EVIDENCE FOR POST-TRANSCRIPTIONAL REGULATION OF INDUCTION OF NADP-SPECIFIC GLUTAMATE DEHYDROGENASE BY ACCUMULATION OF ITS mRNA IN UNINDUCED SYNCHRONOUS *CHLORELLA* CELLS

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GDH	Glutamate dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
tRNA	Transfer ribonucleic acid
hnRNA	Heterogenous nuclear ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
BSA	Bovine serum albumin
DMSl	Dimethyl Suberimidate dihydrochloride
Tris	Tris (hydroxymethyl) aminomethane
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA	Ethylenedinitrilo tetraacetic acid
EGTA	Ethylene-glycol-bis-(2-aminoethyl ether)N,N^1-tetracetic acid
PMSF	Phenylmethyl-sulfonyl fluoride
PAB-cellulose	Para-aminobenzyl cellulose
PBS	Phosphate buffered-saline (0.01M NaH_2PO_4, 0.015M NaCl, pH 7.2 at 22°)
0.1SSC	A tenfold dilution of Standard Saline Citrate buffer (0.15M NaCl and 0.015M sodium citrate, pH 7.0 at 22°)
IgG	Immunoglobulin G

Log Rot	Log product of RNA sample concentration (moles of nucleotide/liter) x time (seconds)

INTRODUCTION

Messenger RNA has the central role in the expression of structural genes in eucaryotes. Therefore, an important objective of many studies of the molecular basis of the mechanism and control of gene expression is a greater understanding of the role of mRNA in transcription and post-transcriptional events. By comparison with mRNA in procaryotes, the majority of eucaryotic mRNA is monocistronic and has a greater metabolic stability (1, 2). The mRNA coding for many proteins which include globin (3), immunoglobulin (4), ovalbumin (5,6), histones (7), vitellogenin (8), dihydrofolate reductase (9) and the large and small subunits of ribulose 1,5-bisphosphate carboxylase (10, 11) have been isolated and purified. From the study of these purified mRNAs, it is now clear that eucaryotic mRNA may contain significant amounts of untranslated nucleotide sequences (12), elements of secondary and tertiary structure (13), and are modified after transcription (14, 15).

In procaryotes, inducible enzymes have been shown to be regulated primarily by transcriptional control mechanisms (16). Transcription and translation of an mRNA chain occur simultaneously, and the two processes may be coupled (17). However, in eucaryotes, transcription and translation are essentially independent processes. The mRNA transcribed from the DNA in the nucleus is transported into the cytoplasm for translation by cytoplasmic ribosomes (18). Therefore, in eucaryotes, regulation of enzyme synthesis can also occur at the post-transcriptional level. A number of laboratories are attempting to determine whether the accumulation of mRNA in the cytoplasm is regulated

only by the rate of transcription or whether genes are transcribed at similar rates and expressed differentially by regulation at the post-transcriptional level (19, 20, 21).

The use of cell free-protein synthesizing systems, capable of translation of heterologous mRNA, has given insight into the mechanisms which determine the availability of the translatable mRNA in the cytoplasm. The detection and quantitation of individual mRNAs is usually achieved by immunological isolation of its translation products by monospecific antibodies. The amount of radioactivity in the immunoprecipitate is directly proportional to the concentration of translatable mRNA. By use of the translation assay, the amount of specific mRNA has been measured during induction of enzymes (21) and proteins (22, 23) and during charges in physiological and growth conditions (24, 25).

A combination of immunological, nucleic acid, and cell synchrony techniques have been employed in this laboratory, to study the regulation of an ammonium-inducible nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase (NADP-GDH) in the eucaryotic microorganism Chlorella sorokiniana. This organism also contains an NAD-GDH isozyme which is present in cells cultured in either ammonium- or nitrate-containing medium. Both isozymes have been purified to homogeneity and partially characterized in this laboratory (26, 27, 28) and have been shown to be two different gene products.

To study the regulation of the inducible NADP-GDH in synchronous cells, two different experimental approaches have been employed. The first approach is to add the inducer to previously uninduced cells and

then measure the inducibility of the enzyme activity and the maximal initial rate of enzyme induction (i.e., enzyme potential) at frequent intervals during the cell cycle (29, 30). The second approach is to culture previously induced synchronous cells in the continuous presence of inducer and then measure the pattern of enzyme accumulation throughout the cell cycle (31). A comparison of either the pattern of enzyme potential or enzyme accumulation with the pattern of DNA accumulation can give valuable insight into the availability of genes for transcription (30, 32).

By use of the first approach, Talley et al (29) showed that the NADP-GDH was inducible by ammonium throughout the cell cycle of cells cultured in the absence of inducer. When NADP-GDH potential was measured at frequent intervals during the cell cycle, Turner et al (30) showed that NADP-GDH potential increased continuously throughout the cell cycle with a major rate-change occurring during the DNA replication period. This rate change could be blocked by a DNA synthesis inhibitor. Moreover, the timing of the rate change in enzyme potential was insensitive to large changes in the cellular growth rate (30). These results suggested that the structural gene of the enzyme is continuously available for transcription and is expressed shortly after its replication during the S-phase.

