CONTROL OF ASPARTATE TRANSCARBAMYLASE ACTIVITY

BY NORIT-A ADSORBABLE COMPOUNDS

DURING SYNCHRONOUS GROWTH OF

CHLORELLA PYRENOIDOSA

by

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PREFACE

The author has elected to place in this thesis the latter segments of his graduate research, which have not been previously published.

The research carried out during the initial two and one-half years of his graduate career, under the advisement of Dr. Kendall W. King, is represented in two full-length publications:

1) Site of Hydrolysis of Cellulodextrins and Reduced Cellulodextrins by Purified Cellulase Components, Biochim. Biophys. Acta, 81 (1964) 122.

2) Mode of Action of a Cellulase Component from C. gilvus, Biochemistry, 2 (1963) 1106.

In addition to these two papers the author is proud to have co-authored with Dr. King a symposium bibliography from the A.C.S. meetings held in Washington, D.C., in March of 1962, which now appears in Advances in Enzymic Hydrolysis of Cellulose and Related Materials, Pergamon Press, (1963).

The research results appearing in this thesis, representing work conducted under the advisement of Dr. R. R. Schmidt during the last year and one-half of the

author's graduate training, have also given rise to two proposed publications: a preliminary note, Control of Aspartate Transcarbamylase Activity During Synchronous Growth of _Chlorella pyrenoidosa_, _Biochim. Biophys. Acta_, in the press; and one full-length paper, bearing the title of the thesis, submitted for publication to _Biochim. Biophys. Acta_.

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INTRODUCTION

Synchronized mass cultures of microorganisms have now become a widely accepted means of studying the metabolic changes that occur throughout the life history of developing cells (see reviews by Burns[1] and Scherbaum[2]). It is now clear that many of the biochemical changes which take place in the developing cell occur as periodic functions of the age of the cell (Baker and Schmidt[3], Herrmann and Schmidt[4,5]). From the periodism in the intracellular levels of certain metabolites, one can predict that there must be periodism in the _active_ levels of the enzymes controlling the synthesis and/or breakdown of these metabolites. At least three control parameters must be dealt with in considering the _active_ level of an enzyme _in vivo_: control of its synthesis at the gene level, control of its activity by small molecule metabolites once it has been synthesized, and control of its activity imposed by its structural orientation within the cell and/or its organelles (which will influence permeability of cofactors, substrates, etc.) or any

combinations of the three parameters. Excellent dis-

cussions of control phenomena have been made by Pitot

and Heidelberger[6], Changeux[7], and Jacob and Monod[8].

DNA and RNA (% of total cellular-P or cellular dry

weight) have been shown to exhibit periodism in syn-

chronous cultures of C. pyrenoidosa[4,5]. According to

the rationale developed above, one would therefore expect

periodism in the active levels of one or more of the

enzymes controlling the synthesis or breakdown of these

macrometabolites. Aspartate Transcarbamylase, the first

enzyme in pyrimidine (nucleic acid precursors) bio-

synthesis, has been widely studied as an enzyme subject

to control by a phenomenon known as "product-inhibition"

(Bresnick[9], Yates and Pardee[10], and Neumann and Jones[11]).

Recently the activity of this enzyme has been shown to

vary directly with an increase in DNA synthesis (Consigli

and Ginsberg[12]). In the present study, Aspartate Trans-

carbamylase activity when followed through two consecutive

synchronous growth cycles of C. pyrenoidosa, proves to

be an interesting model enzyme for studying control

parameters in the developing cell.

MATERIALS AND METHODS

Enzyme assay

The Aspartate Transcarbamylase assay used in these studies was almost identical to that described by Bresnick[9]. The incubation time was shortened to 15 min and 1 mM GSH and EDTA were included in the Tris-HCL buffer. After neutralization of the deproteinized incubation mixture with 5 N KOH, the cold neutral extract was diluted to 3.0 ml using deionized H_2O. After thorough mixing (and recentrifugation to remove the potassium salt and coagulated protein), 2.0 ml of the neutralized extract were put on a 1 x 6 cm (internal diameter x length) Dowex-50-H^+ (X 8, 100-200 mesh) column and 10 ml of H_2O was found to be sufficient to elute off all of the radioactive product. An aliquot of 3.0 ml was taken from the 10 ml column eluate fraction, and its radioactivity was measured using a Model 500 B Tri-Carb liquid scintillation spectrometer. The radioactivity in this column eluate moved as a single radioactive peak with an R_f value identical to that of authentic carbamyl aspartic acid on

paper chromatograms. Controls without carbamyl phosphate as well as controls without enzyme were routinely performed and appropriate corrections were made.

