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COORDINATE AND NON-COORDINATE ACCUMULATION OF ASPARTATE TRANSCARBAMYLASE AND DIHYDROOROTASE IN SYNCHRONOUS *CHLORELLA* CELLS GROWING ON DIFFERENT NITROGEN SOURCES

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Summary

Regulation of the levels of aspartate transcarbamylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) and dihydroorotase (L-5,6-dihydro-orotate amidohydrolase, EC 3.5.2.3) was studied in synchronous cultures of the eucaryotic microorganism *Chlorella*. Analytical polyacrylamide gel electrophoresis and sucrose density-gradient centrifugation studies revealed that these cells contain a single aspartate transcarbamylase and a dihydroorotase with apparent molecular weights of 160 000 and 88 000, respectively. In synchronous cells cultured in nitrate medium, these two enzymes accumulated in single step-patterns over different periods of the cell cycle. In contrast, these enzymes accumulated in a coordinate manner throughout the cell cycle in ammonium medium. Experiments with inhibitors of protein and RNA synthesis indicated that dihydroorotase is stable in vivo and suggested that cell cycle changes in the turnover rate of aspartate transcarbamylase might determine whether or not these enzymes accumulate in a coordinate manner. Although uracil and uridine could be absorbed and metabolized by the cells, synthesis of these two enzymes could not be repressed by culturing synchronous cells in medium, containing high concentrations (29—40 mM) of uracil or uridine, for an entire cell cycle.

Introduction

End-product repression of the synthesis of the pyrimidine biosynthetic enzymes appears to play a major role in regulation of the levels of these

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enzymes during the bacterial cell cycle [1—5]. In eucaryotes, cellular compartmentalization [6,7] of these enzymes and their existence in multienzyme complexes [8—12] and enzyme turnover [13,14] offer additional possible mechanisms for regulation of pyrimidine enzyme levels during the cell cycle.

In this laboratory, synchronous cultures of the highly compartmentalized eucaryotic microorganism, *Chlorella*, are being used to study the manner in which the levels of the pyrimidine biosynthetic enzymes are regulated at different times during the eucaryotic cell cycle. In an earlier publication [15], enzyme turnover, enzyme stabilizer(s), and gene replication were implicated in the regulation of aspartate transcarbamylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) levels during the *Chlorella* cell cycle. In the present paper, studies on the cell cycle patterns of *Chlorella* aspartate transcarbamylase and dihydroorotase (L-5,6-dihydroorotate amidohydrolase, EC 3.5.2.3) show that, although these enzymes are adjacent on the same pathway their apparent turnover rates in vivo are different and the nutritional status of the cell determines whether or not the accumulation of these enzymes is coordinately regulated during the cell cycle.

Materials and Methods

Organism and growth conditions

Cells of the thermophilic green alga, *Chlorella sorokiniana* [16,17] were cultured and partially synchronized by intermittent illumination [15,18—20] and their synchrony was improved further by use of isopycnic centrifugation [19,21,22]. The growth conditions and synchronization procedures for cells cultured in nitrate medium and ammonium medium have been described by Vassef et al. [15] and Israel et al. [23], respectively.

Preparation of cell material for analyses

The harvest, wash, and cell breakage procedures were as described by Vassef et al. [15]. The activities of aspartate transcarbamylase and dihydroorotase were assayed in 100 000 × *g* supernatants of cell homogenates or in frozen-thawed whole cells prepared by a modification of the procedure of Vassef et al. [15]. After washing twice with 0.01 M Tris · HCl buffer, pH 8.5, cells were resuspended to a concentration of 0.05 ml packed cells (i.e., $4 \cdot 10^9$ daughter cells) per ml of 0.2 M Tris · HCl buffer (pH 8.5, containing 1 mM UMP and 1 mM EDTA), frozen at −20°C for 48 h as 0.5-ml aliquots in glass tubes (1.3 × 6 cm), and then thawed on ice. After thawing, the cells were washed three times at 3°C with 0.2 M Tris · HCl buffer (pH 8.5, containing 1 mM UMP) to remove endogenous small molecules prior to assay. As discussed by Schmidt [14], the addition of UMP to the freeze-thaw and wash buffers is essential for stabilization of total aspartate transcarbamylase activity prior to assay. Upon addition of the cells to the assay mixture, the final concentration of UMP is 0.4 mM. UMP has no effect on the activity or stability of dihydroorotase.

Assay of dihydroorotase and aspartate transcarbamylase activity

The enzymic production of carbamyl aspartate by dihydroorotase and aspartate transcarbamylase, was measured in their reverse [24] and forward reac-

tions, respectively, by a radioactive [25] and a colorimetric [26] procedure.

In both procedures, the final concentration of compounds in the dihydroorotase assay mixture was 30 mM dihydroorotate, 1.5 mM reduced dithiothreitol, and 0.2 M Tris · HCl buffer (pH 8.5).

Sulfhydryl compounds inhibit color development in Method II of the Prescott and Jones [26] colorimetric procedure. Therefore, when dithiothreitol was present in the dihydroorotase assay mixture, it was removed by Norit-A prior to color development. After the reaction had been stopped with formic acid, approximately 20 mg of dampened Norit-A was added to the 100 μl assay mixture. The resulting suspension was mixed, allowed to stand for 10 min, centrifuged, and 20—50 μl of supernatant analyzed by the colorimetric procedure.