When NADP-GDH activity was measured in synchronous cells cultured in the continuous presence of inducer, the timing of a positive rate change in enzyme accumulation was observed to be sensitive to changes in the growth rate of the cells. When the overall growth rate of

synchronous cells was 26% per h, the timing of this rate change was displaced outside of the period of DNA replication (31). By use of a specific inhibitor of DNA synthesis, Israel et al (31) showed that the displacement was caused by a delay of expression of newly replicated genes. Pulse chase studies with [^{35}S]sulfate have recently demonstrated that the positive rate change is due to a change in rate of degradation of the enzyme (33).

Recent immunological studies by Bascomb (33) showed that in uninduced cells, the NADP-GDH was continuously being synthesized and degraded. When the inducer was added to uninduced cells, enzyme-antigen accumulated without an induction lag; however a 35 min lag was observed before the onset of accumulation of enzyme activity (33). He also showed that the accumulation of NADP-GDH catalytic activity was accompanied by a parallel increase in enzyme-antigen. During the initial induction phase, the NADP-GDH enzyme was found to be unstable with a calculated t 1/2 of 1.5 h (33). Since the enzyme is unstable in vivo and is continuously turning over, even in the continuous presence of inducer, its mRNA would be predicted to accumulate during periods of NADP-GDH accumulation. Moreover, one possible explanation for the pattern of enzyme potential during the cell cycle is that the NADP-GDH mRNA accumulates in uninduced cells at a rate proportional to gene dosage.

The purpose of this study was to examine (a) the pattern of accumulation of NADP-GDH mRNA during the initial induction phase in synchronous cells and (b) the pattern of accumulation of NADP-GDH mRNA

throughout the cell cycle of synchronous cells cultured in the absence of inducer.

Throughout this disseration, the term "post-transcriptional regulation refers to regulation of gene expression at any step beyond the level of RNA synthesis (i.e., transcription). For example, it is proposed that regulation at the level of post-transcription can occur (a) by modification of RNA structure, (b) by control of translation or (c) by post-translation modification of protein structure. These three examples are not intended to be all inclusive but serve to illustrate the various steps beyond transcription which may be subject to regulation in a eucaryotic cell.

LITERATURE REVIEW

In recent years, it has become increasingly apparent that in procaryotes and eucaryotes, genome organization and control of gene expression are very different. Whereas in procaryotes, transcription of genes and translation of mRNA are tightly coupled, the two processes do not occur simultaneously and are independent events in eucaryotes (17, 18). The study of the structure and function of eucaryotic mRNA has provided much insight into the control of gene expression. Results have shown that control of RNA synthesis (i.e., transcription) and of RNA processing (i.e., post-transcription) are two of the most important control mechanisms in the overall regulation of enzyme synthesis (18, 34).

Many eucaryotic mRNA molecules have been shown to be transcribed as high-molecular weight precursor molecules (i.e., hnRNA, pre-mRNA) which are rapidly cleaved and spliced after transcription (19, 35) Recent reports have demonstrated that some genes, including globin (35), ovalbumin (36), and dihydrofolate reductase (9) genes contain intragenic sequences within the structual gene coding region which are present in the initial transcript but are spliced out after transcription. Many other post-transcriptional modifications have been shown to occur before the mRNA can function efficiently in translation in the cytoplasm. For example, most eucaryotic, mRNAs have been shown to contain a 3' end polyadenylic acid sequence (15) and a 5' end cap (14). The 5' terminal 7-methyl 5' guanosine monophosphate nucleotide has been demonstrated by Shatkin (14) to be required for correct

initiation and efficient translation of many mRNAs. The 3' poly(A) sequence has been shown to have a role in mRNA stability (18) and is thought to be involved in the mechanism of transport of some mRNAs from the nucleus into the cytoplasm (18). Methylation of adenosine residues has been reported to occur at internal residues within the mRNA molecule (18). The precise role of RNA methylation is unknown at present.

A major experimental approach which has been employed to study the regulation of synthesis of specific proteins is to purify the mRNA and to prepare radioactively labelled cDNA from the mRNA template (5, 9, 37). The cDNA can then be utilized as a specific hybridization probe to quantify the concentration of mRNA and gene dosage in the cell.

To purify individual mRNAs, several methods of isolation have been developed in the last decade (6, 38, 39). For mRNAs in high concentration in the cell or tissue, such as the mRNAs coding for ovalbumin (37), vitellogenin (40), and the large subunit of ribulose 1,5-bisphosphate carboxylase (41), physical fractionation methods have been employed. Messenger RNAs have been purified by electrophoresis in polyacrylamide gels (38), centrifugation in sucrose gradients (37) and by oligo(dT)-cellulose column chromatography (40). However, for mRNAs in low concentration, which are undistinguishable from total RNA on the basis of size, immunological methods of isolation have been employed (6, 38). The success of these procedures depends on the antigenicity of the nascent polypeptides being synthesized on poly-

somes and the ability to prevent nonspecific trapping of polysomes. By use of indirect immunological procedures, such as indirect immunoprecipitation (6) and indirect immunoadsorption (38), non-specific adsorption and trapping of polysomes have been minimized. These procedures have been employed to purify several mRNAs which include lysozyme from chicken oviduct (38), vitellogenin from Xenopus (8), and rat liver albumin (6). The use of the indirect immuno-precipitation procedure has also resulted in partial purification of galactokinase mRNA which is only 1.5% of the total cellular mRNA in Saccharomyces cerevisiae (42).

