CELL CYCLE CONTROL OF ASPARTATE TRANSCARBAMYLASE LEVELS IN *CHLORELLA SOROKINIANA*

by

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LIST OF ABBREVIATIONS

c chain - the catalytic polypeptide chain of *E. coli* aspartate transcarbamylase

r chain - the regulatory polypeptide chain of *E. coli* aspartate transcarbamylase

R state - the relaxed conformation of an enzyme

T state - the tight conformation of an enzyme

INTRODUCTION

Regulation of enzyme synthesis in eucaryotic cells may occur at many different levels such as transcription, post-transcriptional processing, or translation. The activity or level of an enzyme within a cell is also dependent on other factors such as enzyme stability, the presence of small molecule effectors or stabilizers, and the physical state of the enzyme as in enzyme aggregates or multi-enzyme complexes.

The eucaryotic microorganism, *Chlorella sorokiniana*, was chosen as a model system in which to study metabolic control and regulation of enzyme levels in a eucaryotic cell. This organism is unicellular and can be cultured in highly synchronous cultures, yet it has the complexity, in terms of organelles and compartmentalization, found in higher eucaryotic cells. The *Chlorella* system was used to study the regulation of the level of a key regulatory enzyme, aspartate transcarbamylase, in an important biosynthetic pathway, the pyrimidine pathway.

LITERATURE REVIEW

Aspartate transcarbamylase from *Escherichia coli* has been studied in great detail, and this work has been the subject of several reviews (1, 2, 3, 4). In *E. coli* aspartate transcarbamylase is the first enzyme unique to the pyrimidine biosynthetic pathway and is a key regulatory enzyme. Yates and Pardee (5) showed that accumulation of pyrimidine intermediates decreased in the presence of uracil and proposed end-product inhibition of the first enzyme on the pathway as a mechanism of feedback control. Aspartate transcarbamylase activity was found to be subject to inhibition by cytidine and CMP. In 1962 Gerhart and Pardee (6) showed that CTP was the most effective inhibitor among the pyrimidine nucleotides and was probably the intracellular feedback inhibitor. The effect of CTP was antagonized by ATP, an activator of the enzyme.

Purification of the enzyme by Shepherdson and Pardee (7) led to characterization of the enzyme and determination of its molecular weight as 310,000 (4). The enzyme was dissociated by p-mercuribenzoate into two kinds of subunits, one retaining catalytic activity and the other having the CTP binding site. The catalytic subunit was found to be a trimer of molecular weight 100,000 and the regulatory subunit was found to be a dimer with 34,000 molecular weight. A single c chain then has a molecular weight of 33,000 and a single r chain, 17,000.

Aspartate transcarbamylase is an allosteric protein (1,4). Positive homotropic effects were demonstrated for both aspartate and carbamyl-P binding. Heterotropic effects were seen upon binding of

the nucleotide effectors, ATP and CTP, with CTP increasing and ATP decreasing the sigmoidicity of the substrate saturation curve. The isolated catalytic subunit has enzymic activity but shows no homotropic effect with aspartate nor heterotropic effect with CTP. The regulatory subunit retains the capacity to bind CTP but has no catalytic activity. The native c_6r_6 enzyme can be reassociated in the presence of mercaptoethanol with the full restoration of both homotropic and heterotropic effects. There is some evidence that a zinc ion in the regulatory subunit is involved in the association of catalytic and regulatory subunits into the native enzyme.

The proposed mechanism of catalysis for *E. coli* aspartate transcarbamylase has been discussed in detail by Jacobson and Stark (4). Carbamyl-P binds to the enzyme first, followed by L-aspartate. The carbamyl group is transferred directly to aspartate without the formation of an intermediate. A conformational change in the catalytic subunit accompanies binding of carbamyl-P, and a second conformational change occurs upon binding of L-aspartate. During catalysis, the compression of the amino group of L-aspartate and the carbonyl carbon of carbamyl-P lowers the activation energy. The product carbamyl aspartate leaves the enzyme site first, followed by P_i. Thus, the reaction mechanism "is ordered by Bi Bi" (8).

Two models have been proposed to explain how binding of ligands to an allosteric protein alters the catalytic activity through conformational changes in the protein. The Monod, Wyman, and Changeaux model (9) proposes that allosteric enzymes are composed of two or more

identical subunits, each having a site for the substrate, activator, or inhibitor. The enzyme can exist in at least two conformational states, R and T, with substrates and activators binding preferentially to the R state and inhibitors to the T state. The binding of ligands influences the equilibrium between the R and T conformations of the enzyme. Conformational changes in all the subunits occur in a concerted manner with consequent conservation of structural symmetry.

The sequential model of Koshland (8) assumes that the binding of a ligand alters the conformation of the subunit to which it binds, thereby altering the binding of additional ligands through subunit interactions.

Gerhart (1) proposed a rotational model for the changes in quaternary structure of aspartate transcarbamylase that occur during allosteric transitions. The catalytic trimers were proposed to exist in two planes with the regulatory dimers positioned around the periphery of a triangle formed by the catalytic subunits. During the R to T transition the two planes of catalytic trimers were proposed to rotate 60° with respect to each other.

Recently, Chan (10, 11, 12, 13) has proposed a new model to account for the allosteric properties of aspartate transcarbamylase in terms of subunit interactions. The model is based on experiments with a c_3r_6 complex which lacks one of the catalytic trimers. This complex shows Michaelis-Menton kinetics and is not inhibited by CTP. Since the c_3r_6 complex exhibited neither homotropic nor heterotropic effects, the presence of both c_3 subunits appears to be necessary for these

interactions. Therefore, Chan proposed an important role for r:r interactions in the allosteric mechanism. In the new model the binding of substrates causes an obligatory conformational change resulting in an alteration in the c:r domain . As a consequence of conservation of symmetry, the r:r domain is shifted to a position of higher potential energy. The allosteric effectors, CTP and ATP, favor different r:r domains and consequently either enhance or reduce the sigmoidal character of substrate saturation.

The aspartate transcarbamylase isolated from *E. coli* is not typical of the enzymes isolated from all bacterial sources. There is a class of enzymes having molecular weights in the range of 100,000 to 140,000 that are not inhibited by pyrimidine nucleotides (14). The best studied example is aspartate transcarbamylase from *Streptococcus faecalis* (15, 16, 17). This enzyme has been purified by Chang and Jones and shown to have four apparently identical subunits. An activator site was found to be sensitive to many anions. In contrast to the *E. coli* enzyme, the mechanism of the enzyme reaction appears to be random sequential rather than ordered.

In most bacteria, the enzymes of the pyrimidine pathway, including aspartate transcarbamylase, are under a repression-derepression type of control (3) in which the end product of the pathway acts as a corepressor to coordinately repress the synthesis of all five enzymes of the pyrimidine pathway. The difference in rates of enzyme synthesis between basal or repressed synthesis and full derepression may be as great as 200-fold (18).

Synchronous cultures of *E. coli* and *Bacillus subtilis* have been used to study the regulation of synthesis of pyrimidine enzymes by repression and derepression (19, 20, 21). In the absence of added exogenous corepressors, aspartate transcarbamylase was synthesized at a fluctuating rate intermediate between its basal and fully derepressed rate. It was proposed that the level of the enzyme oscillates through the cell cycle because the end product of the pathway is a corepressor of the structural gene, and as corepressor levels increase, the structural gene is repressed, enzyme synthesis is slowed, and the level of the enzyme decreases. The consequent lowering of enzyme level results in a slower rate of accumulation of the end product of the pathway, and the gene is again derepressed. This proposed mechanism of regulation was termed oscillatory repression. Enzymes thought to be controlled by oscillatory repression exhibit periodic bursts of synthesis during the cell cycle alternating with periods during which enzyme synthesis occurs at a very slow rate. Kuempel, Masters, and Pardee (19) described such enzymes as autogenous and defined the rate of autogenous enzyme synthesis as the amount of enzyme made per min in a growing autogenous culture. Halvorson (22) prefers the term autoregulated to autogenous unless a specific cellular mechanism has been shown to regulate the rate of formation of the enzyme. The term autoregulated is more general and applies to all endogenous regulatory systems. Unfortunately, the term "autogenous regulation" has recently been used in an entirely different context to describe a regulatory mechanism in which a protein specified by a given structural gene is itself a regulatory element

which modulates expression of that very gene (23).

Kuempel, Masters, and Pardee (19) used synchronous *E. coli* cultures to study derepression of aspartate transcarbamylase to determine whether the structural gene could be derepressed at any time during the cell cycle or only at discrete times during the cell cycle. The enzyme was derepressed by the addition of 6-azauracil. This compound is converted to 6-azaUMP which then acts as a competitive inhibitor for OMP decarboxylase, another enzyme on the pyrimidine pathway, and effectively blocks further synthesis of CTP, the corepressor of aspartate transcarbamylase. Samples were removed at different times during the growth of a synchronous culture under autogenous conditions, and 6-azauracil was added to attempt to derepress aspartate transcarbamylase synthesis. After a short lag, the enzyme began to accumulate in samples taken at any time during the cell cycle. Thus, transcription of the structural gene and consequent synthesis of the enzyme could be made to occur at any time during the cell cycle.

If the structural gene for an enzyme is fully derepressed, the rate of accumulation is assumed to be the maximal rate at which that enzyme can be synthesized. This rate was defined as the potential (19). One would expect the potential for synthesis of an enzyme to increase in proportion to an increase in the number of structural genes for that enzyme. Correlation between increases in potential and gene dosage was demonstrated for sucrase in *Bacillus subtilis* (21). Such a correlation has not been shown for aspartate transcarbamylase.

If oscillatory repression is responsible for periodic synthesis

of aspartate transcarbamylase under autogenous conditions, it should be possible to change the timing of the step increases in enzyme level by altering the levels of corepressors experimentally. Aspartate transcarbamylase synthesis in a synchronous culture of *Bacillus subtilis* was repressed by the addition of uracil (24). Aliquots removed at different times during the cell cycle and washed free of uracil exhibited an increased rate of enzyme accumulation. This experiment showed that derepression could occur at any time during the cell cycle and that release of repression resulted in specific alteration in the timing of subsequent autogenous enzyme steps. When uracil was added to a synchronous culture, aspartate transcarbamylase activity decayed with a half-life of 90 min. Thus, aspartate transcarbamylase from *Bacillus subtilis* was shown to be an unstable enzyme.

In both procaryotes and eucaryotes carbamyl-P, the substrate for aspartate transcarbamylase, is provided by the enzyme carbamyl-P synthetase (18). Carbamyl-P is also a precursor for the arginine biosynthetic pathway. In bacteria a single carbamyl-P synthetase provides carbamyl-P for both the arginine and pyrimidine biosynthetic pathways. but in *Neurospora* and *Saccharomyces* there are two enzymes, one producing carbamyl-P for the pyrimidine pathway, the other producing carbamyl-P for the arginine pathway (18, 25). In *Neurospora* the carbamyl-P produced by a given enzyme is available for only one of the two pathways (26). This phenomenon is known as metabolic channeling. Thus, the carbamyl-P produced by the carbamyl-P synthetase specific for the pyrimidine pathway is further metabolized only by aspartate

transcarbamylase, the next enzyme on the pyrimidine pathway (27). The genes for the two enzymes map at the same locus and the enzymes exist in a complex. Since carbamyl-P synthetase and not aspartate transcarbamylase is the first enzyme unique to the pyrimidine biosynthetic pathway in *Neurospora*, it is not surprising that it is the carbamyl-P synthetase and not aspartate transcarbamylase activity which is subject to feedback inhibition by the end product of the pathway (28). The feedback inhibitor in *Neurospora* is UTP rather than CTP as in *E. coli*. In the pyrimidine pathway of both organisms UTP is converted to CTP.

The first two enzymes of the pyrimidine pathway in *Saccharomyces cerevisiae* are similar to the corresponding enzymes in *Neurospora* in that these two enzymes form a complex (18). A single gene, ura-2, controls the biosynthesis of both enzymes, and the functioning of this gene is repressed by UTP. However, both of these enzymes are controlled by feedback inhibition by the end product of the pathway, UTP, whereas in *Neurospora* only the first enzyme is feedback inhibited (29). In *S. cerevisiae* the channeling of carbamyl-P into either the arginine or pyrimidine biosynthetic pathway is not as strict as in *Neurospora* (30). Genetic studies have shown that either enzyme can provide carbamyl-P for both pathways in mutants lacking one or the other of these enzymes.

The remaining enzymes of the pyrimidine pathway in yeast are not coordinately repressed as they are in bacteria but are controlled by sequential induction (29). The genes corresponding to these enzymes are not linked to the ura-2 gene coding for the carbamyl-P synthetase-aspartate transcarbamylase complex. The type of induction exhibited

by dihydroorotase, dihydroorotate dehydrogenase, and orotidine-5'-phosphate decarboxylase is thought to be sequential induction, a type of metabolic control in which the product of one enzyme in the pathway induces the synthesis of the next enzyme in the pathway and so on until all the enzymes in the pathway have been induced in sequence.

Two multi-enzyme complexes are found in the pyrimidine pathway of mammalian cells (18, 31). In Ehrlich ascites cells the first three enzymes of the pyrimidine pathway, carbamyl-P synthetase, aspartate transcarbamylase, and dihydroorotase are associated in a complex as are the fifth and sixth enzymes, OMP pyrophosphatase and OMP decarboxylase (31). The other enzyme, dihydroorotate oxidase, is particulate and does not appear to be associated with any enzyme complex. When sedimented in a sucrose gradient containing 30% dimethyl sulfoxide and 5% glycerol, the complex comprised of the first three pyrimidine enzymes sedimented with an apparent molecular weight of 800,000-850,000. Under the same conditions the other complex had an apparent molecular weight of 105,000-115,000.

Both the Ehrlich ascites carcinoma and mouse spleen carbamyl-P synthetases were inhibited by UTP and activated by phosphoribosylpyrophosphate (31,32). Aspartate transcarbamylase from ascites cells was not inhibited by nucleotide mono-, di-, and triphosphates.

Aspartate transcarbamylases that have been studied in higher plants, including lettuce seedlings, Alaskan pea, mung bean, and wheat germ, are inhibited by UMP (33, 18). Thus, the particular nucleotide which acts as feedback inhibitor of pyrimidine enzymes seems to be consistent

among a group of organisms but varies within the plant and animal kingdoms. The carbamyl-P synthetase from Alaskan pea and mung bean is activated and stabilized by L-ornithine and inhibited by UMP, suggesting that a single carbamyl-P synthetase functions for both the pyrimidine and arginine biosynthetic pathways in higher plants (18).

The wheat germ enzyme is the smallest aspartate transcarbamylase reported to have regulatory properties (33). Yon has studied the kinetic behavior of the wheat germ enzyme and found that the symmetrical model of Monod *et al.* (9) is consistent with the data. He suggests that the enzyme exists predominantly in the T state in the absence of ligands and that carbamyl-P and UMP bind almost exclusively to the R and T states, respectively. Analysis based on the Monod model suggests that the enzyme is a trimer, and since the total molecular weight was found to be 100,000, each subunit would then have a molecular weight of 33,000.

It is interesting to compare the wheat germ aspartate transcarbamylase to the *E. coli* enzyme. The size and subunit structure of the wheat enzyme is similar to the catalytic subunit from *E. coli*. There are major differences between the two enzymes, however. The feedback inhibitor in *E. coli* is CTP, whereas UMP is the inhibitor of the wheat germ enzyme. No activators of wheat germ aspartate transcarbamylase have been found that would correspond to ATP in *E. coli*. Finally, if the model of Monod *et al.* (9) is assumed to apply to both enzymes, the *E. coli* enzyme exists predominantly in the T state and the wheat germ enzyme in the R state.

The wheat germ aspartate transcarbamylase has a half-life of about 24 hours at pH 10 and 25° (34). The enzyme is inactivated more rapidly in the presence of trypsin, heat (60°), alkaline conditions (pH 11.3), or sodium dodecyl sulfate. UMP acts as a stabilizer of the enzyme, protecting it against denaturation by any of these agents. Inactivation by alkali and detergent was abolished by 30μM and 10μM UMP, respectively. Inactivation by heat and proteolysis was not entirely abolished by UMP, but the half-life was increased from 3 to 30 min for heat inactivation and from 10 to 25 min in the case of inactivation by proteolysis. Carbamyl-P alone had no effect on enzyme inactivation by trypsin or detergent, but it antagonized the effect of UMP. L-aspartate had a slight protective effect, but only at high concentrations. The stability studies are consistent with the cooperative conformational model suggested by the kinetic data. Yon suggested that the conformational state favored in the presence of UMP is more stable than the conformational state that predominates in the absence of ligands. The enzyme is more susceptible to inactivation by a variety of agents when it is in the R state, the state which predominates in the absence of added ligands and in the presence of carbamyl-P. Carbamyl-P would antagonize the protective effect of UMP by shifting the equilibrium from the stable T state to the R state.

The control of aspartate transcarbamylase in *Chlorella sorokiniana* has been studied in this laboratory for some time (35). Vassef *et al.* (36) found that the enzyme accumulated in a step pattern during the cell cycle in synchronous cultures with an increase in enzyme level

occurring during the period of DNA replication, proportional to the increase in gene dosage. A step pattern during the S-phase is consistent with the hypothesis that the enzyme is unstable and its structural gene is transcribed continuously and is under a constant level of repression or is free from repression. It was proposed that the enzyme level remains constant during most of the cell cycle, due to a steady state between enzyme synthesis and breakdown, and then increases during DNA replication as more structural genes become available for transcription. The pattern for aspartate transcarbamylase deviated slightly from the simple case for an unstable enzyme following the pattern of its structural gene. The level of enzyme began to increase gradually just prior to the time of DNA replication. It was proposed that this increase in enzyme level is due to increased enzyme stability at this time during the cell cycle. If the structural gene is transcribed at a constant rate and the rate of degradation of the enzyme is slowed, the enzyme would accumulate. It was also proposed that the change in stability of the enzyme was due to the accumulation of an endogenous stabilizer of the enzyme during the portion of the cell cycle just before DNA replication (between the fourth and eighth hours of the cell cycle).

Alteration of DNA patterns by changing growth conditions led to similarly altered enzyme accumulation. The evidence that aspartate transcarbamylase is stabilized during only a portion of the cell cycle comes from enzyme turnover studies, using cycloheximide as an inhibitor of protein synthesis or actinomycin D as an inhibitor of transcription.

When an inhibitor was added during the early part of the cell cycle, the enzyme decayed. If the inhibitor was added at the ninth hour of the cell cycle, the enzyme level remained constant. Thus, in the absence of protein synthesis the enzyme appeared to be stable at the ninth hour of the cell cycle but not at the beginning of the cell cycle.

The endogenous stabilizer was characterized as being organic, heat stable, acid soluble, and adsorbable to Norit A, and its molecular weight was less than 1,000. Ashing destroyed the activity. Of the two substrates for the enzyme, only carbamyl-P was effective in stabilizing the enzyme *in vitro*, and this compound was eliminated as being the endogenous stabilizer because it is not heat stable and concentrations of 8mM were required for complete stabilization. Other molecules known to bind to the enzyme include feedback inhibitors. The properties of the endogenous stabilizer are consistent with those of nucleotides, and UMP is the feedback inhibitor of aspartate transcarbamylase in *Chlorella*. An endogenous inhibitor of the enzyme accumulated during the same period of the cell cycle in which the enzyme became more stable (35). Cellular acid-extractable, Norit A adsorbable phosphate compounds (e.g., nucleotides) also increased during this period. Of the nucleotides tested, uridine and UMP were most effective in stabilizing the enzyme. Uridine is not on the *de novo* pyrimidine biosynthetic pathway. Therefore, Vassef *et al.* proposed that the endogenous stabilizer was likely to be UMP.

EXPERIMENTAL PROCEDURES

Organism and Culture Conditions - A thermophilic green alga, *Chlorella sorokiniana* (37), previously described (36) as *Chlorella pyrenoidosa* strain 7-11-05 (38) was cultured according to the methods of Hare and Schmidt (39). The culture medium contained either nitrate (39) or ammonium (40) as the nitrogen source. The external light intensity and culture turbidity were adjusted to effect a cell division number of approximately four. The cells were brought into synchrony by alternating periods of illumination and darkness. Three cycles, consisting of ten hours light and eight hours dark, were used for nitrate cultured cells. Since the cells grew faster when ammonium was the nitrogen source, light:dark cycles of 7:5 hours were used with ammonium cultured cells. For cell cycle experiments synchrony was improved by selection of daughter cells by equilibrium centrifugation in aqueous Ficoll (41, 42). For selection of light:dark synchronized daughter cells from the last dark period of the synchronization procedure, the Ficoll concentration was changed from 26.8% to 24.0%.

