USE OF IMMUNOLOGICAL PROCEDURES TO MEASURE RATE OF ACCUMULATION AND DEGRADATION OF INDUCIBLE NADP-SPECIFIC GLUTAMATE DEHYDROGENASE DURING CELL CYCLE OF SYNCHRONOUS *CHLORELLA*

by

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LIST OF ABBREVIATIONS

NADP-GDH	Nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase
NAD-GDH	Nicotinamide adenine dinucleotide-specific glutamate dehydrogenase
SDS	Sodium dodecyl sulfate
IgG	Immunoglobulin G
PPO	2,5-Diphenyloxazole
POPOP	p-bis-[2-(5-Phenyloxazolyl)]-benzene
Temed	N,N,N'N'Tetramethylethylenediamine

INTRODUCTION

The complexity of biochemical and structural organization of the eukaryotic cell gives it the capacity of regulating the cellular level of inducible enzymes in several different ways unavailable to the prokaryotic cell. Other than structural gene replication and transcriptional controls which can vary the amount of precursor mRNA and mRNA, the expression of a eukaryotic gene may be regulated post-transcriptionally by precursor mRNA processing or by mRNA transportation from nucleus to cytoplasm (1,2). When mRNA recognition is regulated to give different rates of enzyme synthesis, control may occur at the translational level (3,4). By varying the stability of the active enzyme with substrate stabilization (5) or covalent modification (6), control may also be post-translational.

In this laboratory, two approaches have been used to facilitate the study of the expression of inducible enzymes in eukaryotic cells. The first approach is to measure enzyme inducibility and enzyme potential (7,8) by addition of the particular inducer to a cell culture that has not been previously exposed to the inducer. Since the level of an inducible enzyme is usually very low or non-detectable in an uninduced cell culture, enzyme potential measurements are not hindered by the presence of a high level of enzyme already accumulated as in fully induced cells. Moreover, these short term initial induction experiments do not involve sufficient enzyme accumulation to levels where the presence of the enzyme or its metabolite might result in feed-back repression of its gene. The second approach is to study the expression of an inducible gene in cell cultures that are continuously exposed to

the inducer (9). This approach might reveal different types of regulatory mechanisms which become operational to prevent the over-production of an inducible enzyme (10). Moreover, by varying culture conditions, which may require the cell to have different levels of an inducible enzyme to support different cellular growth rates, one may cause the cell to shift from one level of regulation to another and thus provide more information about the regulation of the expression of this gene. The work described in this dissertation deals only with the synthesis and degradation aspects of the regulation of one inducible enzyme, the ammonium inducible NADP-GDH (11) in Chlorella. This enzyme is used in this laboratory as a model system to help elucidate at which biochemical level gene expression is regulated under the different conditions mentioned above.

It has long been realized that proteins are constantly being synthesized and degraded in biological systems (12). Even the bacterium E. coli which was believed not to degrade its proteins, but rely on protein dilution by cell division, has been shown to contain 1-7% labile proteins (13). However, the half-lives of only seven E. coli enzymes have been measured in detail (14). Only a small amount of research has been performed on protein turnover in higher plants (15,16) and algae (17). Most turnover studies have been performed on whole rats, their organs, or cell cultures. For example, in the rat liver, the hormone, hydrocortisone, was shown to increase in de novo synthesis rate of tryptophan pyrolase (5). However, in addition to hormonal regulation of enzyme synthesis, the substrate, tryptophan, was observed to play an important role in controlling the tissue level of this enzyme. Tryptophan was

shown to stabilize the enzyme significantly against degradation. When supplied together, tryptophan and hydrocortisone can result in a five-to six-fold increase in tryptophan pyrolase activity per g of rat liver (5). The turnover of tyrosine amino transferase (18,19), acetyl co-enzyme A carboxylase (20), glutamate-alanine transaminase (21), arginase (22), and the dihydrofolate reductase in mouse sacroma cell cultures (23) have also been studied. Collectively, these experiments have shown that (a) an increase of the level of protein in animal cells may reflect enhancement of synthesis and/or stabilization of the protein against degradation, and (b) the decrease in the level of a protein in animal cell may reflect a decrease in the rate of synthesis and/or a shortening of its half-life of degradation. Two recent review articles (14,24) survey the many studies on protein and enzyme turnover in animal cells and bacteria.

A number of enzymes in eukaryotic microorganisms (17,25-34) are being used as models to study the mechanism of enzyme induction in eukaryotic cells. Quinto *et al.* (25) have shown that the glutamine synthetase of *Neurospora crassa* increased in specific activity when the cells were shifted from a good nitrogen source, such as glutamine, to a poor nitrogen source, such as glutamate. By immunoprecipitation of the enzyme with specific antibodies in a pulse-chase experiment, they showed that the increase in enzyme specific activity was proportional to the increase in the amount of immunoprecipitable protein. The enzyme was found to be stable *in vivo*. Changes in the specific activity of the enzyme in the cell homogenate was accounted for by changes in the rate of enzyme synthesis. Moreover, the increase in enzyme synthesis was

accompanied by an increase in the amount of translatable glutamine synthetase mRNA. Hemmings (29) showed that *in vivo* phosphorylation of the *Candida utilus* enzyme NAD-dependent glutamate dehydrogenase during nitrogen starvation resulted in rapid inactivation of the enzyme. Dephosphorylation of the enzyme *in vitro* resulted in reactivation of the enzyme. This organism also has a NADP-GDH which rapidly inactivates upon carbon starvation (28). By specific immunoprecipitation of the enzyme in pulse-chase experiments, Hemmings (29) showed that this loss of enzyme activity was paralleled by a loss of antigenic material to the specific antibody. The mechanism of inactivation was not yet known. By assaying for arginase activity in *Saccharomyces cerevisiae* cultured in the presence of the protein synthesis inhibitor, trichodermin, Bossinger and Cooper (32) have shown that the activity of arginase was stable *in vivo* in the presence of the inhibitor.

In this laboratory, Sitz *et al.* (35) cultured synchronous *Chlorella sorokiniana* (36) in the presence of the protein synthesis inhibitor, cycloheximide, and found that the enzyme ribulose-1,5-bisphosphate carboxylase appeared to have a half-life of 4.5 h *in vivo*. Similarly, Vassef *et al.* (37) demonstrated a half-life of about 3 h for the enzyme aspartate transcarbamylase in *C. sorokiniana*. Under the same culture conditions, Molloy *et al.* (38) showed that phosphoribosylglycinamide synthetase in the same organism appeared to be stable. Covalent modification has been suggested by Solomonson *et al.* (34) for the rapid *in vivo* inactivation of nitrate reductase when ammonium was added to a culture of *C. vulgaris*. The involvement of a cyanide group was demonstrated in the reversible inactivation of the enzyme in crude extract or

in the purified state.

The above short survey is not meant to be an exhaustive review of the literature, but to serve to illustrate the different types of mechanisms which can regulate the levels and activities of enzymes in different types of eucaryotic cells. Moreover, it is apparent from this survey that protein synthesis inhibitors (35), immunological procedures coupled with pulse-chase isotopic techniques (25), and double isotope labeling methods (23) are used to measure the apparent in vivo half-lives of enzymes.

Synchronous cultures can be used to study biochemical events (8,30) which are restricted to discret periods of the cell cycle, e.g., periodic transcription of histone genes (39). Schmidt and Spencer (40) and Schmidt (41) have shown that cell age distributions in asynchronous cultures, growing even under steady-state culture conditions, contain cell-age distributions which are skewed toward daughter cell and intermediate age cells. For example, only 17% of the total cell volume in a steady-state asynchronous culture of Chlorella was associated with cells undergoing nuclear or cellular division. If the increase in activity of an enzyme or sensitivity to a drug only occurs during nuclear or cellular division, this event or sensitivity would appear to be nearly absent if measured in asynchronous cultures. Thus, in synchronous cultures, it is possible to reveal, isolate, and study periodic control-mechanisms (i.e., affecting enzyme levels) which might be otherwise overlooked as "background noise" in asynchronous cultures.

Since Chlorella cells increase in density during cellular development, it is possible to use isopycnic centrifugation, in linear density

gradients of Ficoll (42), to select highly synchronous daughter cells from asynchronous cultures (43) or from cultures which are partially synchronized by intermittent illumination (9). Synchronous cultures obtained by this isopycnic selection procedure, exhibit periodic DNA-replication, nuclear division, and cell division (41). By the use of synchronous cultures, it was shown that Chlorella can be controlled to divide into 2, 4, 8 or 16 daughter cells at cell division at the end of the cell cycle by being illuminated with proportional increases in effective light intensity per cell (11). This increase in cell division number results in proportional increases in DNA content and rates of enzyme and protein accumulation. Thus, whereas in non-photosynthetic organisms the growth rate cannot be easily altered without changing the composition of the culture medium or the culture temperature, the growth rate of synchronous Chlorella cells can be changed simply by increases or decreases in the effective light intensity.

In this laboratory, synchronous cell cultures are being used as an experimental tool to determine whether different regulatory mechanisms control the expression of the structural gene of the inducible NADP-GDH at different stages of the cell cycle of Chlorella sorokiniana.

In addition to the ammonium-inducible NADP-GDH in Chlorella (11,36), this organism also contains a constitutive NAD-GDH isozyme (11,44). This latter isozyme is synthesized in either ammonium- or nitrate-containing culture medium. In this laboratory, both isozymes have been purified, partially characterized, and shown to be physically, chemically, and antigenically distinct from each other (44,45).

Talley et al. (11) showed that the NADP-GDH was inducible through-

out the Chlorella cell cycle. Actinomycin D- and cycloheximide-inhibition experiments indicated that induction of the enzyme was dependent upon both RNA and protein synthesis. In these cell cycle experiments, cells were periodically harvested from a parent synchronous culture, growing in the absence of inducer (i.e., ammonium), and then challenged to synthesize the NADP-GDH. At each stage of the cell cycle analyzed, the enzyme was observed to accumulate in a linear manner, following approximately a 35 min induction lag. The rate of enzyme accumulation between 35 and 60 min was taken as a measure of the initial rate of induction, i.e., enzyme potential (7,8) at each stage of cell development. When enzyme potentials were compared to the pattern of DNA accumulation in a synchronous culture in which each cell was dividing into 4 daughter cells, the enzyme potential abruptly increased fourfold within the S phase of the cell cycle. With improved culture conditions and more highly synchronous cells, Turner et al. (46) showed that enzyme potential also increases continuously during the G-1 phase prior to its abrupt fourfold increase during the S phase. In fact, a close correlation was observed between the increase in enzyme potential and the increase in total cellular protein. The timing of increase in enzyme potential during the S phase was insensitive to large changes (i.e., doubling) in the cellular growth rate. By inhibition of DNA synthesis with 2'-deoxyadenosine, the fourfold increase in enzyme potential normally observed during the S phase was blocked. This compound appeared to be rather specific in that it did not inhibit the increases in culture turbidity, total cellular protein, or the activity of the NAD-GDH isozyme during the period corresponding to the normal S phase.

These results taken collectively suggest that the structural gene of this enzyme is continuously available for transcription (even shortly after its replication) during the cell cycle, and that the abrupt increase in NADP-GDH potential is dependent upon DNA replication within that cell cycle.

Israel et al. (47) used a different experimental approach to study the cell cycle regulation of the NADP-GDH. They wanted to determine whether the regulatory strategy of inducible gene expression would change when preinduced cells were cultured in the continuous presence of an inducer for an entire cell cycle. When preinduced synchronous cells were cultured in the continuous presence of inducer, under conditions to give a fourfold increase in cell number, NADP-GDH activity accumulated in a linear manner throughout the cell cycle with a positive rate change observed within the S phase. However, when the growth rate of the organism was doubled, the positive rate change was displaced from the S phase in the first cycle to approximately the fourth hour of the G-1 phase hour of the subsequent cell cycle. With 2'-deoxyadenosine used as a DNA synthesis inhibitor, the magnitude of the positive rate change was shown to be proportional to the relative increase in DNA in the previous cell cycle. These data support the inference that the timing of expression of newly replicated genes of this enzyme in cells in the continuous presence of inducer is sensitive to the growth rate of the cells. At high growth rates the expression of newly replicated genes can be displaced well beyond the S phase into the G-1 phase in the subsequent cell cycle.

In studies with cycloheximide, Israel et al. (47) provided evidence

that both the inducible NADP-GDH and NAD-GDH were stable *in vivo* during inhibition of protein synthesis. However, when ammonium was removed from the culture medium, the activity of only the NADP-GDH decreased rapidly *in vivo* (i.e., $t_{\frac{1}{2}}$ = 5 to 10 min). The NAD-GDH continued to increase without change in rate of accumulation. The readdition of ammonium during the deinduction period failed to rescue the lost NADP-GDH activity, indicating that this enzyme probably undergoes irreversible inactivation and/or proteolytic degradtation. A very surprising observation was that the addition of cycloheximide at the time of inducer removal prevented the loss in activity of the NADP-GDH during the deinduction period.

Based on the combined observations of (a) the continuous inducibility of the NADP-GDH gene throughout the cell cycle, (b) the apparent differences in the timing of expression of the gene of the NADP-GDH in cells cultured in the absence or continuous presence of inducer, and (c) the sensitivity of the timing of expression of this gene to the growth rate of cells in the continuous presence of inducer, Israel *et al.* (9, 47) proposed a model for the cell cycle regulation of expression of the NADP-GDH gene. In the continuous presence of inducer, the cells were proposed to accumulate a repressing metabolite which then oscillates in concentration during the cell cycle. The metabolite was proposed to repress gene expression or to inhibit some step beyond this process. The timing of the oscillations was proposed to be influenced by the growth rate of the cells. The metabolite was proposed to be absent or at low concentrations in cells growing in the absence of ammonium (or in the presence of this ion for short periods). Thus, in this latter case,

the initial rate of enzyme induction would be directly proportional to the existing gene dosage at any time in the cell cycle. The novel aspect of this model is that oscillatory repression is proposed to be a negative regulatory mechanism which is operative even in cells cultured in saturating levels of exogenous inducer.

To determine the biochemical level at which the apparent delay in expression of newly replicated genes of the NADP-GDH is occurring in fully-induced cells growing in the continuous presence of inducer, it will be necessary to know whether (a) the accumulation of NADP-GDH activity is accompanied by a parallel increase in new enzyme molecules, or represents the activation of pre-existing catalytically-inactive but antigenic precursor, (b) the enzyme is stable in vivo or undergoes degradation (i.e., turnover), (c) the total number of NADP-GDH translatable and untranslatable mRNA sequences and the turnover rate of the mRNA, and (d) the time of replication of the NADP-GDH gene in cells growing at different rates.

The purpose of the present study was to examine (a) and (b) described above by use of immunological procedures and to compare the patterns of accumulation of the NADP-GDH catalytic activity and antigenicity in cells cultured in the continuous presence of inducer, and to determine whether the enzyme undergoes in vivo degradation in fully-induced cells in the absence of protein synthesis inhibitors.

Part of this dissertation research, dealing with purification and in vitro stability of the NADP-GDH and characterization of the conditions affecting the in vivo decrease in NADP-GDH activity after removal of ammonium from synchronous cultures, is described in three papers (9,

45,47) coauthored by myself.

EXPERIMENTAL PROCEDURE

Materials - $NADP^+$ and agarose-hexane-NADP Type 3 were obtained from P & L Biochemicals, Inc.; sheep anti-rabbit IgG, Miles Laboratories, Inc.; DEAE- Sephacel and Sephadex G-200, CNBr activated Sepharose 4B, Protein A-Sepharose CL-4B, Pharmacia Fine Chemicals; electrophoresis reagents, Bio-Rad Laboratories; SDS, highest purity, Gallard-Schlesinger Chemical, Corp; carrier-free $[^{35}S]H_2SO_4$ and L-[3,4,5-^{3}H]leucine 120 Ci/mmole, New England Nuclear Corp; $(NH_4)_2SO_4$, ultra-pure enzyme grade, Schwartz-Mann; nonimmune rabbit serum, Grand Island Biological Co.; New Zealand white rabbits (female), Dutchland Lab Animals, Inc. All other chemicals and materials were of the highest grade available from Calbiochem Corp or Sigma Chemical Co., or otherwise described in Gronostajski et al. (45).

Enzyme and protein assays - In all of the experiments described herein, a spectrophotometric assay (47) was used to measure the deaminating activity of the NADP-GDH. A convenient reaction mixture for 80 assays consisted of 60 ml, 0.0587 M Trizma Base in H_2O (pH unadjusted); 16 ml, 1.02 M L-glutamate in H_2O, pH 7.0, 22°; 4 ml, 20.4 mM $NADP^+$ in 0.02 M Tris-HCl, pH 6.0, 22°. One unit of GDH activity (deaminating) was defined as the amount of enzyme activity required to reduce 1 µmol of coenzyme per min at 38.5°.

Total protein was measured by the method of Lowry et al. (48). To analyze total protein in cell cycle samples, 200 µl of cell homogenate was incubated with 0.8 ml of 1 N NaOH for 12 h and 100 µl of the extract was used for assay of total protein.

