Tight coordination of ribonucleotide reduction and thymidylate synthesis in synchronous algae

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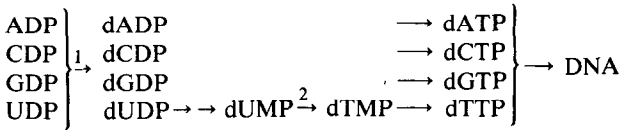
Ribonucleoside diphosphate reductase and thymidylate synthase activities follow a virtually identical peak pattern during the cell cycle of *Scenedesmus obliquus*, coinciding with DNA synthesis. Both enzymes change in parallel when cultures are treated with inhibitors specific for one of them: 5-fluoro-2′-deoxyuridine (a thymidylate synthesis inhibitor) stimulates 30-fold, and hydroxyurea, inhibitory to eukaryotic ribonucleotide reduction, also suppresses thymidylate synthase. We conclude that the two enzymes of deoxyribonucleotide formation are subject to one common intracellular control mechanism.

Ribonucleotide reductase *Thymidylate synthase* *Fluorodeoxyuridine* *Hydroxyurea* *Cell cycle* *Green algae*

1. INTRODUCTION

Functioning of the complete apparatus for formation of deoxyribonucleotides is obligatory for DNA replication to proceed at the correct rate and time during the cell cycle. Two main synthetic enzymes of this sequence are ribonucleoside diphosphate reductase and thymidylate synthase:



1 = ribonucleotide reductase (EC 1.17.4.1)
2 = thymidylate synthase (EC 2.1.1.45)

Cell cycle- or growth-dependence has been demonstrated for both these universal enzyme systems in several organisms, especially in animal cells (e.g., sea urchin eggs or mouse and hamster cells) [1–6]. A regulatory link between them was postulated [7] in which an excess, or absence of thymidine nucleotides represses or derepresses ribonucleotide reductase synthesis. However, both enzymes have not been measured in parallel during cell proliferation, nor have the metabolic parameters under which they affect each other been analyzed in detail. In continuation of our studies of deoxyribonucleotide biosynthesis in synchronous cultures of the green algae, *Scenedesmus obliquus* [8], we have now found that the two activities are tightly coupled under a variety of conditions. These observations support the idea of enzyme cooperation for DNA precursor and DNA synthesis [9].

2. MATERIALS AND METHODS

Axenic cultures of *S. obliquus*, strain D3, were grown and synchronized in a light–dark regime as in [8]; cell rupture and the assay of ribonucleotide reductase activity using [5-^{3}H]cytidine diphosphate as substrate were carried out as described [8]. DNA was determined fluorometrically by the reaction with 3,5-diaminobenzoic acid [10], and protein was determined by the Lowry method.

Reproducible measurement of thymidylate synthase activity in algal extracts, dialyzed in the cold for 15 h against 0.05 M Na-phosphate buffer (pH 7.5) containing 10 mM mercaptoethanol, was performed as follows. Assays contained, in a total

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volume of 0.20 ml of the above buffer, 1–5 μCi [5-^{3}H]dUMP (Amersham Buchler; diluted to spec. act. 1 Ci/mmol); 0.13 mM tetrahydrofolate (prepared after [11] and stored under N_2); 2.5 mM formaldehyde; and up to 0.15 ml of algal extract (0.5–1 mg soluble protein). Controls without extract or without tetrahydrofolate/formaldehyde were always included to correct for non-enzymatic and for extract-catalyzed unspecific tritium exchange. The mixtures were incubated for 1 h at 25°C, and the reaction was then terminated by addition of 200 mg acid-washed charcoal suspended in 1 ml 2% trichloroacetic acid; 0.20 ml of the centrifuged, clear supernatant was used for liquid scintillation counting to measure dTMP synthase-catalyzed tritium release to water.

3. RESULTS AND DISCUSSION

The close parallelism of thymidylate synthase and ribonucleotide reductase activity during the S-phase of light–dark synchronized *Scenedesmus* cultures is shown in fig.1. Induction of both enzymes occurs at the same time, 8 h after illumination. Their maxima may vary by about 1 h from experiment to experiment due to inevitable growth variations in different cultures, but in general the two enzyme activity peaks always coincide with the midpoint of DNA synthesis at 12 h. Both these enzymes are difficult to determine in crude plant extracts [12,13]. We have used assay procedures individually optimized for pH, substrate and cofactor concentrations and find activities comparable within an order of magnitude. As the reductase is certainly underestimated more than dTMP synthase, true enzyme activities may be even closer to each other.