The measurement of aspartate transcarbamylase activity by the aforementioned radioactive and colorimetric procedures was described earlier by Vassef et al. [15].

In general, the radioactive assay was employed with frozen-thawed cells and crude cell homogenates, and the colorimetric assay with enzyme-containing fractions from electrophoresis and sucrose density gradient experiments.

Analytical disc gel electrophoresis

Electrophoresis was performed with 7% polyacrylamide separating gels of approximately 70 mm in length by the procedure described by Talley et al. [18]. Approximately 150 μl (0.9 mg protein) of enzyme preparation (90 mU dihydroorotase) concentrated by ammonium sulfate precipitation (40—70%) was polymerized within the stacking gel. Essentially all of the dithiothreitol-dependent and -independent dihydroorotase activity present in the original crude homogenate was recovered in the 40—70% ammonium sulfate precipitate. Immediately following electrophoresis, the gels were sliced into 1.27 mm transverse sections with the gel slicer describded by Chrambach [27]. Each gel slice was cut in half and each half placed in an Eppendorf microcentrifuge tube with 100 μl of 0.2 M Tris · HCl buffer (pH 8.5), and assayed for dihydroorotase activity in the presence or absence of reduced dithiothreitol by use of the aforementioned colorimetric procedure.

Sucrose density gradient centrifugation

12-ml linear gradients from 5 to 20% sucrose were prepared from 20% (w/v) sucrose in 0.2 M Tris · HCl buffer, pH 8.5, containing 1 mM UMP, 20 mM $MgCl_2$, 6 mM glutamine, and 5% glycerol. Some of the gradients contained 2 mM dithiothreitol and 30% dimethylsulfoxide. The enzyme preparations (0.5 ml) layered on these gradients were 100 000 × *g* supernatants (1.5 h) prepared from cells ruptured in buffer containing all compounds also present in the gradients. The gradients were centrifuged in a Beckman model L2-65B ultracentrifuge (SW 40 rotor) at 34 700 rev./min for 20 h at 3°C. Fractions of 250 μl were taken at the end of the centrifugation period.

Approximately 10 μg of bovine liver catalase was used an an internal marker. Catalase activity in gradient fractions was assayed by a method similar to that employed by Martin and Ames [28]. The molecular weight of the enzymes were calculated from sedimentation rates by the method of Martin and Ames [28].

One enzyme unit of aspartate transcarbamylase or dihydroorotase activity is defined as that amount which catalyzes the formation of 1 μmol of carbamyl aspartate per min.

Total DNA, RNA, protein and cell number measurements

Total DNA was measured using the Burton [29] diphenylamine procedure as modified for *Chlorella* by Hopkins et al. [30]. Total protein was determined by the method of Lowry et al. [31] on the material obtained by extraction of the cells with 1 M NaOH for 12 h at 25°C. For total nucleic acid and RNA mea surements (as in Fig. 6B), the cells were extracted two times with cold 10% trichloroacetic acid and then with 0.5 M perchloric acid at 70°C for 40 min with frequent stirring. Total RNA or total nucleic acid was determined by measuring the absorbance at 260 nm of the hot perchloric acid-soluble fraction. The correction for the contribution of DNA was described by Vassef et al. [15]. Cell number was determined with a model B Coulter Counter.

Reagents

All radioactive reagents were obtained from New England Nuclear; cycloheximide and actinomycin D were gifts from Upjohn and Merck, Sharp, and Dohme. Uridine, dihydroorotic acid, dithiothreitol were from Calbiochem and pyridine nucleotides and UMP from P-L Biochemicals. The sources of the other chemicals are cited in previous publications [15,18].

Results and Discussion

Development of assay for Chlorella dihydroorotase activity

The permeability barriers of frozen-thawed whole *Chlorella* cells to proteins and small molecules are selectively changed [15]. Whereas enzymes cannot diffuse from frozen-thawed cells, the permeability barriers to both endogenous and exogenous small molecules (e.g. amino acids and nucleotides) are destroyed. Thus, if frozen-thawed cells are assayed before and after washing with buffer, it is possible to determine if endogenous small molecules are required for activity or stability of a particular enzyme.

In the present study, two experiments with frozen-thawed cells indicated that *Chlorella* dihydroorotase requires endogenous small molecule(s) for maximal activity. First, a suspension of frozen-thawed cells was serially diluted either with additional freeze-thaw extract (after removal of cells by centrifugation) or with fresh 0.2 M Tris · HCl buffer (pH 8.5) and then assayed immediately for dihydroorotase activity. Whereas dihydroorotase activity was directly proportional to cell number in cells diluted with freeze-thaw extract, this relationship was not observed for cells diluted with buffer (Fig. 1A). Second, the endogenous small molecule fraction was removed from frozen-thawed cells with four washes with Tris · HCl buffer. After each buffer wash, the cells were centrifuged and then resuspended either in buffer or in the freeze-thaw extract obtained from the cells prior to the first buffer wash. Although dihydroorotase activity decreased with each buffer wash, the readdition of the original freeze-thaw extract restored essentially complete enzyme activity (Fig. 1B).