Analytical electrophoresis - At the different steps in the subsequent

purification procedure, the homogeneity of the NADP-GDH was examined by native disc-gel electrophoresis (49) and by SDS polyacrylamide gel electrophoresis by the method of Weber and Osborn (50) and Laemmli (51). The SDS gel procedure of Weber and Osborn (52) was modified by incorporation of a 0.2 ml stacking gel, consisting of 2.5% acrylamide, 0.625% bisacrylamide. The stacking gel was prepared from stock solutions which were modified: 1 ml solution A (7.8 g $NaH_2PO_4 \cdot H_2O$, 20.5 g Na_2HPO_4, 2 g SDS, 3 ml Temed, adjust to 1-liter with deionized H_2O); 1 ml solution D (10 g acrylamide and 2.5 g bisacrylamide in 100 ml H_2O); 0.5 ml riboflavin-5'-phosphate solution (4 mg/100 ml H_2O); 1.5 ml 40% sucrose in H_2O. The protein samples were mixed 1:1 (v/v) with a solution of 0.02 M NaH_2PO_4, 2% SDS, 100 mM β-mercaptoethanol, pH 7.0, 22°. After incubation for 2 h at 38°, equal volumes of the aforementioned incubation solution and 40% sucrose were mixed and up to 200 µl were applied to a gel. Approximately 20 to 100 µg protein were added per gel. Electrophoresis was performed for 12 h at 2 mA/gel. The bands of NADP-GDH activity and protein were located by a tetrazolium assay system (described in a later section, and by Coomassie brilliant blue staining, respectively, as previously described (45).

Purification of Nonradioactive NADP-GDH

Preparation of cells - C. sorokiniana (36) was cultured in ammonium-medium (46,52) containing Gentamycin sulfate (30 µg/ml) in a 30-liter Plexiglas chamber (37). The initial culture turbidity was 0.6 (550 nm, 1 cm light path, Beckman DB spectrophotometer) and the culture was harvested after approximately 16 to 17 h at a final turbidity of 11 to

12. By use of a Sharples centrifuge approximately 145 g fresh-weight of cells were harvested from 30 liter of culture. The cells were washed two times with 0.01 M Tris-HCl buffer, pH 8.25, 4°. The resulting pellet of packed cells was resuspended in an equal volume of 0.1 M Tris-HCl buffer (pH 8.25, 4°) and frozen at -20°. The cells were thawed and then ruptured by passage through a 40 ml mechanically driven French pressure cell (model 5-598A, American Instrument Co.) at a pressure of 18,000 to 20,000 p.s.i. The homogenate was centrifuged at 9750 x g for 20 min. The pellet from the first breakage step was mixed with an equal volume of the buffer and passed through the French pressure cell once again. The homogenate from this step was centrifuged for 45 min at 27,000 x g, the supernatant was combined with the supernatant from the first step, and then was frozen at -20°.

35-70% ammonium sulfate fractionation - The frozen supernatant was thawed at 22° and centrifuged for 45 min at 27,000 x g. A solution of saturated $(NH_4)_2SO_4$ was added to bring the supernatant to 35% saturation. If the resulting protein suspension did not have a pH between 6 to 6.2, it was titrated into this pH range with HCl. After 2 h at 4° with occasional stirring, the suspension was centrifuged for 30 min at 27,000 x g and the pellets were discarded. The supernatant was adjusted to 70%, allowed to stand for 2 h, and then centrifuged. The supernatants were discarded and the pellets were dissolved in 0.025 M Tris-HCl, pH 7.4, 4°. The resulting protein solution was transferred into dialysis tubing. Prior to use, the tubing had been boiled in 5% $NaHCO_3$ and then washed in deionized water. The protein solution was dialyzed two times

(8 h, 12 h) against 2-liter each of degassed 0.025 M Tris-HCl, pH 7.4, 4°, under argon in a sealed Erlenmeyer flask.

DEAE-Sephacel ion-exchange chromatography - The protein solution from the previous step was centrifuged for 20 min at 27,000 x g immediately prior to application to a DEAE-Sephacel column (5 x 15 cm) which was equilibrated with 0.025 Tris-HCl buffer, pH 7.4, 4°. The conductivity of the protein solution should not exceed 3 mmho. The column was washed with 400 ml of initial buffer, and then eluted at 3 ml/min with a 1-liter linear gradient from 0 to 0.4 M KCl in 0.025 M Tris-HCl, pH 7.4. The fractions containing 98% of the NADP-GDH activity were pooled and dialyzed two times (6 h, 12 h) against 2-liter each of 0.025 M imidazole buffer, pH 6.0, 4°. The dialysis was performed with degassed buffers under argon in sealed Erlenmeyer flasks.

The dialyzed enzyme preparation was centrifuged and then applied to the same DEAE-Sephacel column described above except it was equilibrated in 0.025 M imidazole buffer, pH 6.0, 4°. The column was washed with 400 ml of the buffer and eluted at 3 ml/min with a 1-liter gradient from 0 to 0.4 M KCl in the imidazole buffer. The fractions containing 98% of the NADP-GDH activity were pooled.

Sephadex G-200 gel-filtration chromatography - The enzyme preparation from the previous step was concentrated by dialysis against a solution of saturated $(NH_4)_2SO_4$. Approximately 170 ml of enzyme solution was dialyzed for approximately 10 h against 600 ml of saturated $(NH_4)_2SO_4$ in the presence of 200 g of crystals of the same salt. At the end of the dialysis period, the contents of the dialysis bag were collected and

the inside of the bag was rinsed with the saturated ammonium sulfate solution. After centrifugation of the protein suspension at 27,000 x g for 30 min, the pellet of protein was dissolved in 10 ml of Tris-glycine buffer (0.19 M glycine, 0.025 M Tris-HCl, pH 8.75, 4°), placed into the same dialysis tubing, and dialyzed against 1-liter of degassed Tris-glycine buffer for at least 4 h, 4°. After this dialysis, an amount of solid dithiothreitol was added to the enzyme preparation, in the dialysis tubing, to bring its concentration to 10 mM. After 30 min at 22°, the enzyme preparation was dialyzed for 4 h against 1-liter of degassed 0.01 M Tris-HCl buffer (0.25 M KCl, 2 mM dithiothreitol, pH 7.4, 4°). It was then dialyzed against a solution of sucrose in the same buffer such that the final sucrose concentration was 20% at the end of the dialysis period. After centrifugation of the dialyzed enzyme solution, 16 ml were applied to a column of Sephadex G-200 (regular; 5 x 75 cm) which had been equilibrated with degassed 0.01 M Tris-HCl buffer (0.25 M KCl, 2 mM dithiothreitol, pH 7.4). The column was eluted with the same buffer by gravity with a 12 cm pressure head, resulting in a flow rate of 0.38 to 0.46 ml/min. Since the void volume of the column was approximately 345 ml, the first 300 ml of eluate was discarded and then 8 ml fractions were collected. The NADP-GDH activity was eluted in 160 ml closely following the void volume. Fractions corresponding to each half (i.e., 80 ml) of the peak of enzyme activity were pooled separately and then dialyzed against saturated $(NH_4)_2SO_4$ in the presence of excess crystals of the salt as described above. The precipitated enzyme was pelleted by centrifugation, dissolved in 0.01 M potassium phosphate, 2 mM dithiothreitol, pH 6.2 (titrated at 22°) and then dialyzed twice

against 500 ml of the same buffer.

Affinity chromatography - An affinity column of NADP-hexane-agarose Type 3 (5 cm diameter x 1.3 cm height, 25 ml with capacity for binding 1250 units of NADP-GDH) was equilibrated at 4° with 0.01 M NaH_2PO_4, 2 mM dithiothreitol, pH 6.2 (titrated at 22°). The enzyme preparation from the previous step was added to the column at 4° and allowed to pass through at a rate of 0.5 ml/min. The column was sequentially washed with 50 ml of the buffer, 30 ml of the buffer with 1 mM NAD^+, and once again with 50 ml of the buffer. The enzyme was eluted with 50 ml of the buffer containing 2 mM $NADP^+$. Enzyme activity was completely eluted from the column in 46 ml of the elution volume. The fractions containing enzyme activity were pooled and ultrafiltration was used to adjust the protein concentration to at least 0.5 mg/ml. At this protein concentration, NADP-GDH activity was completely stable during storage at 4° or -20° in the absence of dithiothreitol.

Preparative polyacrylamide gel electrophoresis - Prior to electrophoresis, the enzyme preparation, containing approximately 600 units (12 mg) of NADP-GDH, was dialyzed into the Tris-glycine electrophoresis buffer (10X) described by Davis (49), pH 8.3, 22°. After dialysis, the preparation was adjusted to 10 mM dithiothreitol and allowed to stand at 22° for 30 min. The preparation was adjusted to 20 to 30% sucrose and subjected to preparative electrophoresis (53) in the Canalco Prep-Disc apparatus in the PD-2/320 column. A 7% separating gel (3 cm) and a 2.5% stacking gel (2 cm) was used. The Tris-glycine electrophoresis buffer contained 2 mM dithiothreitol. The electrophoresis was

performed at 10 mA for 10 min and then 30 mA for the remaining time. The elution rate from the gel was approximately 0.8 ml/min, and 8 ml fractions were collected. The NADP-GDH was eluted between fractions 25 and 41 with an elution volume of 120 ml. Immediately after electrophoresis, the fractions containing enzyme activity were pooled and the pure enzyme was concentrated by ultrafiltration to at least 0.5 mg/ml for storage at -20° in the aforementioned phosphate-NADP buffer.

Purification of Radioactive NADP-GDH

Preparation of cells - The cells were cultured in 2.54 cm glass tubes (18) in 150 ml of ammonium medium (24) containing either 0.11 mM L-[3,4,5-^{3}H]leucine (0.12 mCi/mmol) or 16 mM [^{35}S] sulfate (28 mCi/mmol). The cells were harvested every 12 h, and the culture medium was recycled through another growth period after innoculation with new cells. This growth-harvest regime was repeated 3 additional times with the total yield of 4 g of cells labeled with each isotope. To prevent bacterial contamination in these cultures, the culture medium contained 30 µg/ml of Gentamycin sulfate.

Modifications used for purification of radioactive NADP-GDH - Essentially the same purification procedure was used for the radioactive enzyme as described for the nonradioactive enzyme except all steps were scaled down. The DEAE-Sephacel columns were 3 x 3.6 cm (200 ml, 0 to 0.4 M KCl linear gradient); Sephadex G-200 column, 2.6 cm x 75 cm; NADP-agarose (Type 3) affinity column, 5 ml; preparative polyacrylamide gel electrophoresis, separating gel (3 cm) and stacking gel (2 cm) in the PD-2/70 column. Since the purification began with only 1 mg of enzyme and the

enzyme rapidly denatures at low protein concentration, a ninefold excess of nonradioactive, purified NADP-GDH was added to the radioactive preparation before the Sephadex G-200 chromatography step was performed. Moreover, purified ovalbumin (0.1 mg/ml) was added to the eluate from the Sephadex G-200 column to minimize inactivation of the enzyme on glass surfaces. The elution buffer used in the preparative electrophoresis step also contained ovalbumin (0.1 mg/ml). The purified radioactive NADP-GDH (65 μg/ml, 3.28 units/ml) was stored at -20° in 0.01 M KH_2PO_4, 0.1 mM $NADP^+$, 0.5 mg/ml ovalbumin, pH 6.2. The purification of ^{35}S- and ^{3}H-labeled enzymes yielded 0.1 mg with 205 x 10^6 cpm/mg (10.3 μCi/mg), and 0.15 mg with 3 x 10^5 cpm/mg (0.15 μCi/mg), respectively.

Preparation of antibodies - Preimmune sera and NADP-GDH antisera were prepared from blood obtained from 5, 2 to 3 month old female New Zealand White rabbits. Before immunization of the rabbits with purified NADP-GDH, blood was collected for preparation of preimmune sera. For the initial injection of each rabbit 0.5 ml of a solution of pure NADP-GDH (2 mg/ml) was mixed with an equal volume of Freund's complete adjuvant, and then sonically treated (Sonifier cell disruptor, model W 185D, Heat Systems-Ultrasonics, Inc.; microprobe) twice for 20 s. From the resulting stable emulsion, 0.5 ml was injected intramuscularly into each thigh of the rabbit. This procedure was used for the first and second week injections; however, for the third and subsequent injections, Freund's incomplete adjuvant replaced the complete adjuvant. After the third week, one month booster injections of 0.25 to 0.5 mg enzyme per rabbit were sufficient for maintaining a maximal titer of antibody.

The rabbits were bled from the marginal ear vein. Approximately 20 ml of preimmune serum was collected from each rabbit before the first injection, and subsequent collections were made from 1 to 3 days after each booster injection. From 40 to 50 ml of blood could be collected from each rabbit per bleeding without adversely affecting the rabbit. The blood was allowed to clot for 0.5 h at room temperature in a glass tube, was separated from the sides of the tube with a stirring rod, and allowed to stand overnight at 4°. After centrifuging the clotted blood at 2,000 x g for 10 min, the serum was decanted from the pelleted clot. The serum which constituted approximately 50% of the original blood volume was stored at -20°.

The titer of a given antiserum preparation was determined by measurement of activity of NADP-GDH remaining in solution after a given amount of enzyme and different amounts of antiserum were incubated together under defined conditions and then centrifuged. This procedure was used to determine the mg NADP-GDH precipitated per ml of antiserum preparation at the equivalence point. The defined conditions were 0.05 M Tris-HCl, pH 7.2; 14 µg/ml NADP-GDH (0.7 units/ml); antiserum equivalent to 5 to 38 µg/ml of pure anti-NADP-GDH; 35 min incubation at 22° in 40 µl reaction volume followed by centrifugation at 9,000 x g for 2 min.

The following relationships were used in calculation of immunoprecipitation data. When 100% precipitation of 14 µg NADP-GDH (0.7 units/ml) was achieved by a 5% (v/v) antiserum in the incubation mixture under defined conditions, the antiserum preparation contained 1.15 mg equivalence of pure anti-NADP-GDH per ml. Conversely, 1 ml of the antiserum precipitated 0.28 mg NADP-GDH at the equivalence point. Thus,

1 mg NADP-GDH required 4.11 mg pure anti-NADP-GDH to be completely precipitated at the equivalence point under the defined conditions, i.e., 1 mg pure anti-NADP-GDH precipitated 0.244 mg NADP-GDH.

Purification of rabbit anti-NADP-GDH - The IgG fraction from anti-NADP-GDH antiserum was obtained by two 0 to 40% ammonium sulfate fractionations by the procedure of Palmiter et al. (54). The further purification of the anti-NADP-GDH was attempted by use of three different types of antigen affinity columns in which the NADP-GDH or its subunits were covalently linked to CNBr activated Sepharose 4B.

The covalent attachment of NADP-GDH holoenzyme (non-crosslinked subunits) to CNBr activated Sepharose 4B was achieved by small modifications of the method described by Schimke et al. (55). Approximately 1.5 g CNBr activated Sepharose 4B was washed at 22° on a glass filter with 2 liter of 0.001 M HCl. The ^{35}S-labeled NADP-GDH (6 μg, 2.72 x 10^5 cpm) was added to 0.72 mg of nonradioactive NADP-GDH in 1.5 ml of 10 mM NaH_2PO_4 buffer, pH 7.2, and then 1.5 ml of 0.2 M $NaCO_3$, pH 9.0 was added. To this preparation, 0.6 g (wet weight) of the activated Sepharose gel was mixed in and allowed to react at 22° for 2 h with occasional mixing. The gel was removed by centrifugation for 5 min at 3,000 x g and then reacted with 5 ml of 1 M ethanolamine (pH 8.0) for 2 h, 22°, to block the excess activated sites. The suspension was centrifuged and the gel was resuspended in 10 ml of 0.1 M sodium acetate and 1 M NaCl, pH 4.8. The gel was packed into a 1 cm diameter column and washed with 3 cycles of 10 ml each of the previous buffer alternating with 0.1 M $NaCO_3$, 1 M NaCl, pH 7.6. The amount of ^{35}S which was eluted with each

wash was monitored. The column was equilibrated with 40 ml of 0.01 M NaH_2PO_4, 15 mM NaCl, pH 7.2. By this procedure, 69% of the holoenzyme was bound to the column. Because the enzyme linked to this column was unstable during elution of bound anti-NADP-GDH antiserum, this column could not be used in the purification of anti-NADP-GDH. The specific problems will be described in RESULTS AND DISCUSSION.

The procedure for coupling of NADP-GDH subunits to CNBr activated Sepharose 4B was similar to that described previously for the holoenzyme (non-crosslinked). The ^{35}S-labeled NADP-GDH (4.5 µg, 2 x 10^5 cpm) was mixed with 2.6 mg of nonradioactive enzyme, dialyzed overnight against 400 ml of 6 M urea, 0.01 M β-mercaptoethanol in 0.1 M $NaCO_3$, pH 9.0, 4°, and then incubated at 38° for 2 h. The β-mercaptoethanol was removed by dialysis against the same buffer without the reagent. The enzyme preparation was then reacted with 1.0 g (dry weight) of washed CNBr activated Sepharose 4B for 2 h, 38°. Only 10% of the ^{35}S-NADP-GDH appeared to be unbound after this step. The gel was removed by centrifugation and resuspended in 4 ml of reaction buffer and dialyzed overnight against 2-liter of 0.01 M KH_2PO_4, pH 6.2, 4°. The dialyzed gel was reacted with ethanolamine, packed into a small column, and washed as described for the holoenzyme affinity column. Because the anti-NADP-GDH had very high affinity for the subunit column and could not be eluted from the column without being inactivated, this column could not be used for purification of anti-NADP-GDH. The specific problems encountered will be described in RESULTS AND DISCUSSION.

A completely stable antigen affinity column from which anti-NADP-GDH could be bound and eluted with high efficiency was developed by

Turner (56) in this laboratory. The holoenzyme was coupled to CNBr activated Sepharose 4B as described above and then the subunits were crosslinked with dimethyl suberimidate (57). The NADP-GDH antibody purified by this method is referred to as pure anti-NADP-GDH throughout this dissertation. I supplied Turner (56) with the purified ^{35}S-NADP-GDH which was coupled to and crosslinked on the column, and then used to evaluate the stability of the crosslinked enzyme to anti-NADP-GDH, and to the various buffer washes.