Preparation of Cellular Material for Analysis - Cells were harvested by centrifugation in a Sorvall RC2-B at 14,600 x g for 5 min at 2-5° and washed twice with 0.01 M Tris-HCl buffer, pH 8.5. The cells were then concentrated either 20- or 40-fold by resuspension in a small volume of 0.2 M Tris-HCl buffer (containing 1 mM Na_2EDTA and 2 mM uridine). For enzyme assays, the cells were either frozen and thawed to break permeability barriers to small molecules (36), or cells were broken in a French pressure cell at 18,000-20,000 psi. In some

cases, cellular debris was removed by centrifugation at 100,000 x g for 60 min in a Beckman Model L2-65B ultracentrifuge.

Estimation of Cell Number, DNA, and Protein - Cell number was determined by either direct microscopic counting with a haemocytometer or counting with a Coulter counter (Model B).

DNA was extracted for 12 hours at room temperature with 1 or 2 N NaOH as described by Hopkins *et al.* (43). DNA was then quantified by the diphenylamine procedure of Burton (44).

Total protein in NaOH extracts was estimated by the method of Lowry *et al.* (45).

Enzyme Assays - The radioactive assay for aspartate transcarbamylase activity was described previously (36). One enzyme unit is defined as the amount of enzyme required to synthesize 0.01 μmoles of carbamyl aspartate per min at 38.5°.

Carbamyl-P synthetase activity was assayed in *Chlorella* cells which had been broken in a French pressure cell. Approximately 7.6 x 10^9 cells per ml were broken in the breaking buffer described in Table I.

Since one of the products of the carbamyl-P synthetase reaction, carbamyl-P is labile, the reaction was coupled to the aspartate transcarbamylase reaction to convert carbamyl-P to carbamyl aspartate, a stable compound. The other product of the carbamyl-P synthetase reaction, ADP, proved to be inhibitory to carbamyl-P synthetase. Therefore, a creatine-P-creatine phosphokinase ATP regenerating system was used to prevent accumulation of ADP. A pyruvate kinase ATP regenerating

TABLE I

Buffers for assay of carbamyl phosphate synthetase activity

	Concentration[a] in buffer	Final concentration
Breaking buffer, pH 7.5		
glyclyglycine	200 mM	
UMP	1 mM	0.4 mM
glutamine	0.5 mM	
$MgSO_4$	30 mM	
glycerol	5%	
dimethyl sulfoxide	30%	
ATP	25 mM	
Assay buffer, pH 7.5		
glycylglycine	200 mM	200 mM
glutamine	15 mM	6 mM
dimethylsulfoxide	30%	24%
glycerol	5%	4%
$MgSO_4$	30 mM	24 mM
$NaHCO_3$	50 mM	20 mM
ATP	25 mM	20 mM
creatine phosphate	100 mM	40 mM
creatine phosphokinase	0.25 mg/ml	0.10 mg/ml

[a]All concentrations expressed as % are v/v.

system inhibited the activity of carbamyl-P synthetase. High pressure liquid chromatography showed that ADP was converted to ATP in the presence of either regenerating system.

The assay buffer for carbamyl-P synthetase (Table I) contained creatine-P and creatine phosphokinase plus the substrates for the reaction, glutamine, ATP, and $NaHCO_3$ and several compounds known to stabilize carbamyl-P synthetase in other organisms, $MgSO_4$, dimethyl sulfoxide, and glycerol. The breaking buffer contained the potential stabilizers for carbamyl-P synthetase as well as UMP to stabilize aspartate transcarbamylase. In both buffers glycylglycine was added to maintain a pH of 7.5.

The standard incubation mixture for the assay of carbamyl-P synthetase activity consisted of 20 µl assay buffer, 10 µl radioactive aspartate, and 20 µl broken cell preparation. Each incubation mixture contained 0.25 µCi of L-[4-^{14}C] aspartate to give a specific activity of 0.5 µCi per µmole. The aspartate was prepared in 20 mM glycyl-glycine buffer, pH 7.5.

The reaction was initiated with addition of cell homogenate. After 20 min incubation at 38.5°, the reaction was terminated by addition of 100 µl of 3 N formic acid. Carbamyl aspartate, the radioactive product of the coupled reaction, was quantified by the same method used in the aspartate transcarbamylase assay (36). Aspartate transcarbamylase activity was measured in a control reaction by adding sufficient carbamyl-P to the assay buffer to bring the final concentration of the reaction mixture to 12.5 mM. The accumulation of carbamyl

aspartate was found to be linear as a function of time (Fig. 1) and protein concentration (Fig. 2).

Aspartate Transcarbamylase Decay Studies - The source of aspartate transcarbamylase for decay studies was either frozen-thawed, washed *Chlorella* cells or ammonium sulfate fractionated enzyme. The frozen-thawed cells were pelleted by centrifugation in an Eppendorf 3200 centrifuge and washed three times with 1 ml of 200 mM Tris-HCl buffer, pH 8.5, containing 1 mM Na_2EDTA. The cells were resuspended and allowed to sit for 1 min between centrifugations to allow for diffusion of small molecules. The washed cells were then resuspended in the same buffer with or without different concentrations of uridine or UMP. Alternatively, *Chlorella* cells were broken in a French pressure cell and centrifuged for 1 hour at 100,000 x g. The aspartate transcarbamylase in the supernatant was precipitated by 30-50% saturated ammonium sulfate in the presence of 2 mM uridine, and dissolved in buffer containing 2 mM uridine and stored at -20°. Just prior to a decay experiment the enzyme was precipitated with 50-70% saturated ammonium sulfate in the absence of uridine. The pellet was then dissolved in buffer with or without uridine or UMP.

Decay of aspartate transcarbamylase was usually measured over a 5-hour period with samples taken at the onset of the decay period and at hourly intervals thereafter. Decay was stopped by the addition of carbamyl-P, one of the substrates of the aspartate transcarbamylase reaction and a stabilizer of the enzyme. The enzymic activity was then quantified by initiating the enzymic reaction with radioactive aspartate.

Fig. 1. Effect of a creatine phosphokinase ATP-regenerating-system on the carbamyl-P synthetase assay. Assay conditions are described in Experimental Procedures. With creatine phosphokinase-ATP-regenerating system, ● ; without creatine phosphokinase ATP-regenerating-system, ○ .

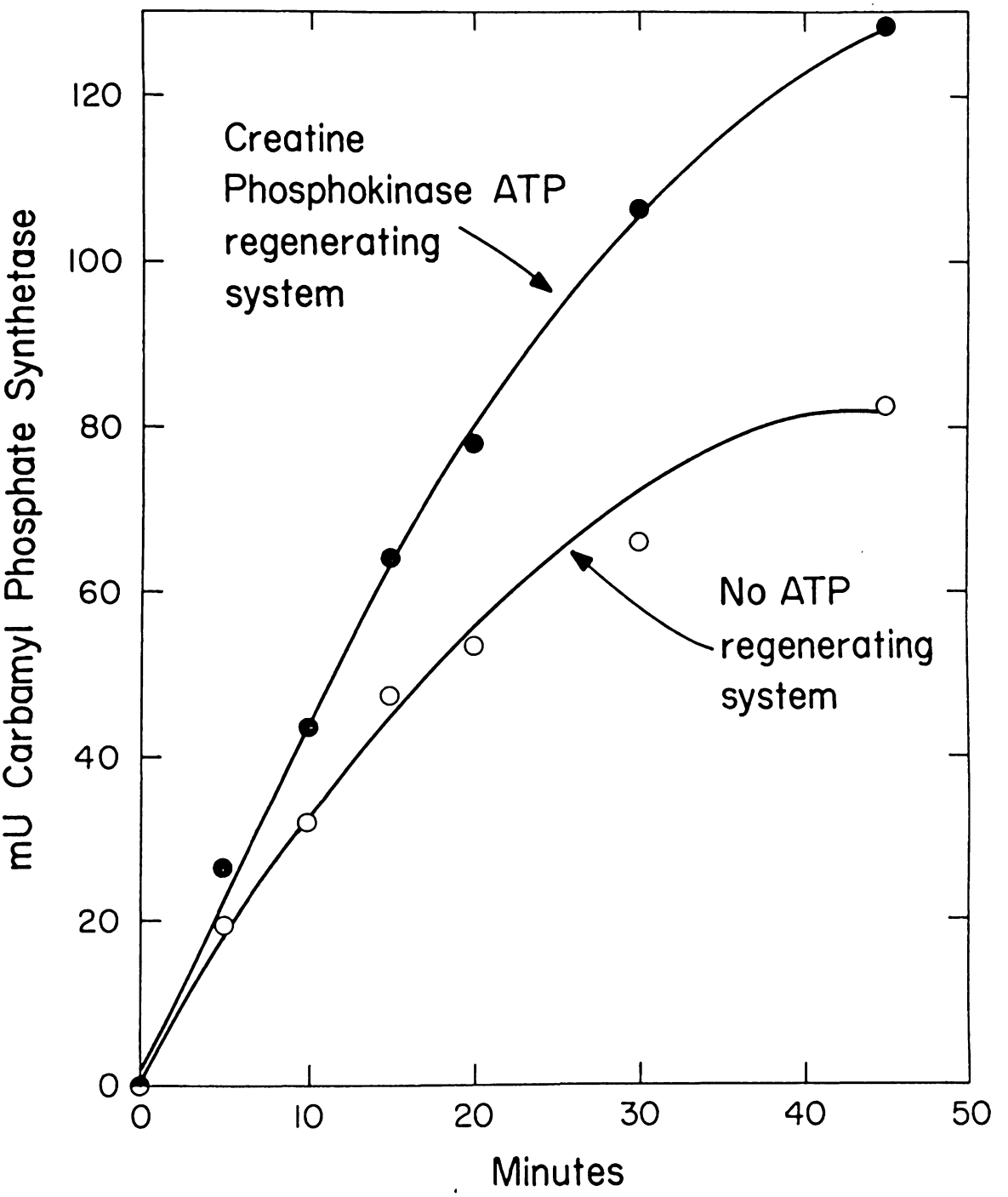
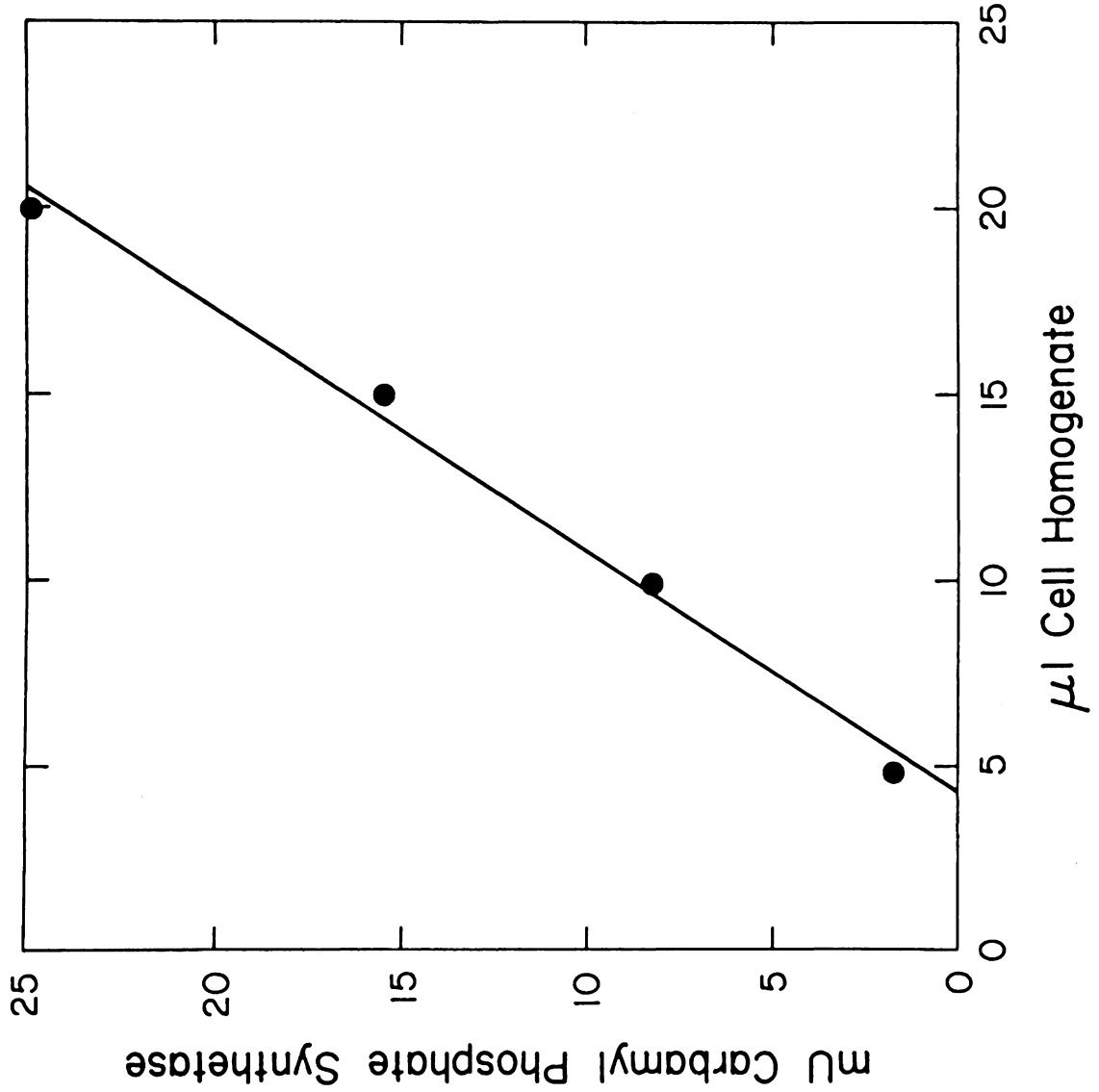

mU Carbamyl Phosphate Synthetase
120
100
80
60
40
20
0
0
10
20
30
40
50
Minutes
Creatine
Phosphokinase ATP
regenerating
system
No ATP
regenerating
system

Fig. 2. Effect of enzyme concentration on the assay of carbamyl-P synthetase. Assay conditions are described in Experimental Procedures.


mU Carbamyl Phosphate Synthetase
0
5
10
15
20
25
μl Cell Homogenate
0
5
10
15
20
25

Reagents - DL-[4-^{14}C] aspartate was purchased from New England Nuclear, Boston, Mass. The following compounds were purchased from Sigma, St. Louis, Mo.: creatine phosphokinase from rabbit muscle, creatine-P, phenylmethylsulfonyl fluoride, and soybean trypsin inhibitor. Protamine sulfate and rabbit muscle pyruvate kinase (both A grade) came from Calbiochem, San Diego, California. Phosphoenol pyruvate was purchased from C. F. Boehringer and Sons, Mannheim, Germany, and 2'-deoxyadenosine from P-L Biochemicals, Inc., Milwaukee, Wisc. All other chemicals were reagent grade or of the highest purity available.

RESULTS AND DISCUSSION

Testing the Model for Regulation of Aspartate Transcarbamylase

The model for regulation of aspartate transcarbamylase proposed by Vassef *et al.* (36) requires that the enzyme be unstable *in vivo* and its stability change during the cell cycle. The stability of aspartate transcarbamylase and its modulation by low molecular weight nucleotide effectors were investigated in both *in vitro* experiments and *in vivo* cell cycle studies. According to the model, the structural gene for the enzyme is either under a constant level of repression, *e.g.*, expressed at a basal level or fully derepressed, or is expressed constitutively. Another feature of the model is that accumulation during S of aspartate transcarbamylase is obligately coupled to DNA replication.

In testing the model, one of the first questions asked was: Are the patterns of accumulation of aspartate transcarbamylase reported by Vassef *et al.* a true reflection of the *in vivo* accumulation of the enzyme? If growth is balanced during the cell cycle, all parameters should increase to the same relative level. However, in the experiments reported by Vassef *et al.* the fold increase in enzyme was greater than that for DNA and cell number. Thus, either growth was unbalanced or the *in vitro* assay for aspartate transcarbamylase activity did not measure the true accumulation of enzyme due to either low recovery of enzyme early in the cell cycle or high recovery at the end of the cell cycle.

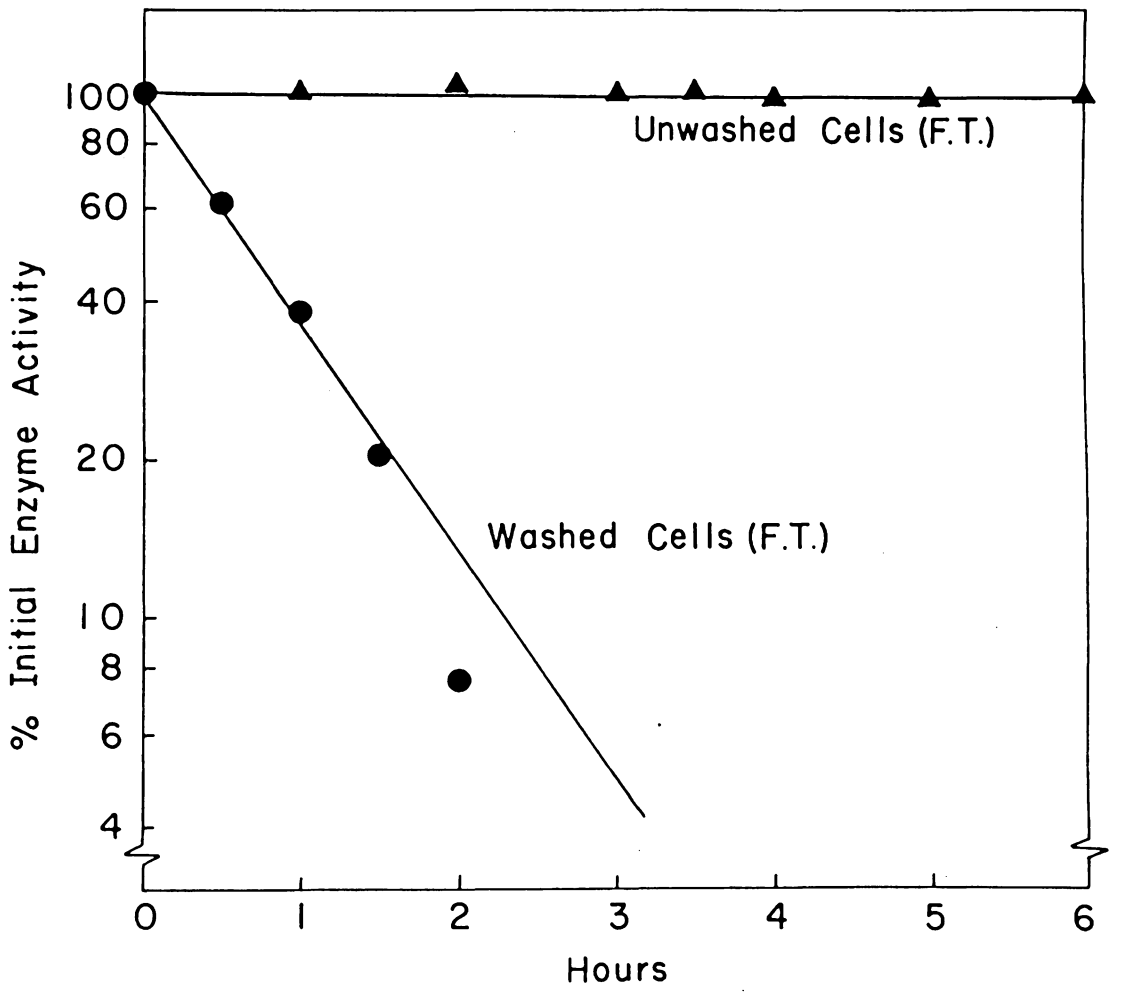
The difference in fold increase of enzyme and DNA or cell number is equal to the accumulation of enzyme during the gradual increase in

enzyme activity prior to DNA replication, suggesting low recovery of enzyme activity in the samples from early in the cell cycle. If the initial level of activity were equal to that seen at the beginning of DNA replication, the fold increase would be equal to that of DNA and cell number.

Possible factors affecting the low recovery of aspartate transcarbamylase early in the cell cycle were investigated. Samples of *Chlorella* cells from each hour in the cell cycle are frozen immediately for enzyme assays. Freezing and thawing cells breaks permeability barriers to small molecule substrates and products so that it is possible to assay the enzyme while it remains in the cell. Is the enzyme stable in these frozen-thawed cells? Cells harvested from the beginning of the cell cycle, frozen and thawed at a concentration of 3.6×10^9 cells/ml or greater showed no loss of enzyme activity over a period of 6 hours (Fig. 3). However, when the same cells were washed after freezing and thawing, the enzyme decayed with a half-life of less than an hour. In a similar experiment cells were harvested at various times during the cell cycle. Aspartate transcarbamylase was stable in the unwashed frozen-thawed cells from early in the cell cycle, but stability decreased in cells harvested later in the cycle. If fewer cells were frozen, as in the experiments of Vassef *et al.*, the enzyme decayed even in cells taken from the first hour of the cell cycle.