Enzyme characteristics

To the authors' knowledge, Aspartate Transcarbamylase has been studied in a photosynthetic organism only by Neumann and Jones[14]. The characterization of Aspartate Transcarbamylase in C. pyrenoidosa was carried out using enzyme obtained from dense cultures of asynchronous cells which had grown for approximately three days in continuous light without dilution.

To avoid duplication of methods contained in the next section, the preparation of the enzyme will be described only briefly in this section. Cells were harvested in the cold, washed with buffer, sonicated for 30 min, and the sonicate centrifuged at 100,000 x g for 2 h. The 100,000 x g supernatant fraction consistently contained from 83 to 87% of the Aspartate Transcarbamylase activity found in whole sonicates and was routinely used as representing the Aspartate Transcarbamylase activity of the cell. The pH optimum, approximately 9.2 for Aspartate Transcar-bamylase in C. pyrenoidosa, can be seen from Fig. 1A.

Fig. 1A. pH optimum of Aspartate Transcarbamylase in

C. pyrenoidosa.

Fig. 1B. [Enzyme] vs. Product curve for Aspartate

Transcarbamylase in C. pyrenoidosa.
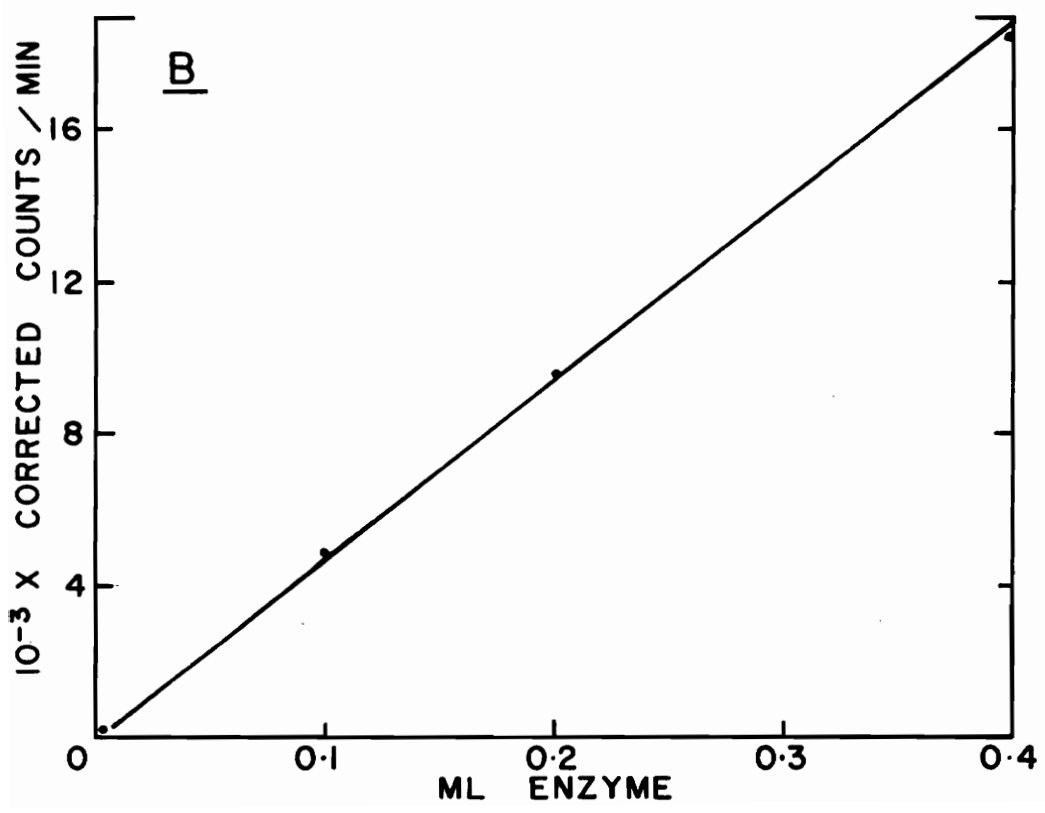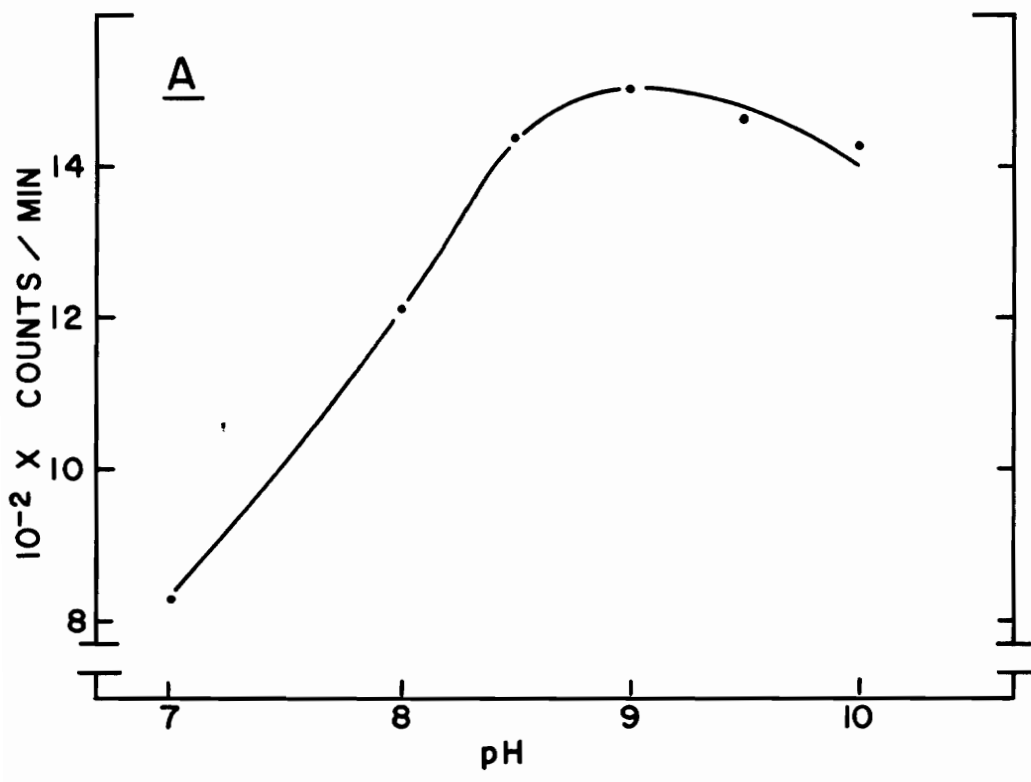
By testing at 5° intervals the temperature optimum for the enzyme was found to be between 35° and 40°.