Microdialysis procedure - During the course of preparing and testing the different antigen affinity columns, with covalently-bound ^{35}S-NADP-GDH, to be used for purification of the rabbit anti-NADP-GDH IgG, a microdialysis procedure was developed. The center of the top of a 1.5 or 0.4 ml Eppendorf centrifuge tube was hollowed out with the narrow blade of a penknife. A 100 µl sample to be dialyzed was pipetted into the bottom of the centrifuge tube. A small square of boiled dialysis tubing (Spectrapor 3 for extra-strength) was capped in place across the opening of the centrifuge tube. With a swift swing of the wrist, the tube was inverted and the sample placed on top of the dialysis membrane. While the tube remained inverted, a drop of dialysis buffer was squirted onto the hollow depression of the centrifuge tube cap and the tube was then lowered into a beaker of the buffer and allowed to dialyze overnight with magnetic stirring. At the end of the dialysis period, the tube was turned upright and centrifuged for a few seconds in an Eppendorf centrifuge to return the dialyzed sample to the bottom of the tube. This method was also used to concentrate small volumes of dilute protein

samples for disc-gel electrophoresis. The samples were dialyzed against a buffer 20% sucrose solution.

Purification of preimmune or nonimmune rabbit IgG - The IgG fraction from 100 ml of nonimmune rabbit serum (lot #0681326), obtained from Grand Island Biological Co., was purified by Turner (56) by affinity chromatography on a column of Protein A-Sepharose CL-4B by a procedure described by Miller and Stone (58).

Purification of sheep anti-rabbit IgG - The sheep anti-rabbit IgG which was obtained from Miles Laboratories, Inc., had been pre-purified from sheep antiserum by DEAE-cellulose chromatography, and was used without further purification.

Rocket immunoelectrophoresis - For this electrophoresis procedure (59), 15 ml of 1% agarose in Bio-Rad Svendsen buffer III was autoclaved for 3 min, allowed to cool to approximately 60°, mixed with 38 µl of $(NH_4)_2SO_4$ fractionated anti-NADP-GDH IgG (equivalent to 87 µg of pure anti-NADP-GDH), and then poured onto a 10 x 10 cm clean glass plate. By use of a Bio-Rad gel puncher and punching plate, 4 mm diameter wells, 7 mm apart were punched 2 cm from one edge. Fifteen microliter samples (2 to 8 µg/ml of NADP-GDH) from 100,000 x g supernatant from cell homogenates were pipetted into the wells, and the "rockets" were formed by 14 h of electrophoresis with Svendsen buffer III at 160 V in a Bio-Rad electrophoresis cell (model 1400) at 4°. Because the NADP-GDH was discovered to remain active after precipitation by rabbit anti-NADP-GDH, Bascomb and Yeung (60) developed a procedure by which the "rockets" could be

visualized by a modified tetrazolium activity stain (61,62). The components of the stain include: 10 ml of incubation mixture described earlier for measurement of NADP-GDH deaminating activity; 10 ml H_2O; 0.25 ml phenazine methosulfate solution (49.8 mg/25 ml H_2O); 0.5 ml Nitro-Blue Tetrazolium solution (126.5 mg/25 ml H_2O); pH 8.3; incubate at 22° for 6 h. Peak heights were measured before the gel was removed from the glass plate for fixing in 7% acetic acid and dry-mounting on white cards.

The tetrazolium activity stain permitted detection of "rockets" from nanogram quantities of NADP-GDH per ml. However, since the pure NADP-GDH was very unstable at concentrations below 90 µg per ml, a standard curve of "rocket" height (or area) versus concentration of the pure enzyme in the lower nanogram range could not be accurately constructed. Ninety-two micrograms of pure NADP-GDH per ml buffer was subsequently shown to give the same size rocket as an equivalent amount of NADP-GDH activity in a 100,000 x g supernatant of a whole-cell homogenate prepared from asynchronous cells. Since the enzyme was stable during dilution in the 100,000 x g cell extract, a linear standard curve of rocket height versus enzyme concentration (15 to 125 ng/ml) was constructed by serial dilution of the extract. For this study, the amount of anti-NADP-GDH IgG in the agarose was increased to 350 µg per 15 ml.

Quantitative immunoprecipitation of NADP-GDH - For direct immunoprecipitation (23), a homogenate of ^{3}H-labeled cells (cultured in ammonium medium containing 0.11 mM ^{3}H-leucine), was frozen (-20°), thawed, and centrifuged for 25 min at 16,000 x g in a 400 µl Eppendorf centrifuge tube in

the HB-4 rotor of a Sorvall refrigerated centrifuge. The supernatant contained approximately 3 units/ml and 60 μg/ml of NADP-GDH. The 210 μl incubation mixture for the immunoprecipitation consisted of: 100 μl supernatant (28.5 μg NADP-GDH/ml); 50 μl 4% Triton X-100, 4% sodium deoxycholate, 50 mM sodium phosphate, pH 7.0; 60 μl $(NH_4)_2SO_4$ fractionated anti-NADP-GDH IgG (equivalent to 650 μg pure anti-NADP-GDH). The above mixture was layered on top of 20% sucrose (containing 1% Triton X-100, 1% sodium deoxycholate, 12 mM NaH_2PO_4, pH 7.0) in a 400 μl Eppendorf centrifuge tube. The immunoprecipitation reaction was allowed to proceed for 90 min at 22°, and then the mixture was centrifuged for 20 min at 16,000 x g in a HB-4 rotor. The supernatant above the sucrose was removed and saved, and the column of sucrose was frozen by placing the centrifuge tube into liquid nitrogen. The tip of the centrifuge tube with the radioactive immunoprecipitate was cut off and dropped into a 1.5 ml Eppendorf centrifuge tube which contained 80 μl of 2% SDS, 60 mM β-mercaptoethanol, 50 mM NaH_2PO_4, pH 7.0. After 3 h at 38° in this solution, the immunoprecipitate dissolved completely. An equal volume of 40% sucrose was added, and the dissolved immunoprecipitate was subjected to SDS electrophoresis for 14 h at 1 mA/gel in a 9% acrylamide gel, pH 7.0, by the method of Weber and Osborn (50). A 10 μl aliquot of the supernatant, removed from the top of the sucrose column, was also analyzed by the same SDS electrophoresis procedure. To locate the position in the gels of radioactive enzyme from cell extracts, purified ^3H-NADP-GDH in buffer was also subjected to SDS electrophoresis in an identical manner.

After electrophoresis, the gels were soaked in 7% acetic acid for

24 h, frozen on dry ice, and then cut into 1 mm slices with the mechanically driven Mickle Gel-Slicer (The Mickle Laboratory Engineering Co., England; distributed in U.S.A. by Brinkmann Instruments). Two gel slices were added to each scintillation vial, and the gels were dissolved by incubation with 0.4 ml of 30% H_2O_2 in the vial tightly capped for 7 h at 60°. After cooling, 0.6 ml H_2O and 10 ml scintillation counting solution (63) were added. The counting efficiency of this mixture was 22.7% for the ^3H-window of a Beckman LS-133 liquid scintillation counter.

For indirect immunoprecipitation of NADP-GDH by a rabbit anti-NADP-GDH (primary antibody) and sheep anti-rabbit antibody (secondary antibody) system, two different procedures were employed. The first procedure was similar to one described earlier (55). This procedure involved the formation of an antigen-primary antibody/secondary antibody complex followed by low-speed centrifugation of this complex through a column of sucrose containing detergents. To evaluate this procedure, a homogenate of ^{35}S-labeled cells was frozen at -20°, thawed, and centrifuged at 20,000 x g. The volume of the primary reaction mixture was 160 µl and contained 2.9 µg/ml NADP-GDH (i.e., 110 µl frozen-thawed supernatant), 4 mg/ml bovine serum albumin, 1% Triton X-100, 1% sodium deoxycholate, 0.25 mM phenylmethylsulfonyl fluoride, 12.5 mM NaH_2PO_4, pH 7.0 and either 63 µg/ml of pure anti-NADP-GDH or an equivalent amount of $(NH_4)_2SO_4$ fractionated anti-NADP-GDH IgG. The primary reaction was allowed to proceed for 90 min at 0°. For the reaction with secondary antibody, 220 µg sheep anti-rabbit IgG was added to bring its final concentration to 1.05 mg/ml in a reaction volume of 210 µl. After 90

min at 0°, this reaction mixture was layered on a discontinuous sucrose gradient composed of 50 µl of 15% sucrose and 200 µl of 30% sucrose. Both sucrose layers contained 1% Triton X-100, 1% sodium deoxycholate, 1 mg/ml bovine serum albumin, 0.25 mM phenylmethylsulfonyl fluoride, 12.5 mM NaH_2PO_4, pH 7.0. After centrifugation for 5 min at 8000 x g, the immunoprecipitate was analyzed by a small modification of the SDS electrophoresis procedure described earlier for direct immunoprecipitation. The modification consisted of boiling the immunoprecipitate in 4% SDS, 300 mM β-mercaptoethanol, 20 mM NaH_2PO_4, pH 7.0 for 20 min at 100° in a tightly capped Eppendorf centrifuge tube . After boiling, an equal volume of 60% sucrose was added to the sample.

The second indirect-immunoprecipitation procedure employed was a modification of one described by Quinto et al. (25). In their procedure, the antigen and primary antibody were reacted before the centrifugation step and this initial antigen-primary antibody complex was reacted with the secondary antibody during high-speed centrifugation of the mixture over a sucrose-detergent column. In an attempt to decrease the immunoprecipitation of nonspecific radioactive proteins, the cell extract was diluted sixfold for both the primary and secondary reactions. To establish the reactions conditions, the following protocol was used. ^{35}S-labeled cell extracts were dialyzed against 50 mM Tris-HCl, pH 7.2, 4°, and then centrifuged at 100,000 x g for 1 h. For the 1.03 ml primary antibody reaction: 0.75 ml of the 100,000 x g supernatant (i.e., 0.267 µg/ml NADP-GDH in final reaction volume) plus 6 to 10 µl of pure ^{35}S-NADP-GDH or cold carrier NADP-GDH to bring final enzyme concentration to 0.672 µg/ml in final reaction mixture; 0.25 ml solution of 4% Triton

X-100, 4% sodium deoxycholate, 16 mg/ml bovine serum albumin, 1 mM phenylmethylsulfonyl fluroide; 50 mM Tris-HCl, pH 7.2; 25 µl pure anti-NADP-GDH (5.75 µg/ml of final reaction volume.) After 90 min at 0° in 1.5 ml Eppendorf centrifuge tube, 50 µl of sheep anti-rabbit IgG (4 mg/ml) was added to give a concentration of 200 µg/ml in reaction mixture of 1.08 ml. This mixture was quickly transferred onto a 10 ml column of sucrose-detergent solution in a polyallomer centrifuge tube and centrifuged at 4° for 90 min at 50,000 x g in a Beckman SW-41 rotor. The sucrose-detergent column was composed of 5% sucrose, 1 mg/ml bovine serum albumin, 1% Triton X-100, 1% sodium deoxycholate, 0.25% phenylmethylsulfonyl fluoride, 50 mM Tris-HCl, pH 7.2, 4°. After centrifugation, the tube was placed in a special holder such that the mid-section of the sucrose-detergent column could be frozen with dry ice without freezing the immunoprecipitate. A hot nichrome wire was used to cut the tube in half through the ice core. The top half of the tube was discarded, and the bottom half was allowed to stand at room temperature for 10-20 min until the ice crown melted. A Pasteur pipette was used to remove the sucrose until only about 0.5 ml remained in the bottom of the tube. The contents of the tube were frozen in liquid nitrogen, lyophilized, and dissolved in 200 µl of the last SDS incubation buffer described earlier in this section. The tube was capped with aluminum foil and a rubber band, boiled for 20 min, and centrifuged for 30 s at 2,000 x g. Sixty microliters of warm 60% sucrose were added and mixed. An 80 µl aliquot was added to a 9% acrylamide gel for analysis by SDS electrophoresis, pH 7.0. The total radioactivity of a second 80 µl sample was measured directly by liquid scintillation. The remaining

sample volume in the tube was quantitatively transferred to a scintillation vial by two 0.5 ml rinses of distilled H_2O. After addition of the Triton-toluene counting solution, the radioactivity of this portion of the sample was measured. By this procedure, the total amount of ^{35}S-NADP-GDH which was immunoprecipitated could be accounted for. The counting efficiency for ^{35}S-labeled proteins was 92% on the 0-100 open window of the Beckman LS-133 counter.

The Triton:toluene 1:2 (v/v) counting solution was composed of 16 g PPO and 400 mg POPOP/4 liter. No water was added for measurement of radioactivity of dry samples and aqueous samples were adjusted to contain 1 ml of H_2O. Ten milliliters of scintillation counting solution were used per vial.

Measurement of ^{35}S-sulfate uptake into cells cultured in ammonium medium - The cells were synchronized by 3 light: dark cycles (7 h:5 h) in ammonium-medium (16 mM sulfate) under conditions (47) in which the culture turbidity increased fourfold (2.1 to 8.4) during each cell cycle in glass 2.54 cm culture tubes. The cells were harvested by centrifugation and washed 3-times with low sulfate medium (100 μM). For the uptake studies, the cells were resuspended in 30 ml of low sulfate medium which had been pre-equilibrated at 38.5° and 4% CO_2-air. The cell suspension (A_{550} = 7.4; 175 x 10^6 cells/ml) was poured into a flat Plexiglas chamber and aerated at a controlled flowrate with 4% CO_2-air. After 10 min equilibration in the dark, the culture was illuminated and 2.45 mCi of $[^{35}S]H_2SO_4$ (carrier-free in 250 μl) was added immediately. The time of addition of the isotope was taken as zero time for the uptake measurements.

The culture turbidity was measured hourly. Based on the hourly increase in culture turbidity, a calculated volume of cell suspension was harvested, and the cells were removed by centrifugation. The supernatant culture medium was rapidly returned to the growing culture. By this procedure, a constant culture turbidity was maintained without greatly perturbing the ^{35}S-sulfate concentration in the culture medium. After removal of the cells, a small aliquot was taken for measurement of culture-medium radioactivity. The harvested cells were washed 3-times with 5 ml ammonium-medium containing 16 mM non-radioactive sulfate, and then twice with 0.1 M Tris-HCl, pH 8.25 at 4°. The washed cells were stored at -20° in 3 ml of 0.1 M Tris-HCl, pH 8.25 at 4°. The cells were thawed and then broken by two passages through the small-volume French pressure cell at 20,000 p.s.i. Ten microliters of cell homogenate were diluted twentyfold with H_2O, and radioactivity was measured in 10 ml of scintillation counting solution. Another 10 µl of cell homogenate were added to 500 µl of 10% trichloroacetic acid and allowed to stand for 20 min at 22°. This mixture was centrifuged for 2.5 min at 9,000 x g and the supernatant was discarded. The cells were then extracted 5-times each with 1 ml of 90% ethanol. The resulting pellet of cells was resuspended in 10 ml of scintillation fluid, and its radioactivity measured. To correct for quenching, pure ^{35}S-labeled NADP-GDH was used as an internal standard in these samples.

Cell cycle studies - The cells were pre-synchronized by light-dark cycles as described in the previous section. At the end of the third dark cycle, 1130 ml of daughter cell suspension were harvested and

quickly chilled on ice to 4°C. The cells were removed from the medium by centrifugation in a Sorvall GSA rotor for 5 min at 10,000 x g. Approximately 6 g (fresh weight) of cells were resuspended in 14 g of ice-cold deionized H_2O. The cells were age-fractionated by isopycnic ultracentrifugation by a modification of the procedure described by Sitz et al. (42). Five grams of cell suspension were layered on top of each of 4 linear density-gradients of aqueous Ficoll (33 ml, 23 to 31.5%, w/w) and centrifuged for 1 h at 100,000 x g in a Beckman SW-27 rotor. These cells were washed 4-times with ice cold 200 ml of ammonium medium (16 mM sulfate) and then resuspended in 100 ml of the same medium. The turbidity was adjusted to 7.22 with pre-equilibrated medium, and 500 ml of cell suspension was poured into a 650 ml flat Plexiglas culture chamber. The culture was illuminated and aerated with 4% CO_2-air (666 ml/min). The culture turbidity was held essentially constant by the continuous dilution procedure described by Turner et al. (46).

The culture turbidity was measured every 0.5 h, and 0.5 ml of cell suspension was fixed with 0.5 ml of 20% formalin and used for a hemacytometer cell count. At each 0.5 h before culture dilutions were made, 10 ml of cell suspension were harvested on Millipore filters, washed twice with 0.05 M Tris-HCl (pH 7.2) resuspended in 3 ml of the same buffer, and frozen at -20°. The cells were thawed, broken in the small French pressure cell, and frozen once again at -20°. The homogenate was thawed centrifuged at 9,000 x g for 2.5 min, and 200 µl and 15 µl of supernatant were taken for measurement of NADP-GDH activity and for rocket immunoelectrophoresis analysis. For the measurement of total cell protein, 200 µl of the whole-cell homogenates was used.

