STUDIES ON THE MECHANISM OF GENE-REGULATED SYNTHESIS OF

DIPHOSPHOPYRIDINE NUCLEOTIDE- AND TRIPHOSPHOPYRIDINE

NUCLEOTIDE-SPECIFIC GLUTAMATE DEHYDROGENASE ISOZYMES

DURING THE CELL CYCLE OF THE EUCARYOTE <u>CHLORELLA</u>

by

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONT.)

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Glutamate dehydrogenase [L-glutamate: NAD$^+$ oxidoreductase (deaminating), EC 1.4.1.2, L-glutamate: NAD(P)$^+$ oxidoreductase (deaminating), EC 1,4.1.3, or L-glutamate: NADP$^+$ oxidoreductase (deaminating), EC 1.4.1.4] via the catalysis of a reaction converting α-ketoglutarate and ammonium to glutamate, is the primary means of nitrogen incorporation into cellular components. The reverse reaction of this enzyme is a very important step in a series of reactions to utilize protein (via the amino acids) for cellular ATP synthesis. An understanding of the cellular control of glutamate dehydrogenase is extremely important in integrating the events which regulate cell metabolism. The control mechanism may involve the specificity of the enzyme for coenzymes, the compartmentation of the enzyme, the response of the enzyme to allosteric molecules, or the actual enzyme level in the cell.

The number of glutamate dehydrogenases, their coenzyme specificity, and their location within the cell varies with the organism studied. Escherichia coli and most other bacteria contain only a NADP$^+$-specific glutamate dehydrogenase (1). Members of the genus Bacillus are thought generally to lack glutamate dehydrogenase activity although Phibbs and Bernlohr (2) have recently reported a NADP$^+$-specific enzyme in both B. licheniformis and B. cereus, and Borris and Aronson (3), a NAD$^+$-independent enzyme in B. thuringiensis.

1

In the fungi, usually two glutamate dehydrogenases are observed. Neurospora (4) contain both a NAD^+- and a $NADP^+$-specific enzyme and a reciprocal relationship often exists between their cellular levels during synthesis and in the presence of catabolites (5). Catabolite repression of the NAD^+-specific enzyme by glucose has been demonstrated whereas the $NADP^+$-specific enzyme is regulated by catabolite induction. The former enzyme is induced by glutamate and ammonium while that of the latter is repressed. The genetic control mechanisms responsible for these observations are not understood. Although Saccharomyces also have the two enzymes, this reciprocal relationship does not seem to hold and the NAD^+-specific enzyme is repressed by ammonium instead of being induced as is the case in Neurospora (6).

Although glutamate dehydrogenase is generally assumed to be a mitochondrial enzyme, further study on the compartmentation of reported isozymes might provide clues to their metabolic roles and aid in formulating models for the differences which have evolved for catabolite repression or induction. The fungus, Fusarium is reported by Sanwal (7) to have a NAD^+-specific enzyme found exclusively in the cytoplasm while the $NADP^+$-specific enzyme is distributed between the cytoplasm and the mitochondria, with a higher concentration in the latter. Sanwal (7) proposes that the two isozymes could act as a transhydrogenase system in Fusarium to generate $NADP^+$ for the pentose phosphate cycle. This cycle is a relatively important pathway of glucose catabolism in this fungus under aerobic conditions.

In the algae, Kates and Jones (8) have reported a single

glutamate dehydrogenase with cross-specificity for coenzymes in Chlamydomonas. Following a partial purification of a similar enzyme in Chlorella pyrenoidosa Pringsheim (cultured in nitrate-containing medium), Kretovich (9) concluded that a single enzyme exists, reactive with NAD^+, and to a lesser extent, with $NADP^+$.

Higher plant and animal cells are generally assumed to contain a single enzyme, reactive with both coenzymes. However, Leech and Kirk (10), following a crude fractionation of chloroplasts and mito-chondria in the broad bean, Vicia faba L. find the chloroplast fraction and the mitochrondrial fraction to be enriched in a $NADP^+$-specific and a NAD^+-specific glutamate dehydrogenase activity, respectively.

A rather astounding report by Yue (11) indicated that several types of higher plant seedlings contain as many as seven mitochondrial isozymes of glutamate dehydrogenase; however, all isozymes detected following disc-gel electrophoresis had the same coenzyme specificity.

In addition to the metabolic regulatory mechanisms invoked by different cell types in regard to isozyme specificity and compart-mentation, the regulatory control exerted by various modulator or allosteric effectors, can also play a role in regulation of enzymic activity. A wealth of literature exists on the modulation of gluta-mate dehydrogenase by effector molecules as well as tendencies of the enzyme from some species to exhibit an aggregating phenomenon. This vast research area will not be discussed since it is difficult to make generalities about these regulatory mechanisms regarding the various organisms discussed. However, it is important to note that

the adenine and guanine nucleotides, which can modulate enzymic activity of glutamate dehydrogenase from the higher forms (12,13) has been shown to have no effect on the activity of the enzyme from some of the lower forms (2,9).

Another mechanism a cell uses to regulate its metabolism is to control its enzyme levels. The type of carbon or nitrogen source metabolized by various organisms has been shown to greatly affect the levels of glutamate dehydrogenase (14,15). This regulation of cellular enzyme(s) levels is probably brought about by induction and/or repression phenomena. Very little research has been done to determine when these phenomena can occur during a cell's life cycle.

Current investigations are underway to determine whether or not a cell has the ability to synthesize specific enzymes at any time during its life cycle. If a cell possesses such a mechanism, its genes should be continuously available for transcription throughout the life cycle. An alternate mechanism would involve restricted times in the cell cycle when transcription could not occur. During these times, decreases in cellular repressor and corepressor levels should have no effect on the availability of the genes for transcription.

Examination of the pattern of synthesis for a given enzyme during the cell cycle of a synchronous culture raises an important question. Does the pattern reflect the availability of the gene (for the corresponding enzyme) for transcription or does it reflect the oscillation of repressor and corepressor molecules in the cell? This

question must be answered to distinguish the two mechanisms previously discussed. Often the observed pattern of enzyme synthesis will be intermediate to that of a minimal rate of enzyme synthesis or basal synthesis and that of a maximum rate of synthesis or derepressed synthesis. Such a condition is termed autogenous synthesis. The autogenous synthesis may or may not show a response to increasing gene dosage during the period of DNA synthesis in the cell cycle.

The most direct approach to answer the question is to examine the synthetic response of enzymes in the presence of inducer or corepressor molecules. If an enzyme level can be increased in the presence of inducer molecules or in the absence of corepressor molecules, then the gene coding for the enzyme is available for transcription. Of course, the increase in enzyme level must show a dependence on both RNA and protein synthesis. A dependence on DNA synthesis is not necessary in this mechanism, although an increase in the synthetic response of enzymes resulting from increasing gene dosage should be observed.

Kuempel et al. (16) have used "potential" to mean the maximum rate of enzyme synthesis of a cell at any given time during its cell cycle, under fully induced or fully derepressed conditions. The potential is not synonymous with inducibility, since the latter refers only to increased enzyme synthesis in the presence of an inducer molecule or in the absence of corepressor. The studies on the bacterial systems (16-21) have indicated that autogenous synthesis of enzymes occurs in a periodic manner and is regulated by oscillatory repression of

end products. The gene dosage of the cell has no effect on the autogenous pattern observed (20). Support for these conclusions is the ineffectiveness of the DNA synthesis inhibitor, 5-fluorodeoxy-uridine, in altering the periodic pattern of enzyme synthesis; however, the removal of end product corepressors from the culture medium at various times during the cell cycle has a very profound effect on the pattern of enzyme synthesis (20).

The maximum rate of induction or derepression of enzyme synthesis, however, does appear to be related to the gene dosage of the cell. This relationship is supported by the observation of Masters and Pardee (18) that the time of maximum sucrase transforming ability (which is related to the number of structural genes per cell for sucrase in B. subtilis) corresponds to the time at which the increase in potential to synthesize sucrase in a synchronous culture occurs. When thymine deprivation is employed to block DNA synthesis, Donachie and Masters (21) have also shown the inducibility of β-galactosidase and D-serine deaminase to remain constant during synchronous growth of E. coli.

A summary of the aforementioned data indicates the following. The autogenous pattern of enzyme synthesis during a bacterial cell cycle does not reflect the availability of genes for transcription but rather reflects an oscillation of corepressor levels in the cell. When corepressor molecules are removed from the culture medium, a burst in enzyme synthesis occurs. Only under fully-induced or fully-derepressed conditions is the size of the burst reflected by the gene

dosage of the cell.

The continuous availability of genes for transcription has also been demonstrated in a number of eucaryotic cells. Mitchison (22,23) has demonstrated this for the genes coding for sucrase and alkaline and acid phosphatases during the cell cycle of the fission yeast, Schizosaccharomyces pombe. In a different eucaryotic organism, Knutsen (24) has examined the induction of nitrite reductase in Chlorella pyrenoidosa (strain 211-8b). He concluded that induction is confined to a limited period of the life cycle. However, the data he presents clearly shows that inducibility occurs at all times during the cell cycle and that the induction rate sharply increases at the time of DNA synthesis. By probably confusing the rate of induction with inducibility, he has neglected to point out the continuous availability for transcription of the nitrite reductase gene in this eucaryotic organism. The measurement of the inducibility of isocitrate lyase during the cell cycle of a different strain of Chlorella pyrenoidosa by Baechtel et al. (25) in our laboratory, supports the hypothesis that continuous availability of genes for transcription occurs in this organism. These workers also observed an increase in potential at the time of DNA synthesis suggesting that the relationship of potential and gene dosage probably holds for the eucaryotic as well as the procaryotic organisms.

The alternate mechanism to that of continuous availability of genes for transcription is that of a temporal mechanism in which induction or derepression of the genes will only occur at limited

times in the cell cycle. Two types of eucaryotic organisms have been reported to utilize this mechanism. In synchronous rat hepatoma cell cultures, Tomkins and coworkers (26) show a period at the end of G_2, during mitosis, and at the early stage of G_1 at which induction of tyrosine transaminase will not occur. They propose that this restriction may be related to changes in the chromosomal configuration during these periods. However, the restricted time during which the structural gene cannot be transcribed is relatively short compared with the restricted period reported for the budding yeast, Saccharomycess cerevisiae (29,30).

Halvorson and coworkers (29) have demonstrated that the basal pattern of enzyme synthesis in S. cerevisiae results from an ordered transcription of the genome which parallels the location of the gene on the chromosome. To test the availability of the genes for transcription at other times during the cycle, they examine the pattern of induced enzyme synthesis. Rather than challenging the genome with inducer molecules at various times in the cycle, Halvorson's group adds the inducer at the beginning of the S. cerevisiae cell cycle.

They observe a periodic pattern of induced synthesis identical to the pattern of uninduced or basal synthesis. These data are interpreted to support restricted times during the cell cycle when transcription cannot occur. However, this periodic pattern would also be seen if the gene were continuously transcribed during the cycle and coded for an enzyme having equal synthetic and decay rates. Halvorson's group has not reported whether or not the enzyme is stable in the absence of its synthesis.

In a recent report on the basal and fully derepressed level of
β-galactosidase in S. lactis and ornithine transaminase in S.
cerevisiae, Carter, Sebastian, and Halvorson (30) state that if the
inducer is added to cells growing synchronously in medium minus
inducer, synthesis of enzyme occurs at any time. This observation is
certainly consistent with continuous rather than limited availabil-
ity of genes for transcription in these Saccharomyces species.

Thus, the only conclusive demonstration of a temporal mechanism
is that of Tomkins in the mammalian system and even in this system,
the genes are available for transcription during a large part of the
cell cycle. It therefore seems that procaryotes and eucaryotes have
a similar mechanism for the control of gene expression since trans-
scription can occur during most, if not all, of the cell cycle. The
autogenous pattern of synthesis probably reflects an intermediate
condition between the fully repressed and full derepressed gene for
the given enzyme and the oscillation of corepressor molecules in the
cell would govern whether the autogenous pattern were continuous or
periodic during the cell cycle.

Studies on the pattern of glutamate dehydrogenase in synchronized
Chlorella pyrenoidosa (strain 7-11-05) were initiated in our laboratory
by White (31) to determine if continuous or periodic synthesis of this
enzyme occurred during the cell cycle. A continuous pattern of syn-
thesis would suggest continuous availability of the gene for glutamate
dehydrogenase for transcription whereas a periodic pattern would sug-
gest either limited availability of the gene for transcription or an

oscillation in corepressor levels for this gene. Once the autogenous
pattern were established, the regulatory mechanism involved could be
studied. The enzyme was not observed to increase during the first
4-6 hours of the cell cycle of light-dark synchronized cells; however,
its activity when assayed with either NADH or NADPH, was continuous
for the remainder of the cycle and into the next cycle. A deviation
in the relative glutamate dehydrogenase activity with each coenzyme
near the end of the cell cycle and a difference in the fold increase
of the activity with each coenzyme led White (31) to suggest the
possibility of glutamate dehydrogenase isozymes in this organism.
Further support for this possibility was a report by Kretovich (32)
of an increased NADPH-glutamate dehydrogenase activity relative to
that of NADH-activity, when Chlorella pyrenoidosa Pringsheim (strain
82T) were cultured on ammonium medium. Determination of whether
glutamate dehydrogenase isozymes existed in this organism was neces-
sary before cell cycle patterns and further studies on gene regulation
could be meaningfully interpreted.

The present study was undertaken to determine if glutamate dehy-
drogenase isozymes exist in Chlorella pyrenoidosa Chick (strain 7-11-
05) and to study the mechanism regulating the synthesis of this
enzyme(s) during the cell cycle.

MATERIALS AND METHODS

Organism and Growth Conditions. The thermophilic strain 7-11-05 of
the unicellular algae (33), Chlorella pyrenoidosa, was cultured under
the conditions described by Hare and Schmidt (34); however, the light
intensity was reduced to either 550 or 1,100 footcandles to yield
division numbers of 4 and 8, respectively. The cells were synchronized
by 3 alternating light-dark cycles of 10:8 and 8.5:7 hours for cells
with division numbers of 4 and 8, respectively. The synchrony of cells
(division number of 4) from the end of the third dark period was im-
proved further by selection of a uniform population of daughter cells
by equilibrium centrifugation with a modified Ficoll procedure (35).

The flat Plexiglass chambers used for culturing cells had an
internal thickness of 0.31 cm, whereas the glass tubes used for cultur-
ing cells had an inside diameter of 2.54 cm.

To give equal nitrogen equivalents in nitrate- and in ammonium-
containing medium, 22.8 mM KNO_3 or 11.4 mM $(NH_4)_2SO_4$ were used in the
two media with a pH of 6.8 and 7.3, respectively. The change in
initial pH from 6.8 of nitrate-containing medium to 7.3 for ammonium-
containing medium was made because of the tendency of the pH to shift
upward during growth of the cells on nitrate- while downward on
ammonium-containing medium.