Another method of isolation which has been employed to isolate mRNAs present in low concentration in the cell is a selective hybridization procedure, described in detail by Alt et al (9). By this procedure, Alt et al (9) prepared purified cDNA from metho-trexate-resistant murine Sarcoma 180 cells which showed a high specificity for dihydrofolate reductase mRNA. The use of this method was based on the relative abundance of dihydrofolate reductase mRNA sequences in the methotrexate-resistant compared with the methotrexate-sensitive cells. To obtain the purified cDNA, homologous and hetero-logous mRNA:cDNA hybridizations were performed with poly(A)-containing RNA from the two cell types. This procedure could be used to prepare DNA complementary to mRNA which is subject to dramatic change in concentration in the cell (i.e., induced versus uninduced).

If the mRNA is in so low a concentration that none of the procedures described above would result in a purification, several

new techniques have been developed in which the coding region of the DNA, isolated from the cell, is employed as a specific probe for the particular mRNA (39, 43). Ricciardi et al (39) have recently described a method which relies on incubation of total cytoplasmic RNA to individual restriction fragments of cellular DNA which have been immobilized on nitrocellulose filters. After the RNA was incubated with the DNA on the filters, the filters were washed. The hybrid RNA molecules bound to the filters were then eluted from the DNA. The specific mRNAs were identified by in vitro translation and immunoprecipitation of the products with specific antibody. By use of this method, Ricciardi et al (39) demonstrated that the mRNAs from adenovirus-2-infected cells were specifically eluted and identified by their translation products in a cell-free protein synthesizing system.

Having obtained a purified mRNA, the next step is to prepare cDNA from the mRNA template (9, 37, 44). By specific mRNA:cDNA hybridization, it is then possible to study the regulation of specific genes by direct quantitation of the transcription product. For example, by specific mRNA:cDNA hybridization, McKnight et al (45) demonstrated that nutritional iron deficiency in chicks was accompanied by a two- to fourfold increase in the level of hybridizable transferrin mRNA sequences and a corresponding increase in the level of serum transferrin. Their results showed that the induction of transferrin, by administration of estrogen to chicks, was also accompanied by a specific increase in the rate of transcription of the transferrin gene (46).

The regulation of gene expression by steriod hormones has been studied extensively by use of specific hybridization methods (37, 47, 48). The induction of the four egg white proteins, ovalbumin, ovomucoid, conalbumin, and lysozyme in the hen oviduct, by administration of estrogen, has been demonstrated to be the result of a specific increase in the rate of transcription of the four genes (49, 50). From these studies, it was concluded that regulation of the initial induction phase by steroid hormones is regulated at the level of transcription.

Since these studies utilize a specific cDNA probe, they have been limited in scope by the availability of the purified mRNA. An important alternative approach for studying molecular mechanisms of gene expression in eucaryotes is based on the measurement of the amount of translatable mRNA in a cell-free mRNA-dependent protein synthesizing system (20, 21, 25). One advantage of this approach is that the amount of specific mRNA can be assayed without extensive purification as long as a monospecific antibody is available to detect and immunoprecipitate the products synthesized in vitro. Moreover, the comparison of the amount of hybridizable mRNA with the amount of translatable mRNA is an excellent method to determine if mRNA is present in the cell which is unavailable for translation in the cytoplasm (50). One disadvantage of the use of cell-free protein synthesizing systems is that the results obtained may depend on factors other than the level of the specific mRNA, such as the monovalent or divalent cation concentration. For example, Tse and Taylor (51)

reported that translation of purified rat liver albumin mRNA in a wheat germ protein synthesizing system required higher concentrations of K^+ and Mg^{++} than were required for translation of total liver mRNA. At higher K^+ and Mg^{++} concentrations, only intact albumin molecules were synthesized. When the K^+ and Mg^{++} concentrations were decreased, only immunoprecipitable fragments of albumin were synthesized. The rabbit reticulocyte lysate protein synthesizing system has been shown not to be as dependent on the K^+ and Mg^{++} concentrations compared with the translation system derived from wheat germ (52). However, by analysis of the products of translation, this approach is becoming an important technique for examination of translational control of specific mRNAs and for mechanisms of post-transcriptional processing (51).

The *in vitro* translation assay has been employed to quantify specific translatable mRNA which is present at a concentration as low as 0.02% of the total cellular mRNA. Schutz *et al* (53) demonstrated that administration of hydrocortisone to rats resulted in an increase in the cellular concentration of tryptophan oxygenase mRNA from 0.023% in the control-animal to 0.079% in the induced-animal. The translation assay has been used to measure the levels of specific mRNA coding for a variety of enzymes such as glutamine synthetase from *Neurospora crassa* (54), carbamyl phosphate synthetase from tadpole liver (55), rat liver tyrosine aminotransferase (21) and fatty acid synthetase (25). The overall conclusion from these studies was that after the treatment (i.e., addition of inducer, change of diet), the

rate of synthesis of the enzyme was closely correlated with the increase in the amount of translatable mRNA. Therefore, the expression of the genes coding for these mRNAs is believed to be regulated primarly at the level of transcription (21, 24, 53, 54).

