experiments involving the use of the above
precipitates with the partially purified enzyme
preparation, in all cases, did not inhibit or add to the
activity of these preparations in terms of the amount
of product formed per sample. To overcome these
difficulties, we freeze-dried the extracts to prepare
more highly concentrated preparations of enzyme
extract, and this process was adopted as a standard
procedure in the preparation of all extracts. The
freeze-dried extracts were stored at $-20^\circ$C until
assayed. No loss of activity was found with this
stored material.

Enzyme assay procedure
Ribonucleotide reductase activity was measured
as the reduction of ATP to dATP. All other
requirements for the assay were as described by
Gleason & Hogenkamp (1970). The amount of
dATP formed was determined by the diphenyl-
amine procedure as described by Blakley (1966). On
the basis of the protein content of the freeze-dried
extracts, sufficient freeze-dried material was dis-
solved in 5 mM-dithiothreitol to give the appro-
imate desired protein concentration, centrifuged at
39,000 g for 45 min at 4°C, and the supernatant
was used for the enzyme assay. Our preliminary studies
with extracts prepared in this manner demonstrated
that the production of dATP proceeded linearly with
time up to about 20 min and with initial enzyme
concentrations in the ranges 0.25–6.0 mg/ml for extracts of vitamin B$_{12}$-deficient cells, 1.0–8.0 mg/
ml for those of exponential-stage cells and 4.0–
16.0 mg/ml for those of plateau-stage vitamin
B$_{12}$-sufficient cells.

Our standard assay procedure consisted of
incubating the reaction mixture at 37°C with
mixing, with samples taken at 0 and 10 min of
incubation and treated as described by Gleason &
Hogenkamp (1970), for the determination of dATP
production. Samples were also taken for the deter-
mination of protein at the end of the assay period.
Results are expressed in terms of specific activity,
defined as units (nmol of dATP formed/h) per mg of
protein.

Protein determinations
Protein concentrations were determined by the
method of Lowry et al. (1951), with purified bovine
serum albumin as a standard.

All results are the average of three determinations
with triplicate samples taken for each determina-
tion.

Results and Discussion
Ribonucleotide reductase activity in vitamin B$_{12}$-
sufficient Euglena
We measured the ribonucleotide reductase ac-
tivity in both the wild-type Euglena gracilis Z strain
and the aplastic mutant Y$_2$ZHL under different
growth conditions. The results (Table 1) show that
both cell types harvested in the exponential stage of
growth demonstrate the same specific activity, of
about 20 units/mg, whether grown in the light or in
the dark and at either pH 3.6 or 6.8. Both types of
cells at the plateau stage of growth show very low or
no detectable activity under the different growth
conditions, even though we used very highly
concentrated extracts.

Our results are comparable with the findings
obtained by Gleason & Hogenkamp (1970) with
respect to exponential-stage cells. However, these
workers have shown exponential-stage Euglena
gracilis var. bacillaris to possess about twice as
much activity as compared with our results. This
difference may be due to the cell type used, since
Z-strain cells have been shown to demonstrate
approx. 40% less activity than var. bacillaris cells
(Gleason & Hogenkamp, 1972; F. K. Gleason,
personal communication). In addition, their media
enhanced a lower division rate, which may be a
factor in increasing the activity.

Ribonucleotide reductase activity in vitamin B$_{12}$-
deficient Euglena
Table 2 shows the ribonucleotide reductase
Table 1. Ribonucleotide reductase activities in exponential-stage and plateau-stage vitamin B12-sufficient Euglena

The enzyme was extracted from Z-strain and mutant Euglena cells grown under different conditions in vitamin B12-sufficient medium and assayed as described in the Experimental section. The data are the mean (+ ± S.D.) values for three determinations with triplicate samples taken for each determination. N.D., No detectable activity.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>pH of medium</th>
<th>Growth condition</th>
<th>Exponential stage</th>
<th>Plateau stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (Z strain)</td>
<td>3.6</td>
<td>Light</td>
<td>21.8 ± 1.5</td>
<td>3.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>Dark</td>
<td>20.4 ± 2.4</td>
<td>3.3 ± 0.0</td>
</tr>
<tr>
<td>Aplastic mutant (Y7ZHL)</td>
<td>3.6</td>
<td>Light</td>
<td>23.1 ± 1.8</td>
<td>3.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>Dark</td>
<td>22.5 ± 0.0</td>
<td>3.7 ± 0.6</td>
</tr>
</tbody>
</table>

Table 2. Relationship between the degree of deficiency and the ribonucleotide reductase activity in Euglena