The physical stability of Aspartate Transcarbamylase in C. pyrenoidosa was similar to that observed by Neumann and Jones for lettuce seedlings[11]. Five repeated freezings and thawings of the 100,000 x g supernatant, each thawing on a successive day, resulted in less than a 5% loss of total activity. Greater than 95% of the initial activity could be accounted for by assaying the 100,000 x g super-natants that had been stored for one month at -15°. Either dialysis or Sephadex gel-filtration (G-25-med) resulted in total loss of Aspartate Transcarbamylase activity in the crude 100,000 x g preparations; however, no evidence of cofactor or activator requirements could be demonstrated for this enzyme.

Cell harvest procedure and enzyme preparation during synchronous growth studies

Cells were harvested at one and two h intervals during two successive synchronous growth cycles. Cell concentration per ml of culture (using a Levy-Hausser hemacytometer) and total cellular-P per ml of culture (using the method of Fiske and SubbaRow[15]) were determined

at each sampling period using suitable aliquots of the
culture. The cell pellets (approx. 1 ml packed-cell-volume
at each sampling period) were washed three times with 10
mM Tris-HCL buffer (pH 7.0), and the cells were stored at
-15° in 15 ml of 0.2M Tris-HCL buffer (pH 9.2) containing
1 mM GSH and EDTA. Total cellular-P per 15 ml cell sus-
pension was determined (along with total cellular-P per
ml of growing culture) and used to relate units of
Aspartate Transcarbamylase activity in the 15 ml cell
suspension to the units of enzyme per ml of growing
culture.

The frozen cell suspensions, which were demonstrated
to contain the same Aspartate Transcarbamylase activity
as fresh cells, were thawed in the cold and sonicated in
a Raytheon 10 KC sonic oscillator for 30 min using maximum
amperage. Cell rupture was greater than 99.9% at all
periods of synchronous growth as determined by cell counts
before and after the sonication treatment. The sonicates
were centrifuged for 2 h at 100,000 x g in a Spinco
Model L ultracentrifuge. The resulting supernatant was
used for the Aspartate Transcarbamylase studies in this
paper.

A typical plot of enzyme concentration vs. product
(counts/min) is shown in Fig. 1B. At each sampling period
during the two synchronous growth cycles, 0.2 ml of crude
enzyme (100,000 x g supernatant) were found to be well
within the range where enzyme concentration is propor-
tional to product accumulation.

Heat-treated enzyme supernatant preparations, used
in studies for Tables I, II, and III were prepared by
heating an aliquot of the 100,000 x g supernatant from
various sampling periods for 2 min at 100°, cooling in
ice, and centrifuging the coagulated protein at room
temperature. These supernatants lost all Aspartate
Transcarbamylase activity. Fractions possessing in-
hibitory properties retained 100% of their inhibitory
activity during 4 min exposures to 100°.