In contrast, there are reports which show that the amount of translatable mRNA does not correlate with the rate of synthesis of the protein. These proteins comprise a wide variety of cellular protein and include inducible enzymes (56), structural proteins (57), transport proteins (58) and adaptive enzymes (20). Since the synthesis of mRNA and protein are not coordinately regulated, post-transcriptional regulation has been invoked to account for the difference in levels of mRNA and protein. A well characterized example of one from of post-transcriptional regulation is the presence of untranslatable mRNA which is associated with ribunucleoprotein particles (57, 58, 59). For example, Olsen et al (60) reported that in reticulocytes, a significant amount of globin α chain mRNA was present in the post-ribosomal supernatant fraction as ribonucleoprotein particles. However, globin β chain mRNA was associated predominantly with polyribosomes (60). Although the precise function of ribonucleoprotein particles has not been demonstrated, they are thought to be a storage form of precursor mRNA which is destined to become polyribosomal mRNA (59). Another example of this form of control has been described by Zahringer et al (59). When rats were treated with a high iron-containing diet, a two-fold increase in the amount of ferritin mRNA was observed to be associated with polysomes. The increase in

ferritin mRNA accumulation was not inhibited by prior treatment of the rats with actinomycin D which suggested that de novo synthesis of ferritin mRNA did not account for the increase in specific mRNA. However, the decrease in amount of ferritin mRNA in the post-ribosomal fraction correlated exactly with the increase in the amount of ferritin mRNA associated with polysomes. Zahringer et al (59) proposed a model in which, prior to administration of iron, ferritin subunits were proposed to be bound to the ribonucleoprotein particles which prevented translation of ferritin mRNA. After the rats were fed on a high-iron containing diet, the inhibition of translation of ferritin mRNA was removed by an iron-promoted aggregation of subunits into ferritin.

Post-transcriptional regulation has also been observed to occur during the development of sea urchin embroyos (61, 62). These embryos have been shown to contain nuclear RNA transcripts which are unavailable for translation in the cytoplasm. Galau et al (61) have demonstrated that most of the nuclear RNA species present in the gastrula stage were also present throughout development of the egg to the mature oocyte. Moreover, Lee et al (62) observed that one mRNA sequence, derived from a cloned single-copy DNA fragment was continuously expressed during development in nuclear RNA. However, the amount of mRNA in the cytoplasm decreased from 850 molecules per cell in the blastula stage to less than 0.03 molecules per cell in the pluteus stage (62). Therefore, an enormous change in the cytoplasmic expression of this sequence occurred during the development of the egg. Lee et al (62) concluded that the nuclear transcripts were subjected to stage-specific differences

in post-transcriptional processing. The exact nature of the post-transcriptional mechanism is currently unknown.

Evidence for post-transcriptional control of enzyme synthesis has been reported to occur in Chlorella. Scragg et al (20) demonstrated that Chlorella cells, which did not contain detectable isocitrate lyase catalytic activity, contained high levels of mRNA coding for isocitrate lysae. The synthesis of isocitrate lyase has been shown to occur in Chlorella cells cultured in the dark in acetate-containing medium (20). When poly(A)-containing RNA, from dark-adapted cells, was placed in a wheat-germ protein synthesizing system, isocitrate lyase was detected in the products by immunoprecipitation with anti-isocitrate lyase antibodies. However, when poly(A)-containing RNA, isolated from a light-limited culture which did not contain enzyme activity, was placed in the translation system, the same amount of isocitrate lyase was synthesized in vitro and immunoprecipitated with anti-isocitrate lyase IgG. These authors concluded that during light limitation, a post-transcriptional mechanism became operative which prevented the expression of the isocitrate lyase mRNA in the cytoplasm (20).

Funkhouser and Ramados (56) have recently reported that in Chlorella, the nitrate-inducible nitrate reductase was synthesized in ammonium-containing cells as a protein precursor which was catalytically inactive. Ammonium-cultured cells contained almost no active enzyme. By Ouchterlony double diffusion analysis (63) of cell extracts from ammonium- and nitrate-cultured cells, crossreacting

material which was precipitated by anti-nitrate reductase IgG was observed to be present in the extracts isolated from both sources of cells. Funkhouser and Ramadoss (56) concluded that the nitrate reductase mRNA was present in uninduced cells (i.e., ammonium-cultured cells) and was actively being translated into a precursor protein which was subject to rapid turnover.

In conclusion, while the synthesis of many proteins has been shown to be regulated by transcriptional control mechanisms, there is increasing evidence that the synthesis of other proteins may be regulated at the post-transcriptional level. With the increasing availability of sensitive biochemical techniques, future studies of the control of gene expression should result in a greater understanding in the relative roles of transcription and post-transcription in the regulation of enzyme synthesis.

MATERIALS AND METHODS

Materials - $NADP^+$ was obtained from P-L Biochemicals Inc.; Preimmune rabbit serum, Grand Island Biological Company; CNBr-Sepharose-4B and Staphylococcus Protein A-Sepharose Cl-4B, Phamacia Fine Chemicals; para aminobenzyl cellulose, Accurate Chemical Corp.; SDS, highest purity, Gallard-Schlessinger Chemical Corp.; ammonium sulfate, ultra-pure enzyme grade, and sucrose, density gradient grade, Schwarz Mann; Sarkosyl, S1-nuclease, micrococcal nuclease, salmon sperm DNA, and Sigmacoat, Sigma Chemical Co.; oligo(dT)-cellulose and oligo$(dT)_{12-18}$ Collaborative Research; Chelex-100 and Enzymobeads, Bio-Rad Laboratories; dimethyl suberimidate dihydrochloride, Pierce Chemical Co.; calf liver tRNA, Boehringer Mannheim; sodium heparin, Riker Laboratories Inc.; [^{35}S]methionine, [^{3}H]deoxycytidine triphosphate, and [^{125}I]NaI, New England Nuclear Corp.; graduated micro-capillary tubes (5 µl), Clay Adams; RNase A, Worthington Enzymes; glass beads, VWR Scientific Inc. Purified avian myeloblastosis virus RNA-dependent DNA polymerase was supplied by Dr. J. W. Beard (Life Sciences Inc., St. Petersburg, Florida). All other chemicals and materials were of the highest grade available from Calbiochem Corp. or Sigma Chemical Co.