For the subsequent series of experiments, frozen-thawed cells were washed

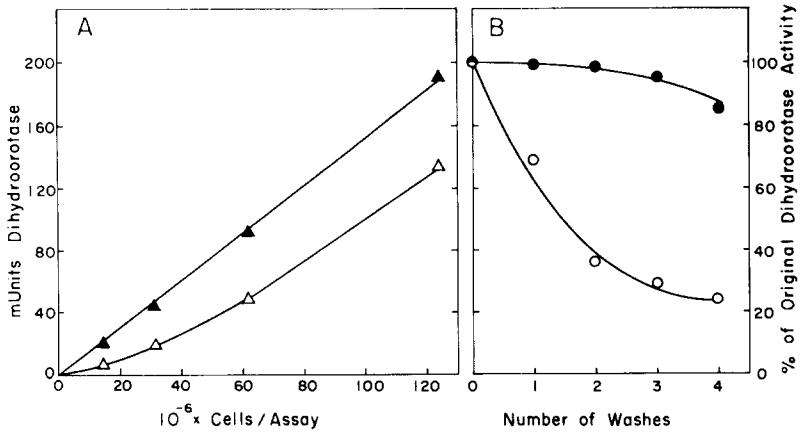

Fig. 1. Dependence of dihydroorotase activity upon endogenous small molecules in cells of *Chlorella sorokiniana*. A, enzyme activity in frozen-thawed cells serially diluted with extract from frozen-thawed cells (▲) or with Tris · HCl buffer (△). B, enzyme activity in frozen-thawed cells after being washed with Tris · HCl buffer (○), and after readdition of original freeze-thaw extract (●) to these washed cells. The cells were frozen and thawed in 0.2 M Tris · HCl buffer, pH 8.5, to obtain the freeze-thaw cell extract.

three times with Tris · HCl buffer and then used as a standardized assay system for testing the effects of different compounds or fractionated cell extracts on dihydroorotase activity. These washed cells contained only 30% of the dihydroorotase activity of unwashed cells. The first small molecules tested in this assay system were inorganic ions. Since dihydroorotase from *Zymobacterium oroticum* [32] is activated by Zn^{2+}, the effect of this metal ion and Ca^{2+}, Co^{2+}, Cu^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Mo^{2+}, Na^{+}, Ni^{2+}, NH_4^{+}, PO_4^{2-}, and SO_4^{2-} were individually tested in the concentration range of 10^{-6}—10^{-1} M in Tris · HCl buffer (pH 8.5). None of these ions enhanced *Chlorella* dihydroorotase activity; however, Cu^{2+}, Mn^{2+}, and Ni^{2+} inhibited enzyme activity. This experiment did not exclude the possibility that at least one of these ions might act in concert with some other endogenous small molecule which had been washed from the cells used in the standardized assay. Therefore, to test this possibility, EDTA was added directly to unwashed frozen-thawed cells in freeze-thaw extract. Over a concentration range of 10^{-6}—10^{-1} M, this metal ion chelator enhanced rather than inhibited enzyme activity. When EDTA was added to buffer-washed cells suspended in Tris · HCl buffer, it had little effect on enzyme activity, indicating that EDTA does not directly affect enzyme activity. Thus, the *Chlorella* dihydroorotase seems to be similar to the enzyme in *Escherichia coli* B [33] and pea seedlings [34] in that free metal ions are not required for maximal enzyme activity.

The increase in activity of dihydroorotase, observed after addition of EDTA to cells in freeze-thaw extracts, suggested that in crude-extracts metal ions might be binding to sulfhydryl-groups on the enzyme or to those on compounds involved in activation of the enzyme. Therefore, reduced forms of dithiothreitol and glutathione were tested as possible in vitro activators of the enzyme. Whereas 100% of original enzyme activity could be restored by addition of reduced dithiothreitol (1.5 mM) to frozen-thawed washed cells (Fig. 2A), reduced glutathione (1—8 mM) could activate the enzyme to only 90% of

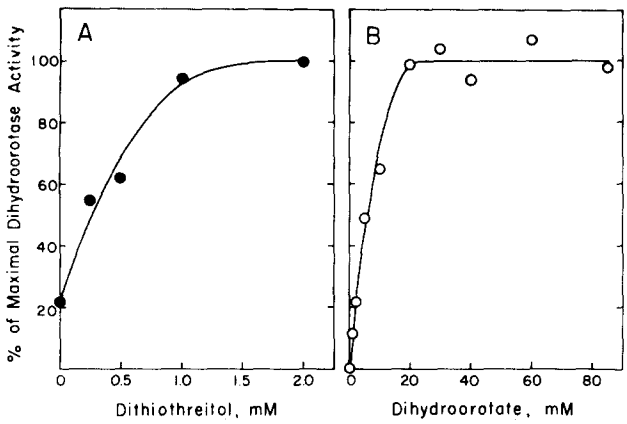

Fig. 2. The activity of dihydroorotase in cells of *Chlorella sorokiniana* in different concentrations of reduced dithiothreitol and dihydroorotate for a 20-min assay period. A, the assay mixture contained 30 mM dihydroorotate in addition to different concentrations of dithiothreitol. B, the assay mixture contained 1.5 mM reduced dithiothreitol in addition to different concentrations of dihydroorotate. Buffer washed frozen-thawed cells were used as the source of enzyme. The same saturation levels with these compounds were obtained with dihydroorotase isolated by ammonium sulfate fractionation (see Materials and Methods). With 1.5 mM dithiothreitol and 20 mM dihydroorotate, the enzymic production of carbamyl aspartate was linear for at least 20 min.

its maximal activity. Since preliminary experiments indicated that the endogenous activator was not saturating the enzyme at all times in the cell cycle, the enzyme was assayed in frozen-thawed washed cells in the presence of 1.5 mM dithiothreitol and 20 mM dihydroorotate (Fig. 2B) in all cell cycle studies reported herein.