It now appears that in the experiments of Vassef *et al.* (36) the enzyme harvested early in the cell cycle decayed *in vitro* and the enzyme harvested just prior to DNA replication was relatively stable

Fig. 3. *In vitro* decay of aspartate transcarbamylase at 0-3° in washed and unwashed frozen-thawed cells of *Chlorella sorokiniana*. Cells were frozen in 0.5 ml aliquots at a concentration of 6.7×10^9 cells/ml in the presence of 0.2 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA. One aliquot of cells was washed three times with an equal volume of buffer and resuspended. ▲, unwashed cells; ● washed cells.

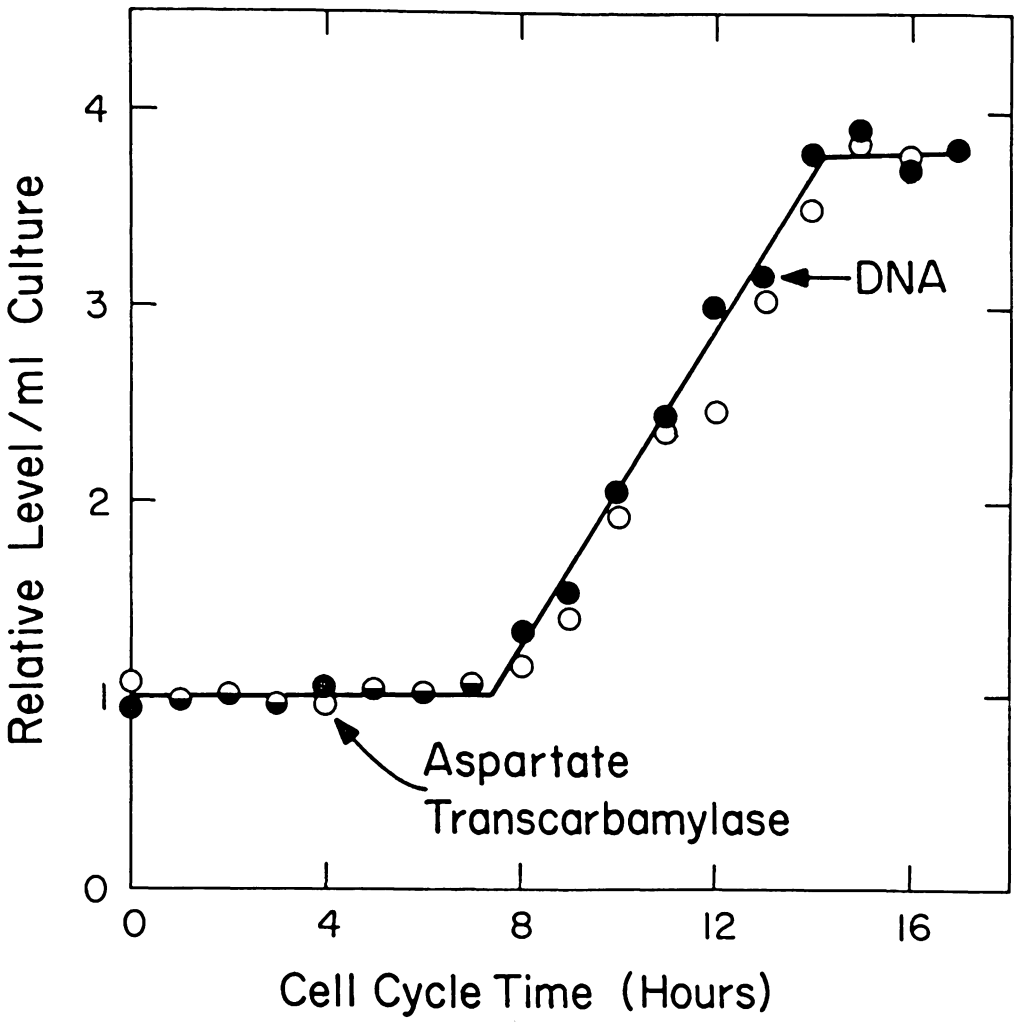

100
80
60
40
20
10
8
6
4
% Initial Enzyme Activity
Unwashed Cells (F.T.)
Washed Cells (F.T.)
0
1
2
3
4
5
6
Hours

in vitro. If the enzyme level early in the cell cycle were equal to that just before DNA replication, the recalculated fold increase in enzyme level would equal the fold increases in DNA and cell number. One way to avoid *in vitro* decay of the enzyme is to freeze and thaw the *Chlorella* cells in the presence of a stabilizer of the enzyme.

Since uridine stabilizes the enzyme *in vitro*, cells frozen and thawed in the presence of uridine exhibit stable enzyme. During a cell cycle in which uridine prevented *in vitro* decay of the enzyme, aspartate transcarbamylase accumulated in a step pattern with the step occurring during DNA replication (Fig. 4). Thus, the small molecule stabilizer effect proposed to operate *in vivo* by Vassef *et al.* is incorrect since the small increase in enzyme level prior to DNA replication can be explained by differential *in vitro* lability of aspartate transcarbamylase taken from various times in the cell cycle. The true pattern of enzyme accumulation under these conditions (nitrate cultured cells dividing into four daughter cells) is a step pattern.

The hypothesis that the level of UMP and/or some other stabilizer(s) fluctuates during the cell cycle may be valid. Such fluctuations could affect the *in vitro* stability of the enzyme frozen in the presence of only endogenous stabilizers. However, other explanations for changes in *in vitro* stability are possible. The differential stability of the enzyme *in vitro* may be only indirectly related to small molecule effects. For example, aspartate transcarbamylase could exist in a multienzyme complex with other enzymes on the pyrimidine pathway as it does in other eucaryotes (18). The presence of the nucleotide effector UTP

Fig. 4. Patterns of DNA and aspartate transcarbamylase during the cell cycle of *Chlorella sorokiniana*. Cells were frozen and thawed in the presence of 0.2 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 2 mM uridine. The turbidity of the culture was held essentially constant by continuous dilution with fresh culture medium. Initial cell number: 1.85×10^{8} cells/ml. Initial enzyme concentration: 5.4×10^{-9} units/cell. DNA, ● ; aspartate transcarbamylase, ○ .


Relative Level/ml Culture
0
1
2
3
4
0
4
8
12
16
Cell Cycle Time (Hours)
DNA
Aspartate
Transcarbamylase

causes either aggregation or disaggregation of aspartate transcarbamylase complexes in *Saccharomyces* and *Neurospora*, respectively. Aggregation may also affect enzyme activity. The catalytic subunits of aspartate transcarbamylase from *E. coli* have greater activity than the native enzyme (46). Thus, the activity of the enzyme measured *in vitro* may be influenced by variations in activity or stability due to formation of enzyme complexes or association of subunits, and complex formation or aggregation in turn may be influenced by small molecule effectors. Another factor which might influence *in vitro* stability of aspartate transcarbamylase is the formation of UMP by nuclease action. Nucleases may be more active during periods of the cell cycle when enzyme is most stable *in vitro*.

The step pattern for accumulation of the enzyme *in vivo* is consistent with the inference that aspartate transcarbamylase is an unstable enzyme whose gene is continuously transcribed and is under a constant level of repression or free from repression. If this inference is true, the rate constant for decay must also be constant through the cell cycle; otherwise, enzyme accumulation would deviate from the step pattern. A step pattern can also be generated by accumulation of a stable enzyme whose gene is transcribed intermittently (22). In this case, a burst of enzyme synthesis occurs as a result of periodic transcription and translation. If the enzyme is stabilized by an endogenous compound such as UMP which turns over continuously and the addition of cycloheximide results in a lower level of the stabilizer, the enzyme would decay in the presence of cycloheximide.

Studies on *In Vitro* Decay of Aspartate Transcarbamylase

Since it was desirable to freeze and thaw cells in the presence of uridine or UMP to prevent *in vitro* decay of the enzyme, it was important to determine the concentration of these stabilizers needed for complete *in vitro* stability. Uridine and UMP are also inhibitors of enzyme activity. The question was asked: Is there a concentration of uridine or UMP that can stabilize without inhibiting aspartate transcarbamylase *in vitro*?

An assay was developed to measure the effects of uridine and UMP on the *in vitro* stability of aspartate transcarbamylase. Frozen-thawed *Chlorella* daughter cells were washed three times in assay buffer and resuspended in buffer containing different concentrations of uridine or UMP (Figs. 5 and 6). The rate of decay of the enzyme was then measured. Decay was stopped by adding carbamyl-P, a substrate of the enzyme which also stabilizes. The initial rates of decay were first order, and the rate of decay was slower as the concentration of uridine or UMP increased.

After about three hours the rates of decay decreased abruptly in the uridine stabilized samples. This change in rate could be accounted for by production of endogenous UMP by nuclease action. High pressure liquid chromatography showed that the nucleotide content of frozen-thawed washed cells increased with time. After about three hours the concentration of UMP produced by nucleases plus the uridine added to the sample reached a level sufficient to stabilize aspartate transcarbamylase. The reason for uridine stabilized samples being more

Fig. 5. *In vitro* stabilization of aspartate transcarbamylase in frozen-thawed *Chlorella sorokiniana* cells by uridine at 0-3°. Cells were frozen in 0.5 ml aliquots in the presence of 0.2 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA, at a concentration of 6.7×10^9 cells/ml. The cells were washed three times with an equal volume of buffer and resuspended in buffer containing 0.40, 0.20, 0.10, 0.5, or 0 mM uridine.

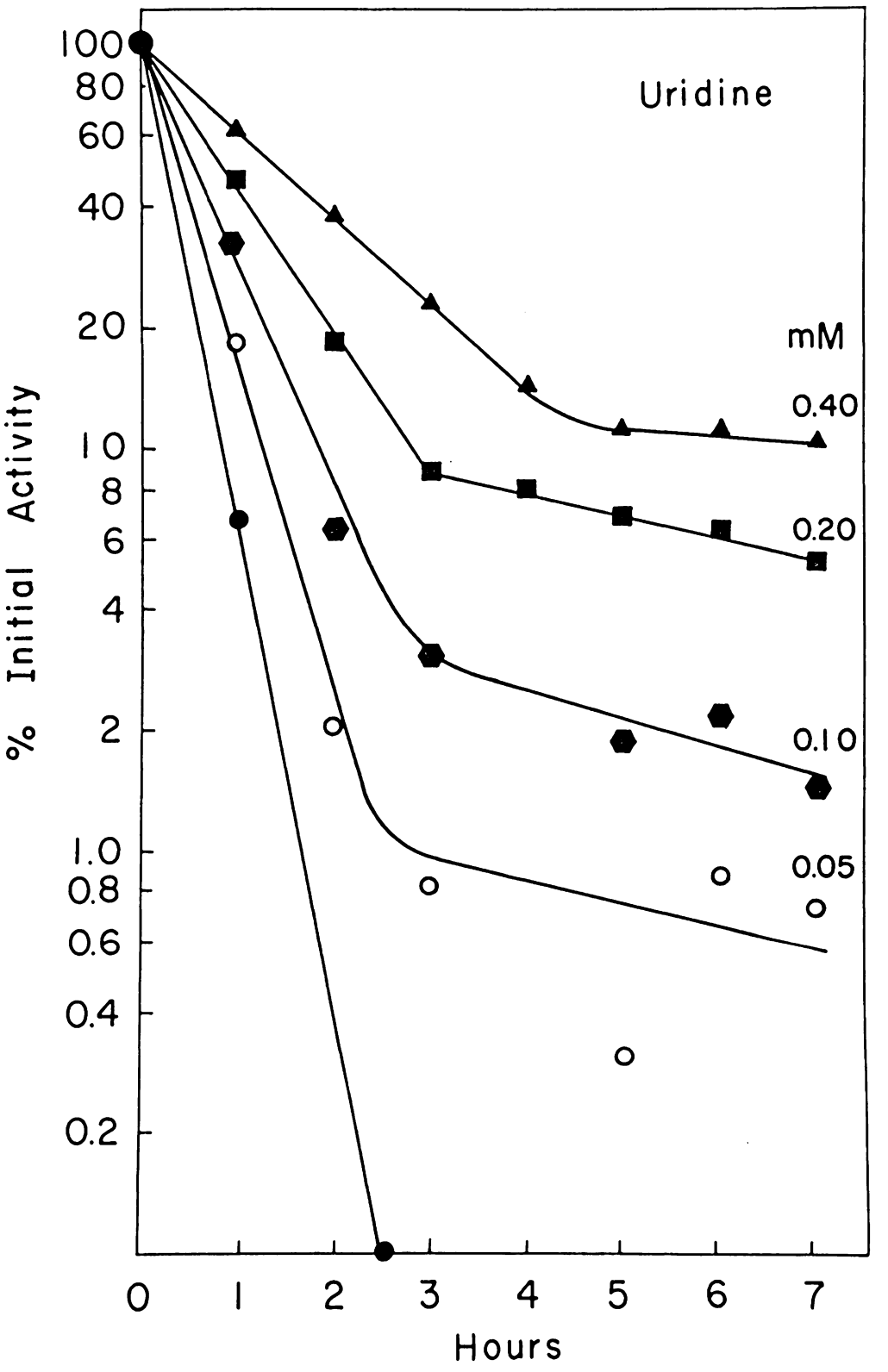
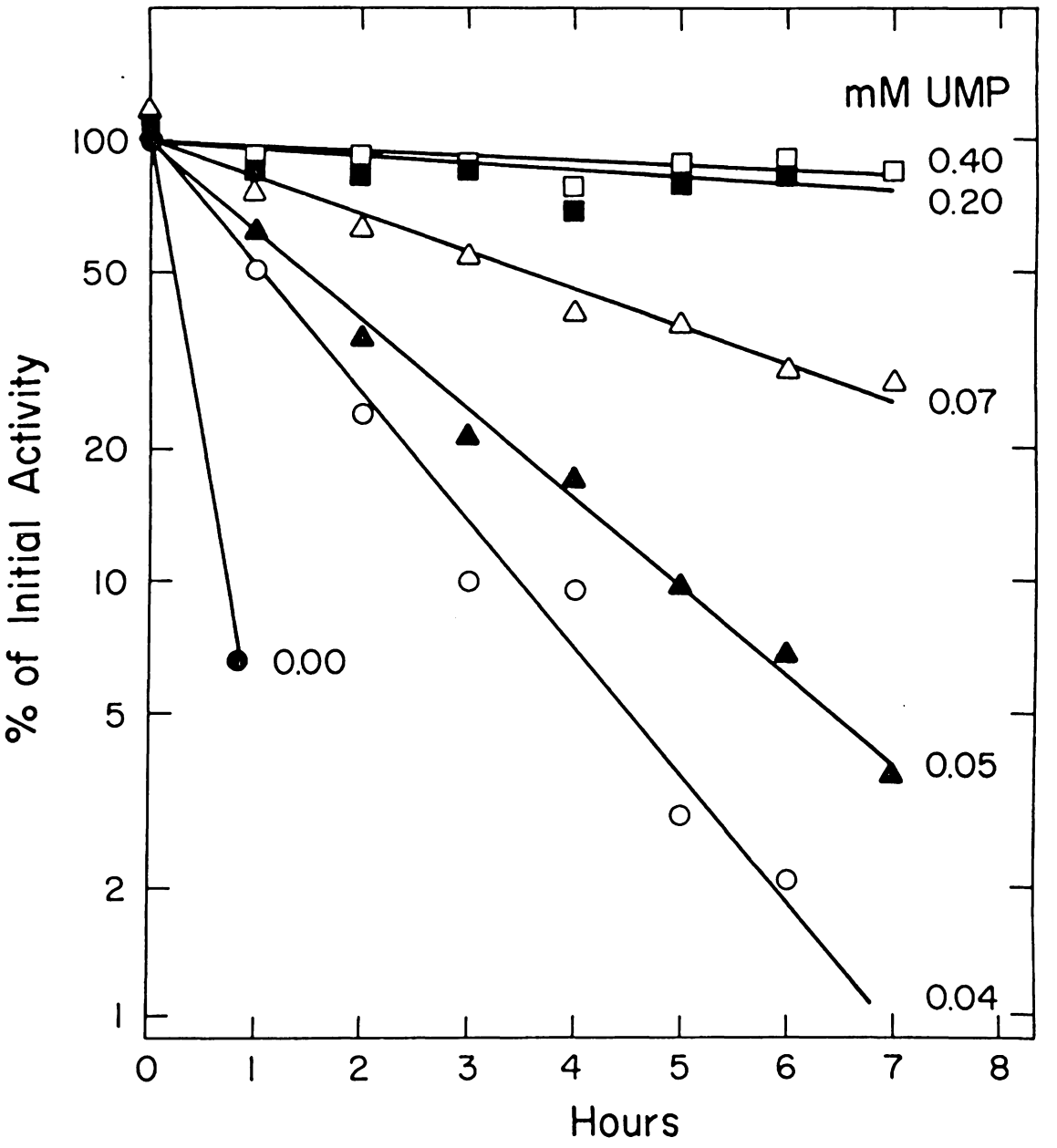

Uridine
mM
0.40
0.20
0.10
0.05
% Initial Activity
100
80
60
40
20
10
8
6
4
2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
Hours

Fig. 6. *In vitro* stabilization of aspartate transcarbamylase in frozen-thawed *Chlorella sorokiniana* cells by UMP at 0-3°. Conditions are the same as in Fig. 5 except that the buffer used to resuspend the cells contained 0.40, 0.20, 0.067, 0.05, 0.04, or 0 mM UMP.


mM UMP
0.40
0.20
0.07
0.05
0.04
0.00
100
50
20
10
5
2
1
% of Initial Activity
0
1
2
3
4
5
6
7
8
Hours

sensitive than UMP stabilized samples is unknown. Perhaps the binding of both nucleotide stabilizers to the enzyme is more effective in stabilizing aspartate transcarbamylase than binding either uridine or UMP alone.

Weeks (47) tested the effect of aging a sonicate of *Chlorella* cells on production of stabilizers. Sonicates were aged for different lengths of time, and then heated to denature enzymes. Dilutions of heated sonicate were tested for stabilizers of aspartate transcarbamylase by measuring their capacity to prevent decay of a test enzyme. These studies demonstrated that a stabilizer was produced during the aging process. In view of the high pressure liquid chromotography data, it seems likely that the stabilizer produced in the experiment of Weeks was UMP.