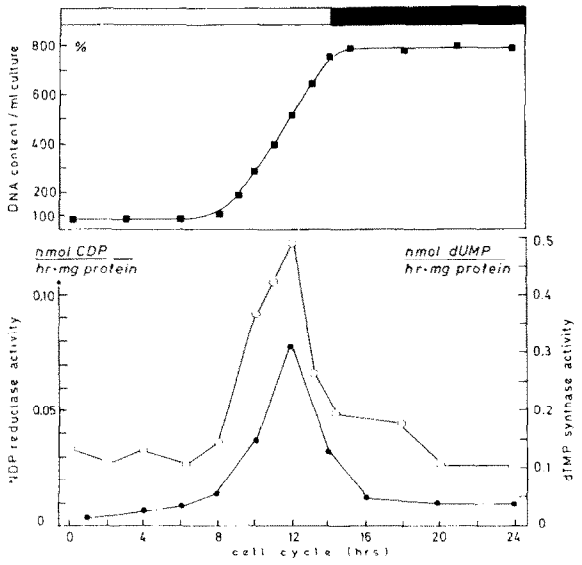

Fig.1. Enzyme activities and DNA synthesis (upper panel) in a light–dark (14:10 h) synchronized culture of *S. obliquus*. Cell-free extracts were prepared from samples harvested at various times of the cell cycle and analyzed for ribonucleotide reductase (●---●, left scale) and thymidylate synthase (○---○, right scale).

Our approach to gain insight into possible links between the DNA precursor-producing enzymes was to follow both under the influence of inhibitors which, by their mechanism, affect only one of them. One such compound is 5-fluorodeoxyuridine (FdU) which, as the 5′-phosphate, is a specific inhibitor of all thymidylate synthases previously studied. We have confirmed inhibition by FdUMP of the enzyme newly isolated from *Scenedesmus* (fig.2). FdU or FdUMP are not ribonucleotide reductase effectors in vitro, but it has been observed in animal cell cultures and in algae that in vivo FdU greatly stimulates ribonucleotide reductase activities [7,8] while blocking DNA synthesis and cell proliferation through lack of thymidylate. When measuring

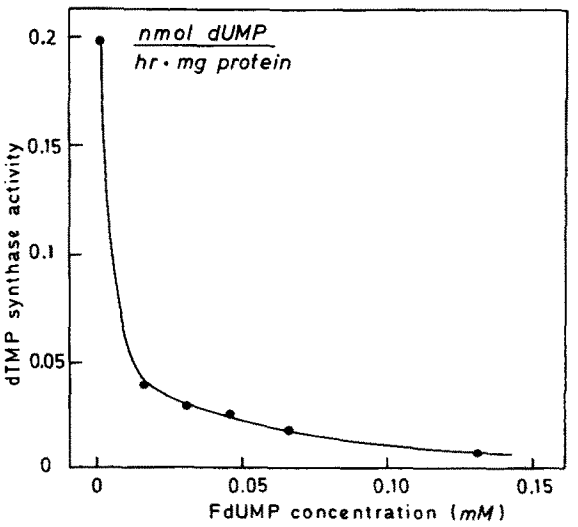

Fig.2. Inhibition of thymidylate synthase in cell-free, dialysed extracts of *Scenedesmus* by increasing concentrations of 5-fluorodeoxyuridine 5′-phosphate (FdUMP) added to the assay medium.

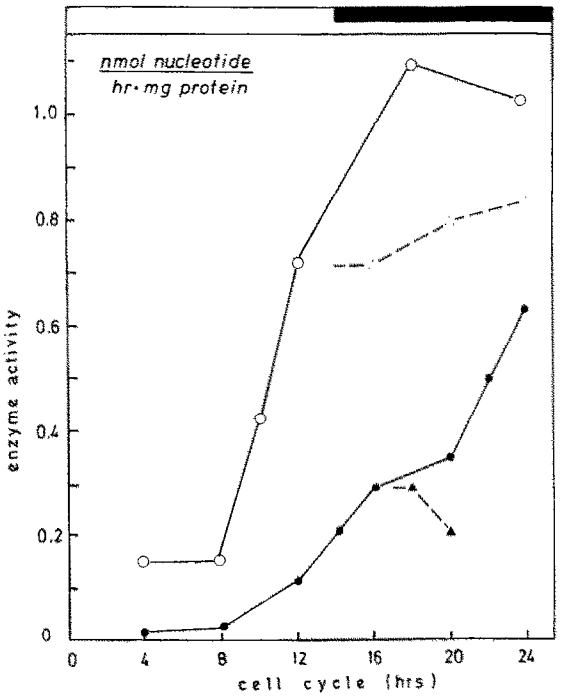

Fig.3. Overproduction of ribonucleotide reductase (●---●) and thymidylate synthase (○---○) in *S. obliquus* treated with 20 μg 5-fluorodeoxyuridine/ml culture. See fig.1 for the untreated controls. FdU may be added from 0–9 h of a cell cycle without much difference in the results. Enzyme activities rise highest under continued illumination after the 14th h and level off earlier in the dark (▲,△), probably due to the energy requirement of protein synthesis [8].