Organism - Chlorella sorokiniana (64) was cultured in either nitrate- or ammonium-containing medium as the sole nitrogen source as previously described (30).

NADP-GDH and Protein Assays - The deaminating activity the NADP-GDH was measured by a spectrophotometric assay which consisted of 52 mM

Tris-HCl, 195 mM L-glutamate, 0.97 mM $NADP^+$, and 10 to 50 µl of enzyme sample in a maximum volume of 1.05 ml and a pH of 8.75 at 38.5° (31). One unit of enzyme activity was defined as the amount of NADP-GDH activity required to reduce 1.0 µmol of $NADP^+$ per min at 38.5°. Total protein was measured as described by Lowry et al (65).

Cell Growth, Harvest, and NADP-GDH Purification - To obtain cells for NADP-GDH purification, Chlorella was cultured in ammonium-containing medium in a 35 liter-Plexiglas chamber (66) as described by Gronostajski et al (26). The cells were harvested with a refrigerated Sharples centrifuge at 50,000 rpm and passed twice through a mechanically driven French Pressure cell (Model 5-598A, American Instrument Co.) at a pressure of 18,000 to 20,000 p.s.i., to achieve 100% cell breakage (26). For use as an antigen in the preparation of monospecific antibody in rabbits and for preparation of the crosslinked-NADP-GDH holoenzyme antigen affinity column, the enzyme was purified to homogeneity as described by Yeung et al (27). When NADP-GDH was added as a carrier antigen to immunoprecipitation reaction mixtures, the enzyme was partially purified up to and including the DEAE-Sephacel pH 7.4 ion-exchange step (27). Fractions containing NADP-GDH activity were pooled, adjusted to contain 14 units/ml, and frozen at -20° in 500 µl aliquots.

Preparation and Purification of Rabbit Anti-NADP-GDH IgG - Rabbit anti-NADP-GDH IgG was prepared in New Zealand white rabbits as previously described (26). At 3 weekly intervals, which commenced

2 weeks after the primary immunization, NADP-GDH antiserum was obtained from whole blood by collecting 50 ml blood from the lateral ear vein at each bleeding (26). The IgG fraction was obtained from antisera by 2 ammonium sulfate precipitations from 0 to 40% concentration as described by Palmiter et al (67). The procedure for preparation of a completely stable NADP-GDH-Sepharose-4B affinity column was modified from that described previously by Shapiro et al (6). The modification employed the use of DMS1 to crosslink the NADP-GDH subunits after the holoenzyme was covalently bound to Sepharose-4B. Initial experiments were performed to examine the effects of the DMS1 on the specificity and extent of crosslink-formation of purified NADP-GDH in solution. One milligram of pure NADP-GDH (1.2 mg/ml) was dialysed against 50 ml crosslinking-buffer (80 mM triethanolamine, 10 mM magnesium acetate, 1 mM EDTA (free acid), 100 mM NaCl, and 1 mM dithiothreitol, pH 8.0) for 4.5 h at 4°. Immediately before the crosslinking reaction was to commence and prior to the addition of protein, 12 mg DMS1 was added to 1.3 ml crosslinking buffer and the pH was adjusted to pH 8.25 at 22° by the addition of approximately 50 µl of 4N NaOH. The dialysed NADP-GDH was then added in 700 µl to the crosslinking-buffer, so that the final concentration of DMS1 was 6 mg/ml. The sample was mixed and the crosslinking reaction was continued for 1 h at 22° with occasional mixing. The reaction was terminated by placing the sample on ice. The extent of crosslinking was examined by SDS polyacrylamide gel electrophoresis described by Weber and Osborn (68) in a 5% polyacrylamide separating

gel with a 2.3% stacking gel. The crosslinking buffer and pH employed were slightly modified from those described by Davies and Stark (69). To prepare NADP-GDH-crosslinked-Sepharose-4B, 10 mg of pure NADP-GDH was dialysed against 500 ml 0.1 M Na_2CO_3, pH 9.0 for 4.5 h at 4°. The dialysed protein was added to 1 g CNBr-Sepharose-4B which was previously swelled and washed with 300 ml 1 mM HCl. The gel was shaken gently overnight in a Dubnoff Shaker incubator at 4° to complete the binding reaction. Unreacted binding sites were reacted with 8 ml of 1 M ethanolamine in 0.1 M Na_2CO_3, pH 8.0 for 5 h at 4°. The NADP-GDH-Sepharose-4B gel was washed twice with 25 ml each of crosslinking-buffer which was modified to contain 2 mM dithiothreitol. Immediately prior to the crosslinking reaction, 150 mg DMSl was added to 23.7 ml crosslinking buffer and the pH was rapidly adjusted to 8.25 at 22° with 4M NaOH. After centrifugation, the crosslinking buffer, with 6 mg/ml DMSl, was added to the NADP-GDH-Sepharose-4B pellet and the gel was suspended in the buffer by gentle shaking for 1 h at room temperature. At the end of the 1 h incubation, the crosslinked gel was placed on ice and centrifuged to recover the NADP-GDH-crosslinked-Sepharose-4B. The gel was washed once in 40 ml PBS buffer, and then 35 ml gel was packed in a 10 ml syringe used as a column. To remove non-covalently absorbed protein, the gel was washed with 2 cycles of 20 ml each of 0.1 M $NaHCO_3$, 1 M NaCl, pH 7.6, and 0.1 M sodium acetate, 1M NaCl, pH 4.8, respectively (6). Rabbit anti-NADP-GDH IgG (150 A_{280} units), partially purified by ammonium sulfate fractionation, was applied to the column at room temperature