The observation that Norit-A can remove the endogenous activator(s) from cell extracts led to the discovery that reduced dithiothreitol also can be removed with Norit-A from assay mixtures.

Evidence for single forms of aspartate transcarbamylase and dihydroorotase in Chlorella

In studies on enzyme regulation during the cell cycle, it is essential to know how many enzyme forms (i.e., isozymes) contribute to a given total cellular enzyme activity. The dithiothreitol-dependent and -independent dihydroorotase activities observed in frozen-thawed cells suggested that either (a) a single form of the enzyme exists which is partially active (i.e., 30%) in the absence of sulfhydryl activators, or (b) multiple forms (isozymes) of dihydroorotase exist in *Chlorella*. To distinguish between these two possibilities, the proteins from cell homogenates were fractionated by polyacrylamide gel electrophoresis and by sedimentation in sucrose gradients. Fractions were then analyzed for dihydroorotase activity in the presence and absence of reduced dithiothreitol. The results from the electrophoresis (Fig. 3A) and sedimentation (Fig. 3B) studies are consistent with *Chlorella* cells containing a single form of dihydroorotase with a molecular weight of 88 000, which has partial activity in the absence of a sulfhydryl activator. In both studies, the dithiothreitol-dependent and-independent activities moved as single and coincident peaks (Fig. 3A, B). Although aspartate transcarbamylase activity was inactivated by electrophoresis, its sedi-

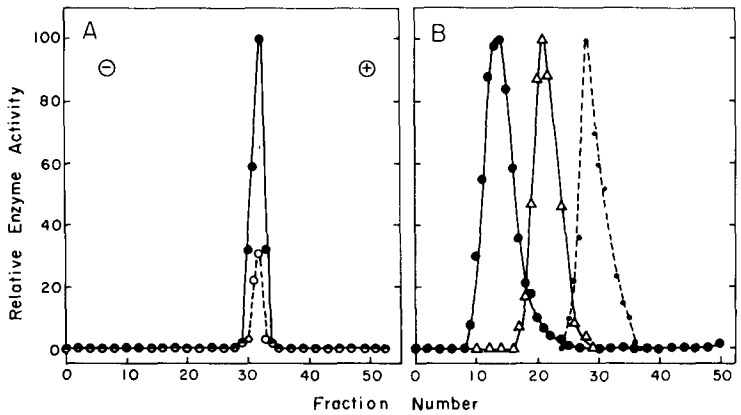

Fig. 3. Analytical disc gel electrophoresis and sucrose density gradient centrifugation of dihydroorotase from *Chlorella sorokiniana*. A, After electrophoresis, the gel was sliced and then assayed for dihydroorotase activity either in the presence (●) or absence (○) of dithiothreitol. B, the sedimentation rates of *Chlorella* dihydroorotase (●) and aspartate transcarbamylase (△) were compared to bovine liver catalase (● - - - - - - ●) used as a marker in linear gradients of sucrose (5—20%, w/v) containing 1 mM UMP, 20 mM $MgCl_2$, 6 mM glutamine, and 5% glycerol. The peak fractions of dihydroorotase and aspartate transcarbamylase activity corresponded to 3.3 and 30 mU, respectively. All gradient fractions were assayed for dihydroorotase activity in the absence and presence of dithiothreitol (1.5 mM). However, for visual clarity only those assayed with the dithiothreitol are plotted. The activity of dihydroorotase in the absence of the reducing agent was 30% of maximal activity and was coincident in position with the activity measured in the presence of the agent.

mentation pattern in sucrose gradients (Fig. 3B) is also consistent with *Chlorella* containing only a single form of this enzyme with a molecular weight of 160 000. This molecular weight is identical to the one determined in an earlier study [15].

Cell cycle patterns of aspartate transcarbamylase and dihydrootase activity

In synchronous *Chlorella* cells cultured in nitrate medium, the activities of aspartate transcarbamylase and dihydroorotase increased as single step-patterns over different periods of the cell cycle (Fig. 4). As reported earlier from this laboratory [15,22], the step pattern for aspartate transcarbamylase was nearly coincident with that for total cellular DNA. However, the onset of the period of accumulation of dihydroorotase activity preceded the S-phase by several hours. Because the activities of these enzymes were measured in frozen-thawed cells which had been washed with fresh buffer prior to assay, the activities of these enzymes were not affected by possible endogeneous activators or inhibitors during the assay in vitro. Thus, this cell cycle study shows that these enzymes do not accumulate in a coordinate manner in nitrate-cultured cells under these culture conditions.