RESULTS AND DISCUSSION

Aspartate Transcarbamylase activity during synchronous
growth

Although cellular dry weight[13], cellular volume[16],
total cellular-S[17], and total cellular-P (Fig. 2) were
observed to be exponential functions, each parameter
having a constant rate of increase, Aspartate

Fig. 2.   Cell number per ml of culture (o---o) and total

cellular phosphorous per ml of culture (Δ---Δ) during

synchoronous growth of C. pyrenoidosa.
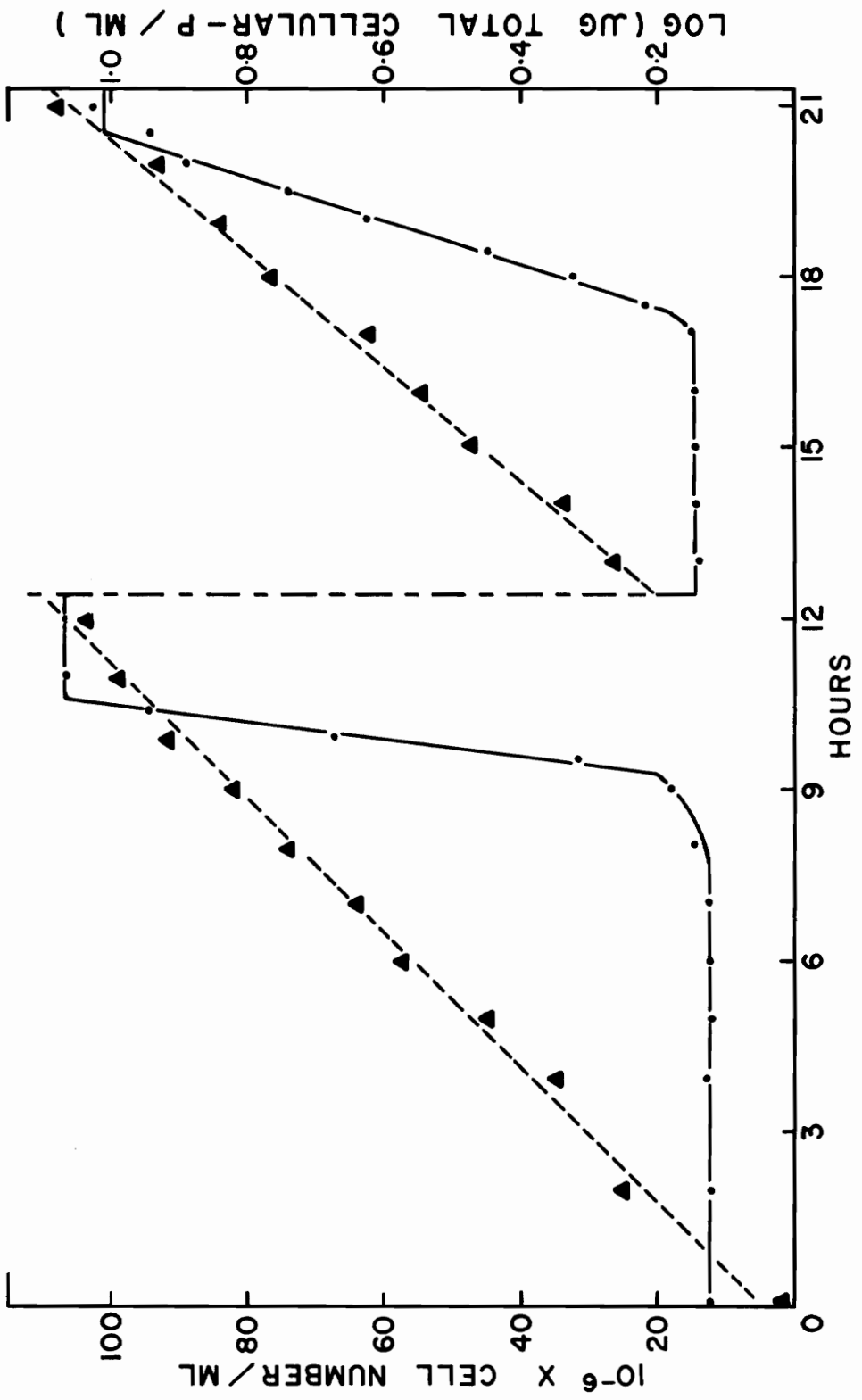
Transcarbamylase activity increased at alternating
exponential rates during synchronous growth of C.
pyrenoidosa (Fig. 3). The trends in Aspartate Trans-
carbamylase activity observed during the first synchronous
growth cycle were recurrent in the second growth cycle,
without repeated exposure of the cells to the synchroni-
zation treatment, suggesting[18] that these trends are
associated with the normal development of the cells.
Therefore, to facilitate discussion, only the trends
observed in the first growth cycle will be considered
in detail.