At 1.5 h in the cell cycle of the parent culture, 74 ml of cell suspension were harvested and used as the source of cells for the pulse-chase experiments. The cells were centrifuged from the culture medium, washed 3-times in ice cold ammonium-medium (containing 50 µM sulfate), and resuspended in 84 ml of the latter medium. The turbidity of the culture was adjusted to 7.1. The culture was transferred to a small Plexiglas chamber, and 4 mCi of ^{35}S-H_2SO_4 (carrier-free, final specific activity 2.7 mCi/µmole) was added to the culture. After 10 min of dark equilibration, the culture was illuminated for 30 min, and then 30 ml were harvested and chilled rapidly. The cells in 5 ml of suspension were washed twice by centrifugation in 20 mM K_2SO_4, 50 mM Tris-HCl (pH 7.2, 0°), and frozen at -20° in 5 ml of the same buffer minus the potassium sulfate. The cells in the remaining 25 ml of suspension were combined with 15 ml of ice-cold ammonium-medium (containing 16 mM sulfate), and then pelleted by centrifugation. The cells were washed twice in the same cold medium, and then resuspended in 25 ml of pre-equilibrated ammonium medium containing 16 mM nonradioactive sulfate (i.e., chase medium). The cells were cultured in the chase medium for 3.5 h with continuous dilution with fresh culture medium. During the chase period, 5 ml samples were harvested at 0.5 h intervals, washed, and frozen as described above.

The experimental details described earlier for characterization of the indirect immunoprecipitation procedure differ slightly from those used during cell cycle studies. Therefore, the exact method used in the cell cycle studies are described below. The cells in 5 ml samples were thawed and broken by 3 passages through the small French pressure cell.

The homogenate was diluted to 11.3 ml with 5 mg/ml of bovine serum albumin in 50 mM Tris-HCl, pH 7.2, 4°C, and centrifuged for 1 h at 100,000 x g. The supernatant was frozen and stored at -20°. After thawing, the supernatant was centrifuged once at 100,000 x g. A small green pellet was discarded. The final supernatant was assayed for NADP-GDH activity and used for immunoprecipitation as follow: 750 µl of 100,000 x g supernatant containing 2 mg/ml bovine serum albumin; 1 to 9 µl of pure NADP-GDH carrier to give a final concentration of 0.67 µg/ml; 250 µl of a solution of 4% Triton X-100, 4% sodium deoxycholate, 12 mg/ml bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl, pH 7.2, 4°; 25 µl of pure anti-NADP-GDH to give a final concentration of 5.75 µg/ml. This 1.03 ml primary reaction mixture was allowed to stand for 90 min on ice. Fifty microliters of sheep anti-rabbit IgG (4 mg/ml stock solution) were then added to give a 1.08 ml secondary reaction mixture. This mixture was immediately layered on top of a 10 ml liquid-column of 5% sucrose (detergent composition described earlier) and centrifuged for 90 min at 50,000 x g at 4°. The immunoprecipitate was processed for SDS gel analysis as described earlier. As background controls, non-immune or pre-immune rabbit serum, which had been purified by a protein A affinity column, was used in place of rabbit anti-NADP-GDH.

RESULTS AND DISCUSSION

Purification and stabilization of NADP-GDH - In several steps of the purification procedure developed for Chlorella NADP-GDH by Gronostajski et al. (14), the enzyme undergoes gradual inactivation. Thus, the possibility exists that the inactivated enzyme might copurify with the active enzyme and result in a final purified enzyme-preparation containing both active and inactive forms of the enzyme. If such a preparation is used for immunization of rabbits, the additional possibility exists that different antibodies might be made against these two forms of the enzyme. Since the objectives of my dissertation research are to measure the cell cycle accumulation of catalytically-active NADP-GDH antigen and to determine whether this form of the enzyme undergoes degradation or inactivation, I have modified the aforementioned purification procedure to achieve at least 95% recovery of active enzyme at each purification step.

In the Gronostajski et al. (14) purification procedure, the major causes of NADP-GDH inactivation were due to oxidation of the enzyme at each purification step, and to dilution of the enzyme to low protein concentrations at the later steps. In the new procedure these problems were circumvented by (a) degassing all buffers, (b) maintaining the enzyme preparation under argon, and (c) designing the purification steps such that small proteins, which can stabilize the enzyme and can be easily separated from it by gel-filtration, are retained until the second from last purification step.

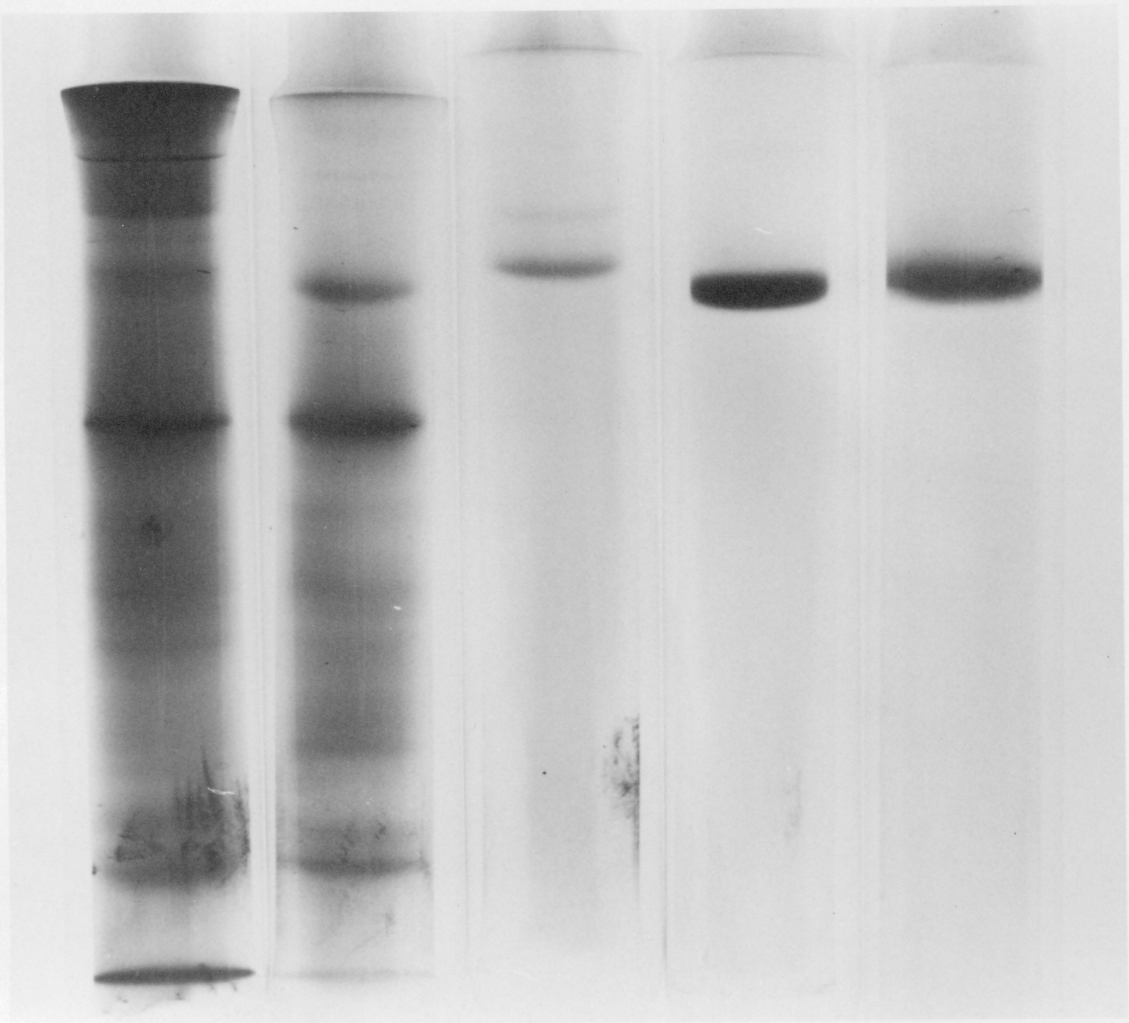
In the former procedure, cell homogenates were centrifuged at

100,000 x g and the resultant supernatant was frozen-thawed three times at -20°. This step was used to remove the green lamellae-protein fraction. The new purification no longer employs the ultracentrifugation step. The lamellae-proteins are removed from the cell homogenate by a single freeze-thaw step. By freezing the homogenate in small volumes of 40 ml each, this step was made more efficient and resulted in the removal of 120 ml packed-volume of precipitated lamellae protein from the homogenate of 140 g fresh weight of cells. The new procedure employs 35 to 70% instead of the 40 to 70% ammonium-sulfate fractionation used in the earlier procedure. The two ammonium fractionation procedures differ in that the pH of the new one is adjusted to approximately 6.0 to accomplish isoelectric precipitation of many contaminating proteins during the 0 to 35% step. The earlier procedure accomplished this isoelectric precipitation in a separate step in which the enzyme preparation was kept at pH 6.0 for 3 days at 0-4°. After the ammonium-sulfate fractionation step, the earlier procedure utilized a 5 x 39 cm Sephadex G-200 column to separate the NADP-GDH (i.e., apparent molecular weight of 410,000 daltons) from smaller proteins. However, because of the high protein concentration of the enzyme preparation at this stage of purification, the preparation had to be divided into 3 samples and applied to the column in 3 consecutive chromatography runs, each one taking 24 h. In the earlier gel-filtration step, a low ionic strength buffer (0.01 M Tris-HCl, pH 7.4, 4°) was also employed. The high protein concentration and low ionic strength buffer promoted protein-protein interaction and also Sephadex-protein interaction and greatly lowered the resolving power of the column. In the new procedure, a DEAE-Sephacel ion-exchange

column of high capacity (5 x 15 cm, functional capacity 12 g protein) was used prior to the gel-filtration step. This ion-exchange step quickly lowers the protein concentration of the enzyme preparation to a level compatible with a subsequent gel-filtration step. However, since the protein concentration was relatively high in the ion-exchange step, the enzyme was stable. In contrast, because the DE-52 cellulose ion-exchange step followed the Sephadex G-200 gel-filtration step in the earlier procedure, the enzyme was unstable during and after ion-exchange chromatography. Instead of using only one ion-exchange chromatography step, the new procedure involves the use of an ion-exchange step at pH 6.0 which immediately follows the pH 7.4 step. This second step is designed to remove a major protein fraction which usually interfers with the resolution of the Sephadex G-200 gel-filtration step, and to remove four minor proteins which compete with the enzyme for binding to the NADP-hexane agarose affinity column. Thus, when the Sephadex G-200 column step is used after the second ion-exchange step in the new procedure, excellent separation of the contaminating proteins from the NADP-GDH was achieved and the enzyme was judged by gel electrophoresis to be 90-95% pure. This gel-filtration step was further improved by (a) stabilization of the enzyme by limited reduction for 30 min at 22° by 10 mM dithiothreitol in 0.19 M glycine, 25 mM Tris-HCl, pH 8.3 at 22°, (b) a longer column (75 cm versus 39 cm), (c) lower protein concentration and smaller number of different proteins due to the use of the two ion-exchange steps before the gel-filtration step, and (d) use of 0.25 M KCl to provide a higher ionic strength to minimize the non-specific protein-protein interaction and the protein-Sephadex interaction.

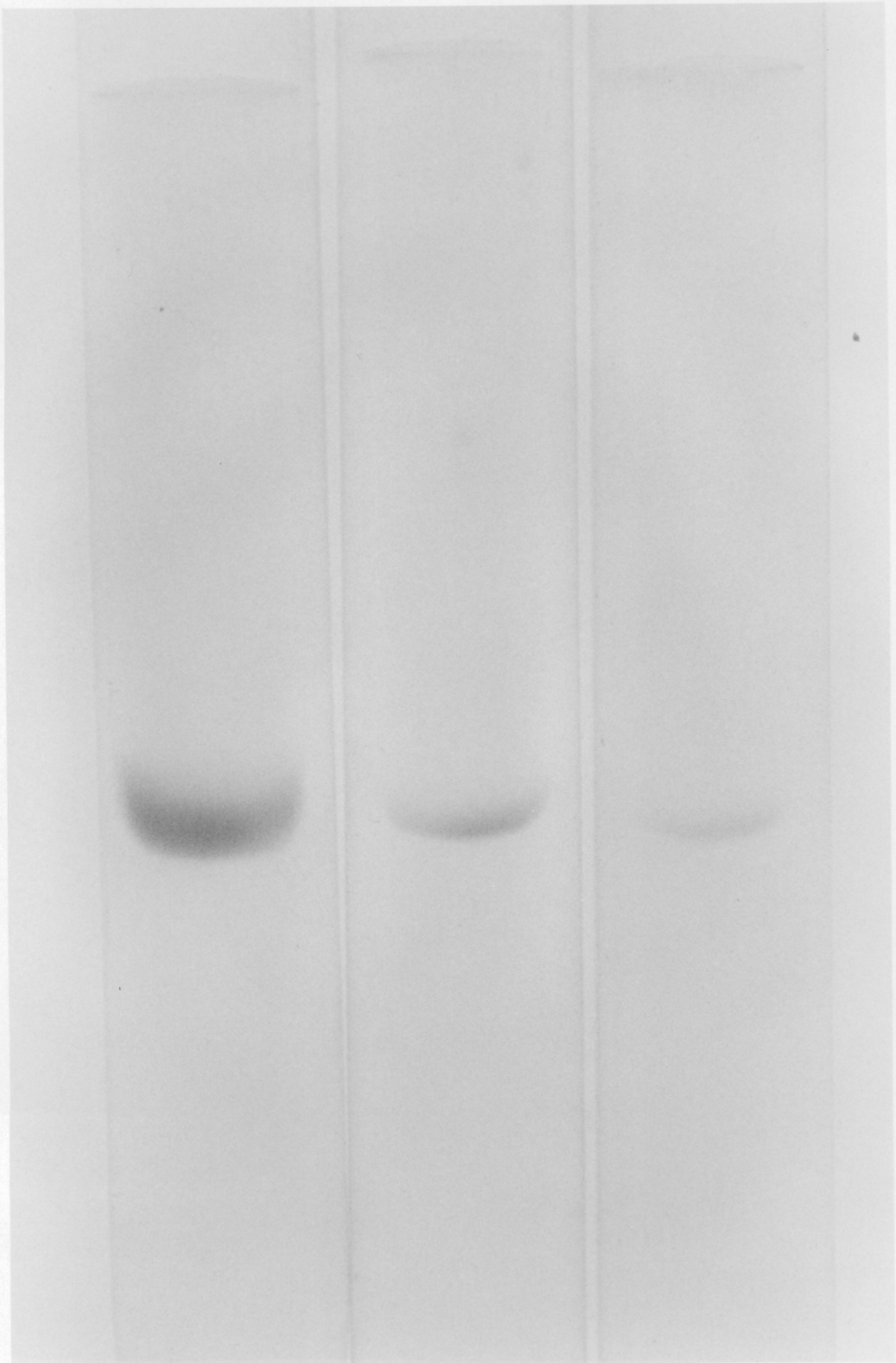
The Gronostajski et al. (14) procedure used a calcium phosphate gel-adsorption chromatography step which was aimed at the removal of a wide and diffuse-looking band of protein(s) seen on native polyacrylamide gels. This diffuse band of protein(s) has a mobility slightly greater than the NADP-GDH and interferred with the final purification step (i.e., preparative polyacrylamide-gel electrophoresis) of the earlier procedure. The calcium phosphate gel-adsorption step was very time consuming and recovery of the NADP-GDH was often low and variable (40 to 75%). Moreover, elution of the enzyme required a large volume of buffer. By use of ultrafiltration, this volume was decreased and the buffer was exchanged for the preparative electrophoresis step. However, a loss in enzyme activity resulted during ultrafiltration, presumably by binding of the enzyme to the membrane filter. To circumvent many of the aforementioned problems, the gel adsorption step has now been replaced with an affinity column chromatography step, using NADP-hexane-agarose Type 3 (with $NADP^+$ coupled to the column via C^8 of the adenine ring). This affinity gel is stable and has high capacity (i.e., 1 mg of NADP-GDH per ml of gel). The enzyme which is eluted from this gel is pure as judged by native polyacrylamide gel electrophoresis (Fig. 1) and by two different SDS gel electrophoresis systems (Fig. 1,2). In the elution buffer for this affinity column, the enzyme is stable to long-term storage (i.e., > 6 months) at -20°. The affinity column is usually the last step in the new purification procedure. However, a preparative disc gel electrophoresis step is employed when polymer-free NADP-GDH is required for injection into rabbits for antibody production. In contrast, in the procedure used by Gronostajski et al. (14), two successive

Fig. 1. Polyacrylamide disc gels before and after selected stages of purification of the NADP-GDH from C. sorokiniana. Native gels (A) through (C) were 7% acrylamide. SDS gel (E) was 9% acrylamide by method of Weber and Osborn. (50). Gels were stained with Coomassie brilliant blue for total protein. (A), 35 to 70% ammonium sulfate fractionation; (B), DEAE-Sephacel pH 7.4 ion-exchange chromatography; (C) Sephadex G-200 gel filtration; (D), (E) NADP-hexane-agarose affinity chromatography.



A B C D E

Fig. 2. SDS polyacrylamide gels of purified NADP-GDH by method of Laemmli (51). From left to right, the gels contained 6, 12, and 50 μg of total protein, which were stained with Coomassie brilliant blue. The gels were composed of 9% acrylamide.



preparative electrophoresis steps were required before the enzyme was brought to electrophoretic homogeneity. Thus, the new purification procedure is substantially different from the earlier one, requiring about one-third as much time, and yields at least 3 times more catalytically-active enzyme. Approximately 55 mg of enzyme was obtained from 145 g fresh weight of cells. In several purification experiments, the enzyme yield ranged between 80 and 87%. In one of these purifications with the new procedure, the percent recovery and fold-purification of the enzyme was monitored at each purification step (Table I). Moreover, at different steps in the purification procedure, both native and SDS gel electrophoresis were used to evaluate the relative purity of the enzyme preparation (Fig. 1).