Preparation of Cell Material for Analysis. Cells were harvested by
centrifugation, washed three times in 0.01 M Tris-HCl buffer (pH 7.7),
and resuspended in 0.1 M Tris-HCl buffer (pH 8.25). Cells were

11

maintained at 4° through the harvest and wash procedure. Cell breakage was obtained by sonic oscillation at 4° by use of Raytheon 10KC sonic oscillator for 15 min or by rupture with a mechanically-driven French press, Model 5-596 (American Instrument Company), at a pressure of 18,000-20,000 psi.[1] Crude sonicates or homogenates were assayed directly or supernatants were prepared from these (as described for each figure in Results and Discussions), and then assayed for NADH- and NADPH-glutamate dehydrogenase activity.

Spectrophotometric Assay of Glutamate Dehydrogenase Activity. The spectrophotometric assay was modified from that of Kates and Jones (8). The modifications in the assay solution were: 33.3 mM Tris-HCl buffer (pH 8.25), 300 mM ammonium sulfate, 25 mM α-ketoglutarate, and either 0.265 mM NADH or 0.249 mM NADPH. The oxidation of NADH or NADPH during the assay was measured by the decrease of absorbance at 340 nm by use of a Gilford Model 1000 recording spectrophotometer with its output connected to a Honeywell recorder. As controls, the endogenous oxidation of each coenzyme was determined in the absence of α-ketoglutarate or of ammonium sulfate. The change in absorbance, in the absence of coenzyme but in the presence of other substrates was used as a control for endogenous coenzyme concentration. The changes in absorbance for both sets of controls were within the experimental error ($\leq$ 5%) of replicate assays.

The assay solutions were equilibrated to temperature in a 38° bath

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[1]Pounds per square inch (psi).

from water which circulated through the cuvette chamber of the
spectrophotometer. An aliquot of 100-500 µl of an enzyme preparation
was added to the assay solution to bring the total volume to 3 ml.
The decrease in absorbance at 340 nm was recorded over a 1-2 min
interval at a chart speed of 10.2 cm per min, and a sensitivity of
0.05, 0.10, 0.20, or 0.50 absorbancy unit at full scale deflection.
The reaction rate remained linear over the time interval of assay and
was proportional to the amount of enzyme assayed. One unit of gluta-
mate dehydrogenase activity is defined as the amount of enzyme required
to oxidize 1 µmole of coenzyme per min.

Analytical Disc-Gel Electrophoresis. The procedure from Davis (36)
was followed as described by the Canalco Co. (37). Solutions used in
the preparation of acrylamide gels are given in Table I. The volume
of stock solution C was adjusted to yield separator gels of 4 or 7%
acrylamide. The gels were polymerized at room temperature in 5 x
75 mm, 5 x 127 mm, or 5 x 150 mm glass tubes. The Tween solution
employed in the standard procedure to coat the walls of the polymeriza-
tion tubes, was omitted after initial experiments to reduce tailing of
the NADPH specific glutamate dehydrogenase during electrophoresis.
Enzyme solutions (2.5-200 µl) were applied in a sample gel. Electro-
phoresis was performed at 4° at an amperage of 1.5-3 ma[2] per tube for
a period of 2-5 hours. Electrophoresis time varied with experiments
due to the different volumes of separator and stacker gels employed.

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[2]Milliamperes (ma).

Table I.   Chemical formulation for analytical disc-gel electro-
          phoretic procedure (Stacks at pH 8.9, Runs at pH 9.5).

Stock Solutions:

(A)	1N HCL	48 ml	(B)	1N HCL	48 ml
	Tris	36.3 gm		Tris	5.98 gm
	*Temed	0.23 ml		*Temed	0.46 ml
	H_2O to make	100 ml		H_2O to make	100 ml
	(pH 8.8 - 9.0)			(pH 6.6 - 6.8)	

(C)	Acrylamide	28.0 gm	(D)	Acrylamide	10 gm
	*Bis	0.735 gm		*Bis	2.5 gm
	H_2O to make	100 ml		H_2O to make	100 ml

(E)	Riboflavin	4.0 mg	(F)	Sucrose	40 gm
	per 100 ml H_2O			H_2O to make	100 ml

(G)	Catalyst		(H)	Electrophoresis Buffer	
	Ammonium Persulphate	0.14 gm		Tris	3.0 gm
	H_2O to make	100 ml		Glycine	14.4 gm
				H_2O to make	1 liter

(I)   Tracking Dye
      .005% Bromphenol Blue Solution

Working Solutions:

Separating gel solution	Stacking gel solution
1 part (A)	1 part (B)
2 parts (C)	2 parts (D)
1 part H_2O	1 part (E)
4 parts (G)	4 parts (F)
(pH 8.8 - 9.0)	(pH 6.6 - 6.8)

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*N,N'-Methylenebisacrylamide (Bis)
 N,N,N',N' Tetramethylethylene-diamine (Temed)

At the completion of electrophoresis, glutamate dehydrogenase activity was detected in the gels using a modified tetrazolium assay (38) solution consisting of the following: 40 ml 0.1 M Tris-HCl buffer (pH 8.25); 3.0 ml 1 M L-glutamate (pH 7.0); 1.0 ml 6.5×10^{-3} M phenazine methosulfate; 2.0 ml 5.7×10^{-3} M nitro-blue tetrazolium; 1.3 ml 2.2×10^{-2} M NAD^+ and/or 1.3 ml 2.2×10^{-2} M $NADP^+$. The assay solutions containing the gels were incubated in the dark either at 37° for 20 min or at room temperature for 1 hour. Gels were rinsed with distilled water and stored in 7% acetic acid until photographed.

Elution of Isozymes from Acrylamide Gels. Cells were cultured 4 hours in ammonium-containing medium in continuous light in glass tubes, and the cells ruptured with the French press. A supernatant preparation was obtained by centrifugation at 100,000 x $\underline{g}$ for 1 hour of the re-sulting 5 ml homogenate containing 1.6×10^6 cells. The supernatant was loaded on each of 12 acrylamide gels. Gels were prepared in 5 x 150 mm tubes using 2.0 ml separator gel (7% acrylamide), 0.5 ml stacker gel, and a sample gel containing 100 µl of the supernatant preparation. Electrophoresis was performed at 2 ma/tube for 2 hours followed by 3 ma/tube for 1 hour 15 min.

To elute the glutamate dehydrogenase isozymes from acrylamide gels after electrophoresis, the isozyme bands were located by incubating one of the set of 12 gels in the tetrazolium assay solution containing both NAD^+ and $NADP^+$. The remaining gels were sectioned and eleven 3 mm sections containing either isozyme were submerged in 5 ml of 0.1 M

Tris-HCl buffer (pH 8.25) and stored at 4° for 30 hours with occasional mixing. The elutants were assayed spectrophotometrically for glutamate dehydrogenase activity.

Sucrose Density Gradient Centrifugation. Twelve ml linear gradients (39) from 5-20% sucrose were prepared from 20% (w/v) sucrose in 0.1 M Tris-HCl buffer (pH 8.25). Enzyme preparations in the same buffer were layered on the gradients with 115 μg bovine liver catalase (A grade, Calbiochem) as an internal marker. After centrifugation (SW-40 rotor) in a Spinco Model-L ultracentrifuge at 40,000 rpm for 24 hours (period of maximum speed), 3-drop fractions were collected by tube puncture with a 20-gauge hypodermic needle, and these fractions assayed for glutamate dehydrogenase and catalase activities.

Catalase Assay. In an assay similar to that employed by Martin and Ames (40), catalase activity in 10 μl of the gradient functions was measured by the decrease in absorbance at 240 nm of a 3.0 ml reaction mixture containing 60 μmoles of H_2O_2 in 0.02 M potassium phosphate buffer (pH 7.5). One unit of catalase activity is defined as a change in absorbance of 1.0 per min per 10 μl of the gradient fraction.

General Methods. Cell number was determined in a Model-B Coulter Counter. Protein determination was by the method of Lowry et al. (41) with crystalline bovine serum albumin as a standard. DNA was measured by the diphenylamine procedure of Burton (42) as modified by Hopkins et al. (35).

Reagents. Cycloheximide was kindly provided by Dr. G. S. Fonken

of Upjohn Company. Actinomycin D was obtained from Schwarz/Mann;

the α-ketoglutarate, Tris, NAD^+ (A grade) were obtained from

Calibiochem, Inc.; the NADH and NADPH (Chromato-Pure) were from P-L

Biochemicals, Inc.; the L-glutamate and the phenazine methosulfate

were from Mann Research Laboratories; the nitro-blue tetrazolium was

from Sigma Chemical Company; the ammonium sulfate was from J. T. Baker

Chemical Company; the sucrose (optical grade) was from Harshaw Chemi-

cal Company; and all reagents for the analytical disc-gel electro-

phoretic procedure (except sucrose) were from Canalco.

RESULTS AND DISCUSSION

Cell Breakage Techniques and the Yield of Glutamate Dehydrogenase
Activity. When cells cultured in ammonium-containing medium were
subjected to sonic oscillation for varying time intervals, an inacti-
vation of both NADH- and NADPH-glutamate dehydrogenase activity was
observed (fig 1). The yield of both activities reach a peak
following 10 min sonication and then decreased thereafter.

The inactivation of glutamate dehydrogenase by sonic oscillation
led to the use of the French press for cell breakage (Fig 2). The
maximum yield of glutamate dehydrogenase activity apparently occurred
at a pressure of 18,000-20,000 psi because a second exposure of the
cellular material to 20,000 psi had no significant effect on enzymic
activity assayed with either coenzyme.

Frozen whole cells (nitrate cultured) could be stored at -20°
for 24-48 hours prior to rupture with the French press without apparent
loss of enzyme activity. Although Hermann (44) and Vassef (45)
observed in frozen-thawed whole cells or a homogenate of an equivalent
cell number of Chlorella, the same activity of aspartate transcar-
bamylase, frozen-thawed whole cells in the present study yielded only
50% of the total glutamate dehydrogenase activity present in ruptured
fresh cells.

Pattern of Glutamate Dehydrogenase During the Cell Cycle of Cells
Cultured in Nitrate-Containing Medium. The relative activity per
ml of culture of the NADH- and NADPH-glutamate dehydrogenase