The enzyme was extracted from Z-strain and mutant Euglena cells grown to various degrees of deficiency in vitamin B12-deficient medium at pH 3.6. Enzyme was assayed as described in the Experimental section. The data are the mean (+ ± S.D.) values for three determinations with triplicate samples taken for each determination.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Growth condition</th>
<th>Early deficiency</th>
<th>Mid deficiency</th>
<th>Late deficiency</th>
<th>Very late deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (Z strain)</td>
<td>Light</td>
<td>248 ± 10</td>
<td>352 ± 3</td>
<td>372 ± 5</td>
<td>418 ± 9</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>278 ± 4</td>
<td>322 ± 6</td>
<td>372 ± 5</td>
<td>424 ± 13</td>
</tr>
<tr>
<td>Aplastic mutant (Y7ZHL)</td>
<td>Light</td>
<td>231 ± 10</td>
<td>315 ± 7</td>
<td>359 ± 7</td>
<td>320 ± 27</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>233 ± 3</td>
<td>308 ± 6</td>
<td>334 ± 23</td>
<td>301 ± 21</td>
</tr>
</tbody>
</table>

activity found in wild-type and mutant cells grown in vitamin B12-deficient medium at pH 3.6 in either the light or the dark. As the cells progress into vitamin B12 deficiency, the ribonucleotide reductase activity increases, reaching maximum values about 20 times higher than those found in exponential-stage vitamin B12-sufficient cells. Similar results were obtained with cells grown in the pH 6.8 medium (results not shown).

Manyfold increases in ribonucleotide reductase activity, as a result of vitamin B12 deficiency, have also been described in other systems. For example, L. leichmannii grown over a nearly 1000-fold range of vitamin B12 concentrations showed about 4-fold increase in the ribonucleotide reductase specific activity with the minimum vitamin B12 concentration (Ghambeer & Blakley, 1965). However, our results compare more favourably with those of Fujioka & Silber (1971), who found a 20-fold increase in the specific activity of the enzyme in megaloblastic bone marrow from patients with vitamin B12 deficiency as compared with normal bone marrow. Previous studies of the human bone-marrow enzyme from patients with pernicious anaemia revealed that the reduction of ribonucleotides occurred at the level of ribonucleoside diphosphates, did not require coenzyme B12 for activity and was inhibited by hydroxuryrea (Fujioka & Silber, 1969). The similarities between vitamin B12-deficiency megaloblastosis in human (cf. Beck, 1975) and vitamin B12 deficiency in Euglena (Carell, 1969; Carell et al., 1970; Christopher et al., 1974) in terms of cell size, RNA, protein and DNA content per cell, extension of the S-phase and the ribonucleotide reductase activity are noteworthy, although the coenzyme does not play the same role in both cases. Much evidence now exists that implicates vitamin B12 deficiency in altered folate metabolism in man (Das & Herbert, 1976), resulting in megaloblastic maturation as a consequence of impaired methylation of deoxyuridylate to thymidylate (Hoffbrand et al., 1976).

A number of studies (Murphree et al., 1969; Lowdon & Vitols, 1973; Elford et al., 1977) with inhibitors of DNA synthesis have shown that a decreased ability to synthesize DNA results in elevated ribonucleotide reductase activity. In addition, a number of studies with E. coli (Biswas et al., 1965; Neuhard & Munch-Petersen, 1966), L. leichmannii (Beck & Hardy, 1965; Ghambeer & Blakley, 1965) and yeast (Lowdon & Vitols, 1973), as well as a study with mammalian cells (Elford et al., 1977), have indicated that elevated ribonucleotide reductase activity results as a consequence of
de-repressed enzyme synthesis $de$ $novo$ rather than enzyme activation, and that a phosphorylated derivative of thymidine, either dTTP or dTMP, may serve as the repressor or co-repressor of synthesis of the enzyme. That this may be the case in vitamin B$_{12}$-deficient Euglena is implied by the fact that ribonucleotide reductases are inducible enzymes and are synthesized just before and during the S-phase (cf. Follmann, 1974). Therefore, since it has been shown that the S-phase in vitamin B$_{12}$-deficient Euglena is differentially extended (Carell et al., 1970; Christopher et al., 1974), the synthesis of this enzyme is considered to continue. In addition, Carell & Goetz (1976) and Goetz & Carell (1978) have shown that (1) vitamin B$_{12}$-deficient Euglena cells have no detectable amounts of dNTP, (2) addition of vitamin B$_{12}$ to deficient cells increases the dNTP pool sizes to 6-fold or more than those found for exponential-stage vitamin B$_{12}$-deficient cells, and (3) addition of cycloheximide to the deficient cells does not affect the expansion of the dNTP pools on replenishment of vitamin B$_{12}$. Therefore we suggest that the increase in enzyme activity is due to an increase in the amount of enzyme protein rather than to enzyme activation. However, a test such as the addition of cycloheximide to cells advancing into the deficiency state was not done.

Furthermore, as indicated in Table 2, whether vitamin B$_{12}$-deficient Euglena cells are grown in the light or in the dark has no significant effect on the activity. However, the activity in the mutant cells is apparently lower than that found for wild-type cells. We have no explanation to account for this difference other than the possibility involving a chloroplastic ribonucleotide reductase. To our knowledge, no such enzyme has been identified. Although this difference may not be significant, it is tempting to speculate on the existence of a chloroplast-specific ribonucleotide reductase. To test this possibility will require a more sensitive method than used here.

It is noteworthy that a mitochondrial ribonucleotide reductase, from regenerating rat liver cells, has already been identified and had its requirements characterized (Larsson, 1969; Danilova et al., 1977), and has been found to resemble the type of enzyme found in E. coli.

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References

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