For use as an internal standard in immunoprecipitation experiments, ^{35}S-labeled NADP-GDH was purified by this new procedure (Fig. 3).

The purified enzyme was unstable to freezing at -20° in 25 mM Tris-HCl (pH 7.4). This treatment was observed to lead to polymerization of the enzyme. Although the largest polymers were inactive and barely entered native polyacrylamide gels during electrophoresis, the smaller polymers still retained enzymic activity. Because the purified enzyme was observed to be completely stable without polymerization in 10 mM KH_2PO_4, 0.1 mM $NADP^+$, pH 6.2, the enzyme was routinely stored in this buffer at -20°.

At concentrations less than 0.1 mg protein per ml, the activity of the NADP-GDH was very unstable in solution (25° to 38.5°) during vigorous agitation. For example, approximately 50% of the activity of the enzyme decayed during 20 s of agitation on a vortex mixer (Fig. 4A). When

TABLE I

NEW PURIFICATION PROCEDURE FOR NADP-GLUTAMATE DEHYDROGENASE FROM CHLORELLA SOROKINIANA

Step	Total units[a]	Specific activity[b]	Recovery (%)	Fold Purification
Supernatant from frozen-thawed cell homogenate	3000	0.25	100	0
1. Ammonium sulfate fractionation (35-70%)	2753	0.5	92	2
2. DEAE-Sephacel, pH 7.4	2695	5	90	20
3. DEAE-Sephacel, pH 6.0	2570	10	86	40
4. Sephadex G-200	2490	47	83	188
5. NADP-affinity column	2450	53	82	212

[a]One unit is defined as the amount of enzyme necessary to reduce 1 μmole of $NADP^+$/min at 38.5°.

[b]Specific activity is defined at units/mg protein.

Fig. 3. SDS polyacrylamide gel analysis of purified ^{35}S-labeled NADP-GDH. The gel was composed of 9% acrylamide. Electrophoresis was by the method of Weber and Osborn (50) modified to include a stacking gel. The sample was 1 µg of ^{35}S-NADP-GDH in 20 µl, containing approximately 17,500 cpm. The gel was soaked 24 h in 7% acetic acid before it was frozen and sliced into 1 mm slices. Each slice was digested with 0.4 ml of 30% H_2O_2 and counted in 10 ml of Triton-toluene scintillation fluid.

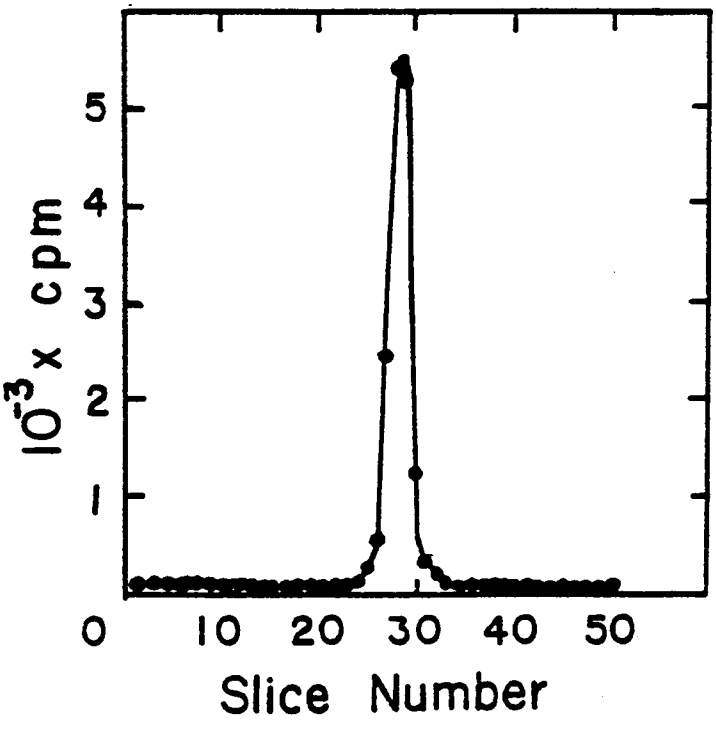
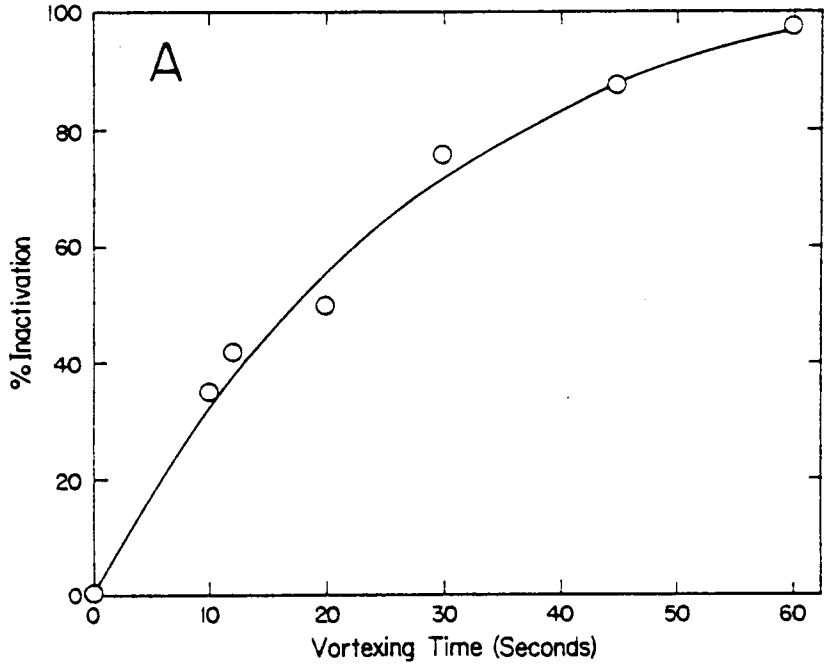
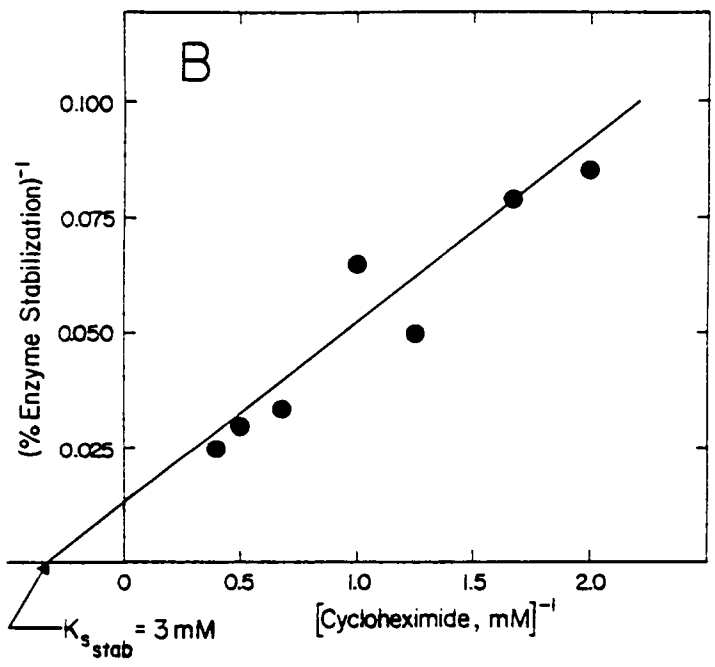

10^{-3} x cpm
5
4
3
2
1
0
10
20
30
40
50
Slice Number

Fig. 4 A,B. Stability of NADP-GDH to aggitation in the absence or presence of cycloheximide. A solution of NADP-GDH (equivalent to 4.8 µg/ml of pure NADP-GDH in 50-70% ammonium sulfate fractionated cell homogenate at 0.7 mg/ml protein concentration) was mixed in an 8 x 100 mm glass test tube with a Vortex-Genie mixer model K-550-G at a speed control setting of "7" for given amounts of time. (A) % NADP-GDH inactivation versus length of aggitation. (B) Double recipricol plot of concentration of cycloheximide versus stabilization of NADP-GDH against aggitation.


A
% Inactivation
Vortexing Time (Seconds)
0
20
40
60
80
100
0
10
20
30
40
50
60


B
(% Enzyme Stabilization)$^{-1}$
[Cycloheximide, mM]$^{-1}$
$K_{s_{stab}}$ = 3 mM
0.100
0.075
0.050
0.025
0
0.5
1.0
1.5
2.0

agitated for 20 s, 3 mM cycloheximide provided one-half maximal stabilization of the activity of the enzyme (Fig. 4B). At 11 mM cycloheximide, the solution could be agitated for 1 min without any detectable loss in activity of the enzyme. This inactivation by agitation could not be prevented by 2 mM dithiothreitol or 2 mM mercaptoethanol. Because cycloheximide has no detectable affect on *in vitro* NADP-GDH activity *per se*, the mechanism by which cycloheximide stabilizes the enzyme is unclear. Since cycloheximide was observed by Israel *et al.* (47) to prevent the normal loss in NADP-GDH activity *in vivo* upon removal of ammonium from induced cells, it seems possible that cycloheximide might bind to the enzyme and induce a conformational change which stabilizes the enzyme against degradation or inactivation *in vivo*. Alternatively, in the *in vivo* situation, cycloheximide might inhibit the activity or synthesis of a protease or enzyme responsible for inactivation of the NADP-GDH. Since cycloheximide has been reported to inhibit the activity of the ribosomal RNA polymerase (64) in eucaryotic microorganisms, perhaps it has the capability of affecting the activity or conformation of other enzymes as well.

Preparation and purification of rabbit anti-NADP-GDH - Five different rabbits were injected with the pure NADP-GDH. When the increase in titer of anti-NADP-GDH was followed over a three month time-course, approximately a fivefold difference was observed in the maximal titers reached among the different rabbits. For the immunological work described herein, the antiserum from the rabbit with the highest final titer was used. By Ouchterlony double-diffusion analysis of pure NADP-GDH and

crude cell extracts, the antiserum was shown to be monospecific for the enzyme (Fig. 5). Moreover, the preimmune serum from this rabbit did not form precipitin bands with crude cell extract nor pure enzyme.

The yield and potency of the ammonium sulfate fractionated anti-NADP-GDH IgG from crude antiserum was similar to that published for anti-ovalbumin (54), i.e., one A_{280} unit of IgG fraction precipitated 63 µg of NADP-GDH at the equivalence point. Initial attempts to further purify this IgG fraction by antigen affinity chromatography were unsuccessful. Although the NADP-GDH holoenzyme could be covalently-coupled to CNBr activated Sepharose 4B gel with high efficiency, it was not possible to link all 6 subunits of this enzyme directly to the gel. By coupling ^{35}S-labeled holoenzyme to the gel, it was shown that the enzyme remained bound to the gel during the antibody-binding-step and the subsequent washing steps before elution of the specific anti-NADP-GDH (Table II). In the absence of antibody, the enzyme was also stable to the buffers normally used to elute antibodies. However, any solution capable of elution of the bound anti-NADP-GDH also resulted in a proportional co-elution of ^{35}S-NADP-GDH (Fig. 6). The enzyme in the eluate was catalytically inactive but remained antigenically reactive. The most likely explanation for column instability is that the bound anti-NADP-GDH weakened the interaction between subunits of the NADP-GDH holoenzyme, such that the normal conditions of low pH or high salt concentration used to dissociate the antibody from its antigen also resulted in dissociation of the enzyme into its subunits. This observation represents the first example of structural destabilization for a multimeric enzyme by binding of its antibody. For elution of anti-

Fig. 5. Ouchterlony double diffusion analysis (65)of the specificity of pure anti-NADP-GDH antibodies and preimmue serum. 4 mm wells were punched in a 1.5 mm thick layer of 1% agar in water on a glass plate. 15 µl samples were placed in each well. The plate was developed for 2 days at 4° in a water saturated atmosphere. After the reaction period, the plate was washed for 24 h with 0.3 M NaCl in 50 mM Tris-HCl, pH 7.4; 24 h in 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4; and 24 h in 50 mM Tris-HCl, pH 7.4. (I) and (II) were stained with NADP-GDH activity stain; (III) and (IV) were stained with Coomassie brilliant blue. Wells (D) (E) each contained a Chlorella homogenate with 0.6 µg of NADP-GDH. Wells (B) (C) each contained 0.6 µg of purified ^{35}S-NADP-GDH. Wells (A) (F), each contained 0.6 µg of purified NADP-GDH. The center wells of (I) and (III) each contained an anti-NADP-GDH IgG fraction that had an equivalence of 2.1 µg of pure anti-NADP-GDH. This amount of IgG corresponded to that in 5% preimmue serum in wells (II) and (IV).

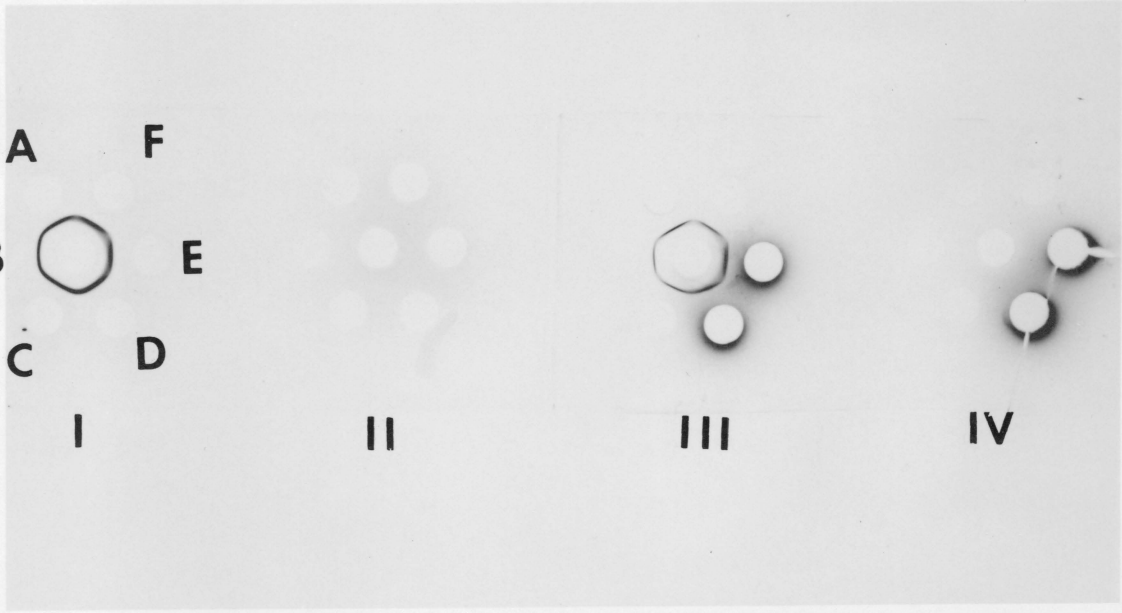

A
F
B
E
C
D
I
II
III
IV

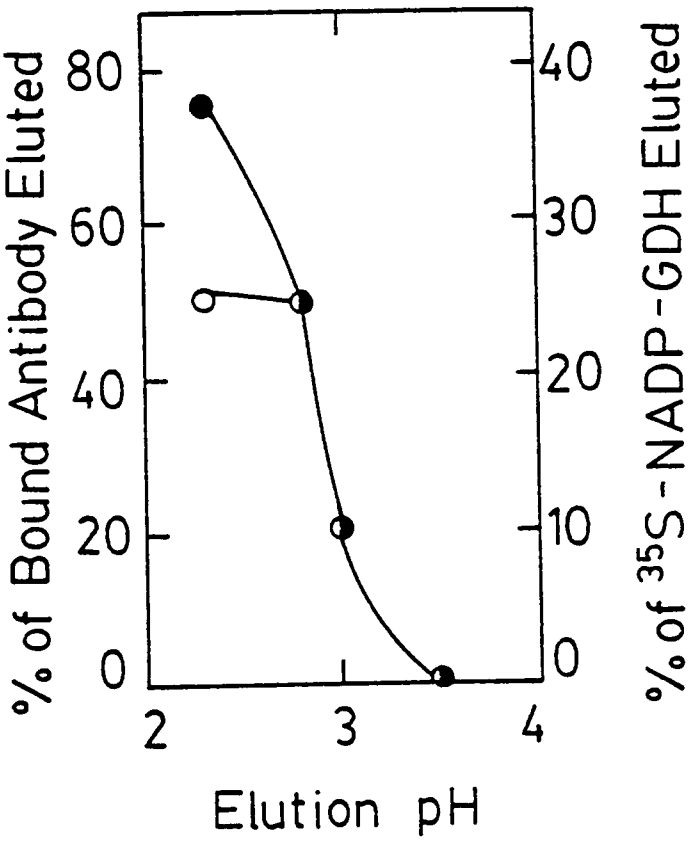
TABLE II

COMPARISON OF THE STABILITIES OF CHLORELLA NADP-GLUTAMATE DEHYDROGENASE COUPLED TO CNBr ACTIVATED SEPHAROSE IN THE PRESENCE OR ABSENCE OF 6 M UREA

	% Radioactive enzyme eluted from column	
	before anti-NADP-GDH elution	during anti-NADP-GDH elution
Native holoenzyme	3.5	50
Enzyme subunits, in 6 M urea*	0	0

*Enzyme assumed to be subunits in 10 mM diothiothreitol and 6 M urea.

Fig. 6. Destabilization of non-crosslinked NADP-GDH antigen affinity column by the elution of bound anti-NADP-GDH. Percent of bound anti-NADP-GDH eluted (○); Percent of coupled ^{35}S-NADP GDH eluted (●). Abscissa: pH of the 0.1 M glycine used to elute the antibodies. Both 0.1 M glycine pH 3.0 and 3 M NaSCN eluted the same amount of anti-NADP-GDH and ^{35}S-NADP-GDH.