The periodism in Aspartate Transcarbamylase activity
(Fig. 3) suggests some interesting facets of regulation
of enzyme activity when correlated with concurrent
metabolic shifts observed in this organism and the
previously reported[9-11] end-product inhibition of the
enzyme itself. Since Aspartate Transcarbamylase
synthesizes both RNA and DNA precursors, one might predict
that the activity of this enzyme would increase during
(or before) periods of accelerated RNA and DNA synthesis.
The rate of increase of Aspartate Transcarbamylase
activity during early cellular development changes

Fig. 3.   Log µ units of Aspartate Transcarbamylase per ml of culture (Δ---Δ) and acid-soluble, Norit-A adsorbable phosphorous[3] (% of total cellular-P) (---) in C. pyrenoidosa.

% OF TOTAL CELLULAR-P

LOG (μUNITS ATC-ase / ML)

HOURS
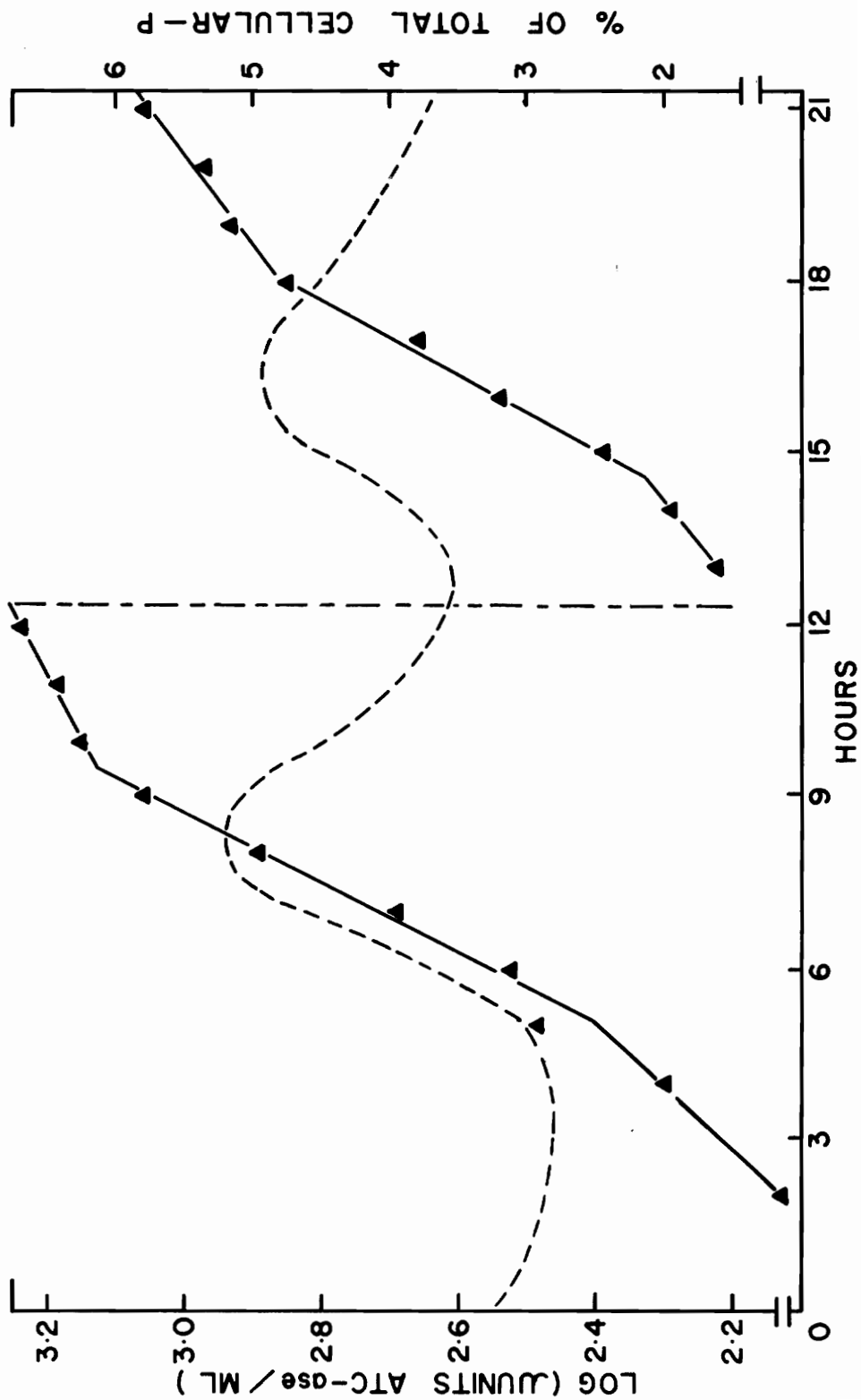
(increases) at the 5th h. This period of increased Aspartate Transcarbamylase activity between the 5th through 9th h of cellular growth (Fig. 3) does indeed correspond to a period of increased DNA and RNA accumulation in this organism as reported by Herrmann[4], and Herrmann and Schmidt[5]. Furthermore, there is an almost concurrent (5th h) increase in the rate of accumulation of acid-soluble, Norit-A adsorbable-P (containing the nucleotide-P) as seen from the data of Baker and Schmidt[3] replotted in Fig. 3.