in a final volume of 8 ml. The IgG was incubated in the gel for 35 min by slowly recycling the sample through the gel, and then the unbound proteins were eluted in 50 ml PBS, followed by 3 cycles of high and low pH buffers as described above. The specific anti-NADP-GDH antibody was eluted in 15 ml with 0.1 M glycine, pH 2.8 and immediately dialysed against 2-liters of PBS. The antigen-purified IgG was concentrated to approximately 2 mg/ml by lyophilization, dialysed against 1-liter of the same buffer to lower the salt concentration, and stored in 500 µl aliquots at -20°. Because the purified anti-NADP-GDH IgG was required for immunological studies of the NADP-GDH mRNA, the last steps in antibody purification (i.e., antibody elution, lyophilization, and dialysis) were carried out with aseptic technique and with autoclaved buffers. To evaluate the stability and performance of the NADP-GDH-Crosslinked-Sepharose-4B during antibody purification, 6 µg ^{35}S-labelled NADP-GDH, purified by Yeung (70), was included in the initial binding of holoenzyme to CNBr-Sepharose-4B. By use of the modified crosslinked NADP-GDH affinity column procedure, an 80% yield of purified antibody was routinely obtained.

Preparation and Purification of Sheep Anti-Rabbit IgG for Indirect Immunoadsorption - Since indirect immunoadsorption was the chosen method for the partial purification of NADP-GDH mRNA, large quantities of purified sheep anti rabbit IgG (i.e., 200 mg) covalently attached to PAB-cellulose was required. Therefore, 90 mg preimmune rabbit IgG was purified and used as an antigen for the preparation of

specific antibodies in sheep and as a ligand for the purification of sheep anti-rabbit IgG. The IgG fraction of rabbit preimmune serum was partially purified by 2 ammonium sulfate precipitations described by Palmiter et al (67). The further purification of preimmune rabbit IgG was achieved by Protein A-Sepharose CL-4B as described by Miller and Stone (71). After collection of the protein pellet from the second ammonium sulfate precipitation step by centrifugation, the protein was dissolved in borate buffer (0.016 M boric acid, 0.012 M NaCl, 0.025 M NaOH, 0.1 mM PMSF and 0.02% NaN_3, pH 8.0) to give a final concentration of 11 A_{280} units/ml and then dialysed against 2-liters of the borate buffer to lower the ammonium sulfate concentration. The IgG fraction was applied in 5 ml to 1 g Protein A-Sepharose CL-4B which was previously swelled, packed in a 5 ml syringe-solumn, and equilibrated in the borate binding buffer. Chromatography was performed at 4°. The sample was applied to the column at a flow rate of 1 ml/min with the use of a Parmacia P3-peristaltic pump. After washing the column with at least 10 bed-volumes of the borate binding buffer at 1 ml/min, the residual A_{280} absorbing material which eluted from the column was negligible. The specific rabbit IgG fraction was eluted in 20 ml 0.1 M glycine, 0.1 M PMSF, and 0.02% NaN_3, pH 3.0 and was immediately dialysed overnight at 4° against 2-liters PBS. The protein was concentrated to dryness by lyophilization, and was stored as the lyophilized powder at -20° or in aliquots of 2 mg/ml in PBS at -20°.

Sheep anti-rabbit IgG was prepared in a yearling, 300 kg,

wethered male sheep by injection of 5 mg Protein A-purified rabbit IgG (in Freund's complete adjuvant) intramuscularly into each thigh. Subsequent booster immunizations were administered at 3 weekly intervals with 2 mg rabbit IgG in Freund's incomplete adjuvant injected intramuscularly into a single thigh. One week after the second immunization, 150 ml blood were collected from the carotid artery into 20 ml Vacutainer tubes. The bleedings and immunizations were continued at 3 weekly intervals over a period of 3 months. The IgG fraction was obtained from sheep antisera by 2 ammonium sulfate precipitations as previously described (67). To purify further the specific sheep anti-rabbit IgG fraction, 20 mg Protein A-purified rabbit IgG was covalently attached to CNBr-Sepharose-4B exactly as described by Shapiro et al (6). Chromatography was performed at 22°. Partially purified sheep IgG in PBS was applied in 8 ml and at a concentration of 16 A_{28} units/ml to 1 g rabbit IgG-Sepharose-4B. The gel was previously swelled, packed in a 5 ml syringe-column and equilibrated in PBS. The protein solution was recycled 3 times at a slow rate (0.5 ml/min). The gel was washed extensively with PBS to remove unbound IgG and the washing was continued until the A_{280} of the unabsorbed material was less than 0.03. The specific IgG fraction was eluted with 0.1 M glycine pH 2.8, 22° and immediately dialysed against PBS as described above. Purified sheep anti-rabbit IgG was covalently attached to PAB-cellulose by use of a diazotization reaction, exactly as described by Schutz et al (38). Approximately 200 mg PAB-cellulose was stored in 20 ml 2N NaOH for 1 h at 22°. The base-