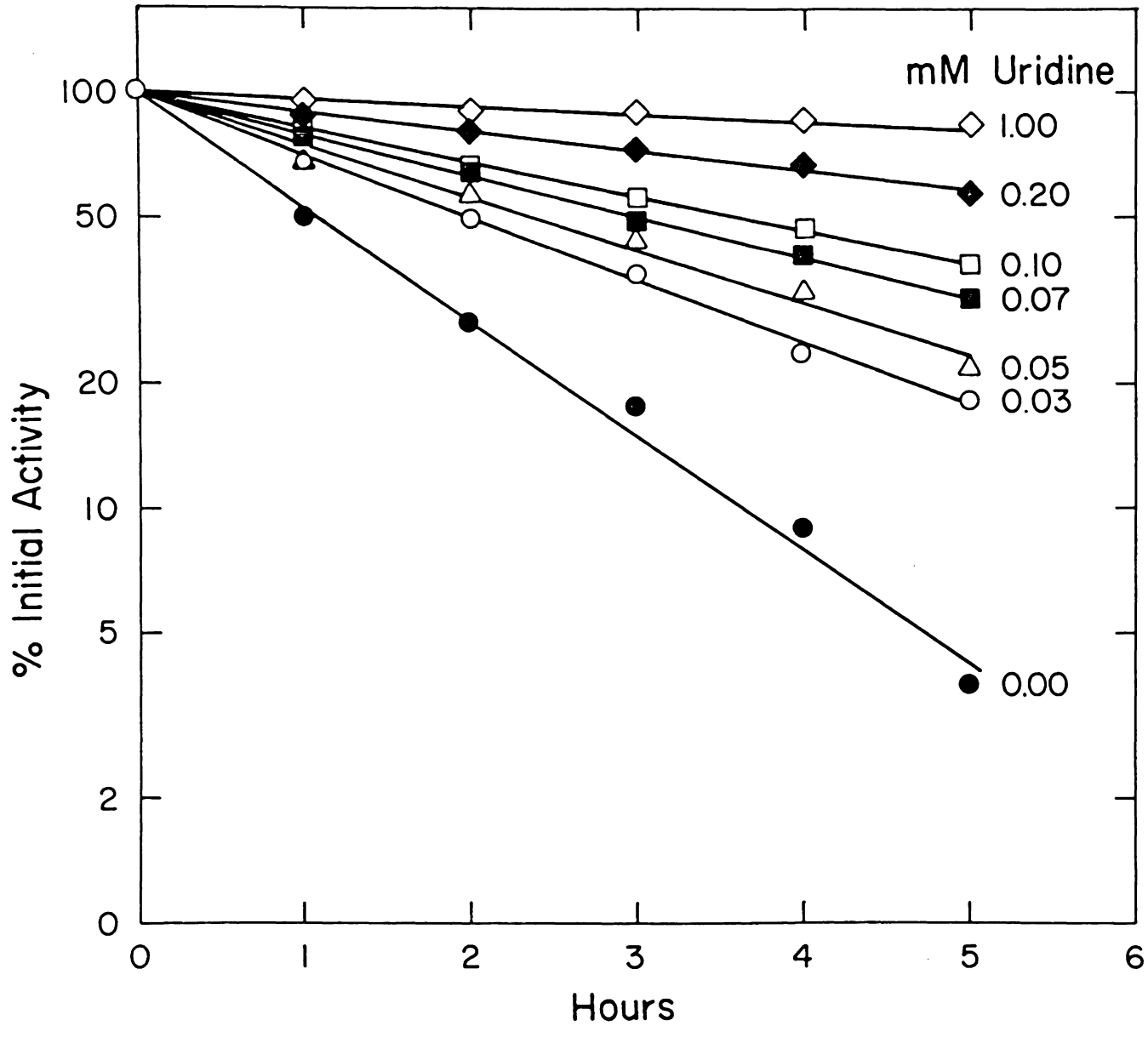
In order to measure the rate of decay of aspartate transcarbamylase as a function of uridine or UMP concentration, it was necessary not only to remove endogenous small molecules from the enzyme preparation to be tested but also to avoid the production of nucleotide stabilizers by nuclease action during the course of the experiment. Two approaches could be used. Either the nucleases could be removed or their substrates, the nucleic acids, could be eliminated from aspartate transcarbamylase preparations. Since both nucleases and nucleic acids are large molecules, it was impossible to remove them from frozen-thawed whole cells. Therefore, broken cell preparations were used as a source of aspartate transcarbamylase, and several methods were investigated as a means of removing nucleic acids and/or nucleases. The method of

choice could not affect aspartate transcarbamylase activity, and if the removal of nucleic acids and/or nucleases could not be carried out in the presence of uridine or UMP, the method had to be rapid enough to minimize loss of aspartate transcarbamylase activity due to decay of the enzyme.

The most successful means of purifying aspartate transcarbamylase was ammonium sulfate fractionation. A broken cell preparation was centrifuged at 100,000 x g to remove debris. Aspartate transcarbamylase was then precipitated between 30 and 50% saturation of ammonium sulfate in the presence of uridine. The enzyme preparation was then stable and could be stored redissolved in buffer containing uridine. The uridine was removed by a second precipitation in the absence of uridine. Under these conditions the enzyme precipitated between 50 and 70% saturation. This procedure removes sufficient nucleic acids and/or nucleases to allow first order rates of decay for a period of at least 5 hours (Fig. 7).

Protamine sulfate was used to try to precipitate additional nucleic acids from the enzyme. Although protamine sulfate reduced the total UV absorbing material remaining in solution, it did not improve the 280/260 ratio. Since these nucleotides adsorb at 280 and 260 nm, this procedure could not be carried out in the presence of uridine or UMP concentrations sufficient to stabilize the enzyme. As a result, there was considerable decay of aspartate transcarbamylase activity. Norit-A was very effective in removing nucleotides and nucleic acids from aspartate transcarbamylase preparations. It also reduced enzyme

Fig. 7. *In vitro* stabilization of ammonium sulfate-fractionated aspartate transcarbamylase from *Chlorella sorokiniana* by uridine at 0-3°. Aspartate transcarbamylase from broken cells was precipitated from a 100,000 x g supernatant between 50 and 70% saturation ammonium sulfate, resuspended in 0.20 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 2 mM uridine, and stored at -20°. Immediately before the experiment the enzyme was precipitated again with 80% saturated ammonium sulfate. The precipitated enzyme was dissolved in buffer containing 1.00 ◇, 0.20 ◆, 0.10 □, 0.07 ■, 0.50 △, 0.03 ○, or 0 ● mM uridine.


mM Uridine
1.00
0.20
0.10
0.07
0.05
0.03
0.00
% Initial Activity
100
50
20
10
5
2
0
0
1
2
3
4
5
6
Hours

activity by about 40%. Either the enzyme was adsorbed to Norit-A, or the charcoal remaining in the enzyme preparation was inhibitory to enzyme activity.

Aspartate transcarbamylase fractionated with ammonium sulfate decayed in a first order fashion with the rate of decay dependent on the concentration of added uridine or UMP. The stabilizing effect can be expressed as $k_{d_o} - k_{d_x}$, the rate of decay in the absence of stabilizer minus the rate of decay in the presence of x concentration of stabilizer. A plot of $k_{d_o} - k_{d_x}$ as a function of uridine concentration gives a hyperbolic curve when the source of aspartate transcarbamylase is ammonium sulfate precipitated enzyme (Fig. 8). A double reciprocal plot $1/(k_{d_o} - k_{d_x})$ vs. 1/[uridine]) is linear (Fig. 9). Similar representations of the UMP data are not linear, and the $k_{d_o} - k_{d_x}$ vs. [UMP] plots suggest that this curve may be sigmoidal (Figs. 10 and 11). Thus, UMP may show allosteric effects on aspartate transcarbamylase stabilization.

In the absence of added stabilizer, aspartate transcarbamylase decayed with a half-life of 15 min in frozen-thawed, washed cells, but the half-life of the enzyme fractionated with ammonium sulfate was over an hour. The mechanism of decay of aspartate transcarbamylase has not been established, but one hypothesis is that the enzyme is degraded by a protease. It is possible that partial purification of aspartate transcarbamylase by ammonium sulfate precipitation removes such a protease. Alternatively, the enzyme might bind uridine tightly during ammonium sulfate precipitation so that the resultant enzyme preparation

Fig. 8. *In vitro* stabilization of ammonium sulfate-fractionated aspartate transcarbamylase from *Chlorella sorokiniana* by uridine at 0-3°. k_{d_o} and k_{d_x}, rate constants for decay in the absence of uridine and at any given concentration of uridine, respectively. ○, data from Fig. 7; ▲, data from a replicate experiment.

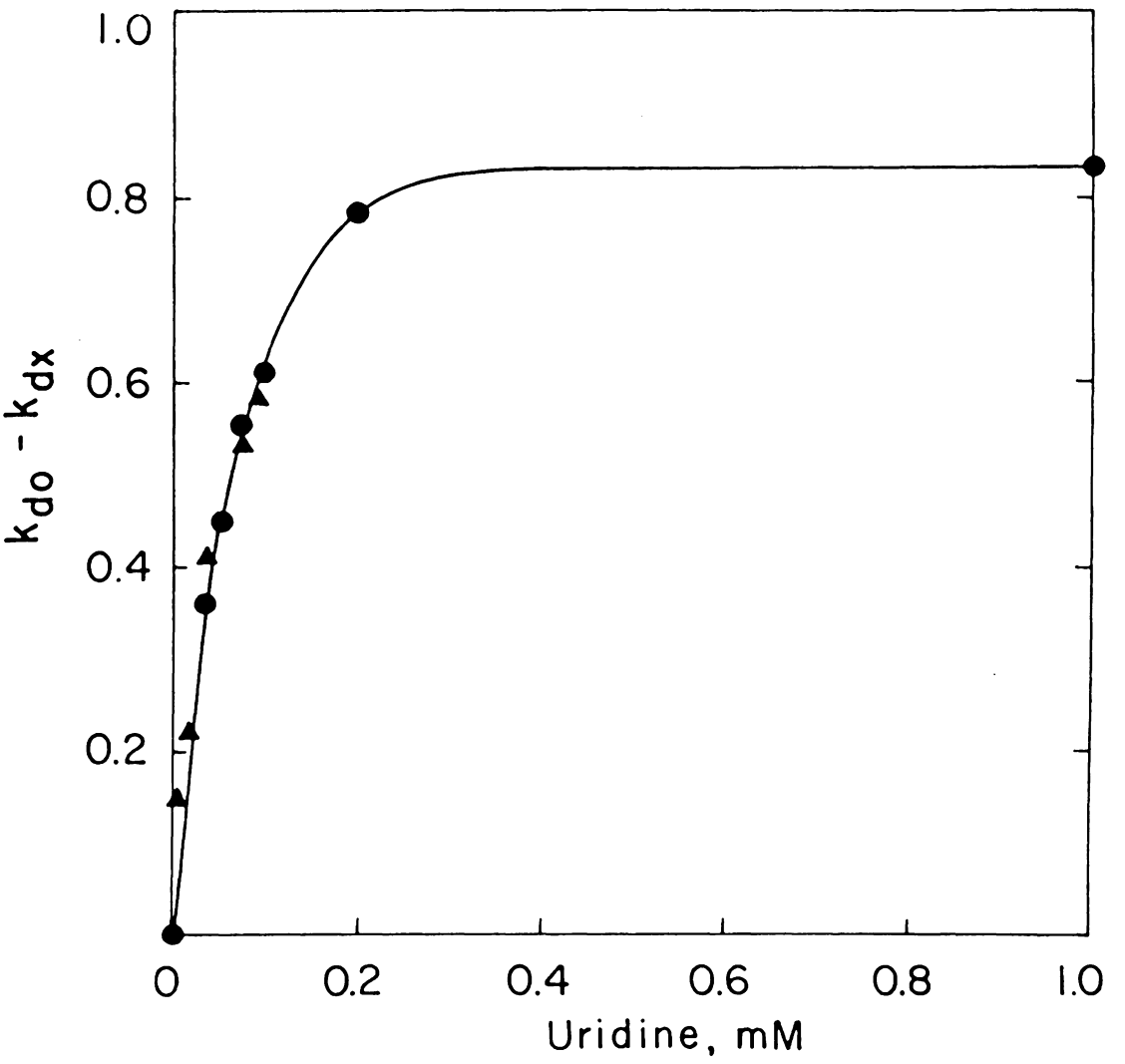

1.0
0.8
0.6
0.4
0.2
$k_{do} - k_{dx}$
0
0.2
0.4
0.6
0.8
1.0
Uridine, mM

Fig. 9. Double reciprocal plot relating the *in vitro* stability of ammonium sulfate-fractionated aspartate transcarbamylase from *Chlorella sorokiniana* at 0-3° to uridine concentration. Data from Fig. 7.

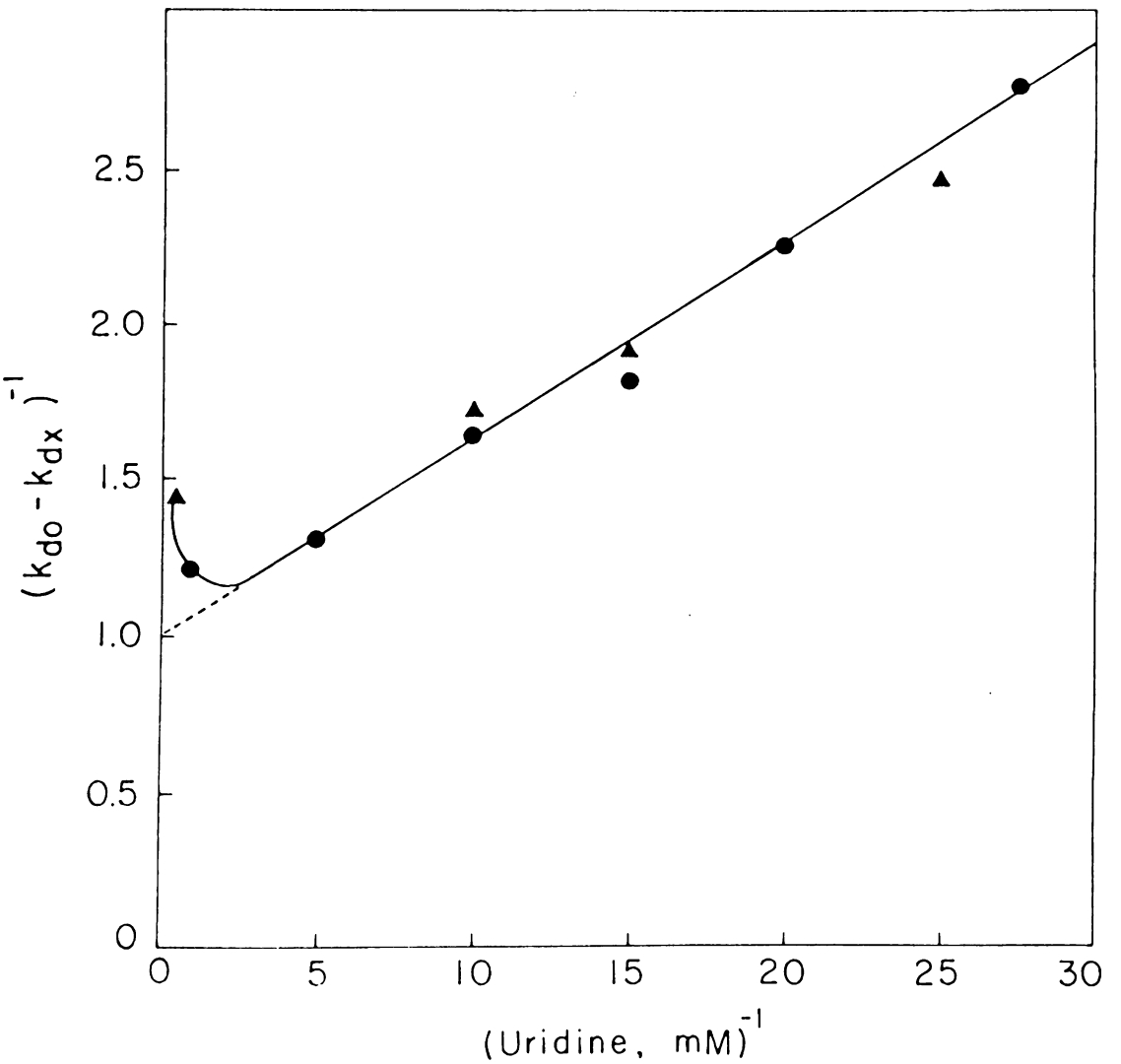

2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
$(k_{do} - k_{dx})^{-1}$
(Uridine, mM)$^{-1}$

Fig. 10. *In vitro* stabilization of ammonium sulfate-fractionated aspartate transcarbamylase from *Chlorella sorokiniana* by UMP at 0-3°. Conditions and definitions described in Figs. 7 and 8, respectively.

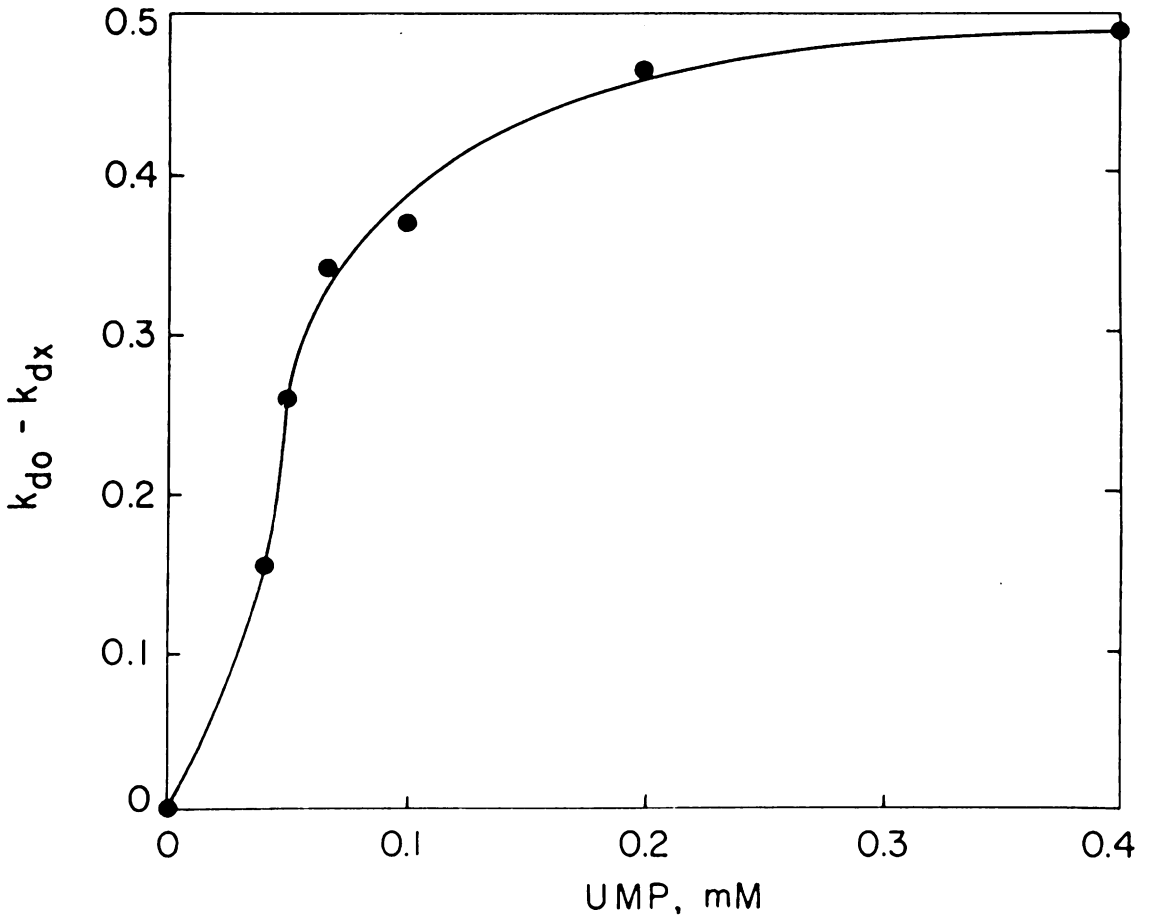
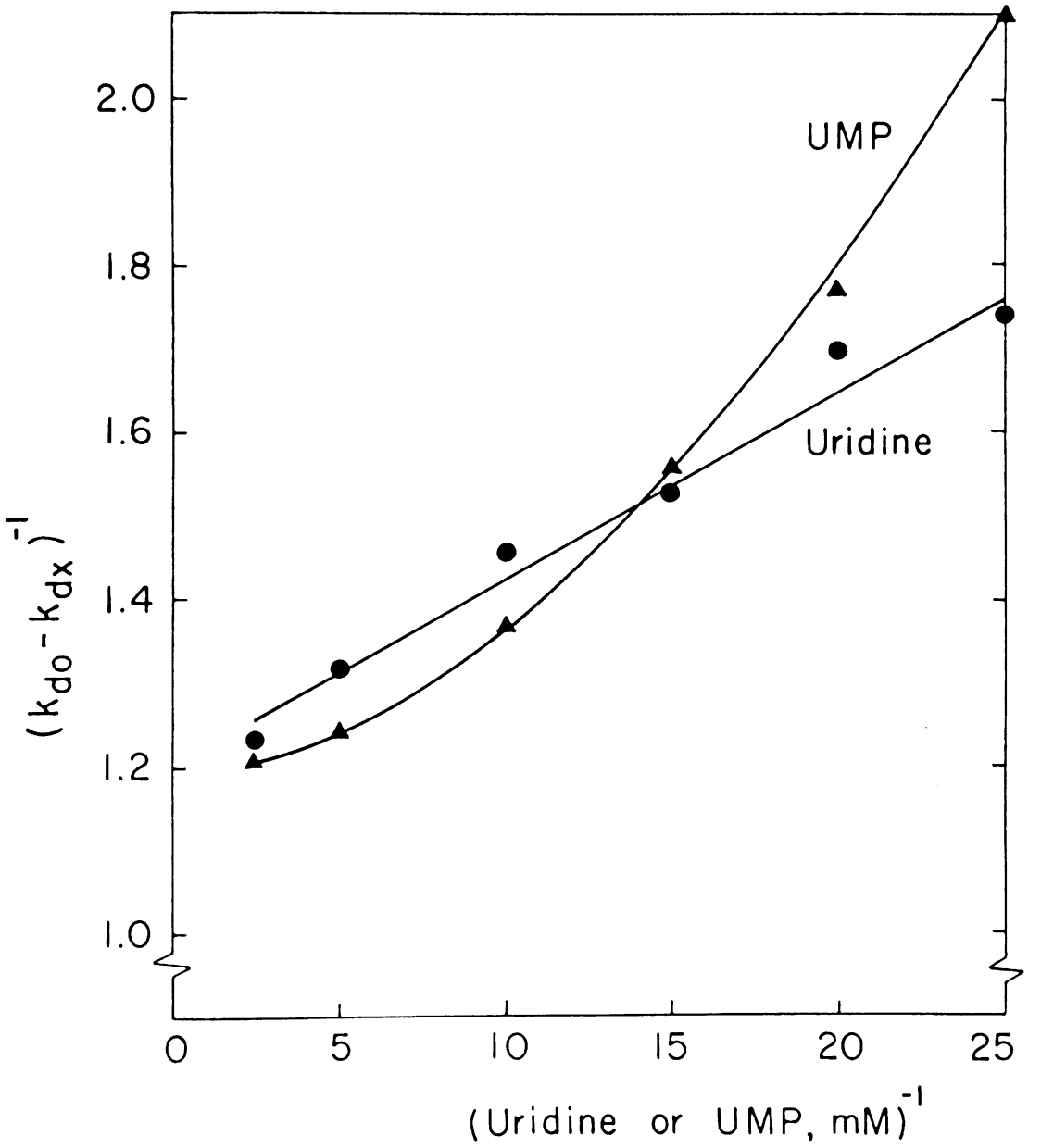

0.5
0.4
0.3
0.2
0.1
0
$k_{do} - k_{dx}$
0
0.1
0.2
0.3
0.4
UMP, mM

Fig. 11. Comparison of double reciprocal plots for the *in vitro* stability at 0-3° of ammonium sulfate-fractionated aspartate transcarbamylase from *Chlorella sorokiniana* in the presence of uridine ● , or UMP ▲ . Conditions and definitions described in Figs. 7 and 8, respectively.