It should be noted that an increase in the rate of accumulation of a component does not necessarily represent an increase in the rate of synthesis of this component. A constant rate of synthesis and a decreased rate of breakdown would result in the same net effect on the accumulation of a component as an increase in its rate of synthesis. Accumulation of a component is therefore best thought of as the condition where the rate of synthesis exceeds the rate of breakdown. Thus, the apparent relationships drawn between Aspartate Transcarbamylase activity, nucleotide-P accumulation, and DNA and RNA accumulation are only suggested by

these data, and must await turnover studies of these components before cause and effect relationships can be inferred.

This period (5th through 9th h) of accelerated Aspartate Transcarbamylase activity is interpreted as representing a period of accelerated synthesis of this enzyme. This conclusion is partially supported by the fact that neither inhibition nor activation effects can be demonstrated during this period (Table I). If small molecule (heat stable) inhibitor(s) were present at either the 5th, 6th, or 7th hours, one would expect a decrease in enzyme activity by increasing the concentration of these factor(s) (by further addition of heat-treated supernatants from these points). Conversely, if (heat stable) activator(s) were present in increasing concentration, one would expect a progressive increase in activation by addition of the heat-treated supernatants during this same time interval. The possibility that an activator is synthesized in saturating amounts is not ruled out; however, in such a case, it would not be controlling the rate of increase in Aspartate Transcarbamylase activity.

TABLE I

INHIBITION OF ASPARTATE TRANSCARBAMYLASE ACTIVITY
AT CERTAIN PERIODS DURING CELL DEVELOPMENT

Untreated Crude Enzyme*	Heat-Treated** Crude Enzyme		% Inhibition[+]
Hour of Growth	Hour of Growth	Amount Added to Untreated Crude Enzyme ml	
5	5	0.2	0
		0.4	0
6	6	0.2	0
		0.4	<1
7	7	0.2	0
		0.4	<1
9	9	0.2	5.4
		0.4	8.4
10	10	0.2	6.1
		0.4	10.8
12	12	0.2	5.5
		0.4	10.2
16	16	0.4	<1
20	20	0.4	7.9
6	12	0.4	13.5
16	20	0.4	10.7
20	16	0.4	0

*For each study 0.2 ml of crude enzyme (2 h, 100,000 x g supernatant) was used.

**2.0 ml of crude enzyme was exposed to 100° for 2 min, cooled in ice, centrifuged, and the specified volumes of the supernatant were used to test for inhibition. The supernatants of the heat-treated crude enzyme were in all cases shown to have an activity not greater than that of controls having no enzyme.

[+]Calculated as follows:

$$100 - (\frac{\text{activity with addition}}{\text{activity without addition}} \times 100)$$

End product inhibition of Aspartate Transcarbamylase activity

At the 9th h of cellular growth there is a marked depression in the rate of increase of Aspartate Transcarbamylase activity (Fig. 3). This depression suggests either a depressed rate of enzyme synthesis, the inception of an inhibition phenomenon, removal of activator(s), or any combination thereof. The peak in nucleotide-P (acid-soluble, Norit-A adsorbable-P) just prior to the depression in Aspartate Transcarbamylase activity (Fig. 3) suggested the possibility of product inhibition, which has been implicated by several workers[9-11] as a factor regulating the activity of this enzyme. To test this possibility the studies reported in Tables I, II, and III were performed. The data presented in Table I clearly demonstrate the functioning of inhibitor(s) during the periods of depressed activity (9th, 10th, and 12th h). This observation is confirmed in the second growth cycle (16th vs. 20th h). Evidence that the inhibition is unique to certain periods of cellular development is obtained from the observation that heat-treated supernatant from the 12th h inhibits enzyme activity

at the 6th h. This observation is also confirmed in the
second growth cycle (16th vs. 20th h). Furthermore, it
should be noted that addition of heat-treated supernatant
from the 16th h had no effect upon 20th h activity.