The in vivo stability of dihydroorotase was examined after inhibition of protein and RNA synthesis by cycloheximide and actinomycin D, respectively. When cycloheximide (25 μg/ml) or actinomycin D (190 μg/ml) was added separately to cells during the period of constant dihydroorotase activity (i.e. first hour) or during the S-phase (i.e. ninth hour), the step-increase or further increase in dihydroorotase activity was prevented and enzyme activity remained constant for a subsequent 5 h in the presence of the inhibitor. Thus,

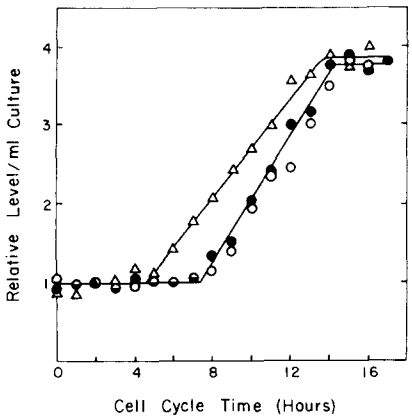

Fig. 4. Patterns of accumulation of aspartate transcarbamylase (○), dihydroorotase (△), and DNA (●) during the cell cycle of synchronous cells of *Chlorella sorokiniana* cultured in nitrate medium in continuous light. The initial values per ml of culture were 75 and 8.2 mU, and 15.1 μg, respectively. The initial cell number per ml of culture was 188 · 10^6 cells. Enzyme activities were assayed in frozen-thawed cells.

based on these inhibitor data, the step increase in dihydroorotase activity appears to result from de novo enzyme synthesis and the enzyme appears to be stable in vivo during the cell cycle. In contrast to the apparent in vivo stability of dihydroorotase throughout the cell cycle, Vassef et al. [15] observed that aspartate transcarbamylase is unstable in vivo early in the cell cycle and stable during the S-phase in nitrate-cultured cells. Thus, it is possible that cell cycle changes in the turnover rate of aspartate transcarbamylase might be responsible at least in part for the non-coordinate accumulation of these two pyrimidine enzymes.

Vassef et al. [15] observed that, when protein or RNA synthesis was inhibited by either cycloheximide or actinomycin D, the activity of aspartate transcarbamylase decayed very rapidly to 50% of its initial activity and then ceased to decay or decayed very slowly. In addition to inhibiting protein synthesis, cycloheximide also was shown [15] to inhibit the accumulation of RNA in *Chlorella*. Since actinomycin D also inhibits RNA synthesis, Vassef et al. proposed that the unusual decay pattern of the enzyme early in the cell cycle, in the presence of either inhibitor, might be related to the accumulation of a nucleotide, such as UMP, during inhibition of RNA synthesis. UMP had been shown to be the only nucleotide which could stabilize the enzyme in vitro.

To test this proposal, we employed 6-azauracil as an inhibitor of UMP synthesis. This compound is converted in vivo to its corresponding ribonucleoside monophosphate derivative which competitively inhibits OMP decarboxylase [35,36]. When RNA synthesis was maximally inhibited by 0.27 mM 6-azauracil at the first hour of the cell cycle, the kinetics of decay of aspartate transcarbamylase were essentially identical to those observed by Vassef et al. [15] in which cycloheximide and actinomycin D were used as inhibitors, i.e., the enzyme decayed to approximately 50% of initial activity and then essentially ceased to decay. The same pattern of decay in enzyme activity was also observed with 0.1 mM 6-azauracil. Thus, since inhibition of RNA synthesis by

this inhibitor results from pyrimidine starvation, the dramatic change in rate of decay in enzyme activity is not likely due to the accumulation of UMP or other pyrimidine nucleotides. This conclusion is further supported by the observation of Wanka [37] who showed that UMP does not accumulate in cycloheximide-inhibited *Chlorella* cells.

The sedimentation profile for aspartate transcarbamylase in sucrose density gradients (Fig. 3B), the single-step pattern for this enzyme during the cell cycle (Fig. 4), and the complete in vivo stability of the enzyme during the S-phase [15], are not consistent with *Chlorella* containing multiple alleles [38,39] coding for different isozymes with different in vivo stabilities. Alternative possibilities include (a) a single form of the enzyme partitioned between cytsol and organelle(s) or between different organelles in which pool levels of endogenous stabilizers change differently in response to inhibitors of protein and RNA synthesis, and (b) a single form of the enzyme which exists in two physical states (e.g. free vs. multienzyme complex or free catalytic and regulatory subunits vs. holoenzyme) which have different in vivo stabilities.

In the experiment described in Fig. 3B, UMP (i.e., the feedback inhibitor of *Chlorella* aspartate transcarbamylase), Mg^{2+}, glutamine, and glycerol were added to the cell breakage buffer of *Chlorella* cells and to the sucrose gradients with the idea of stabilizing a possible multienzyme complex of the enzymes on the pyrimidine pathway. However, these additions clearly gave no evidence for the existence of such a multienzyme complex. In subsequent experiments, the addition of 30% dimethylsulfoxide and 2 mM dithiothreitol or the inclusion of protease inhibitors (e.g., phenylmethylsulfonyl fluoride and benzamidine), along with all of the aforementioned ligands in the cell breakage buffer and in sucrose density gradients, did not lead to cosedementation of the activities of the first three pyrimidine-enzymes.

In the absence of any additions, the activity of *Chlorella* aspartate transcarbamylase decayed very rapidly in vitro in sucrose gradients; however, the activity of the enzyme was completely stabilized and sedimented with an apparent molecular weight of 160 000 in sucrose gradients containing only 1—2 mM UMP. Thus, the addition to sucrose gradients of compounds shown 9—12] to stabilize multienzyme complexes of pyrimidine enzymes from other eucaryotes has failed to show these complexes in *Chlorella*. Moreover, the observation [15] that the activity of *Chlorella* aspartate transcarbamylase is very sensitive to feedback inhibition by UMP also suggests that this enzyme does not exist in a multienzyme complex with carbamyl phosphate synthetase and dihydroorotase. In eucaryotes reported [9—12] to have these multienzyme complexes, aspartate transcarbamylase activity per se is insensitive to inhibition by pyrimidine endproducts. Thus, the reasons for the unusual in vivo stability characteristics of aspartate transcarbamylase still remains obscure.

