Ribonucleotide Reductase Activity in Vitamin B₁₂-Deficient *Euglena gracilis*

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The ribonucleotide reductase activities in vitamin B₁₂-sufficient and -deficient cells of *Euglena gracilis* were measured. We found that as the cells progress into vitamin B₁₂ 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 B₁₂-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 *Escherichia coli* and appears to be characteristic of mammals and some higher plants (cf. Follman, 1974; Reichard, 1978). The enzyme found in *Lactobacillus leichmannii* (Abrams, 1965; Blakley *et al.*, 1965; Beck & Hardy, 1965) and *Pithomyces chartarum* (Stutzenberger, 1974) requires 5'-deoxyadenosylcobalamin as a cofactor and reduces the ribonucleoside triphosphates. The third type is characteristic of *Bacillus megaterium* (Yau & Wachsmann, 1973) and *Rhizobium meliloti* (Cowles & Evans, 1968). This enzyme requires deoxyadenosylcobalamin, but reduces the ribonucleoside diphosphates.

Ribonucleotide reductase of *Euglena gracilis* has been shown to require vitamin B₁₂, 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 B₁₂-deficient and -replenished *Euglena*, have

shown the pools to be undetectable in vitamin B₁₂-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 B₁₂-replenished cells did not inhibit the accumulation of the dNTP, suggesting that vitamin B₁₂-deficient cells may accumulate ribonucleotide reductase apoenzyme, which lacks the vitamin B₁₂ 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 & Vitols, 1973) and fluorodeoxyuridine and methotrexate (Elford *et al.*, 1977) have resulted in elevated ribonucleotide reductase activities.

In the present paper we show that vitamin B₁₂-deficient *Euglena gracilis* displays manyfold 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

Euglena gracilis Klebs strain Z (Pringsheim) and the aplastidic mutant Y₇ZHL (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 K₂HPO₄ as buffer and titrated to pH 6.8 with aq. NH₃. Growth measurements in terms of cell number, production of vitamin B₁₂ 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-chloroacetamide and 2-mercaptoethanol were from Eastman Organic Chemical Co., Rochester, NY, U.S.A.; 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 1×10^8 /ml for vitamin B₁₂-sufficient and 5×10^7 cells/ml for vitamin B₁₂-deficient cultures. After the addition of neutral Norit-A charcoal, the resulting cell slurries were stored at -80°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 supernatant 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 B₁₂-sufficient cells. However, no activity was found in the sediments produced after centrifugation of lysates of either the vitamin B₁₂-sufficient or the vitamin B₁₂-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°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 diphenylamine procedure as described by Blakley (1966). On the basis of the protein content of the freeze-dried extracts, sufficient freeze-dried material was dissolved in 5 mM-dithiothreitol to give the approximate desired protein concentration, centrifuged at 39000g 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 B₁₂-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 B₁₂-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 determination 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 determination.

Results and Discussion

*Ribonucleotide reductase activity in vitamin B₁₂-sufficient *Euglena**

We measured the ribonucleotide reductase activity in both the wild-type *Euglena gracilis* Z strain and the aplastidic mutant Y₇ZHL 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 B₁₂-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 ($\pm$ S.D.) values for three determinations with triplicate samples taken for each determination. N.D., No detectable activity.

Cell type	pH of medium	Growth condition	Sp. activity of ribonucleotide reductase (nmol of dATP/h per mg of protein)	
			Exponential stage	Plateau stage
Wild-type (Z strain)	3.6	Light	21.8 $\pm$ 1.5	3.5 $\pm$ 0.0
		Dark	20.4 $\pm$ 2.4	3.3 $\pm$ 0.0
	6.8	Light	23.1 $\pm$ 1.8	3.3 $\pm$ 0.0
		Dark	22.5 $\pm$ 0.0	3.7 $\pm$ 0.6
Aplastidic mutant (Y ₇ ZHL)	3.6	Light	19.8 $\pm$ 0.0	N.D.
		Dark	20.1 $\pm$ 1.3	4.2 $\pm$ 0.5
	6.8	Light	20.6 $\pm$ 1.6	3.9 $\pm$ 0.5
		Dark	21.0 $\pm$ 1.6	N.D.

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 ($\pm$ S.D.) values for three determinations with triplicate samples taken for each determination.

Cell type	Growth condition	Sp. activity of ribonucleotide reductase (nmol of dATP/h per mg of protein)			
		Early deficiency	Mid deficiency	Late deficiency	Very late deficiency
Wild-type (Z strain)	Light	248 $\pm$ 10	352 $\pm$ 3	372 $\pm$ 5	418 $\pm$ 9
	Dark	278 $\pm$ 4	322 $\pm$ 6	372 $\pm$ 5	424 $\pm$ 13
Aplastidic mutant (Y ₇ ZHL)	Light	231 $\pm$ 10	315 $\pm$ 7	359 $\pm$ 7	320 $\pm$ 27
	Dark	233 $\pm$ 3	308 $\pm$ 6	334 $\pm$ 23	301 $\pm$ 21

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 hydroxyurea (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; Neuhaard & 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 B₁₂-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 B₁₂-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 B₁₂-deficient *Euglena* cells have no detectable amounts of dNTP, (2) addition of vitamin B₁₂ to deficient cells increases the dNTP pool sizes to 6-fold or more than those found for exponential-stage vitamin B₁₂-sufficient cells, and (3) addition of cycloheximide to the deficient cells does not affect the expansion of the dNTP pools on replenishment of vitamin B₁₂. 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 B₁₂-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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