18

Figure 1.   The effect of sonic oscillation on the yield of glutamate
            dehydrogenase from Chlorella pyrenoidosa (strain 7-11-05)
            cultured in ammonium-containing medium. Approximately
            10.7×10^{10} light-dark synchronized cells (division number
            of 8) were cultured in 500 ml of ammonium-containing medium
            in a Plexiglass chamber in continuous light. At the fourth
            hour, 9.1×10^{10} cells were harvested, washed three times
            in 0.01 M Tris-HCl (pH 7.7), and resuspended in 20 ml of
            01. M Tris-HCl, 1 mM Dithiothreitol buffer (pH 8.25). The
            cells were ruptured by sonic oscillation for 2, 4, 8, 12,
            15, 25, and 30 min and 2 ml aliquots removed for spectro-
            photometric assay. Assay solutions also contained 1 mM
            Dithiothreitol. Units of glutamate dehydrogenase per
            100 μl of sonicate, assayed with NADH, ●, and with NADPH,
            O.
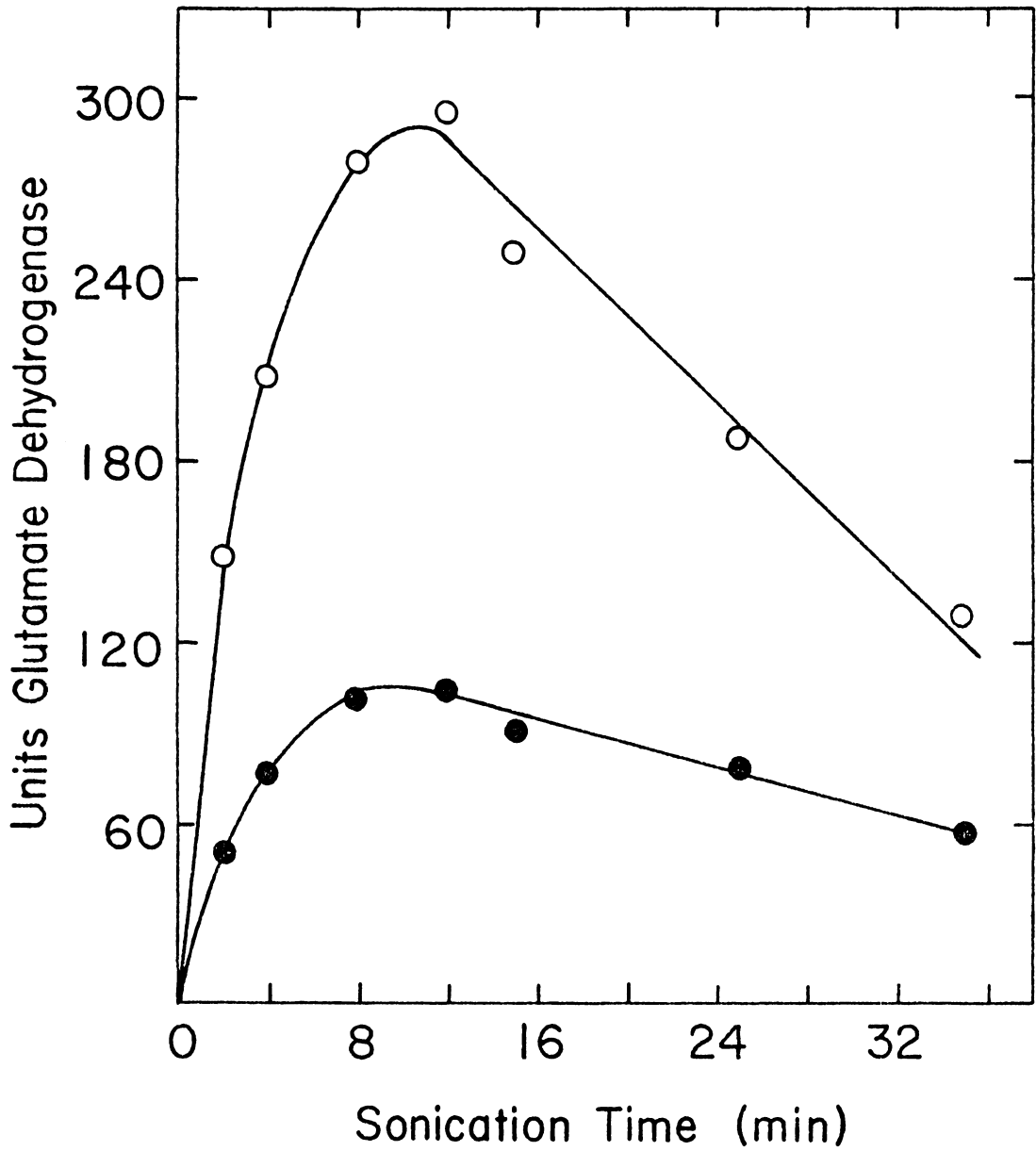
Figure 2.   The yield of glutamate dehydrogenase from <u>Chlorella</u>
<u>pyrenoidosa</u> (strain 7-11-05) ruptured with the French
press at different pressures. Approximately 9.8×10^{10}
light-dark synchronized cells (division number of 8) were
cultured in 500 ml of ammonium-containing medium in a
Plexiglass chamber in continuous light. At the fourth
hour, 9.3×10^{10} cells were harvested, washed three times
in 0.01 M Tris-HCl buffer (pH 7.7), and resuspended in
20 ml of 0.1 M Tris-HCl buffer (pH 8.25). Five ml
aliquots of the suspension were passed through the French
press at pressures of 8000; 12,000; 16,000; and 20,000 psi.
Units of glutamate dehydrogenase in 100 µl of the resulting
homogenates was measured spectrophotometrically with NADH,
●, and with NADPH, ○.
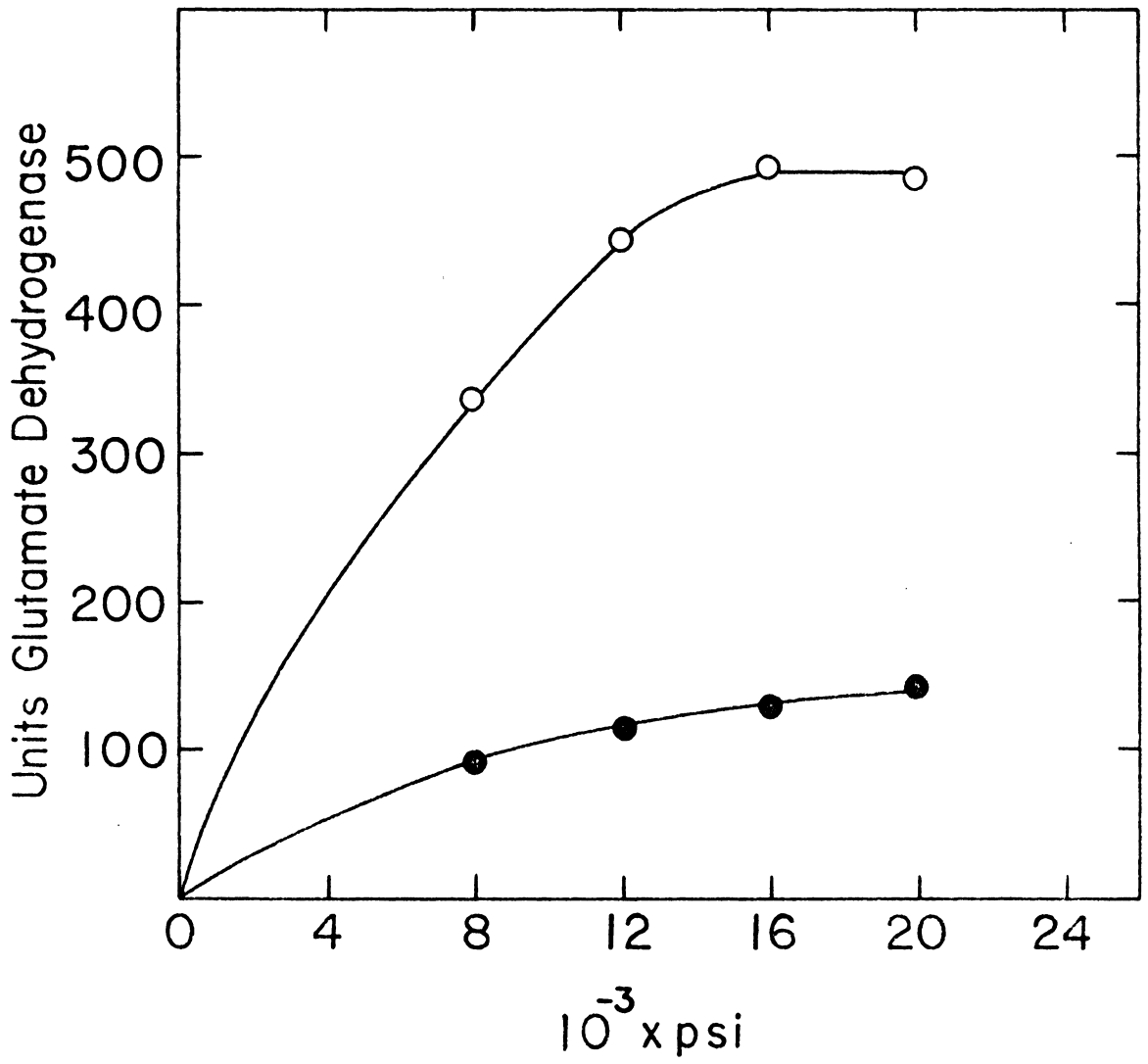
activities remained constant during the first 4 hours of synchronous growth and then increased exponentially into the next cell cycle of Chlorella cultured in nitrate medium (Fig 3). The NADPH: NADH activity ratio was 1:5 at each hour throughout the cell cycle and each activity increased approximately 2-fold by the midpoint of cell division (i.e., the time defined as one cell cycle). Since the fold increase in activities is less than the 4-fold increase in DNA, the gene for the enzyme may be partially or fully repressed during the early period of the cell cycle. A plausible explanation for the continued synthesis of enzyme into the next cycle is that a lower corepressor level in daughter cells cultured in continuous light relative to the corepressor level for daughter cells following a dark period is not adequate to prevent the transcriptional process. This is speculation, however, and must be tested in future experiments.

The observed pattern of glutamate dehydrogenase during the cell cycle in nitrate cultured cells is partially in support of the observations of White (31). The time of increase in enzyme activity and the continuous synthesis of enzyme into the next cycle are in agreement with White's report. However, no differences were observed in the pattern of synthesis of glutamate dehydrogenase when assayed with NADH relative to NADPH.

It is unlikely that the higher division number of cells in White's studies (i.e., 10-fold increase in cell number) accounted for his differences in fold increase of the two activities since in an experiment using synchronized cells with a division number exceeding 12 and

Figure 3. Pattern of glutamate dehydrogenase activity and its relationship to total protein and DNA during the cell cycle of <u>Chlorella</u> <u>pyrenoidosa</u> (strain 7-11-05) light-dark synchronized cells (division number of 4) were gradient selected and cultured in a Plexiglass chamber of nitrate medium in continuous light. The turbidity was held essentially constant by continuous dilution with culture medium. The level of glutamate dehydrogenase assayed with NADH, ●, and with NADPH, O; protein, △, DNA, ▲; and cell number, □, with relative initial values per ml of culture of 16 units, 3 units, 328 μg, 8.43 μg, and 145 x 10^6 cells, respectively.
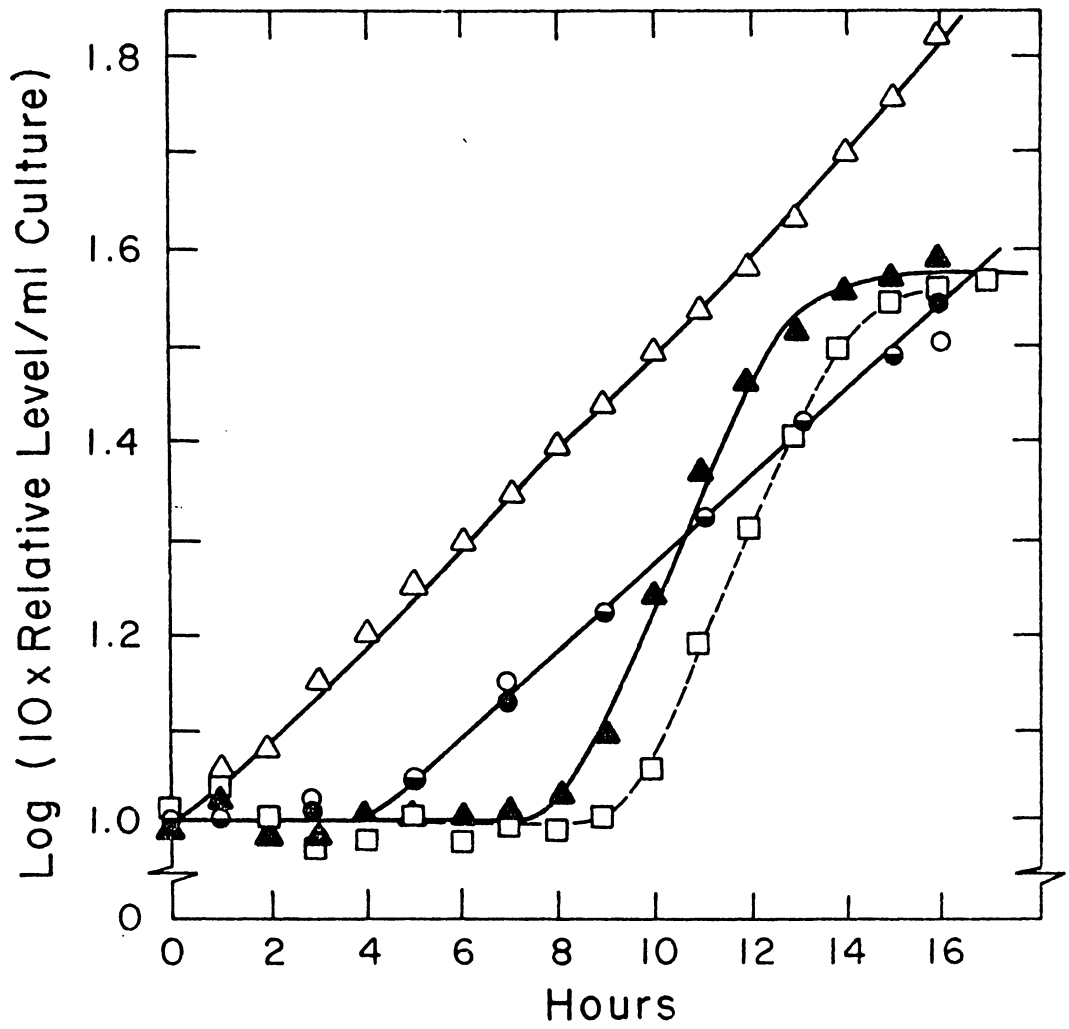
ruptured with the French press, the glutamate dehydrogenase activity assayed with each coenzyme has been shown to be approximately the same (i.e., 6-fold) at the end of the cell cycle (i.e., at the twelfth and thirteenth hours).

Since White had used sonication as a means of rupturing the cells for his studies, his observations may have resulted from a conformational change in the enzyme during sonication near the end of the cell cycle or some other artifact due to the sonication procedure.

Induction of a NADPH-Dependent Glutamate Dehydrogenase Isozyme by Ammonium. The supernatant fractions from cells cultured in either nitrate or ammonium medium were examined for glutamate dehydrogenase activity following analytical disc-gel electrophoresis. A single isozyme was seen in the supernatant from nitrate cultured cells (Fig 4) and the band intensities indicated the isozyme to be more reactive with NAD^+ than $NADP^+$. An additional slower-moving isozyme was observed in the supernatant from ammonium cultured cells and the band intensity indicated a complete specificity for $NADP^+$. Even when 20 times more supernatant was placed on gels, the NAD^+ reactivity of this isozyme was barely detectable. In similar types of experiments involving spectrophotometric measurements, it was found that the extreme sensitivity of the tetrazolium assay and the high resolution obtained due to the concentrating phenomenon of the disc-gel method, permitted visual detection of less than one spectrophotometric unit of glutamate dehydrogenase activity on the gels. One unit of activity

Figure 4. Analytical disc-gel electrophoretic patterns for glutamate dehydrogenase isozymes from <u>Chlorella</u> <u>pyrenoidosa</u> (strain 7-11-05) cultured in either nitrate or ammonium-containing medium. Light-dark synchronized cells (division number of 8) were cultured in nitrate- or ammonium-containing medium in Plexiglass chambers in continuous light at cell concentrations of 183×10^6 cells/ml. The increase in turbidity and in dry weight over a 4 hour period were the same for both cultures. At the fourth hour, 9×10^9 cells were harvested from each culture, washed three times in 0.01 M Tris HCl buffer (pH 7.7), resuspended in 20.0 ml of 0.1 M Tris-HCl buffer (pH 8.25), and ruptured with the French press. The resulting homogenates were centrifuged at 100,000 x $\underline{g}$ for 2 hours, and the supernatants obtained were subjected to electrophoresis. Gels were prepared in 5 x 127 mm tubes using 1.6 ml separator gel (4% acrylamide), 0.4 ml stacker gel, and a sample gel containing 2.5 or 50 µl of supernatants from cells cultured in nitrate or ammonium medium. Electrophoresis was performed at 1.5 ma/tube for 1 hour 20 min followed by 3 ma/tube for 1 hour 30 min.

The specific glutamate dehydrogenase isozymes from supernatants of cells from either culture were located on the gels with the tetrazolium assay by inclusion of either of the specified coenzymes.