% of Bound Antibody Eluted
80
60
40
20
0
40
30
20
10
0
% of ^{35}S-NADP-GDH Eluted
2
3
4
Elution pH

NADP-GDH from this column, 3 M NaSCN and 0.1 M glycine, pH 3.0 were of equal efficiency. Anti-NADP-GDH eluted by 4.5 M $MgCl_2$ was no longer capable of immunoprecipitation of NADP-GDH.

The next type of antigen affinity column was prepared by coupling ^{35}S-NADP-GDH subunits to CNBr activated Sepharose 4B in the presence of 6 M urea. When the enzyme was treated with 10 mM dithiothreitol and 6 M urea at 38°, it was assumed to have dissociated into subunits. The coupling efficiency of the subunits in the presence of urea was about 85% of that for the holoenzyme in the absence of urea. The NADP-GDH subunit column, was able to bind about 50% as much anti-NADP-GDH as its holoenzyme counterpart. However, neither the bound anti-NADP-GDH nor ^{35}S-NADP-GDH subunits were released from the column by elution with 0.1 M glycine, pH 2.8; 10 mM dithiothreitol reduction at pH 8.3 followed by elution with 0.1 glycine, pH. 2.8; and 10 mM dithiothreitol reduction in 6 M urea at pH 8.3 followed by elution with 0.1 M glycine, pH 2.8. If the antibodies were still bound to the antigen on the affinity gel, some of them should still have free antigen binding sites open for binding more antigen in a sandwich type antigen-antibody-antigen complex. Thus, when the gel, after several elution attempts, was mixed with a NADP-GDH solution, it further precipitated an amount of enzyme equal to about 20% of the original binding-capacity of the antibodies. This binding experiment showed that most of the anti-NADP-GDH originally bound to the affinity-gel was still active and was not eluted by the various elution buffers. The reason for this unusual tight-binding of anti-NADP-GDH to subunits of the enzyme is unknown.

By covalently-coupling holoenzyme to CNBr activated Sepharose 4B in

the usual manner and then crosslinking the subunits together with dimethylsuberimidate, Turner (56) in this laboratory prepared a stable holoenzyme affinity column. This column showed high anti-NADP-GDH binding capacity (i.e., 14 mg anti-NADP-GDH/ml of affinity gel). Moreover, all of the bound anti-NADP-GDH could be eluted without detectable loss of enzyme subunits from the column. The column was used repeatedly without loss of binding capacity.

Quantitative immunoprecipitation of NADP-GDH - When ^{3}H-NADP-GDH was precipitated from a ^{3}H-leucine-labeled cell extract by a direct immunoprecipitation procedure (23), SDS polyacrylamide gel electrophoresis showed a single radioactive peak with a high but constant background of radioactivity (Fig. 7A). Analysis of the whole cell extract (after immunoprecipitation) by SDS gel electrophoresis showed that many other soluble cellular proteins (or their subunits) have a size in common with the subunits of NADP-GDH (Fig. 7B). Moreover, as indicated by a second immunoprecipitation in the same extract, the first precipitation appeared to remove only approximately 75% of the enzyme from the extract. This poor recovery was due in part to the presence of the detergents during immunoprecipitation. The detergents which were required to increase the specificity of the immunoprecipitation reaction also decreased the recovery of the NADP-GDH (Fig. 8). Because of the high background and incompleteness of the direct procedure in cell extracts, it was decided to test the completeness and specificity of two different indirect immunoprecipitation procedures (25,55).

Because sulfate is a normal component of the culture medium and

Fig. 7. SDS polyacrylamide gel electrophoresis (50) of the precipitate obtained by direct immunoprecipitation of NADP-GDH by rabbit anti-NADP-GDH from a ^{3}H-leucine-labeled cell homogenate of Chlorella cultured in ammonium-medium. (A), ^{3}H in immunoprecipitate (●); second gel with 10 μg of pure NADP-GDH was stained with Coomassie brilliant blue and scanned with a Gilford model 240 spectrophotometer equipped with a linear transport gel scanner (-----). (B), ^{3}H in supernatant after immuno-precipitation of NADP-GDH.

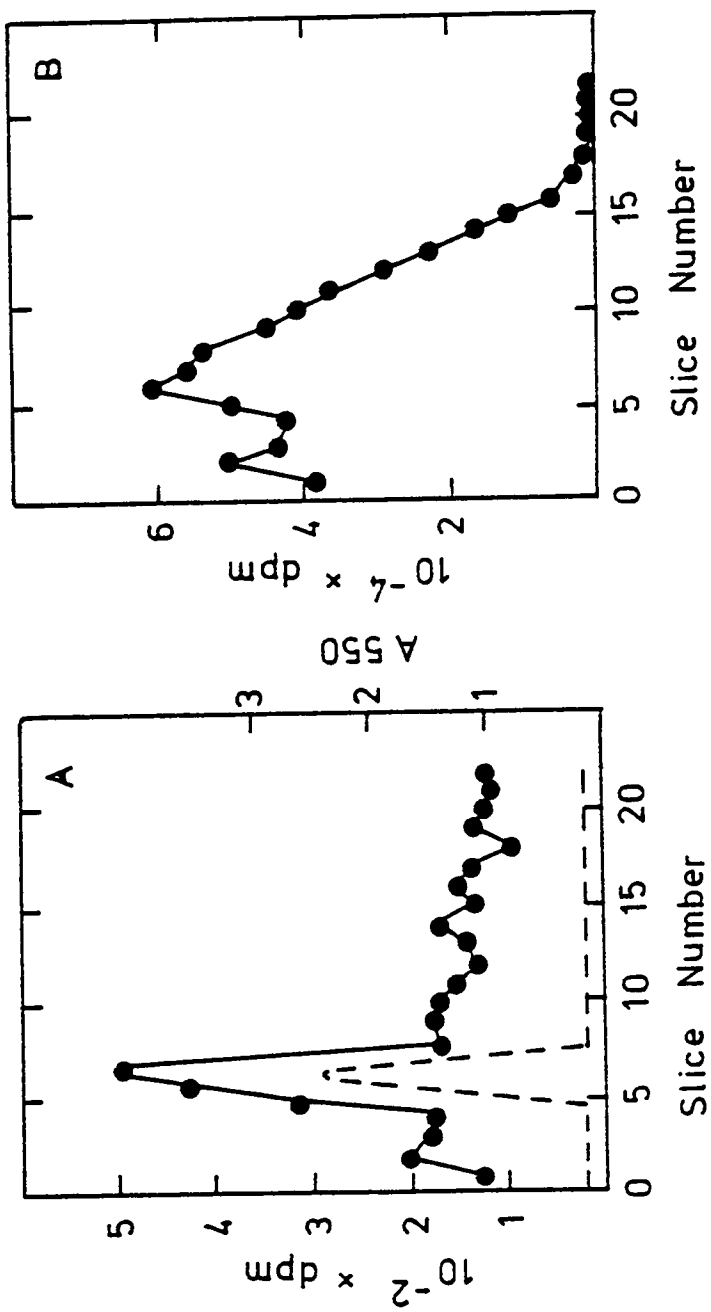
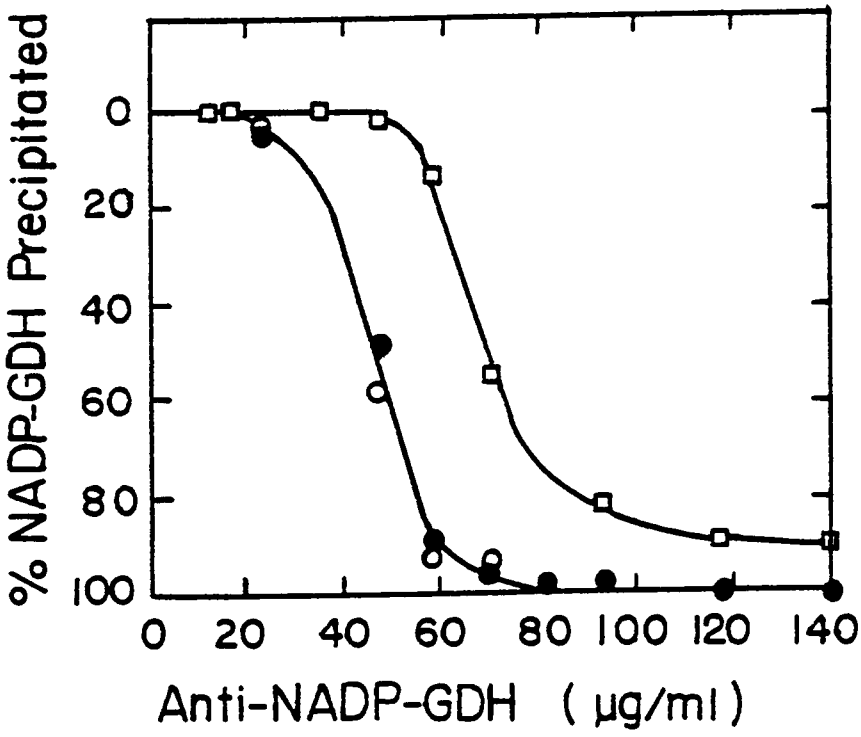

A
B
10^{-2} x dpm
10^{-4} x dpm
A 550
Slice Number
Slice Number
0
5
10
15
20
1
2
3
4
5
6
7
9

Fig. 8. Comparison of the efficiency of direct immunoprecipitation in the presence or absence of detergents. ^{35}S-NADP-GDH was added to a cell homogenate of Chlorella (cultured in NH_4^+ medium) to give a final NADP-GDH concentration of 11.4 µg/ml in the presence (□) or absence (○ , ●) of 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride. The reactions were terminated by 2 min of centrifugation in an Eppendorf centrifuge. Immunoprecipitation was quantified by the amount of NADP-GDH activity (○) or ^{35}S-radioactivity (● , □) in the supernatant, respectively.


% NADP-GDH Precipitated
0
20
40
60
80
100
0 20 40 60 80 100 120 140
Anti-NADP-GDH (µg/ml)

leucine is not, radioactive sulfate was selected instead of ^{3}H-leucine for subsequent labeling studies. In ammonium cultured cells, the uptake of ^{35}S-sulfate and its incorporation into trichloroacetic acid precipitable material (i.e., protein) were linear for 2 h until the medium-sulfate concentration decreased below 20 μM (Fig. 9). The Chlorella total cellular protein was labeled to a specific radio activity (i.e., > 2 x 10^{8} cpm/mg) without perturbing the normal growth rate of the cells.

To achieve greater than 95% precipitation of ^{35}S-NADP-GDH from nonradioactive cell homogenates in the presence of detergents, an indirect immunoprecipitation procedure was used (Fig. 10,11A). In initial studies with radioactive cell extracts, the ammonium sulfate-fractionated rabbit anti-NADP-GDH was employed as the primary antibody along with the sheep anti-rabbit secondary antibody (Fig. 11B). However, when the primary-antibody was purified by antigen affinity chromatography, the specificity of the immunoprecipitation was improved as indicated by a sharper radioactive peak with smaller shoulders (Fig. 11C). However, the background radioactivity was still too high to be completely satisfactory.

Quinto et al. (25) modified the normal indirect immunoprecipitation procedure to make it more specific for glutamine synthetase, which is in low concentration in cellular extracts of Neurospora. In the modified procedure, the enzyme and primary antibody were reacted and this antigen-primary antibody complex was reacted with secondary antibody during high speed centrifugation, such that as the immunoprecipitate forms it is scrubbed by sedimentation through a sucrose-detergent column. This procedure prevents the formation of large aggregates of immunoprecipitate

Fig. 9. Uptake of ^{35}S-sulfate into Chlorella cells cultured in ammonium-medium. Concentration of sulfate in the culture medium (O); Incorporation of ^{35}S into cell homogenate (□), or into 10% trichloroacetic acid precipitable material (■).

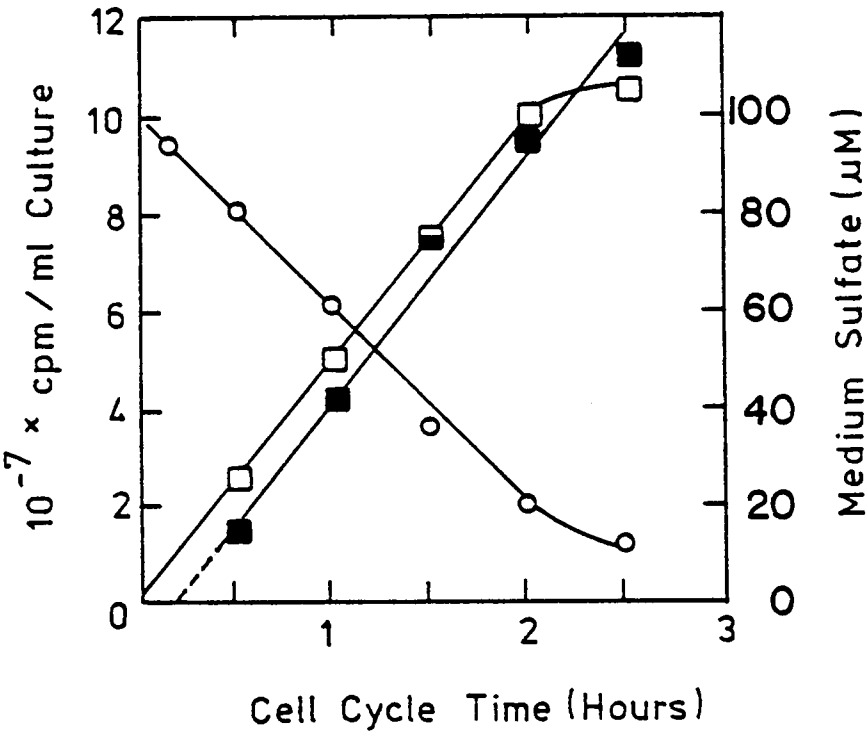

12
10
8
6
4
2
0
10^{-7} × cpm / ml Culture
100
80
60
40
20
0
Medium Sulfate (μM)
1
2
3
Cell Cycle Time (Hours)

Fig. 10. Comparison of the efficiency of direct versus indirect immunoprecipitation of the Chlorella NADP-GDH. The reaction volume was 160 µl, consisting of 100 µl frozen-thawed homogenate of a cell cycle sample of Chlorella (cultured in nitrate medium) which contained no NADP-GDH; 4.2 µg/ml of ^{35}S-NADP-GDH, 1% Triton X-100; 1% sodium deoxycholate; 4 mg/ml bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl, pH 7.2. The reaction temperature was 0°. Pure anti-NADP-GDH (○ , ●); $(NH_4)_2SO_4$ fractionated anti-NADP-GDH anti-serum IgG fraction (△ , ▲). (○ , △) 180 min of primary reaction without addition of secondary antibodies; (● , ▲) 90 min of primary reaction followed by the addition of 100 µg of sheep-anti-rabbit IgG for 90 min of secondary reaction. The reactions were terminated by centrifugation for 2 min in an Eppendorf centrifuge. The ^{35}S radioactivity in the supernatant was then quantified.