Israel et al. [23] observed that in ammonium medium, the cell cycle of this strain of *Chlorella* was 40% shorter and the rate of total protein accumulation was considerably higher than that observed for cells cultured in nitrate medium under the same light intensity. Since the rate of RNA accumulation has been shown [19] to parallel that for total protein accumulation, we decided to determine whether the accelerated rate of RNA synthesis would affect the cell cycle patterns of aspartate transcarbamylase and dihydroorotase

activities in ammonium-cultured cells. In contrast to the non-coordinate and single step-patterns observed for accumulation of these enzymes in nitrate-cultured synchronous cells (Fig. 4), both enzyme activities accumulated in a coordinate manner from the onset of the cell cycle (Fig. 5).

Cell cycle regulation of expression of Chlorella aspartate transcarbamylase and dihydroorotase genes

To determine whether *Chlorella* aspartate transcarbamylase and dihydroorotase synthesis was repressible by pyrimidine endproducts, as reported in procaryotic and other eucaryotic microorganisms [9,41—43], synchronous cells were cultured in nitrate medium in the presence of 0.3 mM uracil for four generations. A concentration of 0.2 mM uracil in the culture medium was observed [4,5] to repress completely the synthesis of aspartate transcarbamylase within a single cell cycle of *Bacillus subtilis* W3. However, at the end of four generations, the enzyme levels per *Chlorella* cell were the same in control and uracil-treated cultures. The ability of 0.5 mM uracil to reverse the inhibition of 0.2 mM 6-azauracil on growth (Fig. 6A) and nucleic acid synthesis (Fig. 6B) was consistent with the absorption and utilization of uracil for nucleic acid synthesis in *Chlorella* cells.

The uptake of uracil and uridine by nitrate-cultured *Chlorella* cells was measured directly by use of radioactive labeled compounds. In a 1-h exposure period of synchronous cells to radioactive uridine, incorporation of radioactivity into total nucleic acids continued to increase until the concentration of uridine in the culture medium reached 40 mM (Fig. 7). Since these incorporation measurements were performed with cells prior to their S-phase, incorporation of radioactivity into the total nucleic acid fraction was equivalent to incorporation into total RNA. Actinomycin D (190 μg/ml) almost completely

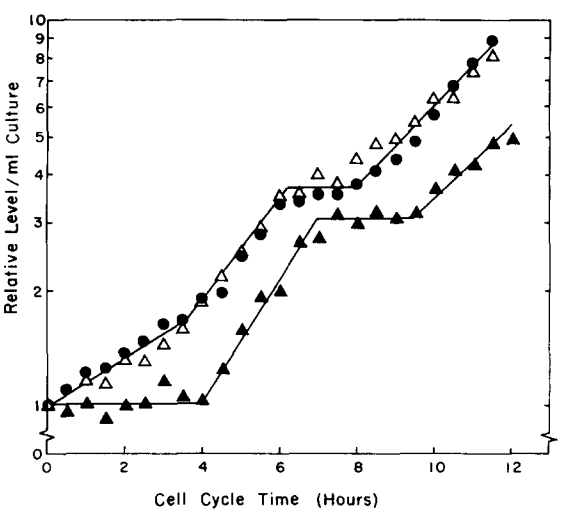

Fig. 5. Patterns of accumulation of aspartate transcarbamylase (△), dihydroorotase (●), and DNA (▲) during the cell cycle of synchronous cells of *Chlorella sorokiniana* cultured in ammonium medium in continuous light. The initial values per ml of culture were 86 and 9.3 mU, 17.3 μg, respectively. The initial cell number per ml of culture was $211 \cdot 10^6$ cells. Enzyme activities were assayed in frozen-thawed cells.

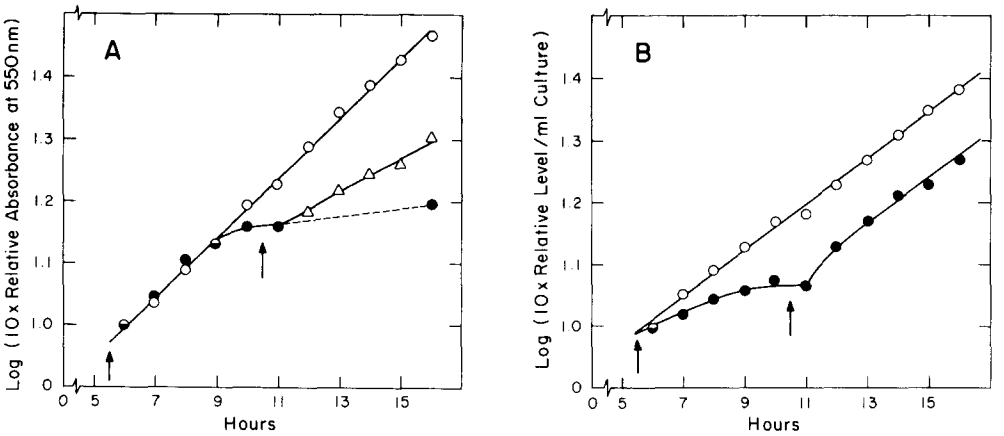

Fig. 6. Effect of 6-azauracil (0.18 mM) and the subsequent addition of uracil (0.54 mM) on (A) cell growth (absorbance 550 nm) and on (B) total nucleic acid accumulation during a portion of the cell cycle of synchronous cells of *Chlorella sorokiniana* in nitrate medium. Experiment A: ○, control culture; ●, 6-azauracil-treated culture; △, 6-azauracil-treated culture after addition of uracil. Experiment B: ○, control culture; ●, 6-azauracil-treated culture before and after addition of uracil. Arrows indicate the times of addition of 6-azauracil and uracil, respectively.