Nitrate: 2.5 µl; A, NAD^+; B, $NADP^+$

50 µl; C, NAD^+; D, $NADP^+$

Ammonium: 2.5 µl; E, NAD^+; F, $NADP^+$
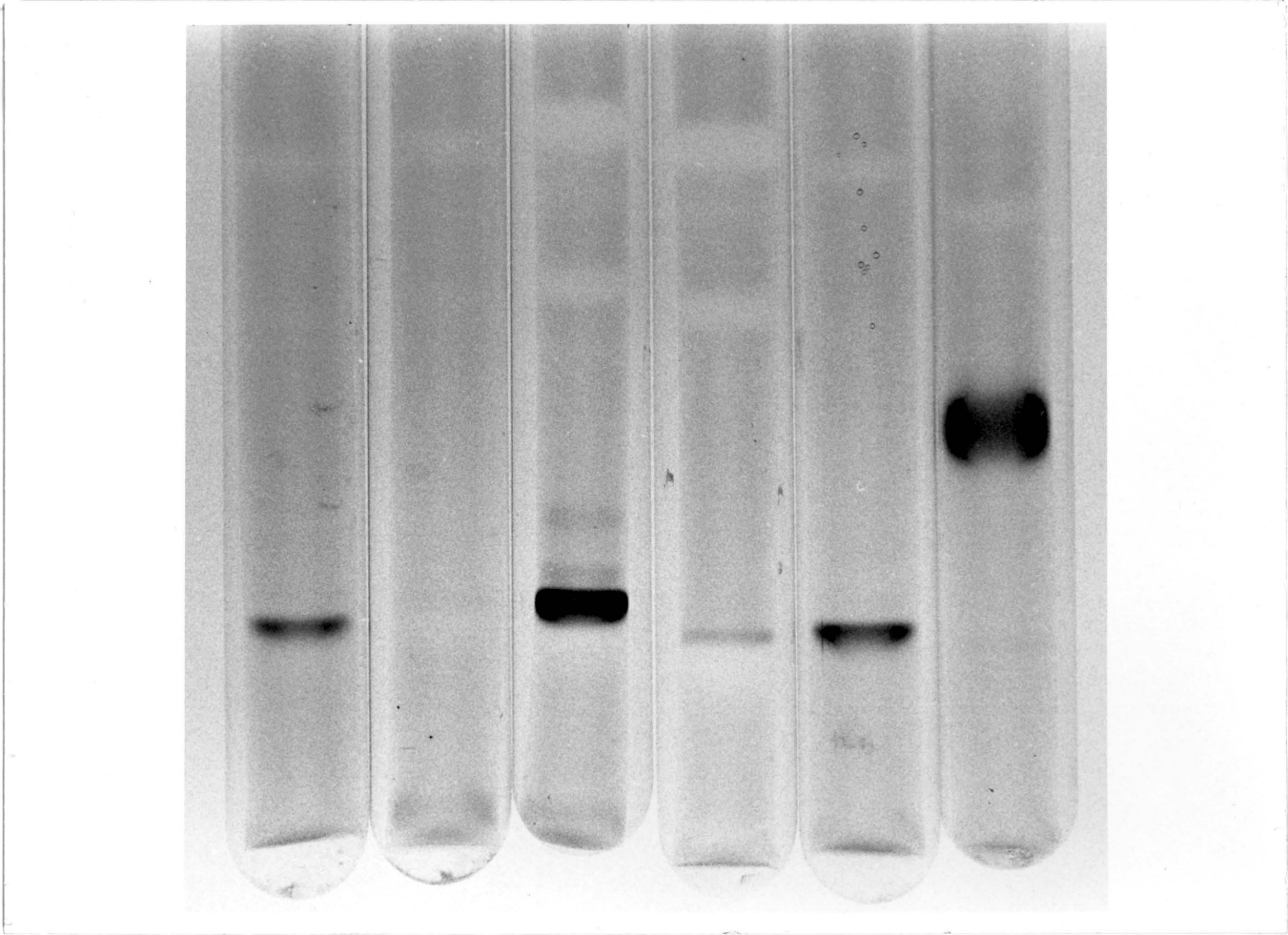
A   B   C   D   E   F

fell within control values for the spectrophotometric measurements.

To substantiate that the glutamate dehydrogenase isozymes were not an artifact of the disc-gel procedure, a comparison of the sedimentation profile of glutamate dehydrogenase from nitrate- with that from ammonium-cultured cells was made (Fig. 5A,B). A single peak of glutamate dehydrogenase was seen in the supernatant from nitrate-cultured cells (Fig. 5A). The NADPH:NADH activity under the peak was 1:5 whereas that of the supernatant placed on the gradient was 1:3.6. The recovery of enzyme units for the NADH- and NADPH-glutamate dehydrogenase activities were 85% and 54%, respectively. Although there is no measurable NADPH-activity in gradient fractions in Fig. 5A which correspond to the NADPH-glutamate dehydrogenase activity peak of the supernatant from ammonium-cultured cells (Fig. 5B), the $NADP^+$-specific isozyme could be detected following disc-gel electrophoresis of the supernatants preparation from nitrate-cultured cells. It is possible that a dilution of the $NADP^+$-specific isozyme (revealed by disc-gel electrophoresis) on the gradient prevented its spectrophotometric detection. The isozyme may have been concentrated enough in the supernatant preparation from the nitrate-cultured cells to contribute to the measured NADPH-activity and therefore to lower the coenzyme ratio (i.e., 1:5 relative to 1:3.6). However, the removal of an activator of NADPH-glutamate dehydrogenase activity during the centrifugation process is also a possibility and could account for the low recovery of NADPH-activity from the gradient. In view of the detection of the $NADP^+$-specific isozyme band in the supernatant preparation, the former conclusion seems more probable. It is important to note here that the

Figure 5A,B. Sedimentation patterns of glutamate dehydrogenase from
Chlorella pyrenoidosa (strain 7-11-05) cultured in either
nitrate- or ammonium-containing medium. Light-dark syn-
chronized cells (division number of 8) were placed in
continuous light in nitrate- or ammonium-containing medium
in glass tubes at concentrations of 153×10^6 cells/ml.
At the fourth hour, 6×10^{10} cells were harvested from each
culture, washed three times in 0.01 M Tris-HCl buffer
(pH 7.7), resuspended in 9.0 ml of 0.1 M Tris-HCl buffer
(pH 8.25), ruptured with the French press, the resulting
homogenates centrifuged at 100,000 x $\underline{g}$ for 1 hour, and
the supernatants obtained, stored at -20°. From these
supernatants, 500 µl aliquots, containing 4.5 mg or 6.9 mg
protein from the cells of nitrate or ammonium cultures,
respectively, were applied on linear gradients of sucrose
(5-20%, w/v; 12.0 ml) prepared in 0.1 M Tris-HCl buffer
(pH 8.25). The gradients were centrifuged at 40,000 rpm
(SW-40 rotor) for 24 hours at 3°, 3-drop fractions collected,
and fractions assayed spectrophotometrically for glutamate
dehydrogenase activity. Glutamate dehydrogenase units per
fraction with NADH, ●, and with NADPH, O, were determined
from the gradients corresponding to cells cultured in A,
nitrate medium and B, ammonium medium.
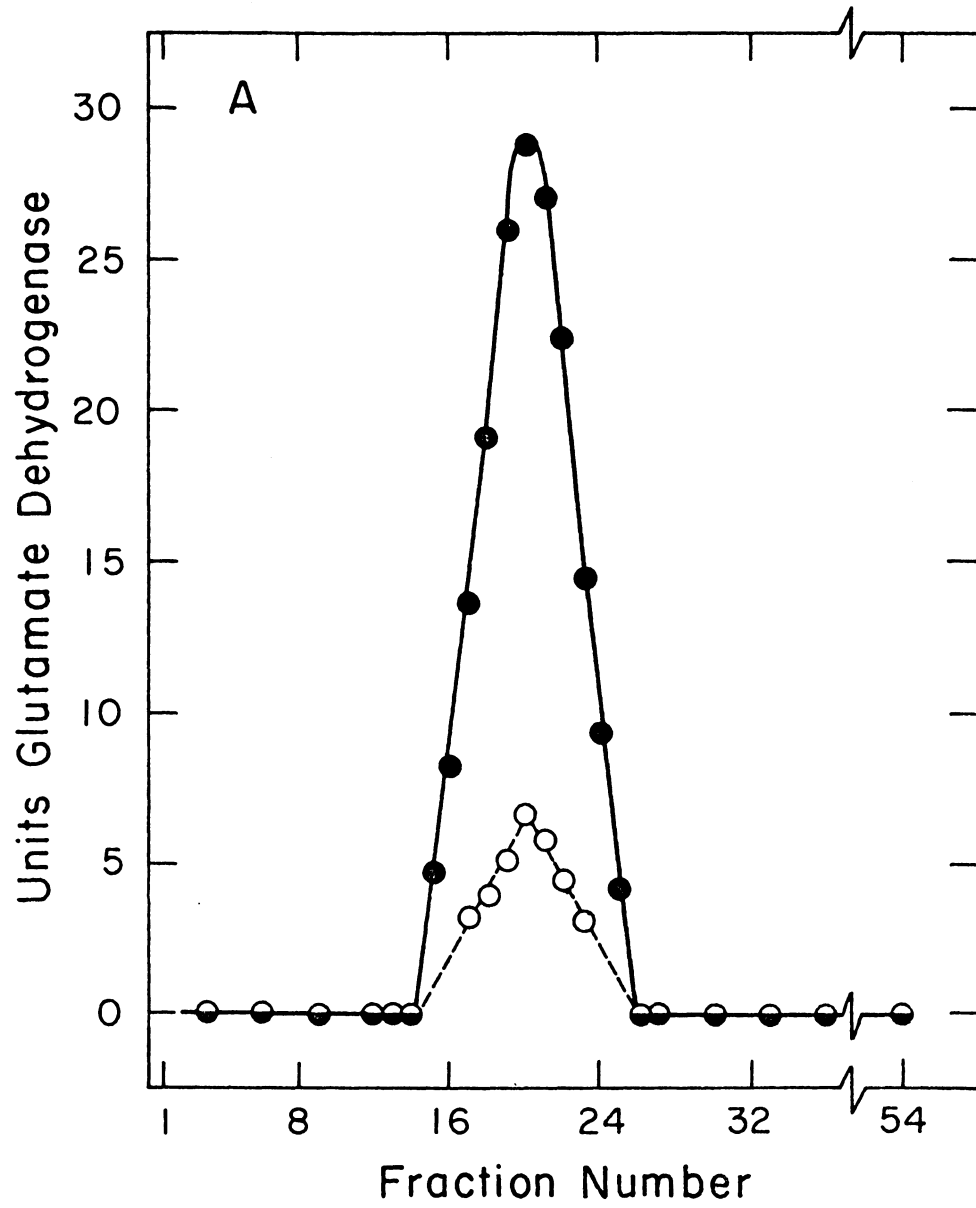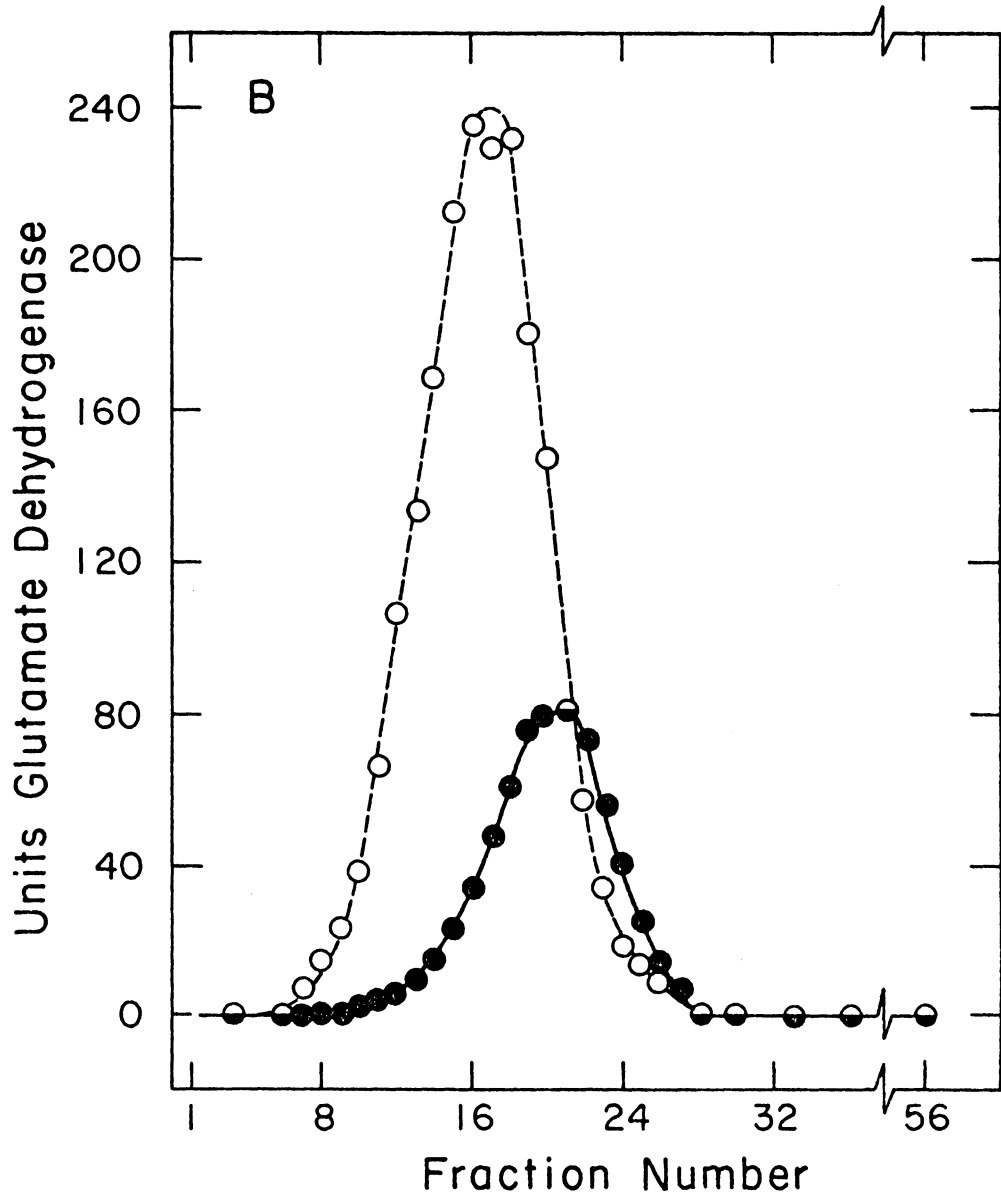
supernatant preparation from the nitrate-cultured cells is at least
15 times more concentrated in cellular material than that used to
measure glutamate dehydrogenase during a cell cycle experiment. The
sedimentation profile for the supernatant from ammonium-cultured cells
has, in addition to the predominant NADPH-activity peak, a NADH-gluta-
mate dehydrogenase activity peak corresponding to that in Fig 5A.
Although the overlapping activities prevent the accurate determination
of the coenzyme activity ratio for the NADPH-reactive isozyme, the very
low level of NADH-glutamate dehydrogenase activity in fractions 10-14
suggests that the isozyme is very specific for NADPH. The recovery
of enzyme units for the NADH- and NADPH-activities were 86% and 72%,
respectively.

To establish the molecular weights of these isozymes, a sedimenta-
tion profile of glutamate dehydrogenase from a supernatant of Chlorella
cultured in ammonium medium was examined in reference to bovine liver
catalase as an internal marker (Fig 6). Relative to the catalase
marker, the molecular weights of the NADPH- and NADH-reactive enzymes
were calculated (40) to be 269,000 and 179,000, respectively.