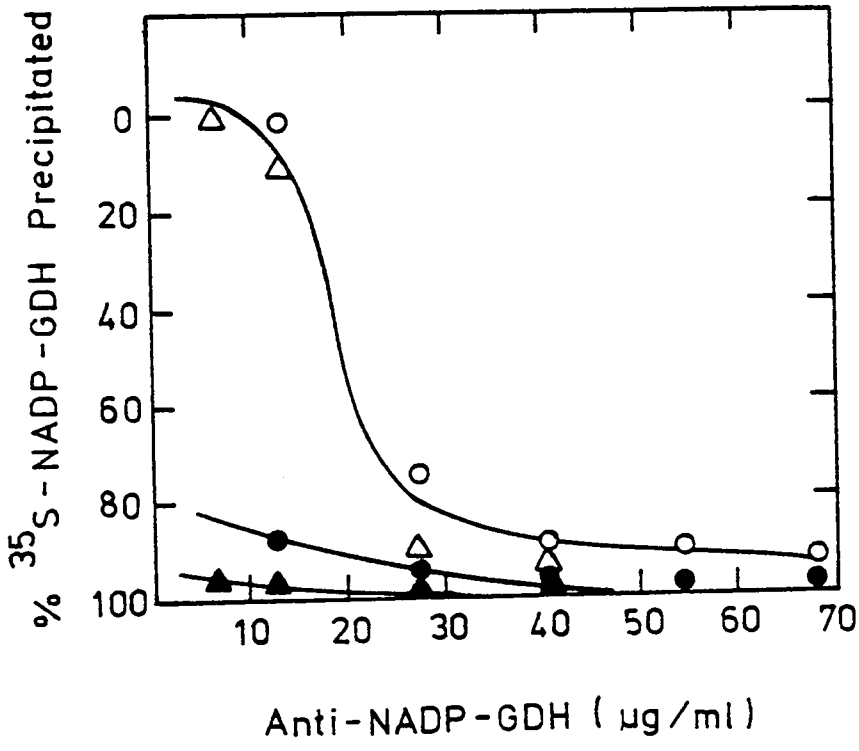
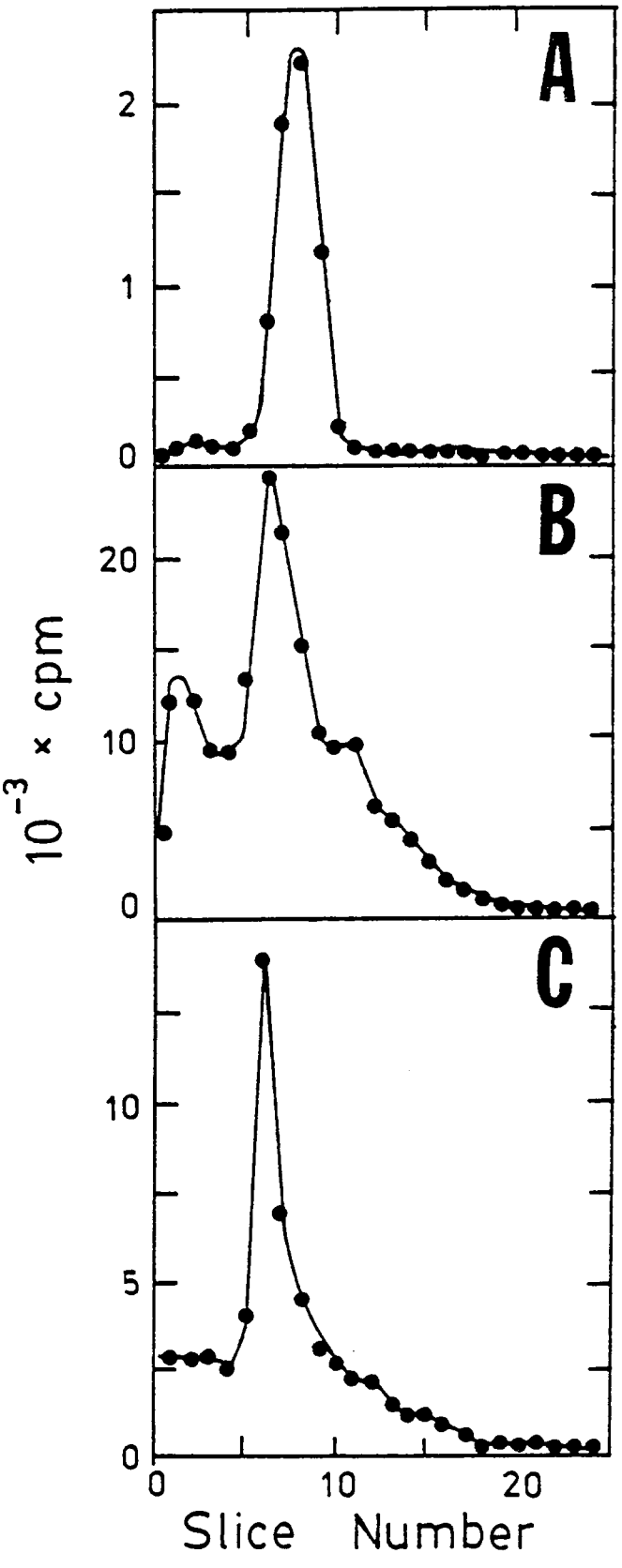

% ^{35}S-NADP-GDH Precipitated
0
20
40
60
80
100
10
20
30
40
50
60
70
Anti-NADP-GDH (µg/ml)

Fig. 11. SDS polyacrylamide gel electrophoresis (50) of the precipitate obtained by indirect immunoprecipitation of ^{35}S-NADP-GDH from cell homogenates of Chlorella. (A), shows the migration of completely dissociated immunoprecipitate obtained with pure ^{35}S-NADP-GDH added to a cell homogenate of Chlorella cells cultured in nonradioactive nitrate medium. (B) ^{35}S in immunoprecipitate obtained by precipitation of NADP-GDH with $(NH_4)_2SO_4$ fractionated anti-NADP-GDH from ^{35}S-labeled homogenate from NH_4-cultured cells. (C) same as (B) except with pure anti-NADP-GDH.


A
B
C
2
1
0
20
10
0
10
5
0
0
10
20
10^{-3} × cpm
Slice Number

which can trap other proteins nonspecifically. With radioactive cell extracts from Chlorella, the amount of pure rabbit anti-NADP-GDH required with a constant amount of sheep anti-rabbit IgG (200 µg/ml) to achieve complete indirect-immunoprecipitation of NADP-GDH by this modified procedure is shown in Figure 12. The percent recovery of the enzyme and the reproducibility of the immunoprecipitation is shown in Figure 13 A,B and in Table III. Although the purified IgG fractions from non-immune serum and the sheep anti-rabbit serum were unable by themselves to precipitate any NADP-GDH activity, they were able to precipitate a radioactive protein(s), which migrated to the same position as authentic NADP-GDH in SDS gels, when used together in the modified indirect procedure (Fig. 13C,D,E).

Relationship between catalytic activity and antigenicity of NADP-GDH during cell cycle - The Chlorella cell cycle in ammonium-medium was characterized by linear increases in culture turbidity (Fig. 14) and total cellular protein (Fig. 15). A sharp rate change in these parameters was seen at the onset of the period of cell release and the end of this period, respectively. A change in the concentration range of the linear density-gradients, employed for the isopycnic cell-selection procedure, resulted in the highest degree of synchrony (i.e., shortest cell-release time; < 1.5 h) reported for this organism in ammonium medium.

Although enzyme potential (i.e., initial rate of induced enzyme accumulation in previously uninduced cells) of the NADP-GDH was previously observed (46) to closely parallel the cell cycle accumulation of

Fig. 12. Optimization of the amount of pure anti-NADP-GDH primary antibody to be used with a fixed concentration (200 µg/ml) of sheep-anti-rabbit secondary antibody to obtain maximal indirect immunoprecipitation of NADP-GDH by procedure of Quinto et al. (25). Each point on the curve was obtained from the amount of net radioactivity associated with the NADP-GDH peak in a SDS gel. Immunoprecipitation was performed from a homogenate of Chlorella cells which had been cultured in ^{35}S-sulfate medium for 3 h.

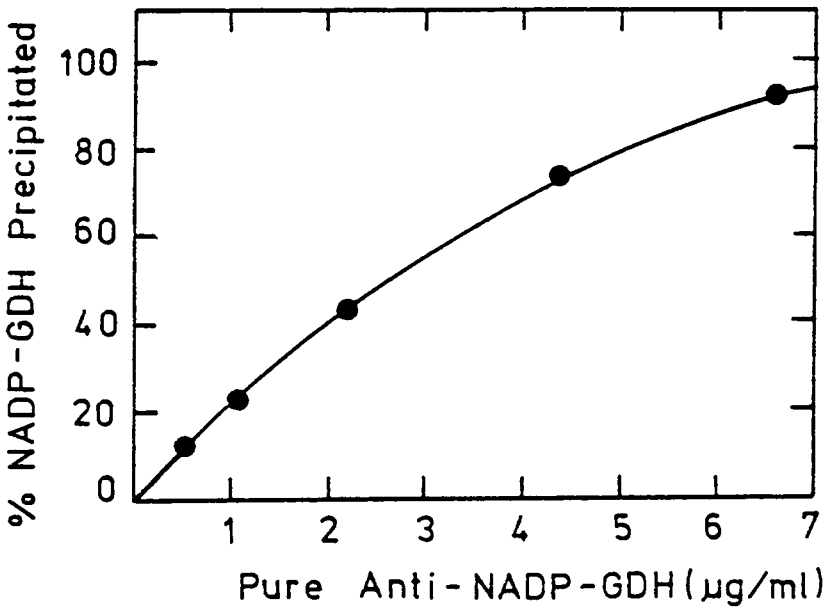

% NADP-GDH Precipitated
100
80
60
40
20
0
1
2
3
4
5
6
7
Pure Anti-NADP-GDH (µg/ml)

Fig. 13. Characterization of the indirect immunoprecipitation procedure of Quinto et al. (25) for Chlorella NADP-GDH. (A), SDS gel electrophoresis of the immunoprecipitate from a homogenate of Chlorella cells (cultured in ^{35}S-sulfate for 3 h) containing pure ^{35}S-NADP-GDH as an internal standard to increase NADP-GDH radioactivity by 30%. (B), Gel-profile of radioactivity of the immunoprecipitate without added internal standard. (C, D, E), Triplicates of the background radioactivity when an equal amount of purified non-immune serum IgG was used to replace the pure anti-NADP-GDH.

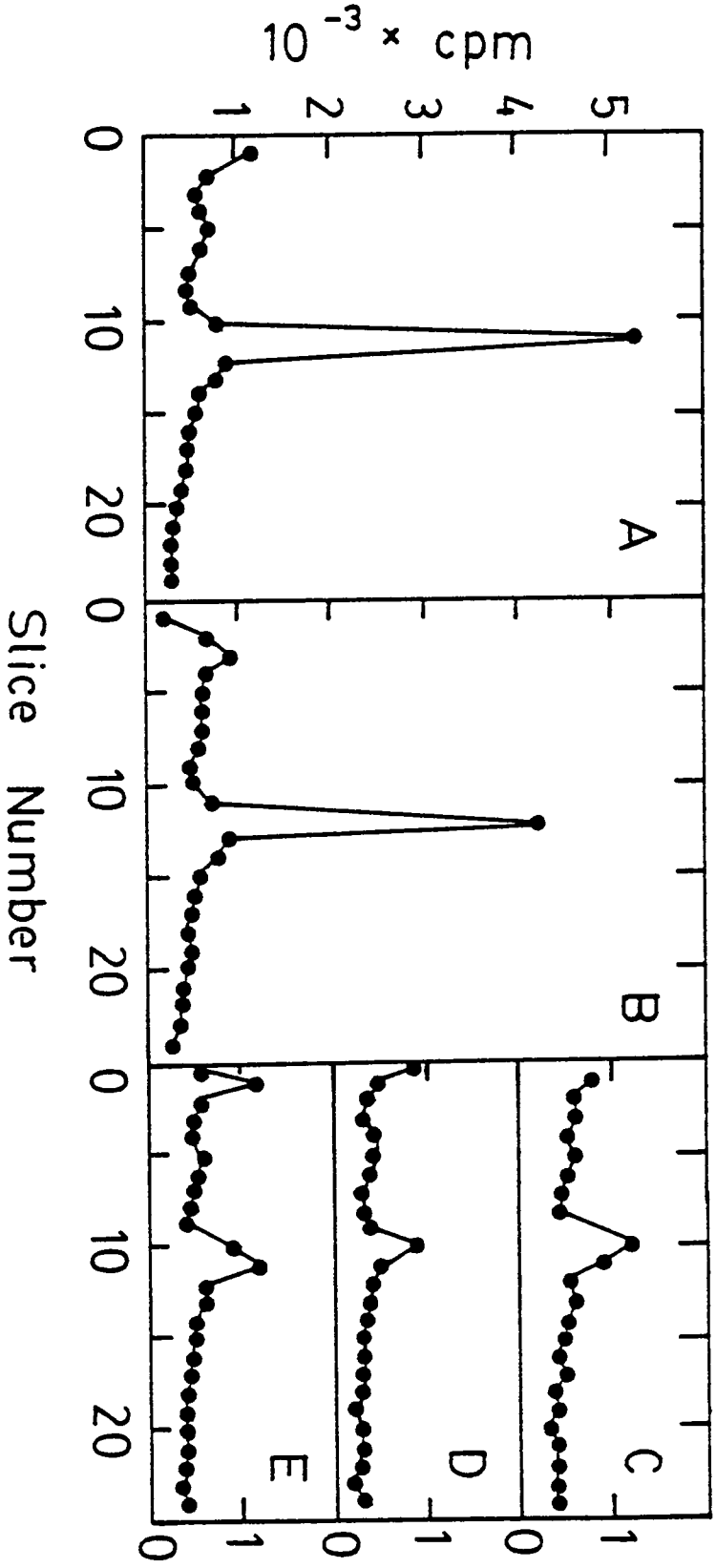

10 -3 x cpm
1
2
3
4
5
0
10
20
A
B
C
D
E
Slice Number

TABLE III

PERCENT RECOVERY AND REPRODUCIBILITY OF INDIRECT IMMUNOPRECIPITATION OF *CHLORELLA* NADP-GLUTAMATE DEHYDROGENASE[a]

	Peak radioactivity (cpm)	Net average (cpm)	Recovery of internal standard (cpm)	% recovery
with internal standard[b]	21,931 23,007 22,693	14,101	4530	96
without internal standard[c]	19,341 17,936 16,764	9,570	----	
background[d]	7,814 9,023 8,492			

[a]Indirect immunoprecipitation by the method of Quinto *et al.* (25).

[b]Internal standard equaled 4,720 cpm of ^{35}S-NADP-GDH.

[c]Radioactive internal standard replaced with equivalent amount of nonradioactive enzyme.

[d]Average background was 8,443 cpm and was subtracted from peak enzyme radioactivity.

Fig. 14. Patterns of culture turbidity and cell number during the cell cycle of synchronous Chlorella cells growing at a rate of 26% per h in the continuous presence of ammonium. Culture turbidity (□); Cell number (■).

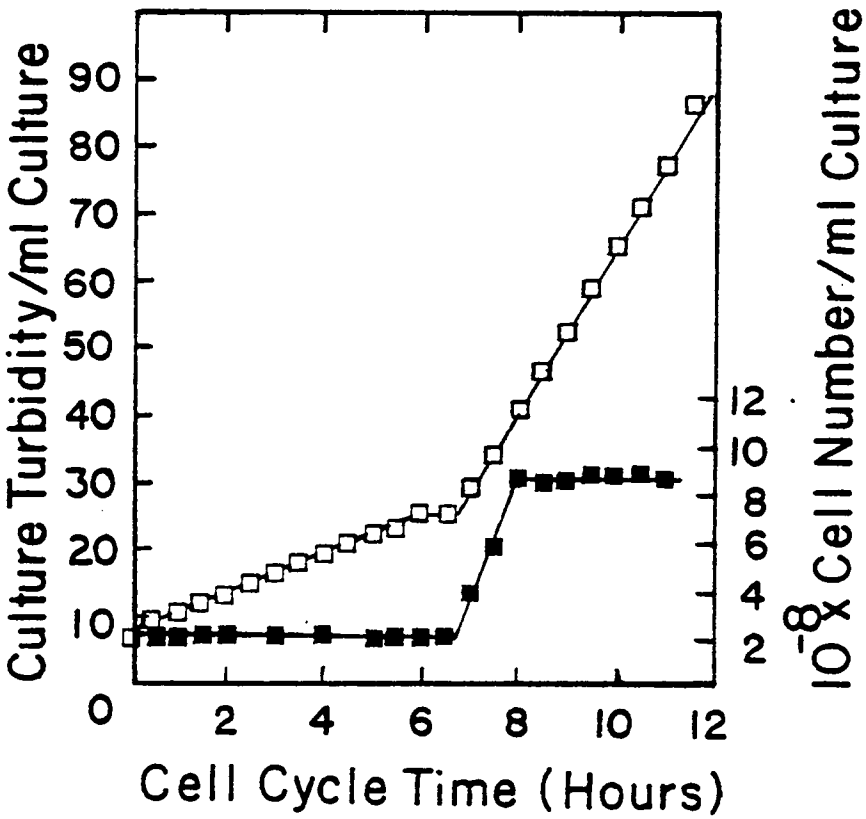
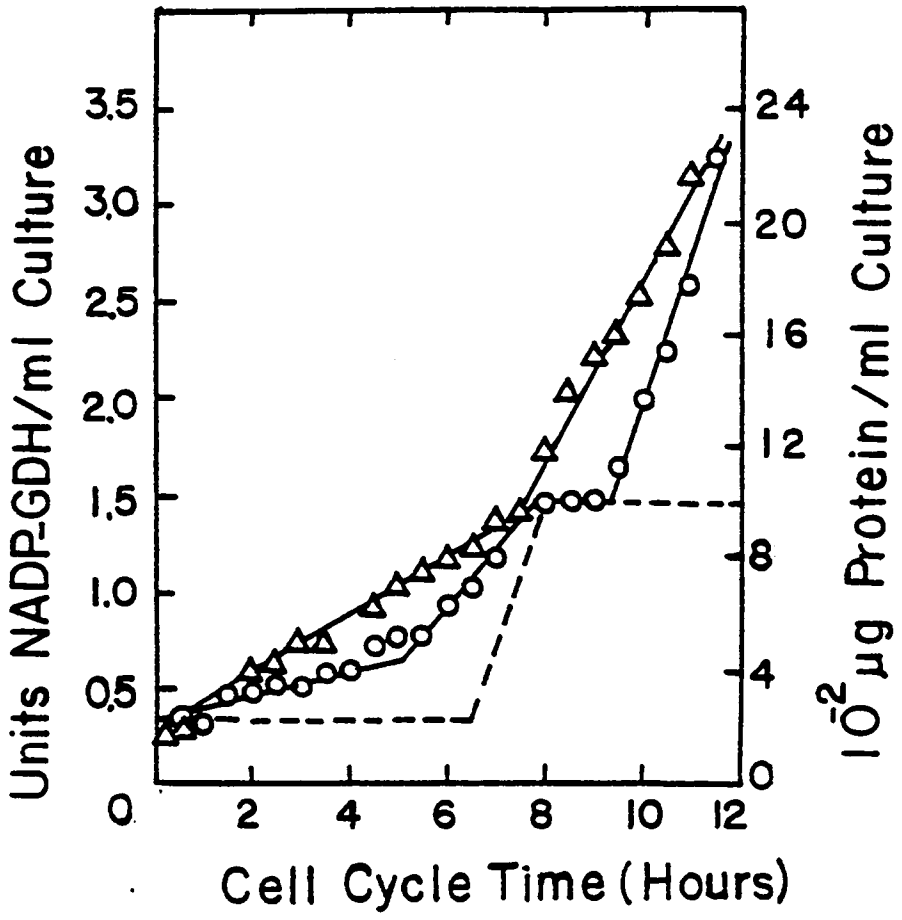

Culture Turbidity/ml Culture
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
Cell Cycle Time (Hours)
12
10
8
6
4
2
10⁻⁸ x Cell Number/ml Culture

Fig. 15. Patterns of accumulation of NADP-GDH and of total cellular protein during the cell cycle of preinduced synchronous Chlorella cells growing at a rate of 26% per h in the continuous presence of ammonium NADP-GDH deaminating activity (O); total cellular protein (Δ); cell number (---).