2.0
1.8
1.6
1.4
1.2
1.0
0
5
10
15
20
25
UMP
Uridine
$(k_{do} - k_{dx})^{-1}$
(Uridine or UMP, mM)$^{-1}$

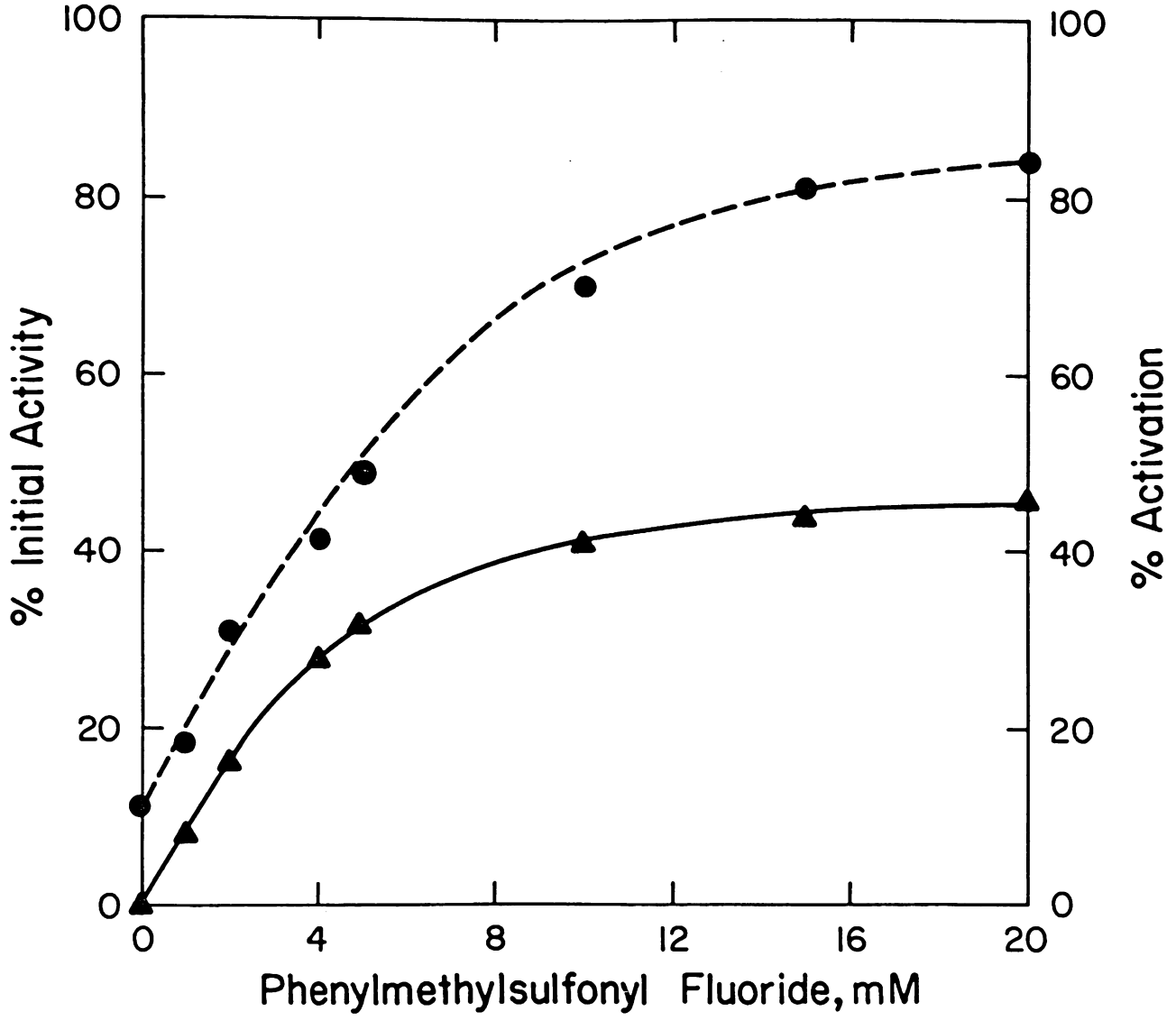
contains some residual uridine.

Mechanism of Aspartate Transcarbamylase Decay

The possibility that decay of aspartate transcarbamylase was due to degradation by a protease was investigated by using inhibitors of proteases and determining their effect on the rate of decay of aspartate transcarbamylase. Frozen-thawed washed cells were used in preference to broken cell preparations because it was reasoned that the protease and aspartate transcarbamylase would be kept in closer proximity to each other if they remained inside the cell. Breaking the cells would not only dilute the effective concentration of the protease, but it also might place aspartate transcarbamylase in contact with proteases not normally encountered *in vivo*. For example, the two enzymes might be compartmentalized inside different organelles so that proteolytic attack on aspartate transcarbamylase does not occur. The disadvantage of using frozen-thawed rather than broken cells is that the protease inhibitor must be able to penetrate frozen-thawed cells. It had been shown that molecules as large as NADH penetrate frozen-thawed cells and that none of the enzymes that have been tested are lost from frozen-thawed cells, but the permeability to molecules intermediate in size was unknown.

The serine protease inhibitor phenylmethylsulfonyl fluoride (48) was tested for its ability to prevent decay of aspartate transcarbamylase in frozen-thawed washed cells (Fig. 12). The rationale for the experiment was that phenylmethylsulfonyl fluoride might bind to and

Fig. 12. Apparent activation and/or stabilization of aspartate transcarbamylase from *Chlorella sorokiniana* by phenylmethylsulfonyl fluoride. Cells were frozen in 0.5 ml aliquots in 0.2 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA, thawed, and washed three times with an equal volume of buffer. The cells were resuspended in buffer at a concentration of 1.34×10^{10} cells/ml. An aliquot of washed cells was mixed with an equal volume of buffer containing different concentrations of phenylmethylsulfonyl fluoride. Samples were taken immediately and after 2 hours at 38.5° to measure the effect of the protease inhibitor on aspartate transcarbamylase activity and stability, respectively. ▲ , % activation; ○, % initial activity.


100
80
60
40
20
0
% Initial Activity
% Activation
0
4
8
12
16
20
Phenylmethylsulfonyl Fluoride, mM

inactivate a protease responsible for breaking down aspartate transcarbamylase. The inhibitor, however, appeared to bind to aspartate transcarbamylase resulting in activation of enzyme activity. Phenylmethylsulfonyl fluoride also stabilized aspartate transcarbamylase against decay. Stabilization could be due to inhibition of a serine protease; however, stabilization is more likely due to the same factor responsible for activation of aspartate transcarbamylase. Both activation and stabilization show a similar dependency upon phenylmethylsulfonyl fluoride concentration.

Soybean trypsin inhibitor, which is a peptide of approximately 20,000 molecular weight (49), was also added to frozen-thawed cells to test its effect on the rate of decay of aspartate transcarbamylase. Both inhibited and control samples decayed to the same activity after 2 hours at 2-5°, room temperature, and 38.5°. It is possible that the soybean trypsin inhibitor did not penetrate the frozen-thawed cells. Alternatively, the inhibitor did not inhibit the protease responsible for aspartate transcarbamylase decay, or else no protease is involved in the decay process.

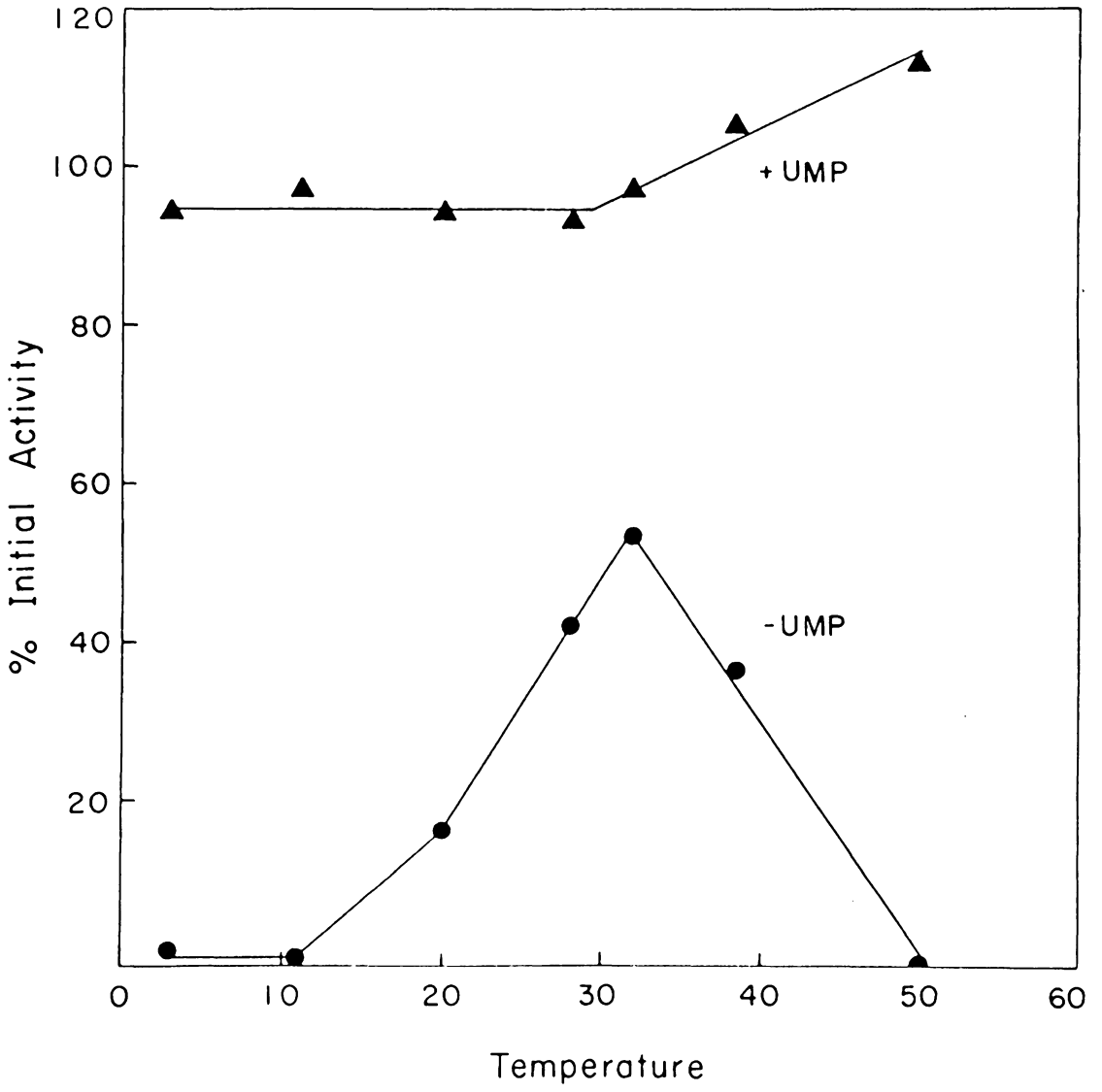
Factors other than proteases might be responsible for the observed *in vitro* decay of aspartate transcarbamylase. All of the decay studies involving the effects of nucleotide stabilizers were carried out at 2-5°. The possibility existed that aspartate transcarbamylase was cold labile. In some organisms carbamyl-P synthetase, the first enzyme on the pyrimidine pathway has been reported to be cold labile (18). In support of the hypothesis that aspartate transcarbamylase is cold

labile, the trypsin inhibitor studies, which were carried out at three different temperatures, indicated that aspartate transcarbamylase was more stable at room temperature than at 2-5°.

The temperature stability optimum of the enzyme was determined by measuring the loss of catalytic activity upon incubation at different temperatures for 90 min (Fig. 13). The temperature optimum for stability was 32°. The addition of 0.4 mM UMP to the enzyme (frozen-thawed cells) resulted in stability of the enzyme at all temperatures tested. In fact, there was a significant increase in activity at the higher temperatures. The increased activity may be due to breakdown of the enzyme into its component subunits or to breakdown of a multienzyme complex. One possible interpretation of the data is that aspartate transcarbamylase is more active but less stable as subunits or outside a complex.

Among the cold labile enzymes that have been reported is phosphofructokinase from chicken liver (50). In studies with this enzyme it was found that chloride ion enhances cold lability. The buffer used for aspartate transcarbamylase assays was Tris-HCl. Therefore, other buffers were tested for their effect on enzyme stability. The enzyme was more stable in glycylglycine buffer (94% of the initial activity remained after 90 min) at 32° than in Tris-HCl buffer. Tris-acetate buffer was similar to Tris-HCl. Therefore, it is the Tris and not the chloride ion that is associated with enzyme instability. Two buffers that are structurally related to glycylglycine, glycine and bicine, were intermediate in their effects. Another study showed that in the

Fig. 13. Effect of temperature on *in vitro* stability of aspartate transcarbamylase in frozen-thawed washed cells of *Chlorella sorokiniana* in the presence and absence of UMP. Cells were frozen in 0.5 ml of 0.2 M Tris-HCl, pH 8.5, at a concentration of 6.7×10^9 cells/ml, thawed, and washed three times with an equal volume of buffer. The cells were resuspended in the original volume of buffer or buffer + 0.4 mM UMP and incubated for 90 min. ○, without UMP; ▲ with UMP.


120
100
80
60
40
20
0
10
20
30
40
50
60
% Initial Activity
Temperature
+ UMP
- UMP

presence of uridine, the enzyme was stable in glycylglycine as well as Tris-HCl buffer (90 min at room temperature).

The effect of the two buffers, Tris-HCl and glycylglycine was further investigated in cells of different ages during subsequent cell cycle experiments. At this point, however, it was clear that the Tris ion was associated with lability of the enzyme in the absence of nucleotide stabilizers.

Inhibition of Aspartate Transcarbamylase Activity

Ammonium sulfate fractionated enzyme was more stable than enzyme in frozen-thawed cells. The difference in stability could be due to removal of a protease, to residual uridine in the ammonium sulfate fractionated enzyme, or to some alteration in the structure of the enzyme itself as a result of the ammonium sulfate treatment. Does the sensitivity of the enzyme to inhibition also change as a result of the treatment? To approach this question, unwashed (Fig. 14) and washed (Fig. 15) frozen-thawed cells and ammonium sulfate fractionated enzyme (Fig. 16) were compared with respect to sensitivity to inhibition by uridine and UMP.

In washed frozen-thawed cells 90% inhibition was seen with about 6 mM uridine or UMP. Sensitivity to uridine inhibition was similar in unwashed cells, but less UMP had to be added to show a given level of inhibition. The enzyme was less sensitive to feedback inhibition by both uridine and UMP after ammonium sulfate fractionation.

There are several possible explanations for the change in sensitivity to inhibition:

Fig. 14. Effect of uridine and UMP on the activity of aspartate transcarbamylase in washed, frozen-thawed cells of *Chlorella sorokiniana*. Cells were frozen in 0.2 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA, thawed, and washed three times with an equal volume of buffer. The cells were resuspended in buffer or buffer containing uridine or UMP. ○ , uridine; ▲ , UMP.

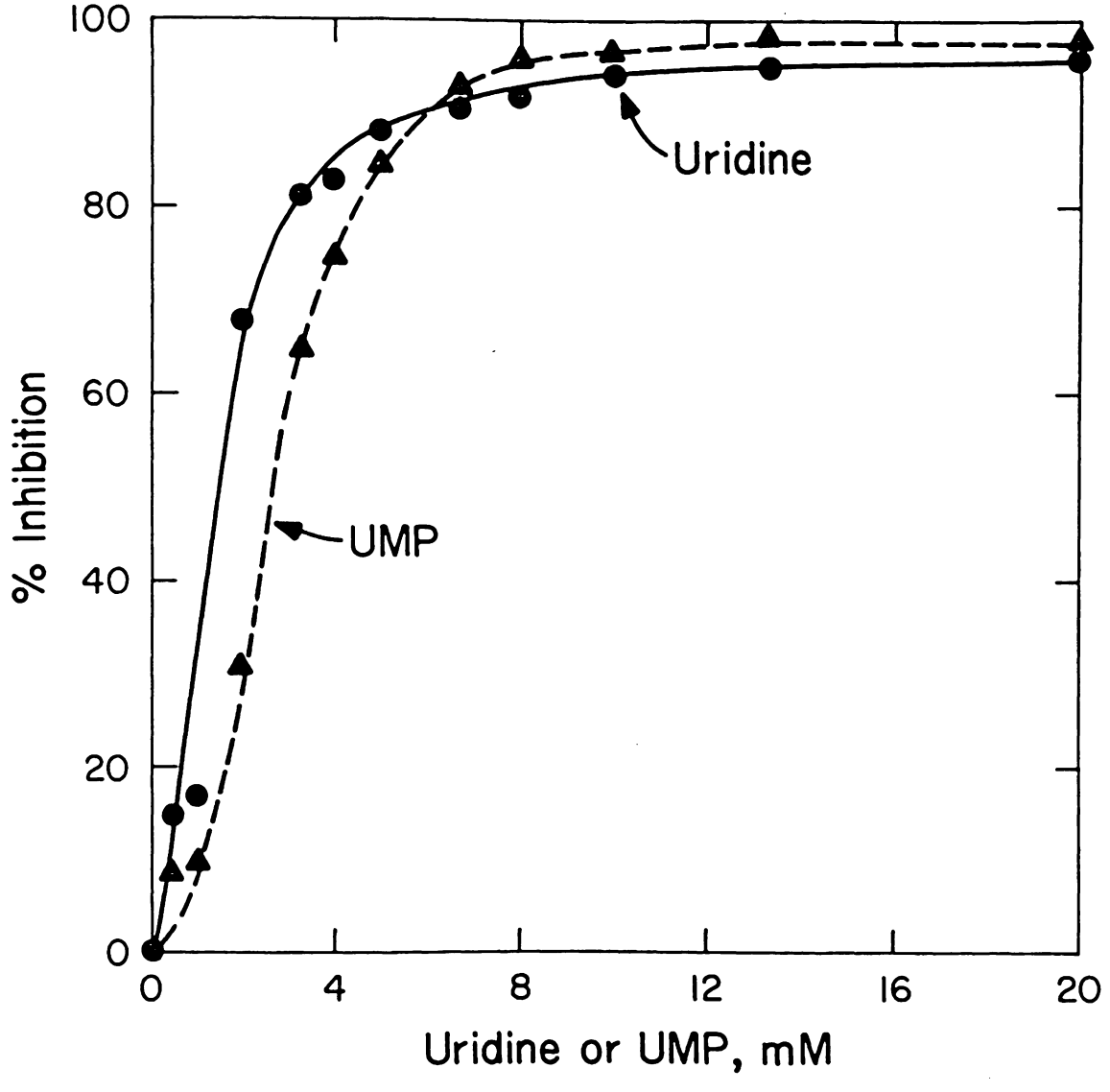

100
80
60
40
20
0
% Inhibition
Uridine
UMP
0
4
8
12
16
20
Uridine or UMP, mM

Fig. 15. Effect of uridine and UMP on the activity of aspartate transcarbamylase in unwashed frozen-thawed cells of *Chlorella sorokiniana*. Cells were prepared and assayed as described in Fig. 14, but were unwashed. ○ , uridine; ▲ , UMP.