The Coenzyme Specificities of the Glutamate Dehydrogenase Isozymes.
The coenzyme activity ratio for both isozymes was established by
separating the isozymes from ammonium cultured cells by analytical
disc-gel electrophoresis followed by elution from the acrylamide gels
as described in Materials and Methods. Each isozyme eluant contained
38% of the activity loaded in the gels as determined from the

Figure 6.   Sedimentation pattern of glutamate dehydrogenase from
            Chlorella pyrenoidosa (strain 7-11-05) cultured in
            ammonium-containing medium, relative to bovine liver cata-
            lase. An aliquot of 200 µl, containing 1.6 mg protein,
            of supernatant obtained at 100,000 x g from synchronized
            cells (division number of 8) cultured 4 hours in continuous
            light in glass tubes in ammonium medium, and 115 µg of
            bovine liver catalase were applied on a linear gradient
            of sucrose (5-20%, w/v; 12.0 ml) prepared in 0.1 M Tris-
            HCl buffer (pH 8.25). The gradient was centrifuged at
            40,000 rpm (SW-40 rotor) for 24 hours at 3°, 3-drop
            fractions collected, and fractions assayed spectrophoto-
            metrically for glutamate dehydrogenase and catalase
            activities. Glutamate dehydrogenase units per fraction
            with NADH, ●, and with NADPH, ○, and catalase, ---.---.
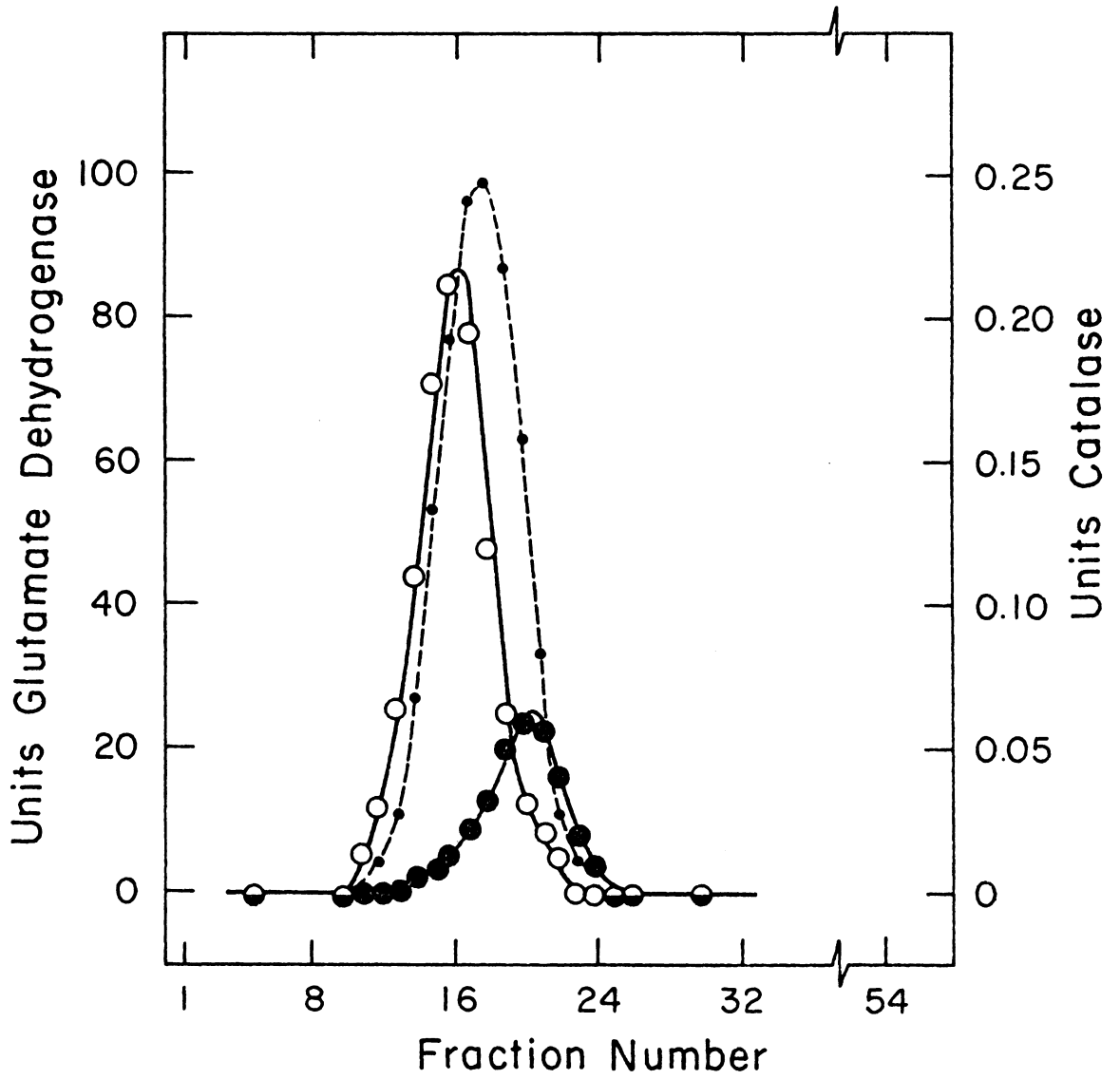
spectrophotometric activity measurement with the more reactive coenzyme for each isozyme relative to the activity with the corresponding coenzyme in the supernatant preparation. When the eluants containing the separated isozymes were resubjected to electrophoresis, no cross-contamination could be detected (Fig 7). The NADPH:NADH gluta- mate dehydrogenase activity ratio in the eluant for the faster-moving isozyme was 1:5; that for the slow-moving isozyme, 33:1.

When the sedimentation profile of either isozyme was examined relative to a catalase marker, the catalase peak in each gradient coin- cided and the sedimentation of each isozyme relative to the catalase peak (Fig 8) was the same as that previously observed in supernatant preparations containing the isozyme mixture (Fig 6). The activity of each isozyme was assayed with each coenzyme for the two groups of gradient fractions. Because of dilution of each isozyme on their respective gradients, the activity of each isozyme could only be de- tected with the coenzyme of highest reactivity. The recovery of the NADPH- and NADH-dependent enzyme units from the gradients were 100% and 89%, respectively.

The coenzyme activity ratios for the isozymes separated by disc- gel electrophoresis are in agreement with the coenzyme reactivities of glutamate dehydrogenase peaks observed in sucrose gradients (Fig 5A,B). Henceforth, the isozyme with a molecular weight of 269,000 as determined on sucrose gradients and with a slower mobility on acrylamide gels is designated the NADPH-specific glutamate dehy- drogenase isozyme; the isozyme with a molecular weight of 179,000

Figure 7.   Analytical disc-gel electrophoretic patterns of glutamate
            dehydrogenase isozymes separated by the disc-gel procedure.
            Gels were prepared in 5 x 75 mm tubes using 0.8 ml separa-
            tor gel (7% acrylamide), 0.3 ml stacker gel, and a sample
            gel containing 20 µl of original supernatants or 100 µl of
            either isozyme eluant. Electrophoresis was performed at
            2 ma/tube for 2 hours followed by 3 ma/tube for 1 hour.
            Supernatant activity assayed with A) NAD$^+$ B) NADP$^+$;
            eluant of the faster-moving isozyme assayed with C) NAD$^+$
            D) NADP$^+$; and eluant of the slower-moving isozyme assayed
            with E) NAD$^+$ F) NADP$^+$.
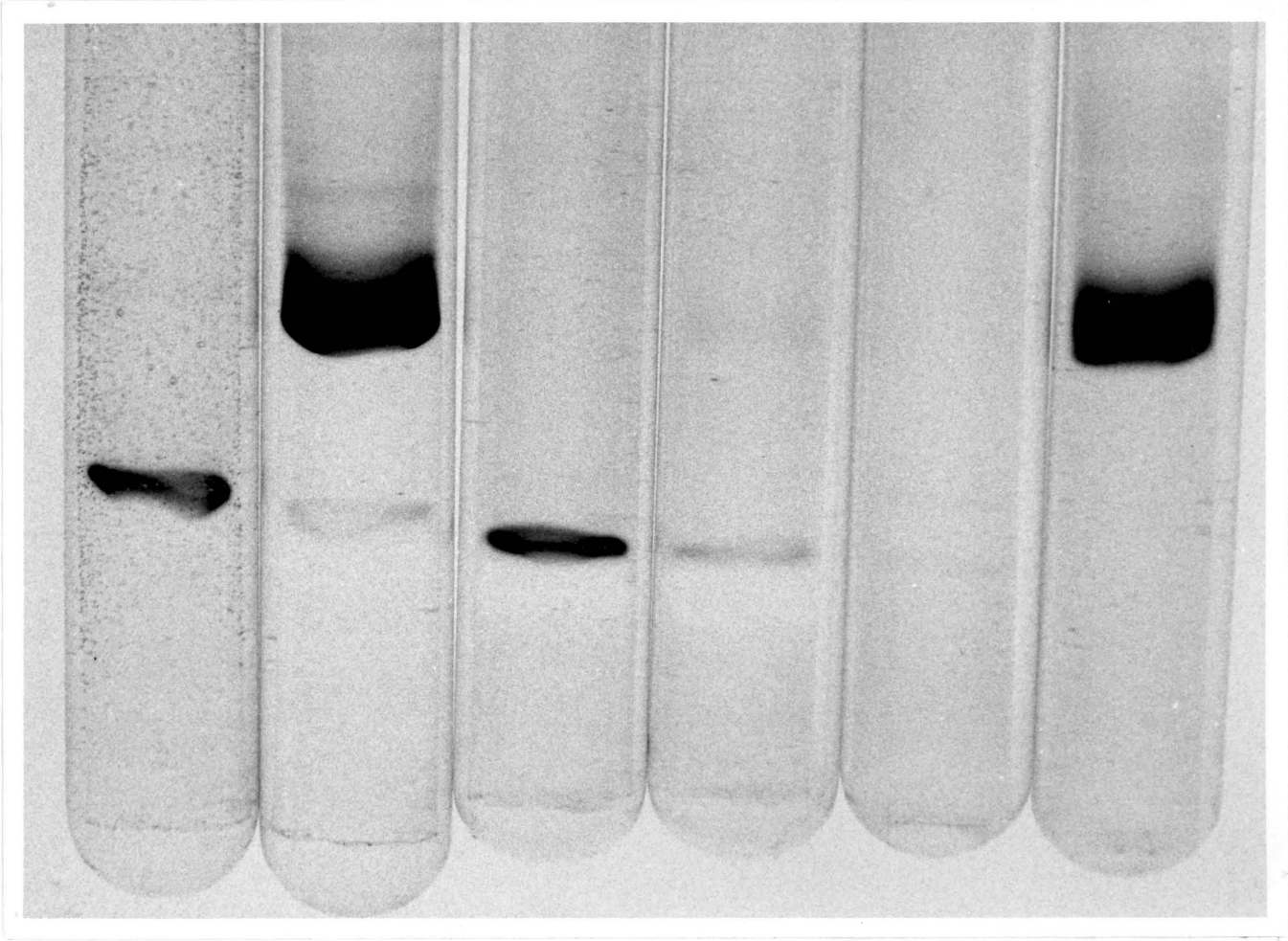
A   B   C   D   E   F

Figure 8.   Sedimentation patterns of <u>Chlorella pyrenoidosa</u> (strain
             7-11-05) glutamate dehydrogenase isozymes, separated by
             analytical disc-gel electrophoresis, relative to bovine
             liver catalase. Isozyme eluants (300 µl) obtained as
             described in Materials and Methods, and 115 µg of bovine
             liver catalase were applied on a linear gradient of
             sucrose (5-20%, w/v; 12.0 ml) prepared in 0.1 M Tris-HCl
             buffer (pH 8.25). The gradients were centrifuged at
             40,000 rpm (SW-40 rotor) for 24 hours at 3°, 3-drop
             fractions collected, and fractions assayed spectrometric-
             ally. Glutamate dehydrogenase units per fraction with
             NADH, ●, or with NADPH, ○, and catalase activity, --·--.
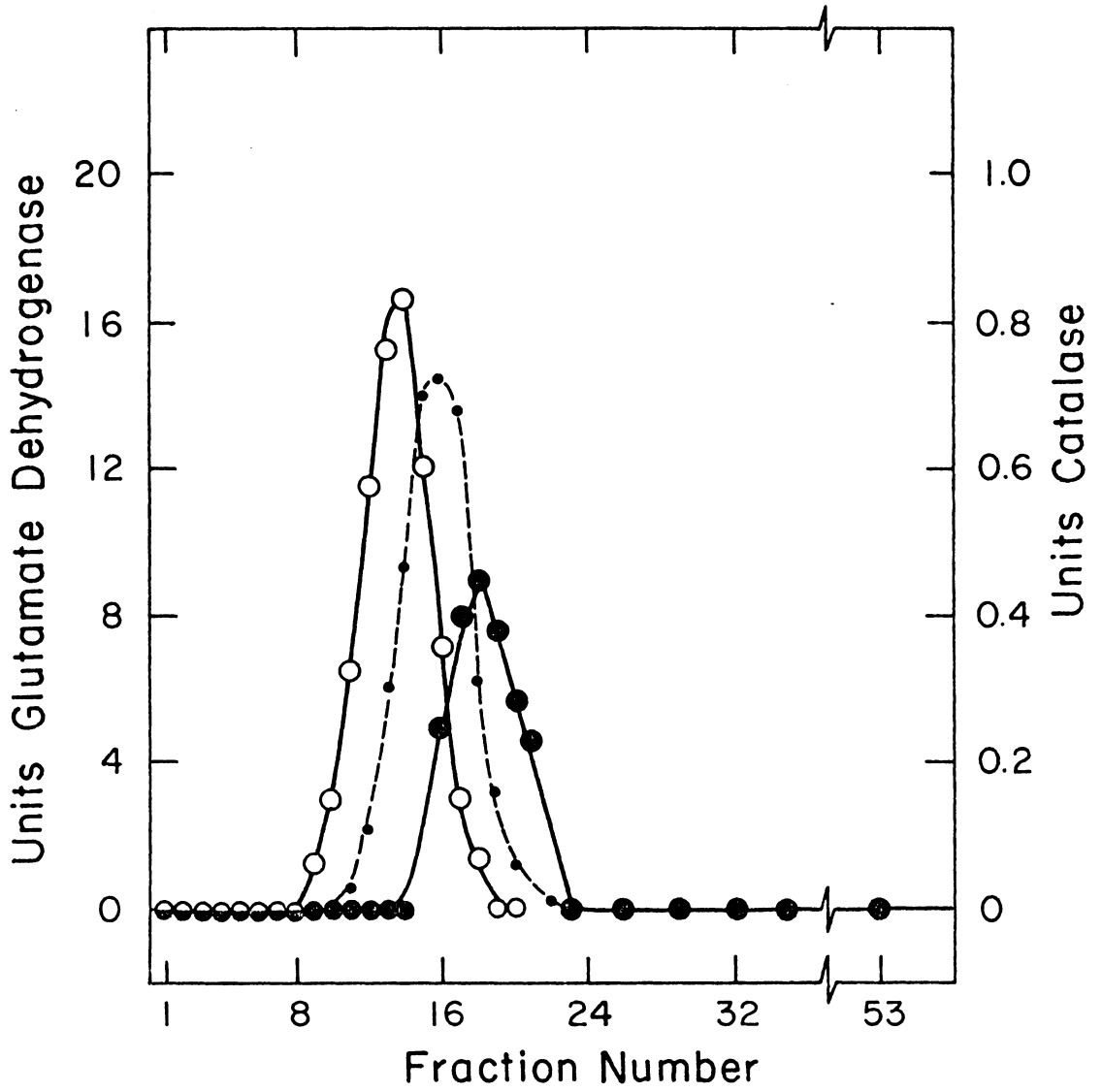
and with a faster mobility on acrylamide gels is designated the NADH-specific glutamate dehydrogenase isozyme.