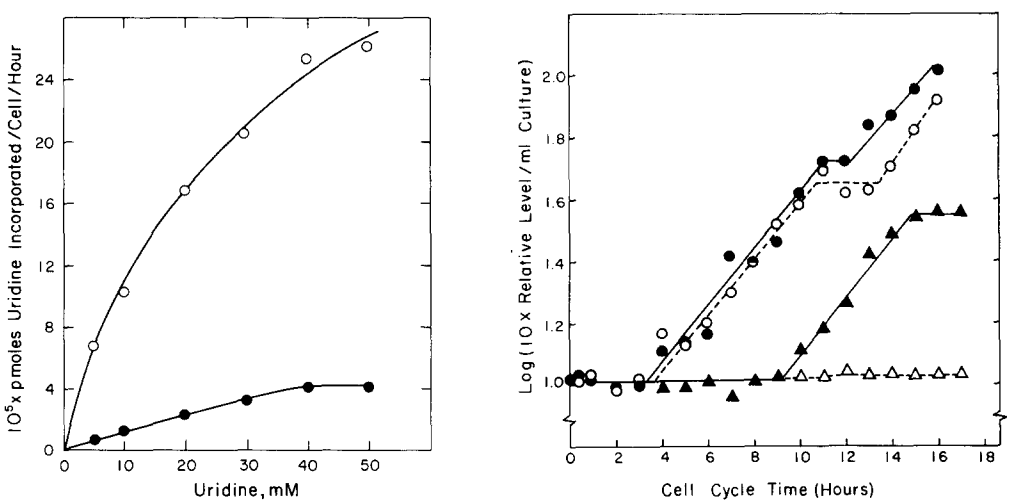

Fig. 7. Incorporation of exogenous radioactive uridine at different concentrations into total cellular compounds and into total nucleic acids during a 1-h growth period of synchronous cells of *Chlorella sorokiniana* in nitrate medium. ○, total cellular compounds; ●, total nucleic acids. After a 1-h incorporation period, the cells were washed five times with cold nonradioactive culture medium and then resuspended in deionized H_2O. An aliquot of suspension was measured for total radioactivity (with and without internal standard) in a scintillation counter. Cells in another aliquot were extracted four times with cold 10% trichloroacetic acid. The residual radioactivity (also corrected for quenching) in the acid-extracted cells was assumed to be in nucleic acids. The difference between the radioactivity in medium-washed cells and in the acid-extracted cells was assumed to be the radioactivity in acid-soluble compounds.

Fig. 8. Patterns of accumulation of dihydroorotase and cell number during a cell cycle of synchronous cells of *Chlorella sorokiniana* cultured in continuous light in nitrate medium in the absence (●, ▲) and in the continuous presence (○, △) of 40 mM uridine. The initial values for dihydroorotase and cell number per ml in both control and uridine cultures were 7.9 milliunits and $180 \cdot 10^6$ cells, respectively. The turbidities of the control and uridine cultures were held constant by hourly dilutions [15] with regular or uridine-containing medium, respectively. The initial addition of uridine was made approximately 30 min prior to placing the cultures in continuous light.

inhibited incorporation into the nucleic acid fraction of the cells. Similar incorporation patterns were obtained with radioactive uracil except that a plateau in rate of incorporation into total nucleic acid was never reached because of solubility problems with uracil over 29 mM. The difference between the radioactivity in medium-washed cells and in that remaining in cells after extraction with cold trichloroacetic acid was taken as the amount of radioactivity in soluble compounds within the cells. In the uridine incorporation experiment (Fig. 7), the average cell volume was calculated from 700 cell diameter measurements. This value was used along with the total radioactivity in the acid-soluble compounds and the specific radioactivity of uridine in the culture medium to calculate the maximum concentration of soluble uridine in the cells. When total acid-soluble radioactivity was assumed to be all in uridine, the maximum concentration of soluble uridine was calculated to be approximately 2 mM in cells exposed to 40 mM uridine for 1 h.

When synchronous *Chlorella* cells were cultured in nitrate medium in the presence of 40 mM uridine for an entire cell cycle, the synthesis of aspartate transcarbamylase and dihydroorotase was not repressed and the patterns of total cellular protein, and cellular DNA were essentially the same in the control and uridine cultures. However, the high uridine concentration did interfer in some manner with cellular metabolism. In the uridine culture (Fig. 8), the mother cell-wall did not rupture to release the four newly-formed daughter cells. However, this inhibition of cell release was not reflected in the total protein or DNA accumulation patterns.

Although it it possible that in *Chlorella* pyrimidine nucleotides do not repress the synthesis or otherwise regulate the levels of aspartate transcarbamylase and dihydroorotase, degradation and/or compartmentalization of exogenous uridine and uracil might prevent these compounds from increasing cellular pool(s) of pyrimidine nucleotides to repressive levels at the site of gene regulation. In certain eucaryotic microorganisms, there is evidence that exogenous compounds are absorbed and partitioned from sites of their normal de novo synthesis and utilization within the cell [44—46].