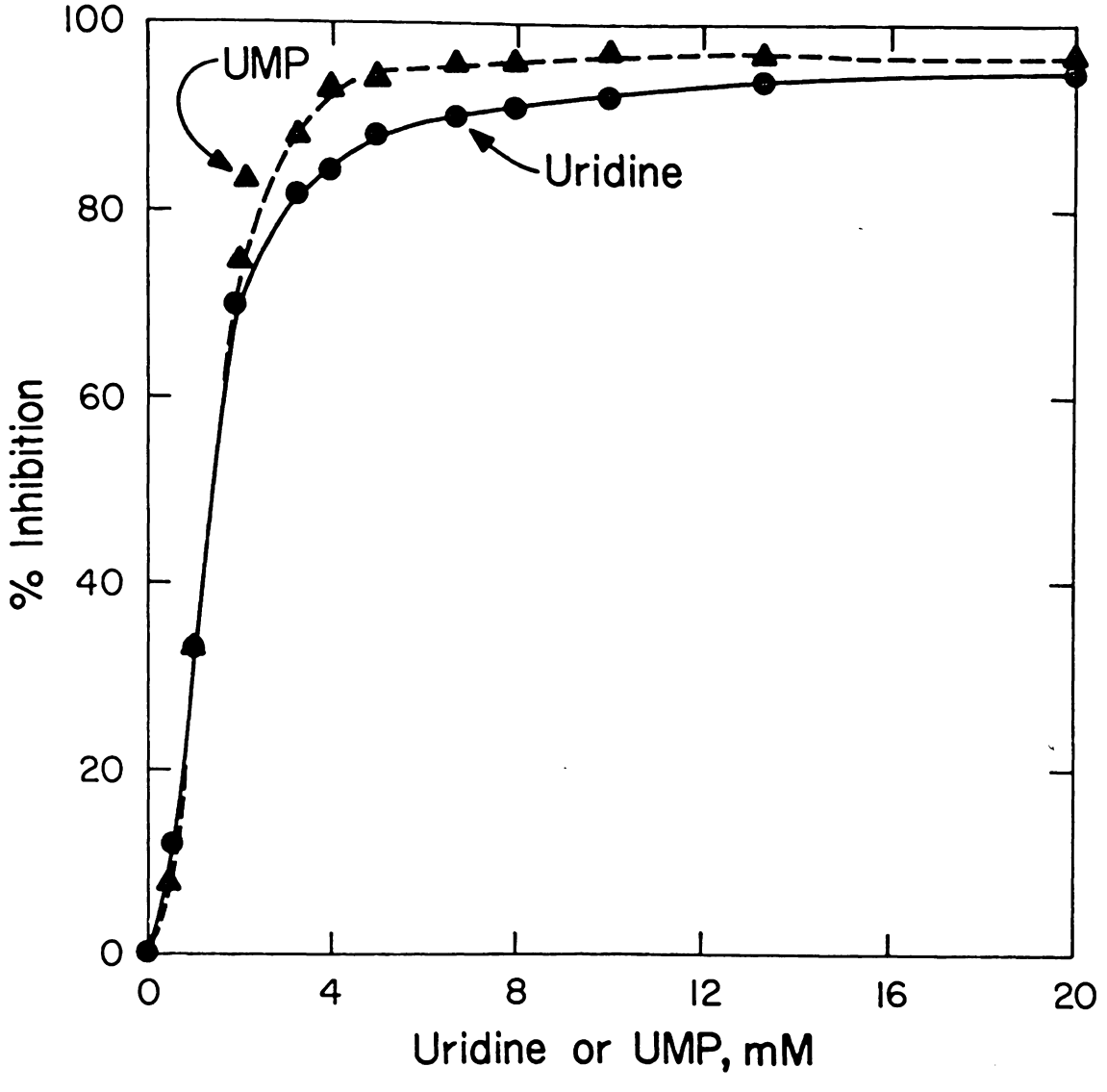
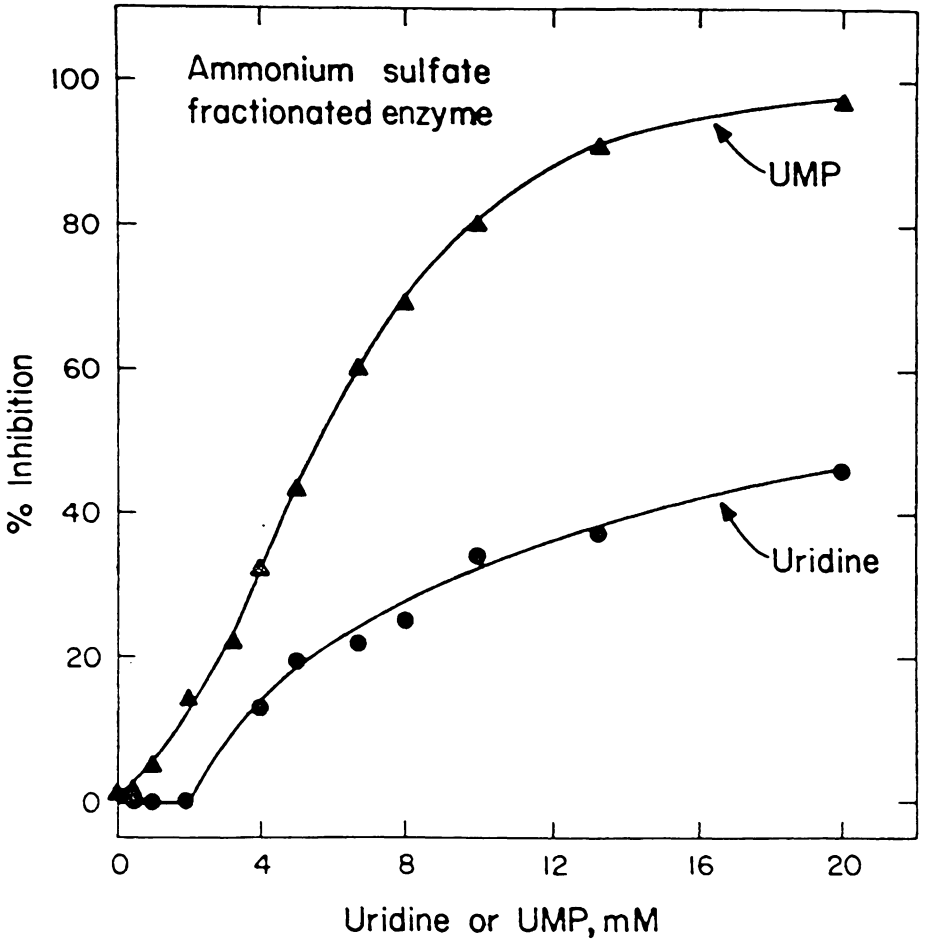

100
80
60
40
20
0
% Inhibition
UMP
Uridine
0
4
8
12
16
20
Uridine or UMP, mM

Fig. 16. Effect of uridine and UMP on the activity of ammonium sulfate-fractionated aspartate transcarbamylase from *Chlorella sorokiniana*. Cells were suspended in 0.20 M Tris-HCl, pH 8.5, containing 1 mM EDTA, broken in a French pressure cell at 18-20,000 psi, and centrifuged at 100,000 x g. Aspartate transcarbamylase was precipitated from the supernatant with 50-70% saturated ammonium sulfate. ○, uridine; ▲, UMP.


Ammonium sulfate
fractionated enzyme
UMP
Uridine
% Inhibition
100
80
60
40
20
0
0
4
8
12
16
20
Uridine or UMP, mM

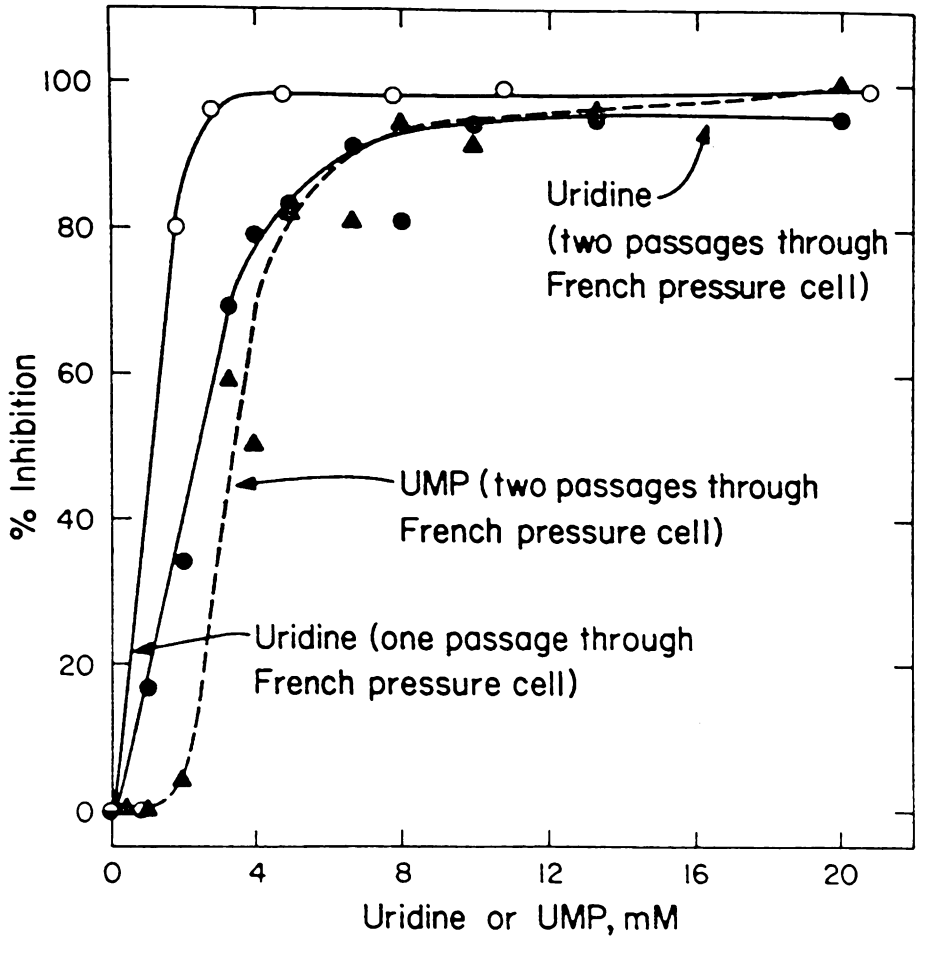
1. Loss of a feedback inhibition site due to dissociation of aspartate transcarbamylase into catalytic and regulatory subunits.
2. Disaggregation of a multienzyme complex.
3. Breaking *Chlorella* cells in a French pressure cell may affect the nature of enzyme subunits or complexes (Fig. 17). When the cells were broken by passing them through the French pressure cell only once, the enzyme was still sensitive to inhibition by uridine. A second passage through the French pressure cell, however, resulted in a partial loss of sensitivity to feedback inhibition.

Attempts to Demonstrate a Multi-Enzyme Complex of Certain Pyrimidine Enzymes

In *Neurospora* and *Saccharomyces*, aspartate transcarbamylase exists in a multienzyme complex with carbamyl-P synthetase, the first enzyme on the pyrimidine pathway (18). In animal cells the first three enzymes carbamyl-P synthetase, aspartate transcarbamylase, and dihydroorotase, form a complex (31). To look for such complexes in *Chlorella*, it was necessary to develop an assay for carbamyl-P synthetase (see Experimental Procedures).

In an attempt to determine whether *Chlorella* contains a complex of aspartate transcarbamylase, carbamyl-P synthetase, and/or dihydroorotase, *Chlorella* cells were broken in a French pressure cell and centrifuged at 30,000 x g to remove debris. The supernatant was centrifuged at 100,000 x g for various lengths of time. The rationale was to sediment

Fig. 17. Effect of uridine and UMP on the activity of aspartate transcarbamylase in 100,000 x g supernatants of *Chlorella sorokiniana*. Cells were broken by passage through a French pressure cell either once or twice. Buffer: 0.20 M Tris-HCl, pH 8.5 containing 1 mM EDTA. ○ and ◒, uridine; ▲, UMP.


100
80
60
40
20
0
% Inhibition
0
4
8
12
16
20
Uridine or UMP, mM
Uridine
(two passages through
French pressure cell)
UMP (two passages through
French pressure cell)
Uridine (one passage through
French pressure cell)

and thus concentrate the complex, resuspend it and apply it to a sucrose density gradient, and then look for co-sedimentation of the enzymes. The complexes described in other eucaryote cells range from 600,000 to 850,000 in molecular weight (18, 31), and sediment at 100,000 x g in 1 or 2 hours. Neither carbamyl-P synthetase nor aspartate transcarbamylase sedimented significantly in 5.5 hours at 100,000 x g. Thus a carbamyl-P synthetase-aspartate transcarbamylase complex of this size did not exist in the *Chlorella* preparation. Since aspartate transcarbamylase did not sediment in the period of time expected for a multi-enzyme complex, the planned assays for dihydroorotase were omitted. Perhaps *Chlorella* does not have a multi-enzyme complex of pyrimidine enzymes or the complex is of low molecular weight. When the cells were ruptured in the French pressure cell, a complex may have been destroyed. The observed loss of sensitivity to inhibition by uridine and UMP might be indicative of enzyme complex destruction.

These experiments demonstrate the importance of seeking conditions for maintaining an enzyme in its native condition during procedures such as enzyme purification. The sensitivity of the enzyme to inhibition might be one way of monitoring enzyme or complex integrity during each step of enzyme purification.

Cell Cycle Regulation of Aspartate Transcarbamylase Levels

The apparent absence of a carbamyl-P synthetase-aspartate transcarbamylase complex in broken cell preparations combined with loss of sensitivity of aspartate transcarbamylase to inhibition indicated that

the enzyme seems to be in a more native state in frozen-thawed cells than in broken cell preparations. Frozen-thawed cells were chosen for subsequent cell cycle studies. When cells were frozen in 2 mM uridine or UMP, the enzyme remained stable. Frozen-thawed cells were then washed in buffer containing 2 mM uridine or UMP to remove any endogenous small molecules that might fluctuate in concentration during the cell cycle and differentially alter enzyme activity or stability. The concentration of uridine or UMP in the final assay mixture was then 0.8 mM which gives minimal inhibition with uridine and almost no inhibition with UMP. The relative advantages of Tris-HCl and glycylglycine buffer were further investigated in cell cycle experiments.

Chlorella cells were synchronized by alternating light-dark periods and harvested at the daughter cell stage. A more homogeneous population of cells was obtained by isopycnic Ficoll centrifugation as described in Experimental Procedures. *Chlorella* cells can be made to divide into different numbers of daughter cells, depending on the light intensity per cell. Because cells tend to shade each other in cell suspensions, the effective light intensity per cell depends upon the cell concentration as well as the external light intensity. In most of the following experiments, light conditions were selected to give an average fold increase in cell number approaching four.

The model proposed by Vassef *et al.* (36) requires that the enzyme is either expressed constitutively or is under a constant degree of repression. A step increase in aspartate transcarbamylase activity

results from a step increase in gene dosage. One way of testing this model is to perturb culture conditions in an attempt to separate enzyme and DNA synthesis. Previous work (36, 51) had shown that DNA could be changed from a step to a semi-continuous pattern by diluting the cells at a faster rate than they were growing so that the culture turbidity gradually decreased throughout the cell cycle. In an experiment of this type, Vassef *et al.* (36) found that aspartate transcarbamylase accumulation was also changed from a step to a semi-continuous pattern. Apparently, changes in the effective light intensity per cell can have profound effects on macromolecular accumulation in synchronous cultures. Conceivably, if light intensity were altered in the proper way, the accumulation of aspartate transcarbamylase and DNA might be uncoupled.

When the culture dilution rate was varied during a cell cycle to cause the culture turbidity to oscillate (Fig. 18) the DNA pattern was semi-continuous but, more importantly, aspartate transcarbamylase accumulation began before the onset of DNA replication (Fig. 19). The separation of the timing of accumulation of these two macromolecules indicates that synthesis is not obligately coupled. The increased rate of enzyme accumulation must be due to something other than an increase in gene dosage. Possibly the gene is derepressed as a result of perturbation of corepressor pools by the changing cultural conditions. However, it is clear that the aspartate transcarbamylase structural gene is not expressed constitutively. Thus, one of the conditions set by the Vassef model, constitutive enzyme synthesis or

Fig. 18. Programmed changes in culture turbidity during a cell cycle in nitrate medium. The culture was continuously diluted with fresh culture medium at a rate designed to cause oscillations in culture turbidity. Initial cell number: 2.62×10^8 cells/ml.

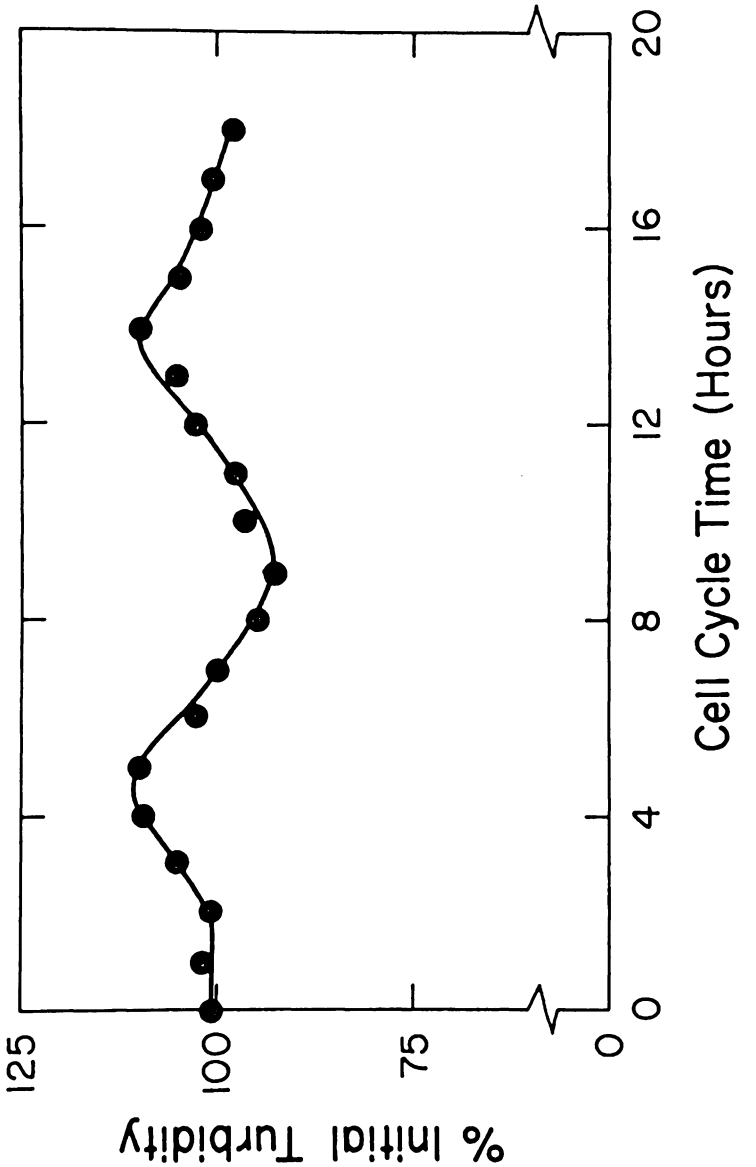
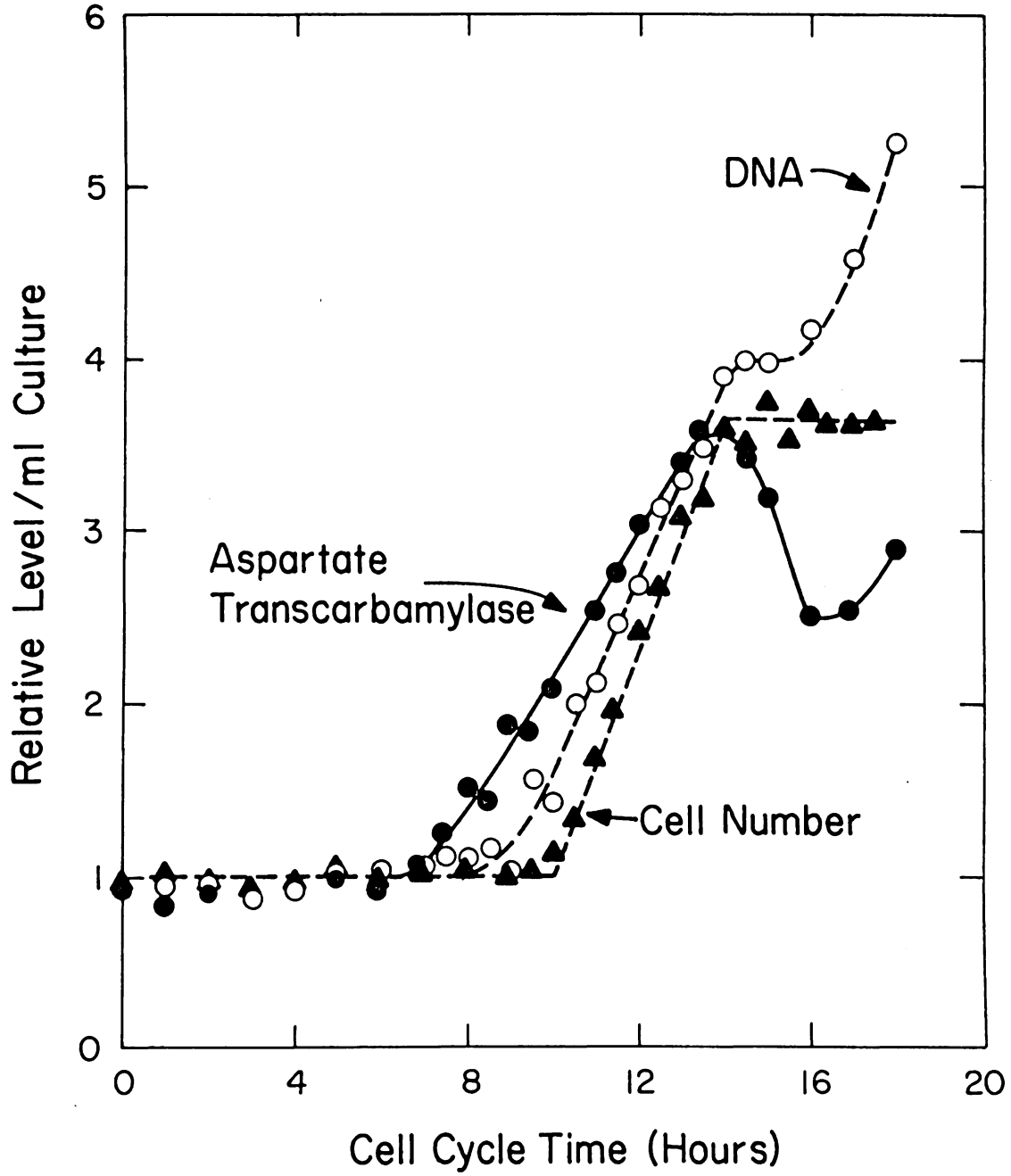

% Initial Turbidity
125
100
75
0
0
4
8
12
16
20
Cell Cycle Time (Hours)

Fig. 19. Pattern of aspartate transcarbamylase accumulation during the cell cycle of *Chlorella sorokiniana* cultured in nitrate medium. The culture is the same as that in Fig. 18. Initial concentration of enzyme: 6.1×10^{-9} units/cell Level of enzyme ● , DNA ○ , and cell number ▲ .