The Dependence of the Induction of the NADPH-Specific Isozyme on Protein and RNA Synthesis. Jacob and Monod (46) have indicated that under fully induced conditions, the rate of synthesis of an inducible enzyme should be proportional to the rate of protein synthesis. The rate of protein synthesis in Chlorella has been shown to be a function of the effective light intensity per cell (47). By inoculating cultures at different turbidities or cell concentrations, it is possible to vary the effective light intensity per cell and as a result, to vary the rate of cellular protein accumulation. When the initial cell concentration ranged from 26×10^6 to 380×10^6 cells/ml, the protein level per daughter cell increased to a lesser degree during a 4 hour growth period, as the initial cell concentration increased. The linear relationship between the increase in the NADPH-dependent glutamate dehydrogenase activity and total protein as the growth rate of Chlorella daughter cells increases (Fig 9), supports the inference that the NADPH-specific isozyme is fully induced at the ammonium level employed (i.e., 11.4 mM $(NH_4)_2SO_4$). Whereas the NADPH-activity has a linear relationship with protein level per cell at the fourth hour, the NADH-glutamate dehydrogenase activity appears to decrease with increasing protein level per cell (Fig 9). It has been shown in Fig 3 that no accumulation of NADH-glutamate dehydrogenase activity occurs during the first 4 hours of the cell cycle for light-dark synchronized

Figure 9. The relationship of glutamate dehydrogenase activity and protein in <u>Chlorella pyrenoidosa</u> (strain 7-11-05) cultured in ammonium-containing medium at different effective light intensities. Light-dark synchronized cells (division number of 8), were placed in continuous light in ammonium medium in glass tubes at cell concentrations of 26×10^6, 88×10^6, 237×10^6, and 380×10^6 cells/ml to achieve different effective light intensities per cell. At the fourth hour, 2.3×10^9 cells were harvested from each culture, washed three times in 0.01 M Tris-HCl (pH 7.7), resuspended in 6 ml of 0.1 M Tris-HCl buffer (pH 8.25), and ruptured with the French press. Protein and glutamate dehydrogenase activity were determined for 100 µl of the homogenates.

Glutamate dehydrogenase activity assayed with NADH, ●, and with NADPH, ○, for cells cultured at 26×10^6, 88×10^6, 237×10^6, and 380×10^6 cells/ml, corresponding to 66, 50, 34, and 28×10^{-6} mg protein/cell, respectively.
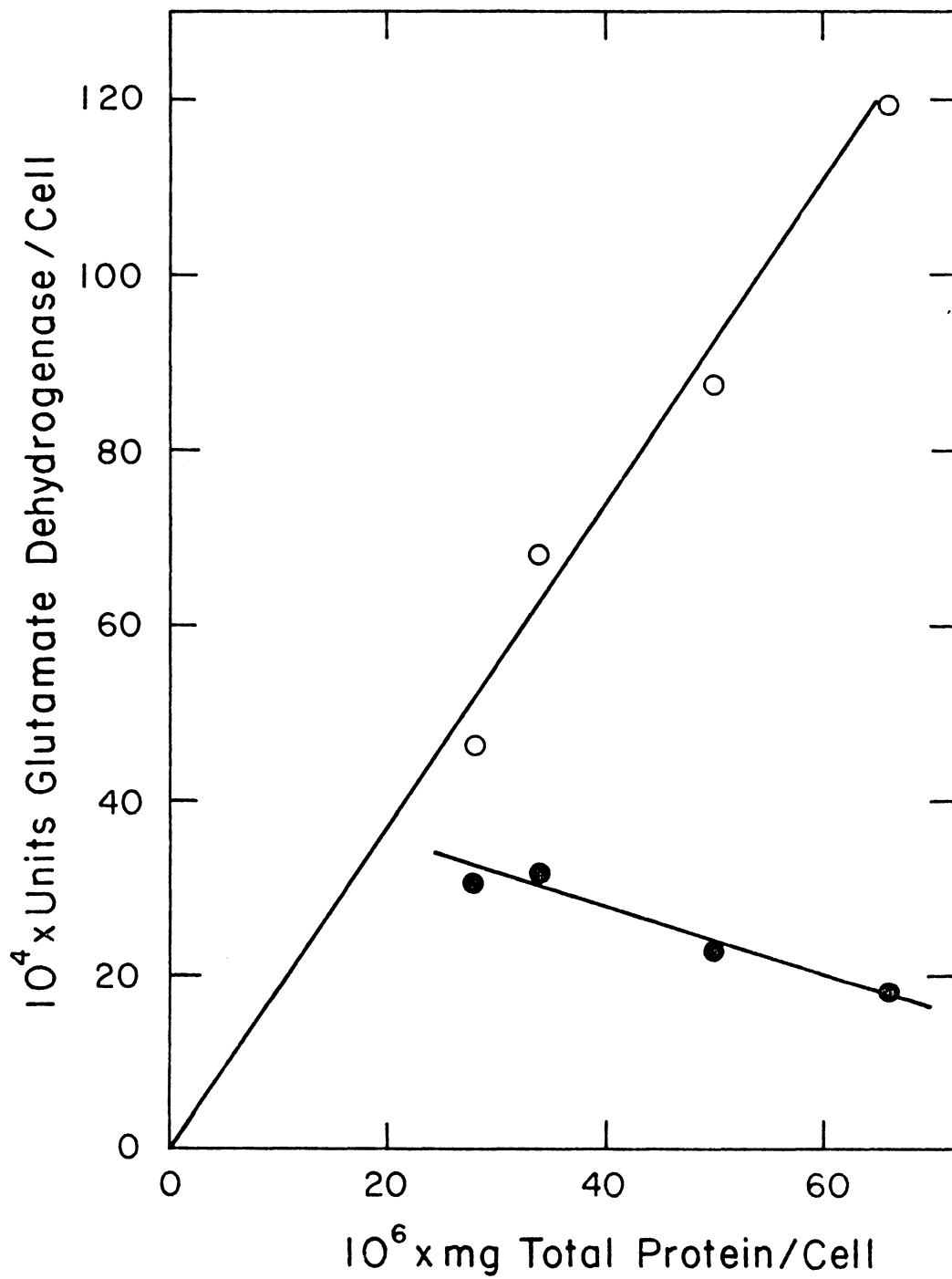
cells. Therefore, it is not surprising that the increase in total protein per cell at the lower cell concentrations in Fig 9 results in a lower specific activity.

When cycloheximide (25 µg/ml) or actinomycin D (200 µg/ml) was present in ammonium culture medium prior to the onset of an 80 min induction period, the increased NADPH-glutamate dehydrogenase activity relative to the control was not seen (Table II). Cycloheximide totally blocked the increase whereas actinomycin D was 85% effective.

The supernatants from cells cultured under these conditions were subjected to analytical disc-gel electrophoresis. The resulting isozyme patterns clearly show the total inhibition of induction of the NADPH-specific isozyme by cycloheximide and to a lesser extent, by actinomycin D (Fig 10). These data demonstrate the dependence of the increased NADPH-glutamate dehydrogenase activity in ammonium-induced cells on both RNA and protein synthesis and show that the increased activity does indeed represent the synthesis of the NADPH-specific glutamate dehydrogenase isozyme rather than an activation phenomenon of endogenous glutamate dehydrogenase activity for NADPH.

Measurement of Inducibility and Potential of the NADPH-Specific Glutamate Dehydrogenase Isozyme During the Cell Cycle. Synchronously growing cells of Chlorella were removed, at hourly intervals, during their cell cycles from nitrate-containing culture medium and were placed on ammonium-containing medium and challenged to synthesize the NADPH-specific GDH isozyme during a 60 min induction period. A

Table II. The inhibition of induced synthesis of the NADPH-specific glutamate dehydrogenase isozyme by cycloheximide and by actinomycin D.

Asynchronous cells cultured in nitrate medium were placed in a small Plexiglass chamber in ammonium medium, ammonium medium plus cycloheximide (25 µg/ml), or ammonium medium plus actinomycin D (200 µg/ml) at cell concentrations of 124 x 10^6 cells/ml and pre-equilibrated (4% CO_2-air; 38.5°) for 15 min in dark. Cultures were then placed in continuous light (550 footcandles) and 3.1 x 10^9 cells were harvested from each culture at 0, 60, and 80 min. The cells were centrifuged, washed three times in 0.01 M Tris-HCl buffer (pH 7.7), resuspended in 6.0 ml of 0.1 M Tris-HCl buffer (pH 8.25), and ruptured with the French press. The resulting homogenates were centrifuged at 100,000 x $\underline{g}$ for 1 hour. The glutamate dehydrogenase activity in 500 µl of the supernatant fractions was assayed spectrophotometrically with NADH and with NADPH, and was used to calculate the level of NADPH-specific glutamate dehydrogenase isozyme. A coenzyme activity ratio of 1:5 for the NADH-specific glutamate dehydrogenase isozyme was used in these calculations.

Treatment	Harvest time (min)	Units total NADH activity	Units total NADPH activity	20% units total NADH activity	Units NADPH-specific isozyme	% inhibition of NADPH
Control	0	31.6	6.4	6.3	0	–
	60	32.0	28.6	6.4	22.2	–
	80	32.7	37.8	6.5	31.3	–
Cycloheximide	0	31.0	6.2	6.2	0	–
	60	28.3	5.6	5.7	0	100
	80	29.2	6.2	5.8	0.4	99
Actinomycin D	0	30.6	6.1	6.1	0	–
	60	27.5	9.0	5.5	3.5	84
	80	26.8	10.1	5.4	4.7	85

Figure 10. Analytical disc-gel electrophoretic patterns showing the
inhibition of induced synthesis of the NADPH-specific
glutamate dehydrogenase isozyme by cycloheximide and by
actinomycin D. Gels were prepared in 5 x 150 mm tubes
using 1.0 ml separator gel (7% acrylamide), 0.5 ml
stacker gel and a sample gel containing 200 μl from
supernatants of cells cultured under the conditions
described in Table II. Electrophoresis was performed at
2 ma/tube for 2 hours 50 min followed by 3 ma/tube for
1 hour 15 min. Glutamate dehydrogenase isozymes detected
using the tetrazolium assay solution containing NAD^+ and
$NADP^+$ in supernatants of cells from A, the ammonium
control culture at 0 min; B, the ammonium control culture
at 60 min; C, the actinomycin D culture at 60 min; and
D, the cycloheximide culture at 60 min.
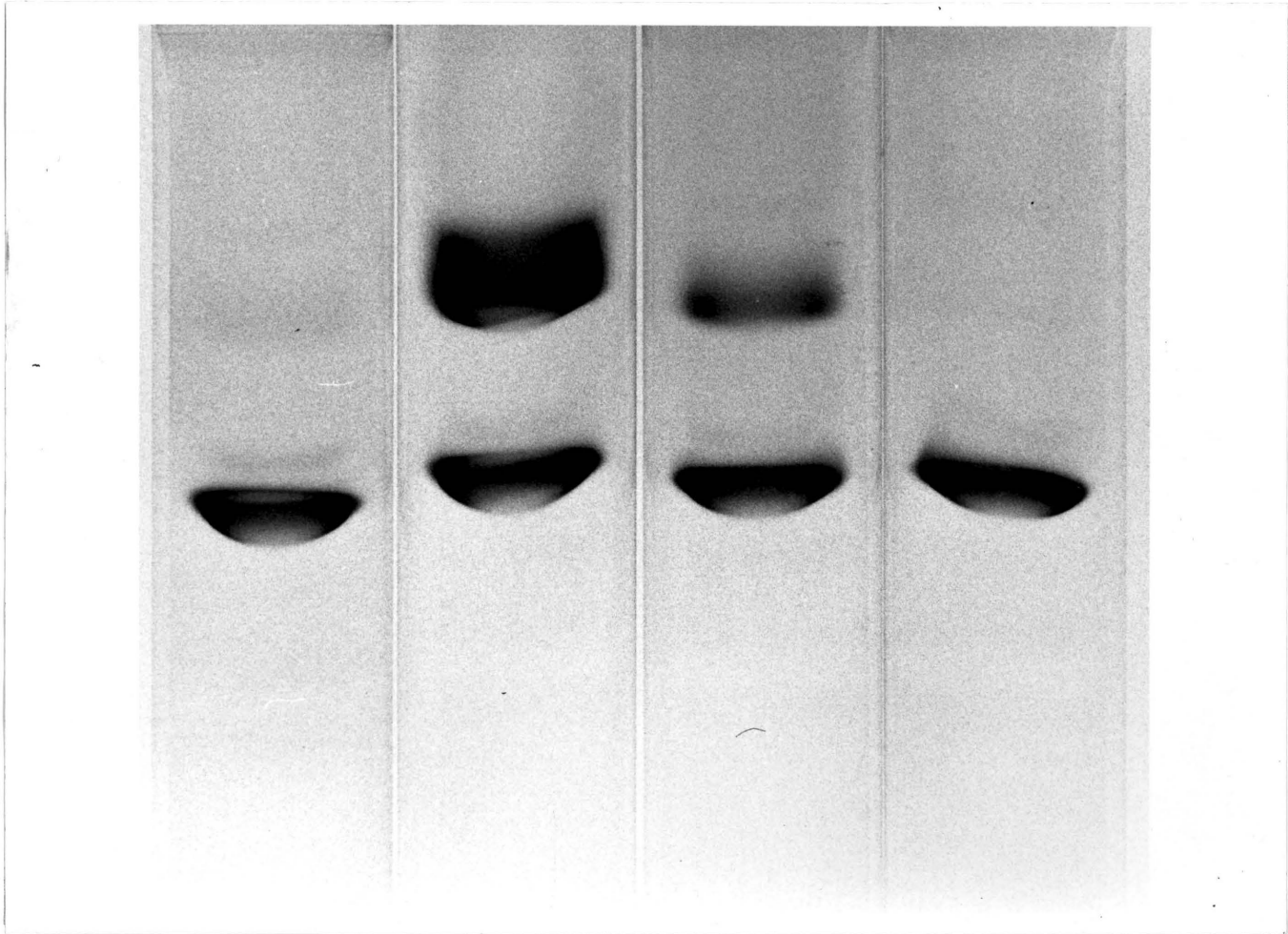
A   B   C   D

visual comparison of the isozyme patterns (on acrylamide gels), from

cells in nitrate medium (Fig 11A) and ammonium induced cultures

(Fig 11B), clearly shows the NADPH-specific isozyme to be inducible

at all stages of the cell cycle, i.e., the isozyme exhibits continu-

ous inducibility during the cell cycle. Since the induction of the

NADPH-specific isozyme is dependent upon both protein and RNA

synthesis, continuous inducibility strongly suggests that the struc-

tural gene of this enzyme is continuously available for transcription

throughout the cell cycle. These results are consistent with those

of Baechtel et al. (25) who showed continuous inducibility of

isocitrate lyase during the cell cycle of this same eucaryote.