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References

1 Beckwith, J.R., Pardee, A.B., Austrian, R. and Jacob, F. (1962) J. Mol. Biol. 5, 618—634
2 Masters, M., Kuempel, P.L. and Pardee, A.B. (1964) Biochem. Biophys. Res. Commun. 15, 38—42
3 Kuempel, P.L., Masters, M. and Pardee, A.B. (1965) Biochem. Biophys. Res. Commun. 18, 858—867
4 Donachie, W.D. (1965) Nature 205, 1084—1086
5 Masters, M. and Donachie, W.D. (1966) Nature 209, 476—479
6 Davis, R.H. (1972) Science 178, 835—840
7 Davis, R.H. (1975) Annu Rev. Genetics, 9, 39—65
8 Williams, L.G., Bernhardt, S. and Davis, R.H. (1970) Biochemistry 9, 4329—4335
9 Lue, P.F. and Kaplan, J.G. (1971) Can. J. Biochem. 49, 403—411
10 Shoaf, W.T. and Jones, M.E. (1973) Biochemistry 12, 4039—4051
11 Mori, M. and Tatibana, M. (1973) Biochem. Biophys. Res. Commun. 54, 1525—1531

12 Kent, R.J., Lin, R-L, Sallach, H.J. and Cohen, P.P. (1975) Proc. Natl. Acad. Sci U.S. 72, 1712—1716
13 Schimke, R.T. and Doyle, D. (1970) Annu. Rev. Biochem. 39, 939—976
14 Schmidt, R.R. (1975) in Intracellular Protein Turnover (Schimke, R.T. and Katunuma, N., eds.), pp. 77—99, Academic Press, New York
15 Vassef, A.A., Flora, J.B., Weeks, J.G., Bibbs, B.S. and Schmidt, R.R. (1973) J. Biol. Chem. 248, 1976—1985
16 Shihira, I. and Krauss, R.W. (1965) Chlorella, Physiology and Taxonomy of Forty-One Isolates, pp. 30—31, Port City Press, Baltimore
17 Sorokin, C. (1957) Physiol. Plant. 10, 659—666
18 Talley, D.J., White, L.H. and Schmidt, R.R. (1972) J. Biol. Chem. 247, 7927—7935
19 Schmidt, R.R. (1974) In Vitro 10, 306—320
20 Hare, T.A. and Schmidt, R.R. (1968) Appl. Microbiol. 16, 496—499
21 Sitz, T.O., Kent, A.B., Hopkins, H.A. and Schmidt, R.R. (1970) Science 168, 1231—1232
22 Hopkins, H.A., Sitz, T.O. and Schmidt, R.R. (1970) J. Cell Physiol. 76, 231—234
23 Israel, D.W., Gronostajski, R.M., Yeung, A.T. and Schmidt, R.R. (1977) J. Bacteriol., 130, 793—804
24 Lieberman, I. and Kornberg, A. (1954) J. Biol. Chem. 207, 911—924
25 Herrmann, E.C., Dunn, J.H. and Schmidt, R.R. (1973) Anal. Biochem. 53, 478—483
26 Prescott, L.M. and Jones, M.E. (1969) Anal. Biochem. 32, 408—414
27 Chrambach, A. (1966) Anal. Biochem. 15, 544—548
28 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
29 Burton, K. (1956) Biochem. J. 62, 315—323
30 Hopkins, H.A., Flora, J.B. and Schmidt, R.R. (1972) Arch. Biochem. Biophys. 153, 845—849
31 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
32 Sander, E.G., Wright, L.D. and McCormick, D.B. (1965) J. Biol. Chem. 240, 3628—3630
33 Sander, E.G. and Heeb, M.J. (1971) Biochim. Biophys. Acta 227, 442—452
34 Mazus, B. and Buchowicz, J. (1968) Acta Biochim. Poloncia 15, 317—325
35 Skoda, J. and Sorm, F. (1959) Biochim. Biophys. Acta 28, 659—660
36 Handschumacher, R.E. (1960) J. Biol. Chem. 235, 2917—2919
37 Wanka, F., Vereijken, P.H., Weijers, W. and Aelen, J.M.A. (1972) K. Ned. Akad. Wet. Proc. Ser. C 75, 441—448
38 Halvorson, H.O., Bock, R.M., Tauro, P., Epstein, R. and LaBerge, M. (1966) in Cell Synchrony—Studies in Biosynthetic Regulation (Cameron, I.L. and Padilla, G.M., eds.), pp. 102—106, Academic Press, New York
39 Tauro, P., and Halvorson, H.O. (1966) J. Bacteriol. 92, 652—661
40 Schimke, R.T. (1975) in Methods in Enzymology (O'Malley, B.W. and Hardman, J.G., eds.). Vol. 40, pp. 241—266, Academic Press, New York
41 Lacroute, F. (1968) J. Bacteriol. 95, 824—832
42 Jund, R. and Lacroute, F. (1972) J. Bacteriol. 109, 196—202
43 Caroline, D.F. and Davis, R.H. (1969) J. Bacteriol. 100, 1378—1384
44 Weiss, R.L. (1973) J. Biol. Chem. 248, 5409—5413
45 Weiss, R.L. and Davis, R.H. (1973) J. Biol. Chem. 248, 5403—5408
46 Subramanian, K.N., Weiss, R.L. and Davis, R.H. (1973) J. Bacteriol. 115, 284—290