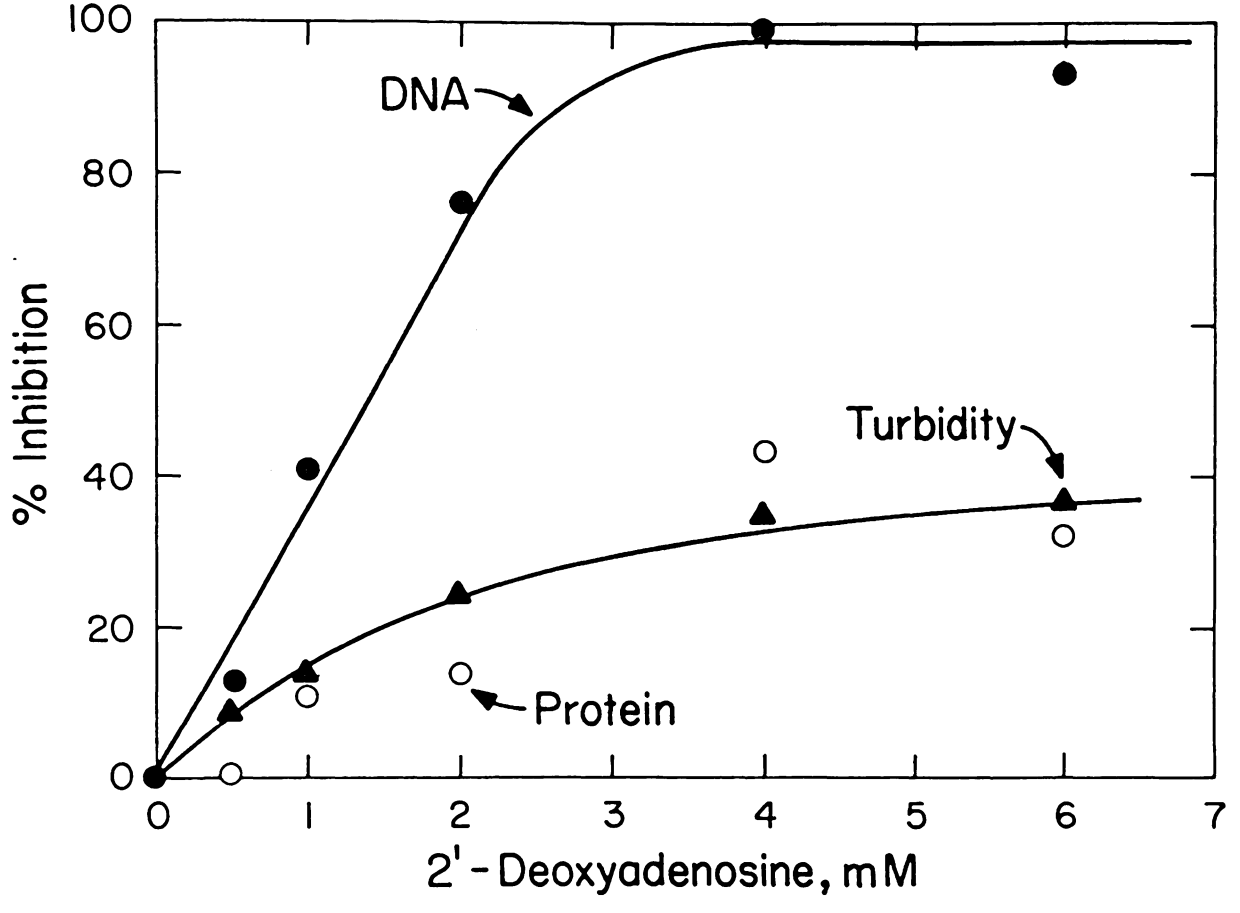

Relative Level/ml Culture
0
1
2
3
4
5
6
0
4
8
12
16
20
Cell Cycle Time (Hours)
DNA
Aspartate
Transcarbamylase
Cell Number

a constant degree of repression with resultant and coincident aspartate transcarbamylase and DNA patterns, is not met under all cultural conditions.

In the same experiment aspartate transcarbamylase activity decreased early in the next cycle. The decreased activity could be due to either a change in *in vivo* or *in vitro* stability or activity. If the decay were due to *in vivo* change in enzyme stability, this experiment would be the first instance in which *in vivo* instability of this enzyme was demonstrated in the absence of inhibitors such as cycloheximide or actinomycin D. However, the observed decrease in enzyme activity could be the result of an alteration in the subunit structure of the enzyme or a change in the components of a multi-enzyme complex. Another possibility is that the effect occurred *in vitro*, even though UMP was present to stabilize the enzyme.

Another experimental approach to determine whether or not aspartate transcarbamylase accumulation is coupled to DNA replication is to inhibit DNA synthesis with specific inhibitors and then determine the effect on enzyme accumulation. An inhibitor of ribonucleotide reductase activity, 2'-deoxyadenosine (52), was found to inhibit DNA synthesis in *Chlorella*. In nitrate cultured cells, a concentration of the inhibitor sufficient to inhibit DNA synthesis by at least 90% also inhibited total cellular protein synthesis by 30 to 40% (Fig. 20). When DNA synthesis was inhibited by only 75%, the inhibition of protein synthesis was still rather high at 20-25%. Thus, a DNA inhibitor which inhibits both DNA and protein synthesis in its effective range is not

Fig. 20. Effect of 2'-deoxyadenosine on the synthesis of DNA and protein in *Chlorella sorokiniana* cultured in nitrate medium. A culture was synchronized by the light:dark procedure described in Experimental Procedures. The culture turbidity was held essentially constant for the first 8 hours of the cell cycle by continuous dilution with fresh culture medium. At the eighth hour the culture was split and centrifuged at 14,600 x g for 5 min and cells resuspended in culture medium containing different concentrations of 2'-deoxyadenosine. The cultures were allowed to grow for an additional 6 hours without further dilution. Samples were then taken for measuring DNA ● , protein ○ , and culture turbidity ▲ . Initial cell number: 1.77×10^8 cells/ml.


100
80
60
40
20
0
% Inhibition
DNA
Turbidity
Protein
0
1
2
3
4
5
6
7
2'-Deoxyadenosine, mM

suited for evaluating the relationship between gene replication and aspartate transcarbamylase accumulation.

Although protein synthesis was sensitive to 2'-deoxyadenosine in its effective concentration range for inhibition of DNA replication in nitrate-cultured cells, complete inhibition of DNA replication without significant inhibition of protein synthesis was subsequently observed in ammonium cultured cells (Fig. 21). Thus, it seemed advantageous to use ammonium cultured cells to examine the relationship between gene replication and aspartate transcarbamylase accumulation.

Before the relationship between enzyme accumulation and DNA replication could be studied with 2'-deoxyadenosine in ammonium cultured cells, the culture conditions of the cells in a different nitrogen source had to be redefined and the cell cycle patterns of the enzyme and DNA reexamined in the absence of inhibitor. The cells were observed to grow at a faster rate in the presence of ammonium, and the cell cycle length was shortened by approximately 40%.

In a preliminary cell cycle experiment with ammonium as the nitrogen source, the cells were frozen in either Tris-HCl plus 2 mM uridine or glycylglycine plus 2 mM uridine (Fig. 22). The enzyme patterns on a relative level basis were similar when the enzyme was assayed in either of the buffers. The relationship between the step increases in aspartate transcarbamylase and DNA was similar to that seen in nitrate cultured cells.

To determine whether ammonium induces isozymes of aspartate

Fig. 21. Effect of 2'-deoxyadenosine on the synthesis of DNA and protein in *Chlorella sorokiniana* cultured in ammonium medium. Experimental conditions were the same as those described in Fig. 20 except that the cells were cultured in ammonium medium and the inhibitor was added 2.5 hours into the cell cycle. Initial cell number: 1.5×10^8 cells/ml. DNA, ● and ○, protein, ■ .

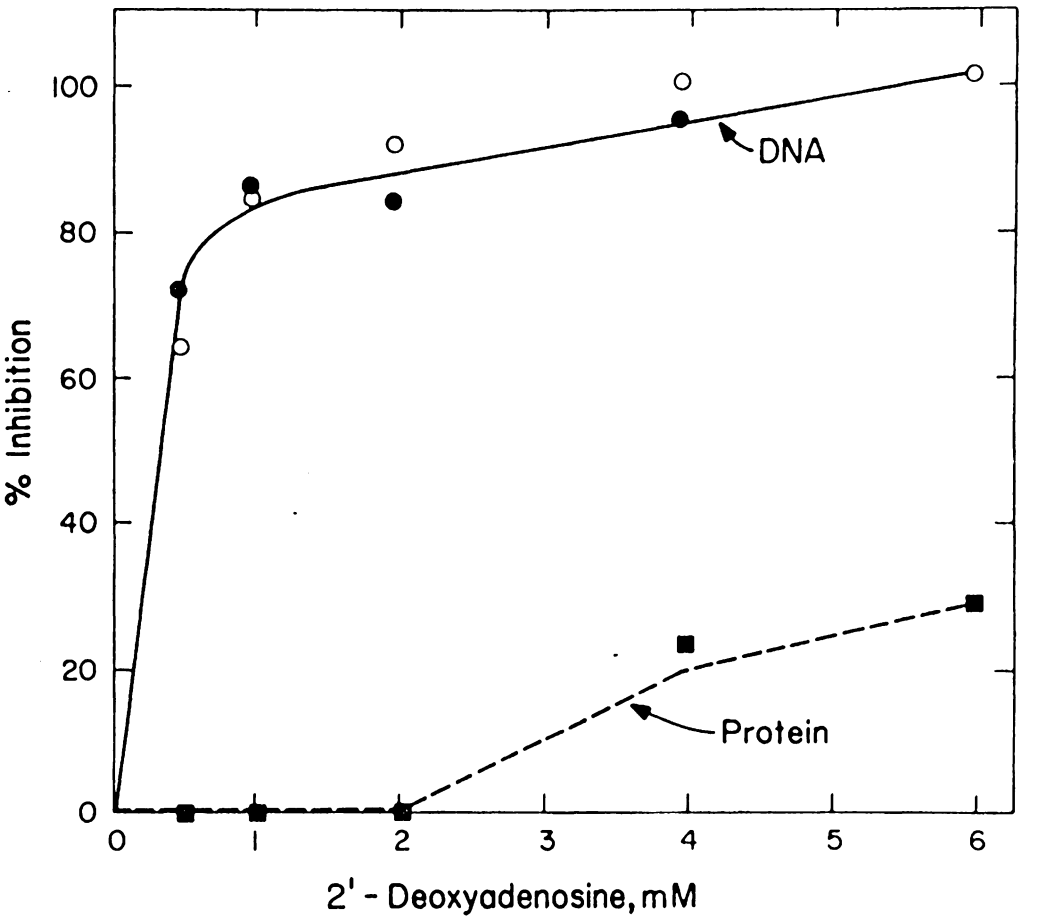
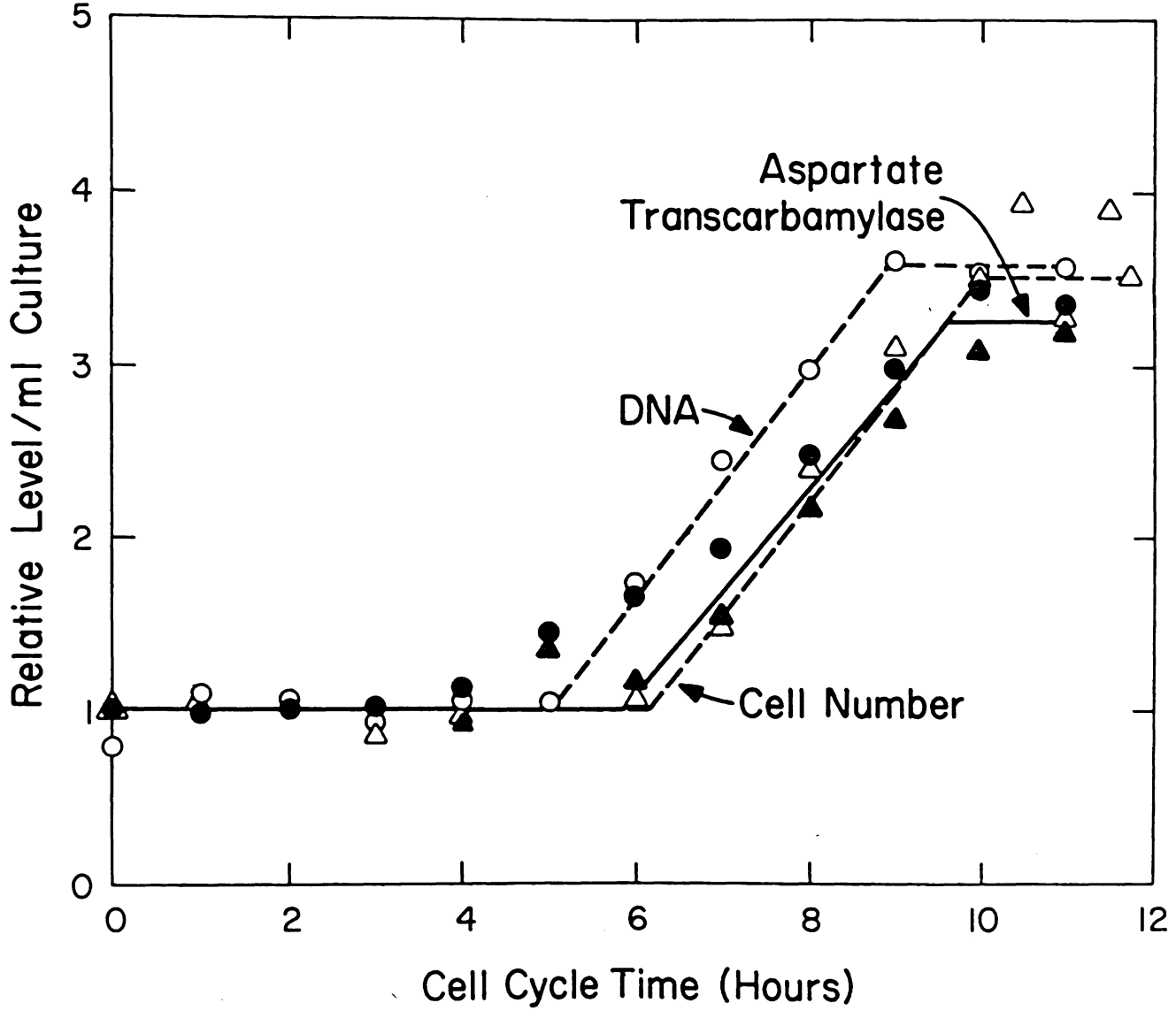

100
80
60
40
20
0
% Inhibition
DNA
Protein
0
1
2
3
4
5
6
2' - Deoxyadenosine, mM

Fig. 22. Patterns of DNA and aspartate transcarbamylase accumulation during the cell cycle of *Chlorella sorokiniana*. The enzyme was assayed in 0.2 M either Tris-HCl or glycylglycine buffer, pH 8.5, containing 1 mM EDTA and 0.8 mM uridine. Cells were frozen in 0.5 ml aliquots and washed in either Tris or glycylglycine buffer containing 2.0 mM uridine. The final concentration of uridine in the enzyme assays was 0.8 mM. The turbidity of the culture was held essentially constant by continuous dilution with fresh culture medium. Initial cell number: 1.53×10^{8} cells/ml. Initial enzyme concentration: 6.1×10^{-9} units/cell. DNA, O ; cell number, Δ ; enzyme assayed in Tris-HCl, ● ; enzyme assayed in glycylglycine, ▲ .


Relative Level/ml Culture
Cell Cycle Time (Hours)
Aspartate
Transcarbamylase
DNA
Cell Number
0
1
2
3
4
5
0
2
4
6
8
10
12

transcarbamylase as seen for certain other enzymes in *Chlorella* (40) the aspartate transcarbamylase levels in daughter cells from ammonium and nitrate cultures were compared. The levels were found to be the same, making it unlikely that ammonium induces isozymes.

In ammonium medium, the enzyme increased in a step pattern beginning during the S-phase, but the enzyme pattern was somewhat displaced in time from the pattern of DNA accumulation (Fig. 22). However, the change in nitrogen source did not make a major change in the pattern or timing of accumulation of the enzyme during the cell cycle. Prior to initiating the deoxyadenosine inhibition studies, it was decided to attempt to perturb culture conditions to determine if timing of enzyme accumulation and gene replication could be separated.

Using the same rationale employed earlier in the cell cycle experiments with nitrate cultured cells, the culture turbidity of the ammonium cultured cells was changed in an attempt to separate the timing of aspartate transcarbamylase and DNA accumulation during the cell cycle (Figs. 23 and 24). The alteration in DNA accumulation was different than that observed in nitrate cultured cells. Although DNA accumulated through most of the cell cycle, a change in rate of accumulation was still seen at the usual time of onset of the S-phase. Changing the culture turbidity in this experiment failed to separate the timing of aspartate transcarbamylase and DNA accumulation as observed earlier in nitrate cultured cells.

The accumulation of the enzyme ribulose-1,5-diphosphate carboxylase in nitrate cultured *Chlorella* (51) can be changed from a step to a

Fig. 23. Patterns of DNA and aspartate transcarbamylase accumulation during the cell cycle of *Chlorella sorokiniana* cultured in ammonium medium. The cells for enzyme assays were frozen in 0.5 ml aliquots and washed in 0.2 M glycylglycine buffer, pH 8.5, containing 1 mM EDTA. The culture was diluted continuously in such a way to cause the culture turbidity to decrease in the early part of the cell cycle and then increase toward the end. Initial cell number: 2.16×10^{8} cells/ml. Initial enzyme concentration: 7.3×10^{-9} units/cell. DNA, ● ; enzyme, ○ .