If a gene is continuously available for transcription, then the

fully induced rate of synthesis, i.e., potential, of the enzyme should

be proportional to the dosage of its structural gene during the cell

cycle. Thus, the potential should exhibit the same fold-increase as

the total DNA, and it should increase during the period of DNA repli-

cation.

The potential of the NADPH-specific glutamate dehydrogenase was

measured at hourly intervals during the cell cycle. The induction kinet-

ics of the NADPH-specific isozyme, from three different hours of the cell

cycle, is shown before (Fig 12A) and after (Fig 12B) correction for the

NADPH cross-reactivity contributed by the NADH-specific isozyme. A

35 min induction lag, followed by a linear increase in the NADPH-specific

isozyme for at least 80 min, was a characteristic of each stage of

the cell cycle. Therefore, the slope determined between 35 and

Figure 11A,B. Analytical disc-gel electrophoretic patterns showing
              the inducibility of the NADPH-specific glutamate, dehy-
              drogenase isozyme throughout the cell cyle of <u>Chlorella</u>
              <u>pyrenoidosa</u> (strain 7-11-05). Gels were prepared in
              5 x 150 mm tubes using 1.0 ml separator gel (7% acryla-
              mide), 0.5 ml stacker gel, and a sample gel containing
              supernatant of an equivalent number of cells induced for
              60 min at each hour by ammonium to those of the parent
              nitrate culture at each hour. Culturing conditions
              were as described in Fig. 13. Electrophoresis was run
              at 2 ma/tube for 2 hours 50 min followed by 3 ma/tube
              for 1 hour 15 min. Glutamate dehydrogenase isozymes
              were detected with the tetrazolium assay mixture contain-
              ing both NAD$^+$ and NADP$^+$. A, supernatants from nitrate
              cultured cells at hours 0, 1, 3, 4, 7, 8, 9, 10, 11, 12,
              13, 14, 15, and 17; B. supernatant from cells cultured
              60 min in ammonium medium at each of these hours.
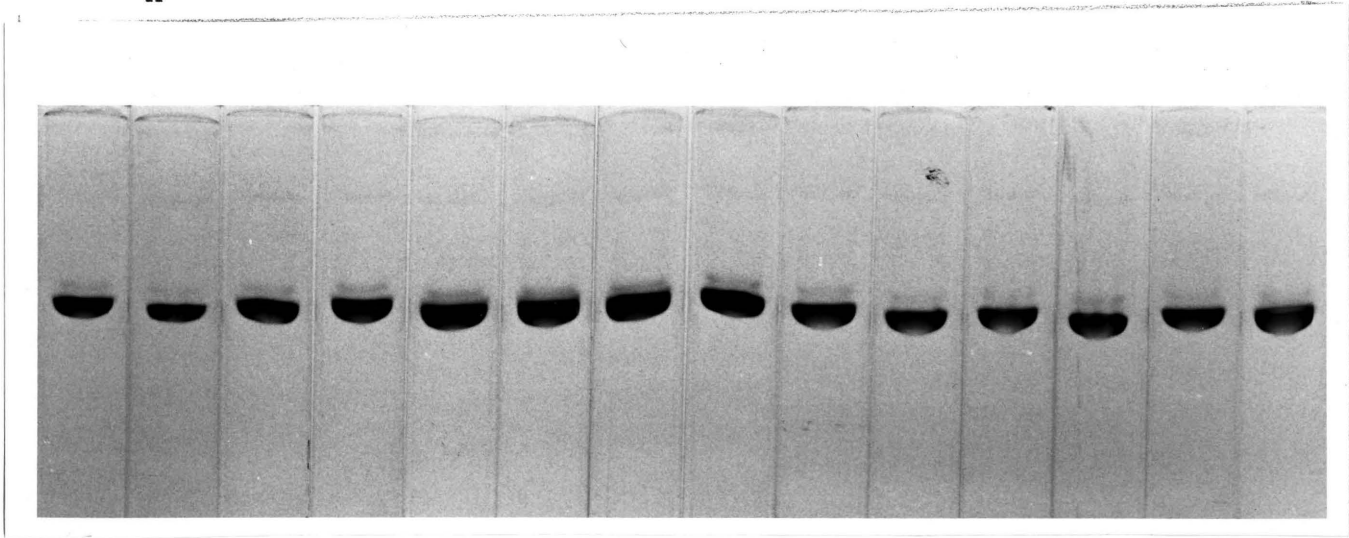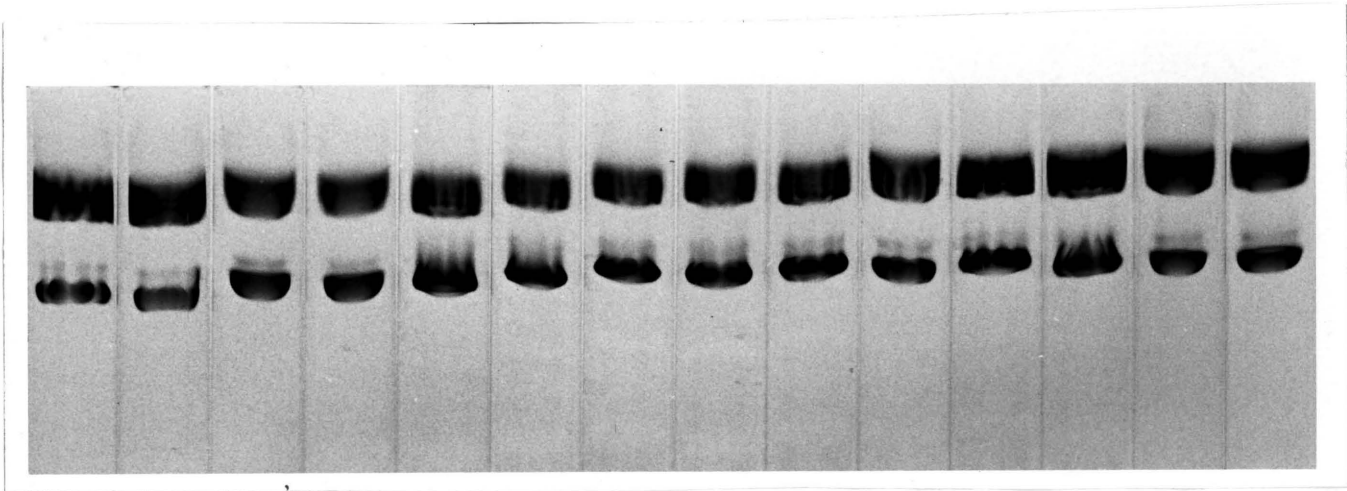
Figure 12A,B. The kinetics of the ammonium induced synthesis of
             the NADPH-specific glutamate dehydrogenase at different
             times in the cell cycle of <u>Chlorella pyrenoidosa</u>
             (strain 7-11-05). Light-dark synchronized cells
             (division number of 4) were gradient selected and
             cultured in a Plexiglass chamber on nitrate medium in
             continuous light at a constant turbidity and an initial
             cell concentration of 191 x 10^6 cells/ml. At the
             second, sixth, and sixteenth hours, cells in 160 ml of
             culture were centrifuged, resuspended in 160 ml of pre-
             equilibrated (4% CO_2-air; 38.5°) ammonium medium, and
             placed in a small Plexiglass chamber of the same
             internal thickness in continuous light. At 10 min
             intervals cells in 15 ml of culture were harvested by
             centrifugation, washed three times in 0.01 M Tris-HCl
             buffer (pH 7.7), resuspended in 6.0 ml of 0.1 M Tris-
             HCl buffer (pH 8.25), and frozen at -20°. The suspen-
             sions were thawed, passed through the French press for
             cell breakage and the resulting homogenates centrifuged
             at 100,000 x <u>g</u> for 1 hour and then frozen at -20°. A,
             the total NADH- and total NADPH-glutamate dehydrogenase
             activities measured spectrophotometrically for cells
             from the second ($\bigcirc$,$\bullet$), sixth ($\triangle$,$\triangle$) and sixteenth
             ($\square$,$\blacksquare$) hours, respectively. B, the NADPH-specific
             glutamate dehydrogenase isozyme induction patterns
             calculated by subtracting 20% of the total NADH activi-
   ties from the total NADPH activities.
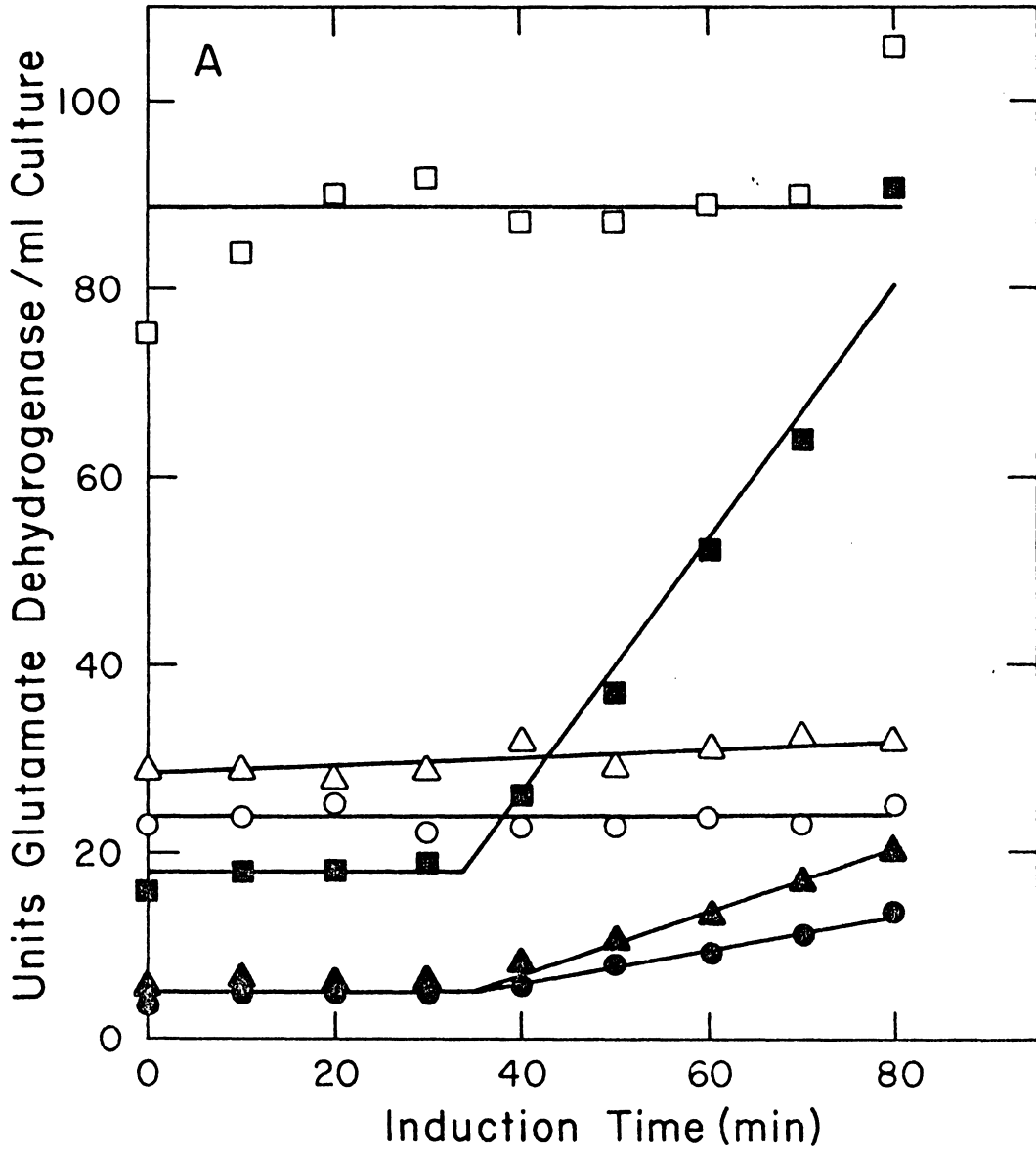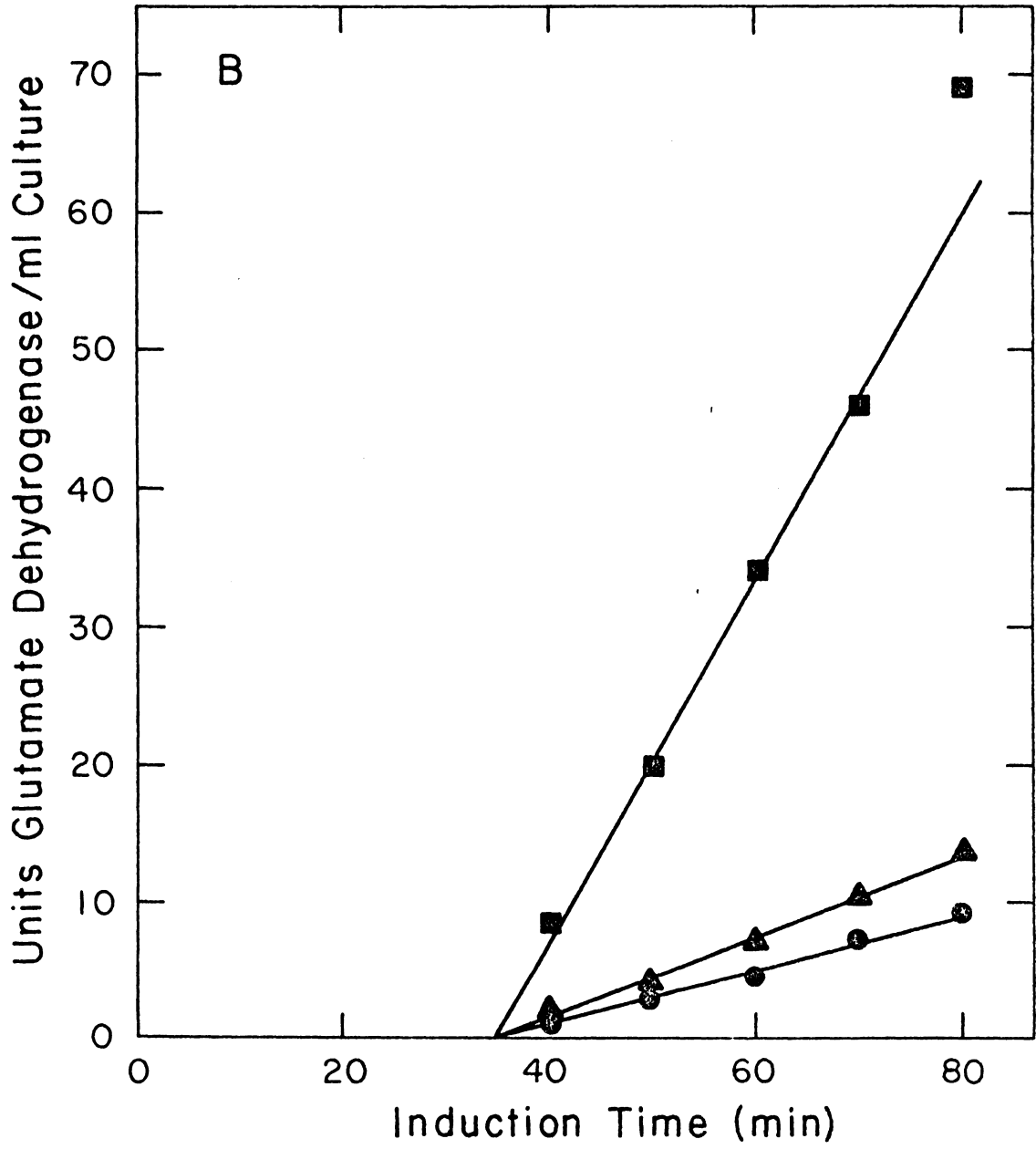
60 min at each hour in the cell cycle was taken as a measure of the potential of the NADPH-specific isozyme at that stage of development. When the slope from each hour was expressed relative to the average of the slopes up to the eighth hour, the potential was observed to increase in a single step during the period of DNA replication (Fig 13). The fold-increase in potential and DNA were essentially the same, 4.15 and 3.75, respectively. The variation in the potential during the first 8 hours of the cell cycle probably contributed to the slight discrepancy in the calculated fold-increase between these two parameters.