The data in Table II demonstrate product inhibition
in C. pyrenoidosa and are in good agreement with the data
of Neumann and Jones[11] in lettuce seedlings. It should
be noted that the principal inhibitors of the enzyme from
these two plant sources are pyrimidine nucleoside
monophosphates which would be stable to the heat-treatment
used in these studies.

If the inhibitory compound(s) present during the
periods of depressed Aspartate Transcarbamylase activity
are indeed nucleotide end-products, it would be expected
that they could be removed from the heat-treated super-
natants by Norit-A adsorption. The data presented in
Table III demonstrate that the inhibition observed in the
studies presented in Table I can be removed by addition
of small quantities of Norit-A. The limited quantities
of crude enzyme available in these studies would not allow
a more definitive identification of the inhibitors present
during periods of depressed Aspartate Transcarbamylase

TABLE II

END-PRODUCT INHIBITION OF ASPARTATE

TRANSCARBAMYLASE

Compound	Final Concen-tration (M)	Percentage* of Inhibition
UMP	.01	88.9
UDP	.01	26.0
UTP	.01	10.9
CMP	.01	22.0
CTP	.01	12.5
GMP	.01	4.8
TMP	.01	11.7
AMP	.01	7.4
Pi	.01	1.9

In these experiments 0.2 ml of enzyme was assayed in the presence of the added compound as described under MATERIALS AND METHODS.

*Calculated as in Table I.

TABLE III

REMOVAL OF INHIBITION OF ASPARTATE TRANSCARBAMYLASE

ACTIVITY BY NORIT-A

Untreated Crude Enzyme*	Heat-Treated* Crude Enzyme	Norit-A** Addition	% Inhibition
Hour of Growth	Hour of Growth		
20	20	−	8.1
20	20	+	0
16	16	−	0
16	16	+	0
16	20	−	10.6
16	20	+	0

*As in Table I.
**0.2 ml of a Norit-A suspension (10 mg ml) was added to 1.0 ml of the heat-treated enzyme supernatants (see methods) and after centrifugation 0.4 ml of clear super-natant used to test for inhibition. Studies without Norit-A addition were performed using 0.4 ml of heat-treated crude enzyme as in Table I.

activity; however, such studies will be undertaken in future work.

Though the inhibition phenomenon alone (by extrapolation) apparently cannot explain the magnitude of the depressed rate of Aspartate Transcarbamylase activity during this period (9th through 12th h), it should be borne in mind that the concentrations of both enzyme and inhibitor are greatly altered during the preparation of the enzyme (dilution to 15.0 ml, disruption of intracellular organelles etc.), and the effect of such treatment cannot be estimated in this study. Therefore, concurrent with the control of Aspartate Transcarbamylase activity by Norit-A adsorbable compound(s) there might also exist a depressed rate of enzyme synthesis during this period (9th through 12th or 17th through 20th h). Since no stimulation of activity was observed at the 20th h by addition of supernatant from the 16th h, coupled with the observation that no activation phenomena could be demonstrated at any point by further addition of their respective heat-treated supernatants, the removal of an activator seems least likely as a control factor in these studies.

The apparent lag in inhibition (1 h) between the peak in the nucleotide-P pool (8th h) and depression of Aspartate Transcarbamylase activity (9th h) is puzzling. Since the nucleotide-P pool represents both purine and pyrimidine nucleotides it is quite possible that pyrimidine nucleotides (the principal inhibitors of Aspartate Transcarbamylase) continue to increase for a sufficient time to induce product inhibition of the enzyme even though the total nucleotide-P pool is decreasing. Supporting this conclusion in part is the observation by Curnutt and Schmidt[19] with this organism that both ATP and ADP increase at a constant exponential rate throughout the life cycle and are thus independent of the fluctuations in the acid-soluble, Norit-A adsorbable-P pool plotted in Fig. 3.

SUMMARY

Aspartate Transcarbamylase (Carbamoyl-Phosphate: L-Aspartate Carbamoyltransferase, EC 2.1.3.2), the first enzyme in pyrimidine biosynthesis, was located in the soluble supernatant of a 100,000 x g preparation of sonicated Chlorella pyrenoidosa cells. The pH optimum, temperature optimum and stability characteristics of

the enzyme from this organism are reported.

Aspartate Transcarbamylase activity when measured during two consecutive synchronous growth cycles in C. pyrenoidosa under continuous illumination was found to increase at alternating exponential rates in each growth cycle. When the increase in Aspartate Transcarbamylase activity was expressed in its logarithmic form, a plot with linear segments was obtained, each segment having a different slope.