soluble cellulose-supernatant was retained after centrifugation at 5,000 x g for 5 min and was collected after neutralization with 3 N HCl and centrifugation at 5,000 x g for 5 min. The activated cellulose pellet was suspended in 3 volumes ice cold 0.5 N HCl for 5 min at 1.5°. One-ninth volume of freshly prepared 2 M $NaNO_2$ was added to the cellulose suspension and the reaction was continued for 10 min at 2° with occasional mixing. After centrifugation, the supernatant was discarded and 25 mg sheep anti-rabbit IgG in 5 ml 0.2 M sodium borate, pH 8.6 was added. The pH of the resulting cellulose-protein mixture was adjusted to 8.6 at 2° and the binding reaction was continued overnight at 2°. By gently shaking the sample in a Dubnoff shaker incubator, the cellulose was kept in suspension. The binding reaction was terminated by centrifugation and the sheep IgG-cellulose was washed 3-times with 40 ml PBS. To remove non-covalently absorbed protein, the cellulose was washed with 2 cycles of 40 ml each of 0.1 M sodium borate pH 8.6 and 0.1 M sodium acetate pH 4.0. After washing the sheep IgG-cellulose 3-times with 40 ml PBS, the resin was stored at a final concentration of 10 mg/ml of cellulose in PBS with 0.2% sodium azide at 4°.

Measurement of Antibody Titer - The titer of rabbit anti-NADP-GDH IgG was determined by incubation of a known amount of NADP-GDH with a series of increasing amounts of antiserum or purified rabbit anti-NADP-GDH IgG. After incubation at 23° for 30 min, the immunoprecipitates were collected by centrifugation at 15,600 x g for 2 min. The percentage of GDH activity which remained in the supernatants was

measured. The standard conditions of the assay were 0.01 M NaH_2PO_4, 0.015 M NaCl, pH 7.2; 14.0 µg/ml NADP-GDH (0.7 units/ml); IgG equivalent to a range from 0 to 40 µg/ml of pure anti NADP-GDH IgG; the reaction was performed in a 40 µl volume.

The titer of sheep anti-rabbit IgG was measured by quantitative immunoprecipitation and determination of the amount of protein in the immunoprecipitate after centrifugation. Similar to the anti-NADP-GDH IgG immunotitration, a known amount of rabbit IgG was incubated with a series of increasing amounts of sheep antiserum or purified sheep anti-rabbit IgG under defined conditions. The standard assay conditions employed were 0.01 M NaH_2PO_4 pH 7.2 0.015 M NaCl; 0.46 mg/ml Protein A-purified rabbit IgG; sheep IgG equivalent to a range from 0 to 340 µg/ml; 35 min incubation at 22° in a 300 µl reaction volume followed by centrifugation at 15,600 x g for 2 min. The pellets were washed 2 times with 1 ml PBS, dried, and solubilized in 50 µl 1N NaOH for protein determination by the method of Lowry et al (65).

Radioiodination of Purified Rabbit Anti-NADP-GDH IgG - Radioiodination of purified anti-NADP-GDH IgG was achieved with high specific activity, carrier free [^{125}I]NaI (17 Ci/mg) in accordance with the procedure described by Bio-Rad (72). One-hundred micrograms of purified rabbit anti-NADP-GDH IgG (10 mg/ml) were incubated in a total volume of 115 µl with 5 µl of [^{125}I]NaI (200 mCi/ml), 0.5 g of Enzymobeads and 12 mM β-D-glucose in 0.09 M NaH_2PO_4, pH 7.2 for 15 min at 23°. The reaction was terminated by centrifugation at 8,000 x g for 2 min. To separate the ^{125}I-labelled antibody from excess unreacted

[^{125}I]NaI, 100 µl of supernatant were applied to a Sephadex G-25 column (1 cm x 15 cm) equilibrated in 0.14 M NaH_2PO_4, pH 7.2, and 10 mg/ml BSA. The flow rate was 0.5 ml/min which was maintained with a Pharmacia P-3 pump. The radioactivity in each 0.5 ml fraction was measured directly by a Beckman Gamma 4000 counter. Radioactive fractions which eluted at the void volume were pooled in a 2.5 ml total volume and stored at 4°. The specific activity of the ^{125}I-labelled anti-NADP-GDH IgG was determined to be 2.7 x 10^9 cpm/mg of protein.

Sterile Technique for Polysomes and RNA Isolation - To minimize degradation of RNA by RNase activity, the following precautions were taken. The glassware was heat-sterilized at 175° for 7 h. All of the buffers for RNA isolation and cDNA synthesis were prepared in deionized H_2O, which was passed through 50 ml of Chelex-100 to remove divalent cations. The buffers, plastic tubing, and small pieces of equipment were sterilized in the autoclave. Heparin and sucrose was added to the sterilized buffers and were not autoclaved. Dialysis tubing was boiled in 5% $NaHCO_3$ and 0.1 mM EDTA, washed in deionized H_2O, and autoclaved. Glassware which came into direct contact with cDNA was siliconized with a solution of Sigmacoat, dried, rinsed with Chelex-treated deionized H_2O, and baked at 175° for 7 h. When RNA samples were handled, plastic gloves were worn. During experiments, RNA-containing samples and buffers were maintained at 4°.