Since the effective light intensity per cell can greatly affect the rate of cellular protein synthesis and thus presumably the rate of induction of the NADPH-specific isozyme, it was necessary because of the increase in cell volume during synchronous growth, to perform the inductions at a constant culture turbidity rather than at a constant cell number at each hour during the cell cycle. To achieve this end, the synchronous culture on nitrate medium, from which the cells were taken for hourly inductions, was diluted at hourly intervals to maintain a constant culture turbidity. By accurately measuring both the harvest and dilution volumes, it was possible to correct the data for the dilution by the method of Hare and Schmidt (34). Thus, when cells were taken for the hourly inductions, they were washed free of the nitrate medium, resuspended in exactly the same volume of ammonium medium and placed in a small Plexiglass chamber of identical internal thickness to the parent culture. Although the turbidity

Figure 13. The induction potential of the NADPH-specific glutamate dehydrogenase isozyme and its relationship to DNA during the cell cycle of <u>Chlorella</u> <u>pyrenoidosa</u> (strain 7-11-05). Light-dark synchronized cells (division number of 4) were gradient selected and cultured in a Plexiglass chamber on nitrate medium in continuous light at a constant turbidity. At each hour cells in 160 ml of culture were centrifuged, resuspended in 160 ml of pre-equilibrated (4% CO_2-air; 38.5°) ammonium medium, and placed in a small Plexiglass chamber in continuous light. At 60 min, the cells were harvested by centrifugation, washed three times in 0.01 M Tris-HCl buffer (pH 7.7), resuspended in 6 ml of 0.1 M Tris-HCl buffer (pH 8.25), and frozen at -20°. The suspensions were thawed, passed through the French press for cell breakage, and the resulting homogenates centrifuged at 100,000 x $\underline{g}$ for 1 hour and frozen at -20° for later assay. The relative induced rate of the NADPH-specific glutamate dehydrogenase isozyme, **O**, calculated from the spectrophotometric activities measured with NADH and with NADPH and assuming a 1:5 coenzyme activity ratio for the uninduced isozyme, and the relative level of DNA, **◐**, and cell number, --**▲**--, with an initial rate of 8 units/25 min and an initial level per ml of culture of 76.33 μg and 191 x 10^6 cells, respectively.
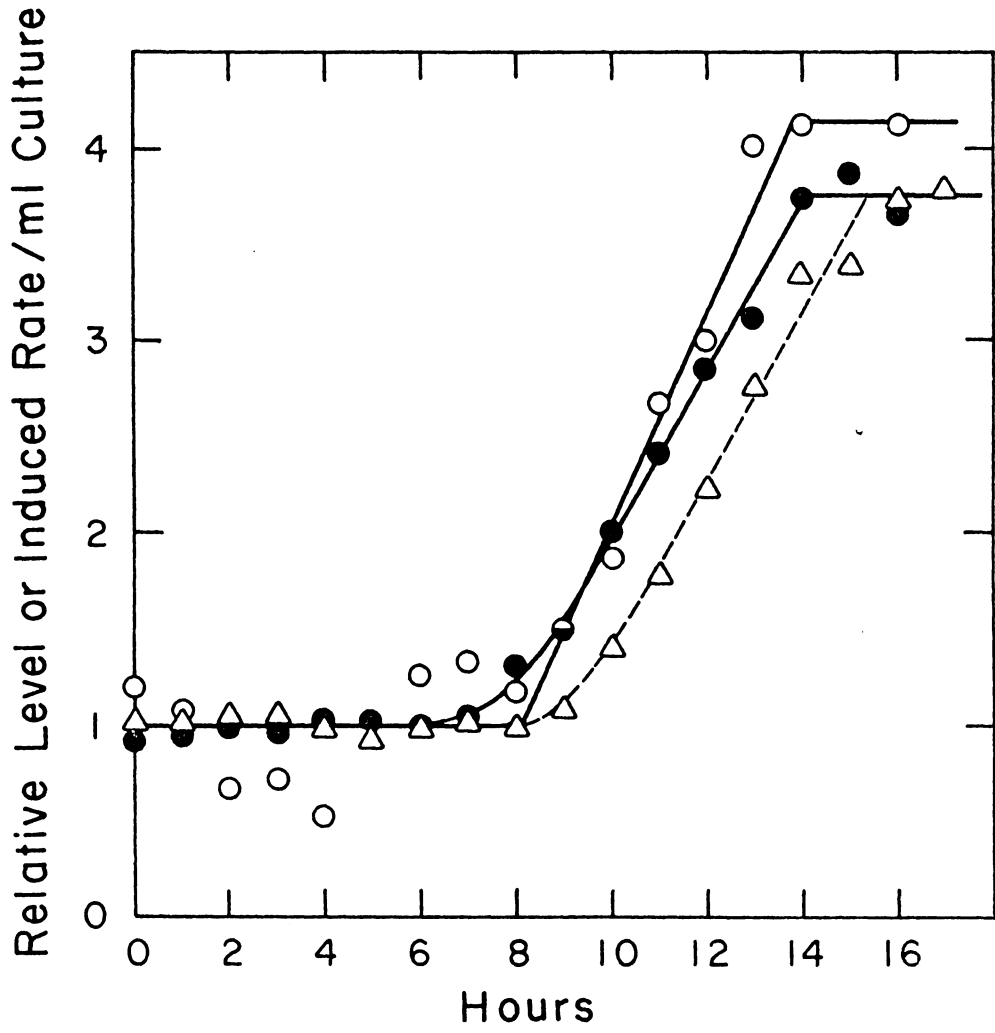
of the culture was maintained within 10% of its initial value during
the cell cycle, the probable source of the variation in measurement
of potential in the present study was the aeration rate of the
cultures between the hourly inductions. Although the aeration rate
of the parent culture was precisely regulated by a flowmeter, the
aeration rate or bubbling rate of the smaller cultures during
induction was regulated visually. It has recently been shown by Dunn
and Schmidt (43) that the aeration rate affects the rate of growth
at the cell concentrations employed in these studies.

Baechtel et al. (25) precisely regulated the culture turbidity
during their measurements of potential of isocitrate lyase during the
cell cycle of this Chlorella. They also observed the potential to
increase in a stepwise manner during the period of DNA replication.
Knutsen (24) used another strain of this Chlorella to measure the
potential of nitrite reductase during the cell cycle. In contrast
to the results reported in this dissertation and those of Baechtel
et al. (25), the potential of nitrite reductase decreased dramatically
near the end of the cell cycle. Knutsen (24) and Mitchison (22)
have interpreted these results to reflect a restricted inducibility
or unavailability of the nitrite reductase structural gene for
transcription during the latter stages of the cell cycle of Chlorella.
Close examination of Knutsen's (24) experimental design reveals that
a constant cell number (after a 6-fold concentration from the parent
culture) was induced for nitrite reductase at each hour in the cell
cycle. Since the cell number and thus culture turbidity increased

approximately 12-fold during the cell cycle, the probable reason for the decrease in potential near the end of the cycle was limiting light.

These studies clearly show that the increase in potential for the NADPH-specific glutamate dehydrogenase coincides with the time of DNA replication and suggests that a relationship exists between the gene dosage of the cell and the potential as is seen in other organisms. The delay period between the increase in gene dosage and the increase in potential reported by Mitchison (23) in Schizosacch--aromyces pombe is not seen. Mitchison (23) has termed this delay period the "critical point" and in studies on the rate of derepression of the sucrase gene at different times during the cell cycle in S. pombe under fully derepressed conditions, he reports that the period occupies one-third of the cell cycle. Changes in sucrase potential during the cell cycle are expressed at the end of each derepression period rather than at the beginning. However, if the rate of derepression of synthesis is related to the gene dosage of the cell, the rate will be a function of the gene dosage at the initial time of derepression rather than at the end.

Since the derepression rate at each time in the cell cycle is measured over a period equal to one-third of the cell cycle, the rates would be shifted by this interval of time if DNA increase and increase in potential were coincident. Therefore, the "critical point" reported by Mitchison may be generated in the way his data is expressed rather than an actual phenomenon related to the

availability of the sucrase gene for immediate transcription after
the replication of the gene.

In the studies on the potential of the NADPH-specific glutamate
dehydrogenase isozyme in this dissertation, the potential is measured
over a 25 min period, an extremely small fraction of the cell cycle.
The rates are expressed at a time in the cycle which corresponds to
the initiation of the induction period, but even if these rates were
expressed at the end of the induction period, as Mitchison has done
for his data, the time at which the increase in potential occurs would
not be significantly changed. It was also possible to measure DNA and
potential in the same experiment, whereas Mitchison is not able to do
this due to limiting cell material for both measurements in a single
experiment. This is possibly another factor which influences the
differences in timing that Mitchison observes for these two events.

The results presented here add additional support to the con-
tinuous availability of genes for transcription in the eucaryotic
organism. By challenging the genome with inducer at hourly periods
during the cell cycle, the induced enzyme synthesis is shown to occur
at all times during the cycle and the potential to reflect the gene
dosage of the cell. This type of experimental approach is critical
in relating the regulatory mechanisms for control of gene expression
in the eucaryotes. It remains to be seen if the temporal mechanism
observed by Halvorson's group (28) in Saccharomyces will be further
supported by this type of study, rather than the experimental approach
they have used in the past (i.e., having the inducer present throughout
the yeast cell cycle).

SUMMARY

The existence of two glutamate dehydrogenase isozymes in Chlorella pyrenoidosa (strain 7-11-05) was demonstrated by analytical disc-gel electrophoresis and by sucrose density gradient centrifugation. The NADH-specific isozyme was synthesized throughout most of the cell cycle of light-dark synchronized cells. The constant level of isozyme during the first 4 hours of the cycle may have represented a repression of synthesis due to a build-up of corepressor levels during the prior dark period. This proposal is offered to explain the continued synthesis of the isozyme at the beginning of the second cycle in continuous light. The level of the NADPH-specific isozyme appeared to be negligible in nitrite-cultured cells.

The NADPH:NADH activity ratios for the NADH-specific and NADPH-specific isozymes were 1:5 and 33:1, respectively; the molecular weights were calculated to be 179,000 and 269,000, respectively. The establishment of these coenzyme activity ratios permitted calculation of the relative activities of the isozymes in a mixture without the necessity of separating the two isozymes for measurements.

The NADPH-specific isozyme was induced by ammonium whereas the NADH-specific isozyme was not significantly affected by ammonium. The induction of the NADPH-specific isozyme was dependent on both RNA and protein synthesis and the isozyme was inducible at all times during the cell cycle. The maximum rate of induction, defined as the potential, increased during the period of DNA synthesis and the fold

60

increase in potential and in DNA were equivalent. This data supports the continuous availability for transcription of the gene for this isozyme in the eucaryote _Chlorella_ and is consistent with the hypothesis that, under fully induced conditions, the gene dosage of the cell governs the potential.

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STUDIES ON THE MECHANISM OF GENE-REGULATED SYNTHESIS OF
DIPHOSPHOPYRIDINE NUCLEOTIDE- AND TRIPHOSPHOPYRIDINE
NUCLEOTIDE-SPECIFIC GLUTAMATE DEHYDROGENASE ISOZYMES
DURING THE CELL CYCLE OF THE EUCARYOTE CHLORELLA

by

Deanna Jean Talley

ABSTRACT

Two glutamate dehydrogenase isozymes were shown to exist in

Chlorella pyrenoidosa (strain 7-11-05) and the cellular level of

these isozymes was regulated by the nitrogen source upon which the

algae were cultured. One isozyme was induced by ammonium and was

specific for the coenzyme, triphosphopyridine nucleotide (TPNH).

The isozyme present in cells cultured in nitrate-containing medium

has a much higher specificity for the coenzyme, diphosphopyridine

nucleotide (DPNH). The TPNH:DPNH activity ratios for the respective

isozymes were 33:1 and 1:5 and their respective molecular weights

were calculated to be 269,000 and 179,000.

The DPNH-specific isozyme was synthesized throughout most of the

cell cycle of light-dark synchronized cells. The level of the TPNH-

specific isozyme appeared to be negigible in the nitrate-cultured

cells.

The induction of the TPNH-specific isozyme was dependent on both

RNA and protein synthesis and the isozyme was inducible at all times

during the cell cycle. The maximum rate of induction or the potential

increased during the period of DNA synthesis and the fold increase in potential and in DNA were equivalent. This data supports the continuous availability for transcription of the gene for this isozyme in the eucaryote <u>Chlorella</u> and is consistent with the hypothesis that, under fully induced conditions, the gene dosage of the cell governs the potential.