Studies designed to elucidate the causes of these shifts in Aspartate Transcarbamylase activity are reported. The data gathered in these studies indicated that Aspartate Transcarbamylase activity is at least partly under the control of Norit-A adsorable compound(s) during cellular development. Inhibition studies by a variety of nucleoside mono-, di-, and triphosphates, as well as fluctuations in intracellular nucleotide levels (represented by the acid-soluble, Norit-A adsorbable-P) during cellular development, indicate that the Norit-A adsorbable compounds regulating Aspartate Transcarbamylase activity in this organism are probably pyrimidine

nucleotides.

Certain relationships between RNA and DNA synthesis, nucleotide levels, and shifts in Aspartate Transcarbamylase activity during cellular development in this organism are discussed.

ACKNOWLEDGEMENTS

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VITA

Francis Eugene Cole was born May 25, 1937, in Lakeland, Florida. After living in several cities in southern Florida he moved to Key West, Fla., in 1951. He graduated from Key West High School in 1955 and after one year at University of Florida, enrolled in Oglethorpe University in Atlanta, Georgia. In June of 1960 he was awarded a Bachelor of Arts degree majoring in Biology and Chemistry. In September of 1960 he entered the Department of Biochemistry and Nutrition at Virginia Polytechnic Institute working toward a Doctor of Philosophy degree in Biochemistry with a minor in Chemistry and Microbiology.

In 1956 he was married to the former Elizabeth Anne Williams of Dania, Florida. A son, Francis, Jr., was born in 1957.

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<u>ABSTRACT</u>

The factors which regulate enzyme activity in the
growing cell have been the subject of active research
during the past decade and are generally considered under
the heading of "metabolic control". At least three control
parameters must be considered as regulating the <u>active</u>
level of a given enzyme: control of enzyme synthesis at
the gene level, control of enzyme activity by small
molecule metabolites once the enzyme has been synthesized,
and control of enzyme activity imposed by its structural
orientation within the cell and/or its organelles (which
would affect permeability of substrates, cofactors, etc.).
It can be hypothesized that periodism in the intracellular
level of a given metabolite must be accompanied by
periodism in the <u>active</u> level of one or more of the enzymes
responsible for the synthesis or breakdown of this
metabolite.

Synchronized cultures of microorganisms afford a
unique tool for studying periodic changes in the

intracellular levels of metabolites during cell growth.
The studies presented in this thesis were designed to
elucidate the relationships between the activity of
Aspartate Transcarbamylase and factors which affect and
are affected by the activity of this enzyme in syn-
chronized cultures of Chlorella pyrenoidosa.

Aspartate Transcarbamylase, the first enzyme in
pyrimidine biosynthesis, has been reported to be controlled
by a phenomenon known as "product-inhibition". This enzyme
was located in the soluble supernatant of a 100,000 x g
preparation of sonicated C. pyrenoidosa cells. The pH
optimum (9.2), temperature optimum (approx. 37°), and
stability characteristics of this enzyme from this organism
are reported.

Aspartate Transcarbamylase when measured during two
consecutive synchronous growth cycles in C. pyrenoidosa
under continuous illumination was found to increase at
alternating exponential rates in each growth cycle. When
the increase in Aspartate Transcarbamylase was expressed in
its logarithmic form, a plot with linear segments was
obtained, each segment having a different slope.

The rate of increase of Aspartate Transcarbamylase
activity during early daughter cell development changes

(increases) at the 5th h.   The 5th through 9th h of cell

growth, where Aspartate Transcarbamylase activity is

increasing at an accelerated rate, corresponds to the

premitotic and nuclear division stages in the cell.

Correlated with this period of increased Aspartate

Transcarbamylase activity (5th through 9th h) there is

an increase in the rate of accumulation of RNA and DNA

concurrent with increase in the rate of accumulation of

intermediates in the acid-soluble, Norit-A adsorbable-P

pool (which contains the nucleotide-P fraction).   At

approximately the 9th h of cell growth there is a decrease

in the rate of increase of Aspartate Transcarbamylase

activity.   Evidence is presented indicating that the

depression of activity during this period is at least

partly due to the presence of Norit-A adsorbable

compound(s) present in the cell (pyrimidine nucleotides, the

principal inhibitors of this enzyme, would be Norit-A

adsorbable).   At the 8th h of cell growth (1 h prior to

the period of depressed Aspartate Transcarbamylase

activity) the acid-soluble, Norit-A adsorbable-P pool

reaches a maximum value (as % of total cellular-P).

These data together with inhibition studies with a

variety of nucleoside mono-, di-, and triphosphates
suggest that pyrimidine nucleotides may be factors
regulating Aspartate Transcarbamylase activity during
cellular development.