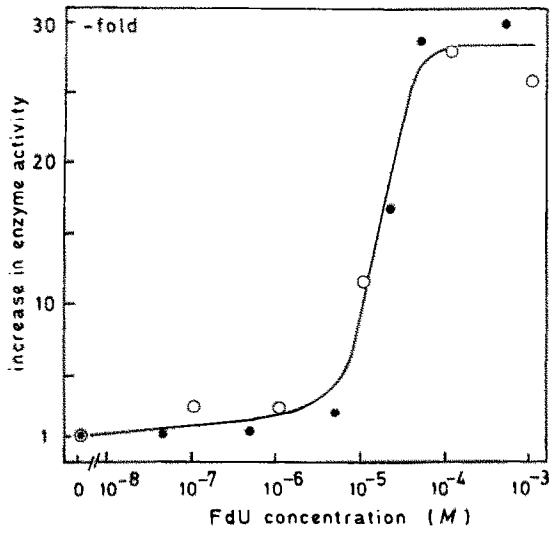

Fig.4. Effect of an increasing concentration of 5-fluorodeoxyuridine upon ribonucleotide reductase (●) and thymidylate synthase activity (○) in *S. obliquus*. FdU was added to the cultures at 0 h; enzyme activities were determined in extracts from cells harvested at the 22nd h and are expressed as increase over the activity found in untreated algae.

thymidylate synthase activity in extracts of such FdU-treated algae we made the remarkable observation that there is a completely analogous stimulation of this enzyme, although it had been expected to be inactivated in a complex with FdUMP (fig.3). The dose–response curve of FdU action in algal cultures is virtually identical for the increase of reductase and dTMP synthase activities (fig.4). Despite elevated enzyme level the intracellular thymidine triphosphate pool is low [8]. These properties taken together indicate that, whereas the algal thymidylate synthase is inhibited in vivo, it does not interact with FdUMP to yield an irreversible complex like bacterial enzymes [14,15]; rather, the active enzyme is recovered upon removal of the nucleotide analog during work-up in vitro. If dialysis is omitted at that stage, thymidylate synthase activity in the cell extract is in fact negligible.

Hydroxyurea (HU) has the reverse effect, but again acts on both enzymes in parallel. HU is a potent inhibitor of the iron-containing algal ribonucleoside diphosphate reductase in vitro [16]; its mode of action most likely combines metal chelation and radical scavenging effects. *Scenedesmus* thymidylate synthase is completely unaffected in vitro by up to 10 mM HU. However, the presence of 0.1–5 mM hydroxyurea in the culture medium during G_1 not only inhibits appearance of ribonucleotide reductase but also strongly reduces thymidylate synthase activity in algal extracts (table 1). It should be noted that hydroxyurea does not impair protein synthesis in general [17].

The strictly parallel effects of FdU and HU let us conclude that the S phase-specific peak of both deoxyribonucleotide-synthesizing enzymes reflects coordinate regulation, through a mechanism which is still unknown. Both compounds reduce all intracellular deoxyribonucleotide concentrations, with the exception of dATP in the presence of FdU [8]. Therefore we feel that DNA precursor production in the algae does not fit a model in which

Table 1

Effect of hydroxyurea on ribonucleotide reductase and thymidylate synthase activities in two different cultures of *S. obliquus*. Algae were harvested at 12 h

HU concentration (mM)	Added at h	Reductase (nmol.mg^{-1}.h^{-1})	dTMP synthase (nmol.mg^{-1}.h^{-1})
0 (control)		0.06	0.82
0.1	0	n.d.	0.18
1	0	n.d.	0.22
5	0	n.d.	0.19
0 (control)		0.06	0.38
5	0	n.d.	0.22
5	3		0.25
5	6	<0.01	0.22

n.d., not detectable

deoxyribonucleotide pools serve as signal for enzyme synthesis [7]. Preliminary experiments with thymidylate-uptake mutants of yeast (M. Lammers, M. Brendel, unpublished) also contradict such form of control.

Evidence has been accumulating in viral and mammalian systems that several enzymes of DNA precursor metabolism and DNA replication (e.g., thymidine and nucleoside diphosphate kinase, dihydrofolate reductase, dTMP synthase, ribonucleotide reductase, and DNA polymerase) exist in loosely organized enzyme aggregates [9,18,19]. Our experience with the purification of *Scenedesmus* ribonucleotide reductase and thymidylate synthase (to be described elsewhere) suggests that the two plant enzymes share similar cooperativity. Except by affinity chromatography on a reductase-specific adsorbent, aminohexyl-dATP Sepharose [8], where thymidylate synthase is not bound, it has not as yet been possible to separate the two enzyme activities from each other by conventional methods. Remarkably, recombination of the fractions obtained after affinity chromatography results in a 2-fold stimulation of specific activities. Considering that the delicate enzymes usually decrease in activity following almost any addition, such an increase can only be interpreted as a consequence of mutually favourable protein–protein interactions. These properties are currently under study.

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