Units NADP-GDH/ml Culture
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
2
4
6
8
10
12
24
20
16
12
8
4
0
10^{-2} µg Protein/ml Culture
Cell Cycle Time (Hours)

total cellular protein, the cell cycle increase in NADP-GDH catalytic activity in fully-induced cells has a pattern quite different from that for cellular protein (Fig. 15). The cell cycle pattern observed in this experiment for NADP-GDH catalytic activity differs from the ones reported earlier by Israel et al. (9,47) from this laboratory. Israel et al. (9,47) did not observe the cessation of accumulation of catalytic activity observed in the present study between the eighth and ninth hours of synchronous growth (Fig. 15,16). The markedly improved cell synchrony (i.e., 1.5 h vs. 2.5 h cell division time), observed in the present study, might account for the difference in the cell cycle patterns. Since catalytic activity of this enzyme does not increase continuously as originally visualized, one must consider additional regulatory mechanisms in proposing models (8) for the regulation of cellular levels of this enzyme (e.g., enzyme turnover).

By use of rocket immunoelectrophoresis (Fig. 16), it was possible to show unequivocally that NADP-GDH catalytic activity was paralleled by an increase in NADP-GDH antigen (Fig. 17). The simplest inference is that the catalytic activity pattern reflects the de novo synthesis of new molecules of NADP-GDH. Thus, no evidence was obtained to indicate that the increase in NADP-GDH activity in the light was due to direct activation of the enzyme by light-coupled redox systems (e.g., thioredoxin-ferridoxin system) such as those reported by Buchanan et al. (66, 67) for several plant enzymes.

Evidence for degradation of NADP-GDH in ammonium-cultured cells - By employing ^{35}S-sulfate in a pulse-chase experiment, it was possible to

Fig. 16. Standard curve of concentration of NADP-GDH antigen versus peak height, assayed by rocket immunoelectrophoresis, on a serially diluted cell extract as described in EXPERIMENTAL PROCEDURE. The electrophoresis was performed with 1% agarose containing 5.8 μg/ml of anti-NADP-GDH. The rockets were visualized with a tetrazolium activity stain.

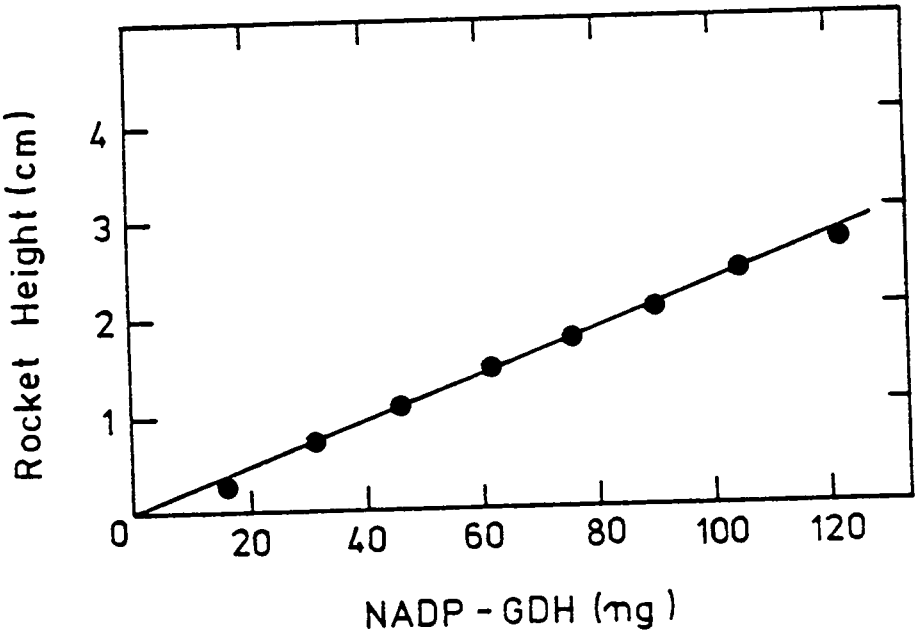
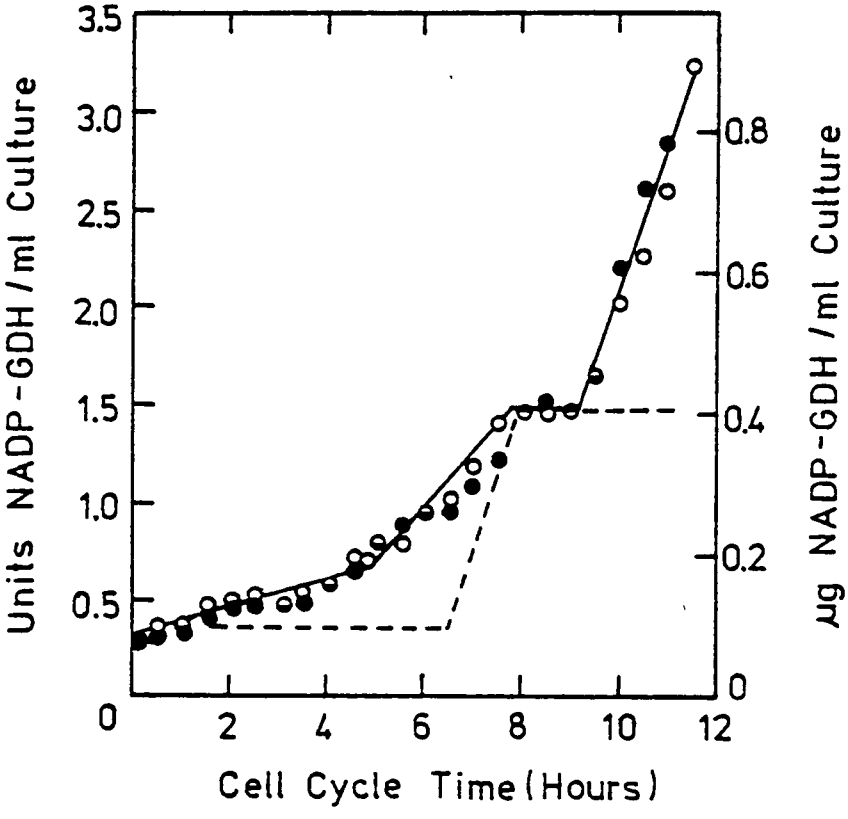

Rocket Height (cm)
4
3
2
1
0
20
40
60
80
100
120
NADP - GDH (mg)

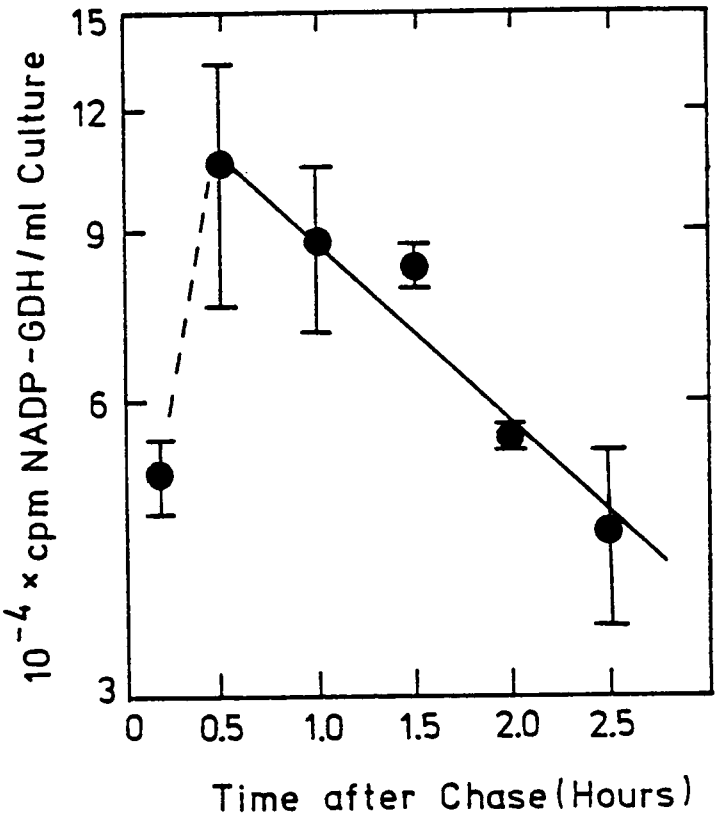
Fig. 17. Patterns of accumulation of the catalytic and antigenic activity of NADP-GDH during the cell cycle of preinduced synchronous Chlorella cells growing at 26% per h in the continuous presence of ammonium. Deaminating activity (O); antigenic activity as determined by rocket immunoelectrophoresis (●), cell number (---).


Units NADP-GDH / ml Culture
µg NADP-GDH / ml Culture
Cell Cycle Time (Hours)
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.2
0.4
0.6
0.8
2
4
6
8
10
12

use a quantitative indirect-immunoprecipitation procedure to estimate the *in vivo* half-life of the ^{35}S-labeled NADP-GDH in synchronous cells growing in the continuous presence of inducer. Although Israel *et al.* (47) showed the activity of this enzyme to be stable *in vivo* in ammonium-cultured synchronous cells in which protein synthesis was blocked with cycloheximide, the enzyme was observed to decay with an apparent half-life of 1.6 h in ammonium-cultured cells in the absence of inhibitors (Fig. 18). Although Hare and Schmidt (67) have shown that the sulfur amino acids are amongst those free amino acids which are in lowest concentration in *Chlorella*, the continued increase in ^{35}S-content of the enzyme observed (Fig. 18) during the first 0.5 h of the chase-period probably reflects continued incorporation of ^{35}S-amino acids into the enzyme from the free amino acid pool. Because of this latter possibility and also of the likelihood that ^{35}S-amino acids from turnover of other cellular proteins recycle through the free amino acid pool, the observed degradation rate of NADP-GDH is probably a minimum estimation of the actual rate, i.e., the $t_{\frac{1}{2}}$ of the enzyme is probably shorter than 1.6 h.

When ammonium was removed from the culture of induced *Chlorella* cells, Israel *et al.* (47) observed that the activity of the NADP-GDH decayed with a $t_{\frac{1}{2}} \simeq 8$ min. This decay was blocked by the addition of cycloheximide at the time of removal of inducer from the culture medium. In an independent study, Bascomb (69) has recently shown, by use of rocket immunoelectrophoresis, that this loss in catalytic activity of the NADP-GDH is accompanied by a loss in antigenicity of the enzyme. These data suggested that the enzyme undergoes degradation after removal

Fig. 18. Degradation rate of NADP-GDH as measured by indirect immunoprecipitation in a pulse-chase experiment. Pre-induced synchronous Chlorella cells, growing at a rate of 26% per h, were pulsed with 50 µM ^{35}S-sulfate (2.7 Ci/mmol) for 30 min at 1.5 h of cell cycle time and then chased with 16 mM sulfate for 2.5 h. Indirect immunoprecipitation was performed in duplicate by the method of Quinto et al. (25). The two intercepts of the line in the graph were determined by linear regression of the data in its logarithmic form before plotting on semi-log coordinates. The half-life of the NADP-GDH was calculated to be 1.6 h.


15
12
9
6
3
10^{-4} × cpm NADP-GDH / ml Culture
0
0.5
1.0
1.5
2.0
2.5
Time after Chase (Hours)

of the inducing agent. Since in the present study, the enzyme was also shown to undergo degradation in synchronously growing cells in the continuous presence of inducer, it can be inferred that cycloheximide also inhibited NADP-GDH degradation in fully-induced cells in the earlier study by Israel et al. (47). Thus, the regulation of the cellular levels of this enzyme might be analogous to that of the hormone-induced accumulation of tyrosine aminotransferase in hepatoma cells (70). The NADP-GDH might undergo continuous degradation, in presence or absence of inducer, with induced changes in the rate of enzyme synthesis being the major determining-factor as to whether or not the enzyme accumulates. Moreover, if the half-life of the enzyme is between 8 min and 1.6 h, the amount of translatable NADP-GDH-mRNA would be predicted to accumulate during periods of induced enzyme accumulation.

In this last experiment, the half-life of the NADP-GDH was examined only during the first one-third of the cell cycle. In future experiments, the half-life measurements should be made before and after the changes in rate of enzyme accumulation during the cell cycle and during the period in which no enzyme accumulation occurs (i.e., 8 to 9 h). If the enzyme is unstable throughout the cell cycle, the cessation of enzyme accumulation might represent a time in which a steady-state exists between enzyme synthesis and breakdown. Alternatively, the stability of the enzyme might change during the cell cycle, and the absence of enzyme accumulation could reflect a period in which the synthesis of the enzyme has ceased and the enzyme has been stabilized by some endogenous factor.

Until the additional turnover measurements are performed, one can

only speculate as to what types of mechanisms are regulating the level of the enzyme at any given stage of the cell cycle. For example, if the enzyme is revealed to have a short half-life (i.e., between 8 min and 1.6 h) which is constant throughout the cell cycle in fully-induced cells, a simple model can be proposed to explain the pattern of enzyme accumulation. It can be proposed that the enzyme is synthesized continuously and the amount of translatable NADP-GDH mRNA accumulates continuously with a pattern very similar to that observed (Fig. 17) for the enzyme during the cell cycle. If an in vitro translation system (71) can be used to show that the concentration of translatable NADP-GDH mRNA is indeed increasing in the predicted pattern, then it will be necessary to determine whether the accumulation of translatable mRNA is being rate-limited at the transcriptional or post-transcriptional level, or by the number of gene copies. For the analysis of these parameters, it will be necessary to synthesize a complementary DNA copy from the purified NADP-GDH mRNA and to use this cDNA as a hybridization probe (72) to quantify the number of NADP-GDH-genes and -mRNAs in fully-induced synchronous cells growing at different rates during the cell cycle.

SUMMARY

A new 5-step purification procedure was developed which could be completed in 5 days with an 80 to 85% yield of electrophoretically pure NADP-GDH from C. sorokiniana. A monospecific, highly purified anti-NADP-GDH was prepared from the antisera of immunized rabbits. The anti-NADP-GDH was purified on a stable antigen affinity column which was prepared by covalent-coupling of the holoenzyme to CNBr activated Sepharose 4B followed by crosslinking of the enzyme subunits together with dimethylsuberimidate. With ^{3}H-leucine and ^{35}S-sulfate, as labeling agents of whole cells, the reproducibility and efficiency of both direct- and indirect-immunoprecipitation procedures were tested with pure labeled NADP-GDH as an internal standard. For these studies, a partially purified sheep anti-rabbit IgG fraction was employed as a secondary antibody. A modified indirect immunoprecipitation procedure was tested in a cell cycle experiment to determine whether it would be feasible to use as a tool for further cell cycle experiments on enzyme turnover. Although the method was somewhat lengthy, it appeared to be reliable and was used to reveal that during the first one-third of the cell cycle of fully-induced cells, the NADP-GDH has a maximum half-life of 1.6 h.

Rocket immunoelectrophoresis was used to show that NADP-GDH catalytic activity and antigen increased in a parallel fashion during the cell cycle of fully-induced cells. These data suggest that the cell cycle catalytic activity pattern reflects de novo synthesis of new molecules of NADP-GDH. Since the enzyme in fully-induced cells undergoes rapid in vivo degradation, changes in the rate of enzyme synthesis probably determine the rate of enzyme accumulation during the Chlorella

cell cycle in the continuous presence of ammonium.

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USE OF IMMUNOLOGICAL PROCEDURES TO MEASURE RATE OF ACCUMULATION AND DEGRADATION OF INDUCIBLE NADP-SPECIFIC GLUTAMATE DEHYDROGENASE DURING CELL CYCLE OF SYNCHRONOUS *CHLORELLA*

by

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(ABSTRACT)

A new five-step purification procedure was developed which could be completed in five days with an 80 to 85 percent yield of electrophoretically pure nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase from *Chlorella sorokiniana*. A monospecific, highly purified antibody against the enzyme was prepared from the antisera of immunized rabbits. The antibody was purified on a stable antigen affinity column which was prepared by covalent-coupling of the holoenzyme to CNBr activated Sepharose 4B followed by crosslinking of the enzyme subunits together with dimethylsuberimidate. With ^{3}H-leucine and ^{35}S-sulfate, as labeling agents of whole cells, the reproducibility and efficiency of both direct- and indirect-immunoprecipitation procedures were tested with pure labeled-enzyme as an internal standard. For these studies, a partially purified sheep anti-rabbit immunoglobulin G fraction was employed as a secondary antibody. A modified indirect immunoprecipitation procedure was tested in a cell cycle experiment to determine whether it would be feasible to use as a tool for further cell cycle experiments on enzyme turnover. Although the method was somewhat lengthy, it appeared to be reliable and was used to reveal that during the first one-third of the cell cycle of fully-induced cells, the NADP-GDH has a maximum half-life of 1.6 h.

Rocket immunoelectrophoresis was used to show that enzyme catalytic

activity and antigen increased in a parallel fashion during the cell cycle of fully-induced cells. These data suggest that the cell cycle catalytic activity pattern reflects *de novo* synthesis of new molecules of enzyme. Since the enzyme in fully-induced cells undergoes rapid *in vivo* degradation, changes in the rate of enzyme synthesis probably determine the rate of enzyme accumulation during the *Chlorella* cell cycle in the continuous presence of ammonium.