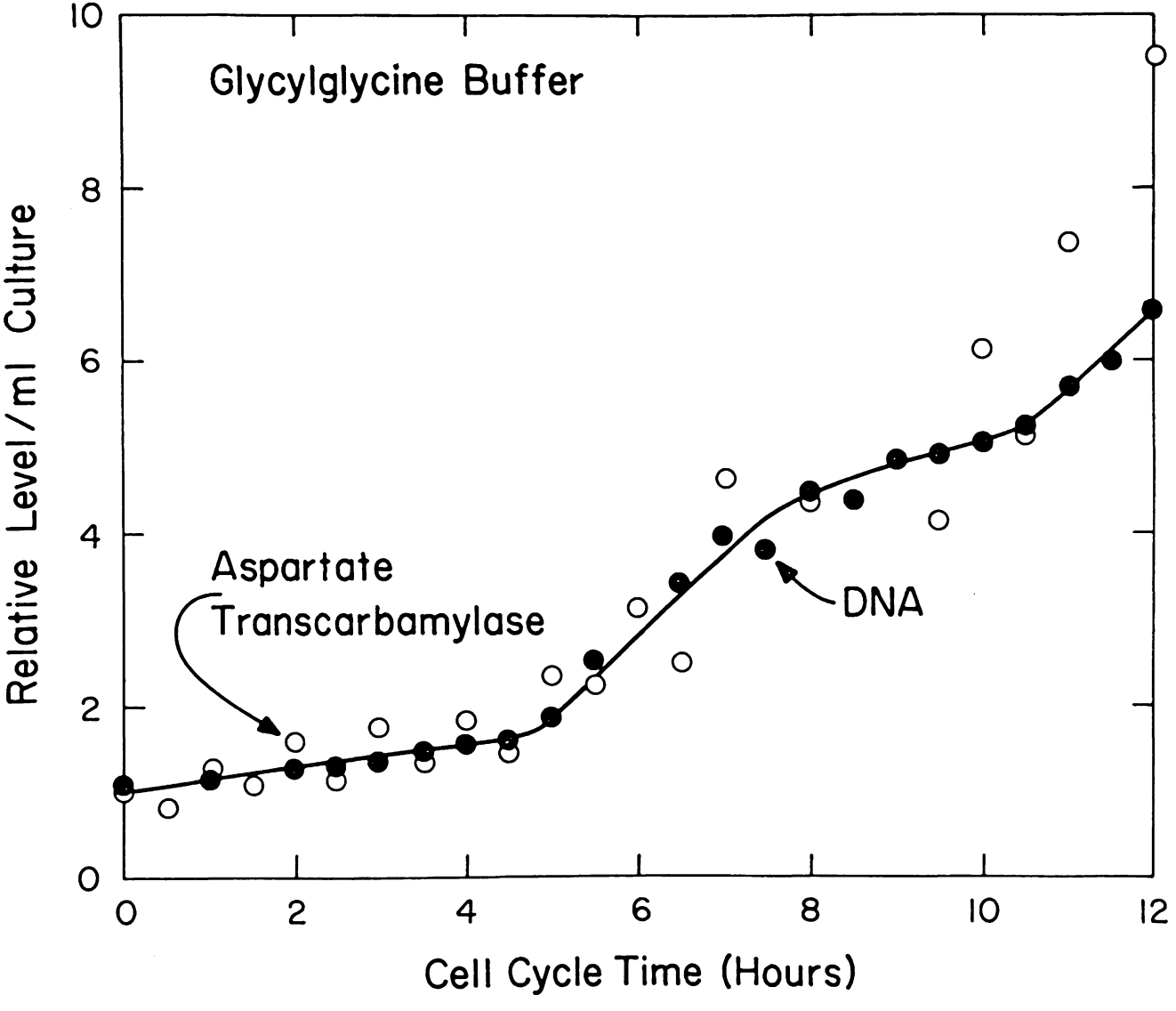
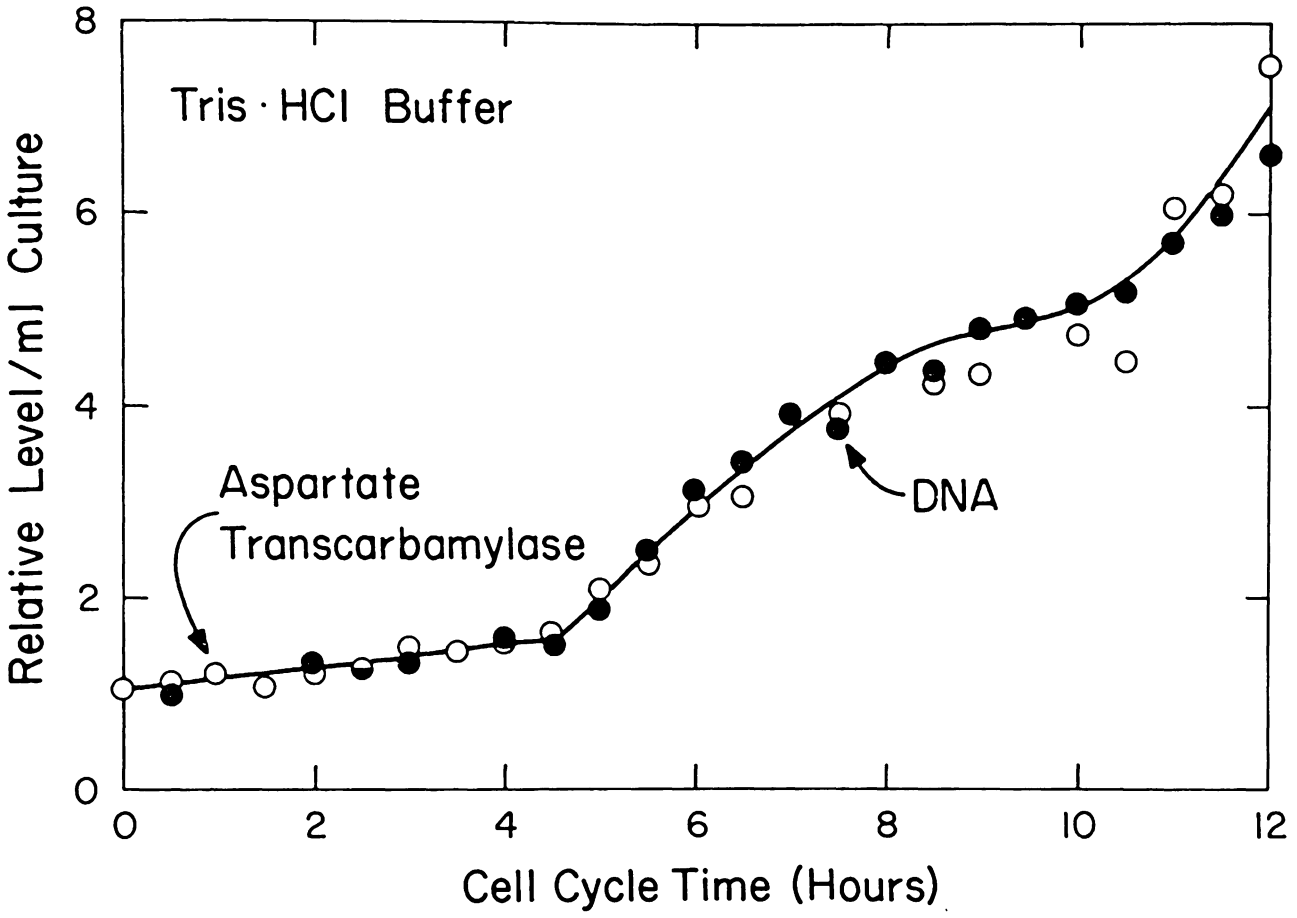

Glycylglycine Buffer
Aspartate
Transcarbamylase
DNA
Relative Level/ml Culture
Cell Cycle Time (Hours)
0
2
4
6
8
10
12

Fig. 24. Patterns of DNA and aspartate transcarbamylase accumulation during the cell cycle of *Chlorella sorokiniana* cultured in ammonium medium. For enzyme assays the cells were frozen in 0.5 ml aliquots and washed in 0.2 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 2 mM UMP. The final concentration of UMP in the enzyme assays was 0.8 mM. Other conditions are the same as described in Fig. 23. DNA, ● ; enzyme, ○ .


Tris · HCl Buffer
Aspartate
Transcarbamylase
DNA
Relative Level/ml Culture
Cell Cycle Time (Hours)
0
2
4
6
8
10
12

semi-continuous pattern by doubling the light intensity. In the case of this enzyme it is not the changing light intensity but the high or low intensity *per se* that determines the pattern of accumulation of the enzyme.

When aspartate transcarbamylase was assayed in either Tris-HCl plus 2 mM UMP or glycylglycine buffer, the enzyme and DNA patterns were coincident. Although in the present experiment, and the previous one, the enzyme patterns were the same in both buffers, the activity of aspartate transcarbamylase was highest in the Tris-HCl plus 2 mM UMP (or uridine) buffer. Therefore, the latter buffer was chosen for subsequent experiments.

Since the patterns of enzyme and DNA accumulation were coincident under those culture conditions tested in ammonium medium, the crucial experiment was to use 2'-deoxyadenosine to determine whether or not enzyme accumulation was obligately coupled to DNA replication.

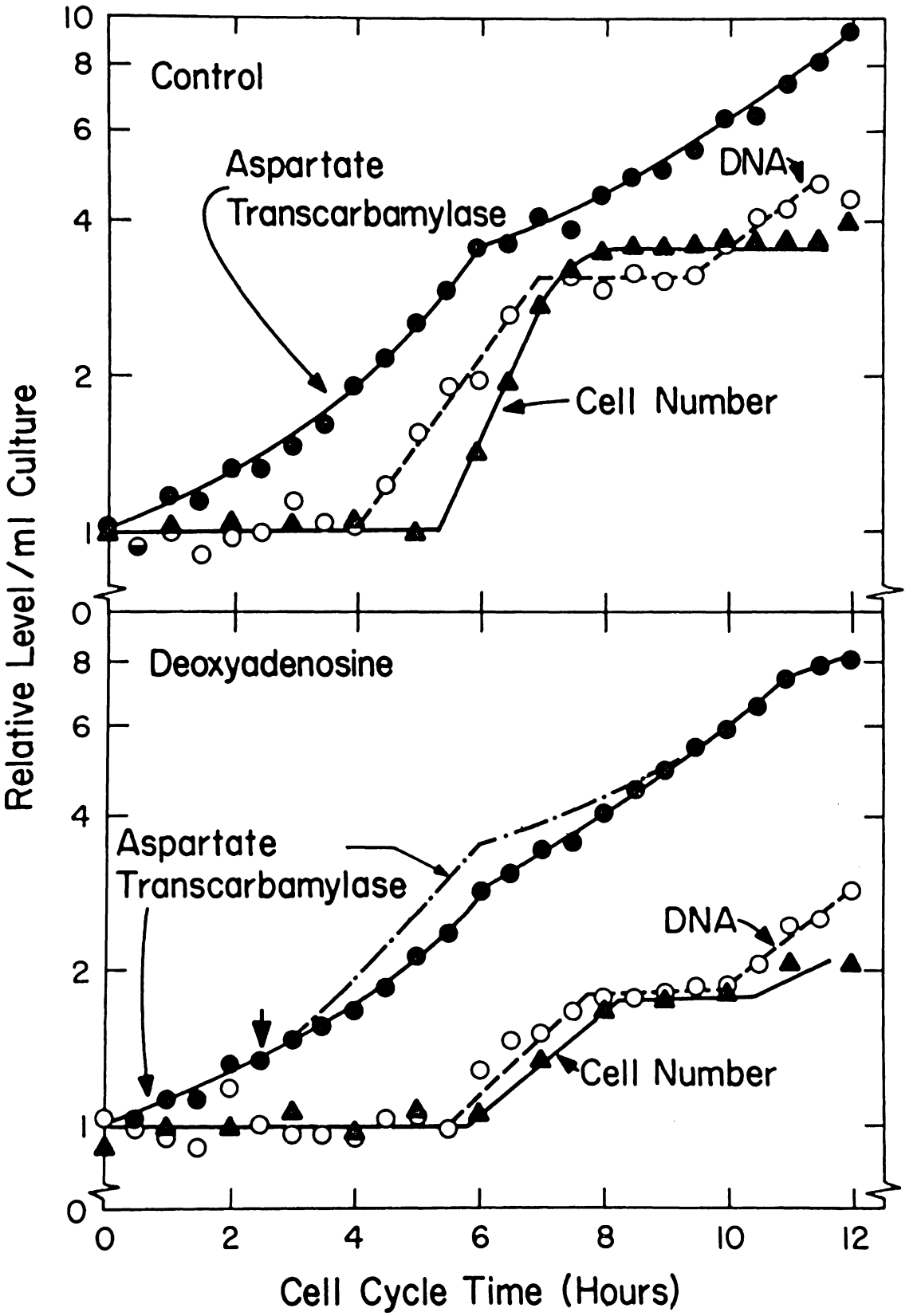
In the first cell cycle on ammonium medium in which the culture turbidity was held constant the overall growth rate of the cells for one cell cycle was approximately 13% and the fold increase in cell number was only 3.5. To obtain a greater difference between the DNA levels in inhibited and uninhibited cells, a decision was made to change the fold increase in cell number from 3.5 to 4.0. In nitrate cultured cells, this increase in division number is accomplished by increasing the effective light intensity per cell. Therefore, in DNA inhibition experiments designed for ammonium medium the effective light intensity was increased and the culture turbidity was held constant during the

cell cycle.

In the control culture aspartate transcarbamylase unexpectedly accumulated in a continuous rather than a periodic manner (Fig. 25). However, DNA accumulated in its usual step pattern. These results alone demonstrate that DNA and enzyme accumulation are not obligately coupled. This was the first instance in which accumulation of the two was uncoupled in ammonium medium.

When 2'-deoxyadenosine was added just prior to DNA replication, DNA synthesis was inhibited by 74%. The difference in accumulation of aspartate transcarbamylase between the control and inhibited cultures was minor. Thus, the inhibition experiment and changing light experiment provide two lines of evidence to indicate that aspartate transcarbamylase and DNA synthesis are not obligately coupled in ammonium cultured cells.

Fig. 25. Effect of inhibition of DNA synthesis with 2'-deoxyadenosine on the pattern of aspartate transcarbamylase accumulation during the cell cycle of *Chlorella sorokiniana* cultured in ammonium medium. Buffer for freezing and washing cells: 0.2 M Tris-HCl, pH 8.5, containing 1 mM EDTA and 2 mM UMP. The final concentration of UMP in the enzyme assays was 0.8 mM. Parallel cultures were continuously diluted to maintain a constant culture turbidity. At 2.5 hours in the cell cycle, one culture was diluted with medium containing sufficient 2'-deoxyadenosine to bring the final concentration to 2 mM. Subsequent dilutions were made with medium containing 2 mM 2'-deoxyadenosine. Aspartate transcarbamylase, ● ; DNA, ○ ; cell number, ▲ . Initial cell number: 2.30×10^{8} cells/ml. Initial enzyme concentration: 4.9×10^{-9} units/cell.


Control
Aspartate
Transcarbamylase
DNA
Cell Number
Deoxyadenosine
Aspartate
Transcarbamylase
DNA
Cell Number
Relative Level / ml Culture
Cell Cycle Time (Hours)
0
2
4
6
8
10
12

SUMMARY

Aspartate transcarbamylase (carbamylphosphate:L-aspartate carbamyltransferase, E. C. 2.1.3.2) from *Chlorella sorokiniana* was stabilized *in vitro* by uridine and UMP with 0.04 mM uridine and 0.05 mM UMP giving half maximal stability. Positive cooperative effects on stabilization were observed for UMP but not uridine. The enzyme was stabilized at all temperatures between 2° and 50°, but in the absence of the nucleotide the enzyme was both cold and heat labile and had a temperature stability optimum of 32° for an incubation time of 90 min. The enzyme was more stable in glycylglycine buffer than in Tris-HCl buffer. The enzyme was inhibited by uridine and UMP, but concentrations of 1.6 mM uridine or 2.6 mM UMP were required for 50% inhibition. Sensitivity to inhibition was diminished by ammonium sulfate fractionation or multiple passages through a French pressure cell. The loss of sensitivity to inhibition may be due to breakdown of the enzyme into subunits or breakdown of a multienzyme complex of pyrimidine enzymes. An assay for activity of carbamyl-P synthetase (E. C. 2.7.2.5), a suspected component of the multienzyme complex, was developed for *Chlorella*. Neither aspartate transcarbamylase nor carbamyl-P synthetase was sedimented by centrifugation at 100,000 x g for 5.5 hours. Either a multi-enzyme complex does not exist, the complex is of low molecular weight, or the complex was destroyed during preparation of the cellular material.

Synchronous *Chlorella* cells were used to study the regulation of aspartate transcarbamylase during the cell cycle. Under certain

culture conditions (constant light intensity per cell and nitrate as the nitrogen source) the enzyme accumulated in a step pattern with the step increase in enzyme accumulation occurring during DNA replication. This pattern is consistent with two hypotheses: The structural gene may be transcribed only during the S-phase, or the structural gene may be transcribed continuously if the enzyme is unstable and either under a constant level of repression or free from repression. In the second case, if the enzyme were synthesized and broken down at the same rate, the enzyme would accumulate only when the gene dosage increased as a result of DNA replication. When culture conditions were altered in such a way as to cause the light intensity per cell to oscillate during the cell cycle, accumulation of the enzyme began before the onset of DNA replication. Therefore, the structural gene for aspartate transcarbamylase is not expressed constitutively, and enzyme accumulation is not restricted to the S-phase as predicted by the first hypothesis. In another experiment, the nitrogen source was changed from nitrate to ammonium, and the effective light intensity was increased but held constant during the cell cycle. The enzyme accumulated in a continuous pattern and DNA in a step pattern, again demonstrating that DNA and enzyme accumulation are not obligately coupled. When DNA synthesis was inhibited by 74%, by the addition of 2'-deoxyadenosine, there was no corresponding effect on enzyme accumulation.

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CELL CYCLE CONTROL OF ASPARTATE TRANSCARBAMYLASE LEVELS IN CHLORELLA SOROKINIANA

by

Judy Harms Wilkins

(ABSTRACT)

Aspartate transcarbamylase (carbamylphosphate:L-aspartate carbamyltransferase, E. C. 2.1.3.2) from *Chlorella sorokiniana* was stabilized *in vitro* by uridine and UMP with 0.04 mM uridine and 0.05 mM UMP giving half maximal stability. Positive cooperative effects on stabilization were observed for UMP but not uridine. The enzyme was stabilized at all temperatures between 2° and 50°, but in the absence of the nucleotide the enzyme was both cold and heat labile and had a temperature stability optimum of 32° for an incubation time of 90 min. The enzyme was more stable in glycylglycine buffer than in Tris-HCl buffer. The enzyme was inhibited by uridine and UMP, but concentrations of 1.6 mM uridine or 2.6 mM UMP were required for 50% inhibition. Sensitivity to inhibition was diminished by ammonium sulfate fractionation or multiple passages through a French pressure cell. The loss of sensitivity to inhibition may be due to breakdown of the enzyme into subunits or breakdown of a multienzyme complex of pyrimidine enzymes. An assay for activity of carbamyl-P synthetase (E. C. 2.7.2.5), a suspected component of the multienzyme complex, was developed for *Chlorella*. Neither aspartate transcarbamylase nor carbamyl-P synthetase

was sedimented by centrifugation at 100,000 x g for 5.5 hours. Either a multi-enzyme complex does not exist, the complex is of low molecular weight, or the complex was destroyed during preparation of the cellular material.

Synchronous *Chlorella* cells were used to study the regulation of aspartate transcarbamylase during the cell cycle. Under certain culture conditions (constant light intensity per cell and nitrate as the nitrogen source) the enzyme accumulated in a step pattern with the step increase in enzyme accumulation occurring during DNA replication. This pattern is consistent with two hypotheses: The structural gene is transcribed only during the S-phase, or the structural gene may be transcribed continuously if the enzyme is unstable and either under a constant level of repression or free from repression. In the second case, if the enzyme were synthesized and broken down at the same rate, the enzyme would accumulate only when the gene dosage increased as a result of DNA replication. When culture conditions were altered in such a way as to cause the light intensity per cell to oscillate during the cell cycle, accumulation of the enzyme began before the onset of DNA replication. Therefore, the structural gene for aspartate transcarbamylase is not expressed constitutively, and enzyme accumulation is not restricted to the S-phase as predicted by the first hypothesis. In another experiment, the nitrogen source was changed from nitrate to ammonium, and the effective light intensity was increased but held constant during the cell cycle. The enzyme accumulated in a continuous pattern and DNA in a step pattern, again demonstrating that DNA and

enzyme accumulation are not obligately coupled. When DNA synthesis was inhibited by 74%, by the addition of 2'-deoxyadenosine, there was no corresponding effect on enzyme accumulation.