Cell Growth, Harvest, and Breakage Conditions for Polysome and RNA Isolation - To obtain cells for the partial purification of NADP-GDH

mRNA by indirect immunoadsorption (38) of NADP-GDH specific polysomes, Chlorella was cultured in ammonium-containing medium under continuous illumination in a 35 liter-Plexiglas chamber (66). However, for analytical polysome and RNA isolation studies and for antibody-polysome binding studies, Chlorella cells were presynchronized with intermittent illumination in 2.54 cm glass tubes (73). Since initial cell breakage studies demonstrated that cell size was an important factor in the percentage of cell breakage obtained, presynchronized cells were employed for small-scale polysome isolation studies in order to maximize the yield of undegraded total polysomes from small quantities of cells. Ammonium-cultured Chlorella cells were presynchronized by 3 successive cycles of intermittent illumination of 7 h light followed by 5 h dark (31). A fourfold increase in culture turbidity was observed at the end of each cell cycle. To obtain a synchronized culture at a stage in the cell cycle, when the maximum number of intermediate cells and the minimum number of daughter cells were present in the culture, the cells were transferred after the third dark period into a 3 liter-Plexiglas chamber and cultured with constant illumination (1,000 foot candles) at an initial optical density of 3.0 (550 nm, 1 cm light path, Beckman DB spectrophotometer). The cells were not diluted further. Cell size was examined by a microscope to determine the optimum time for cell harvest which was approximately 6 h after the onset of the continuous light cycle. Nitrate-cultured cells were cultured under identical conditions, except that the periods of intermittent illumination were lengthened to 10 h light and 6 h dark

(73). To obtain a similar cell size distribution as in ammonium-cultured cells, cells were cultured in the 3 liter-chamber for approximately 2 h longer than the ammonium-cultured cells. The changes were necessary to compensate for the decreased cellular growth rate of nitrate- compared to ammonium-cultured cells.

To harvest cells for the partial purification of NADP-GDH mRNA, the cells in the 35 liter-chamber were incubated with a final concentration of 25 µg/ml of cycloheximide for 30 min prior to cell harvest. This step was included to minimize ribosome "run-off" from mRNA during cell harvest and washing procedures. The culture was harvested at an optical density of 10 with a Sharples centrifuge at 50,000 rpm and the cells were washed twice in 0.01 M Tris-HCl pH 8.25 at 4°. The cells were resuspended in the Tris-HCl buffer (i.e., 4 parts cells: 1 part Tris-HCl buffer, w/v) and the resulting thick suspension was frozen and stored in 125 ml polyethylene bottles in liquid nitrogen. Approximately 120 g cells were obtained by this procedure. On the day of use, the cells were thawed and washed once in 100 ml cell breakage buffer (25 mM Tris-HCl, 250 mM KCl, 25 mM $MgCl_2$, 15 mM EGTA, 250 mM sucrose, 1 mg/ml heparin, 2.5 µg/ml cycloheximide, pH 8.0) by centrifugation at 9,750 x g for 5 min at 4° in a Sorvall GSA rotor. Equal 5 g aliquots of cells were resuspended in 15 ml of cell breakage buffer, and transferred into a 75 ml stainless steel homogenization chamber containing 20 g 0.1 mm glass beads. Cell breakage was obtained by continuous agitation in a cell homogenizer (Braun, Type 2876) for 45 s at 4000 cycles/min with

intermittent cooling with liquid CO_2 (i.e., 5 s on, 5 s off).

When synchronized cells were used as starting material for analytical total polysome isolation and polysome-antibody binding studies, cycloheximide was added to the culture as described above except that the length of incubation was 10 min. The cells were harvested from the 3 liter-Plexiglas chamber with a Sorvall RC-2B centrifuge at 9,750 x g in a GSA rotor. The cells were washed in Tris-HCl buffer in an identical manner except that the cells were frozen at -20° as 4 g cell pellets in 50 ml stainless steel centrifuge tubes. Alternatively, the cells were not frozen and were used without further delay, as starting material for polysome isolation. Approximately 16 g of cells were routinely obtained by this procedure. When the cells were thawed, the cells were washed and resuspended in cell breakage buffer as described above, except that cycloheximide was omitted from the buffer. Up to 5 g cells could be placed in the 75 ml chamber without decreasing the percentage of broken cells. However, the ratio of bead weight to cell weight was kept constant at 4 to 1 in all experiments. Because the cells were synchronized, 50% cell breakage could be obtained with 25 s of homogenization under the same conditions employed for asynchronous cells.

Polysome Isolation for Sedimentation Analysis in Sucrose Gradients - Total cellular, undegraded polysomes were isolated from either ammonium- or nitrate-cultured cells by a modification of the procedure described by Buell et al (37). Immediately following partial cell breakage with the cell homogenizer, the total contents of

the 75 ml chamber was transferred into a 45 ml polyethylene centrifuge bottle and stored on ice. To solubilize membrane-bound polysomes and to lyse partially broken cells (74), the homogenate was incubated for 10 min at 4° with 0.1 volume of freshly prepared 10% sodium deoxycholate and 10% Triton X-100 with occasional gentle mixing. The detergent-treated homogenate was centrifuged at 27,000 x g for 5 min in a Sorvall SS-34 rotor at 4° to pellet the glass beads and the unbroken cells. After the pellet was discarded, the supernatant was centrifuged at 27,000 x g for 25 min to obtain the low-speed supernatant. Total cellular polysomes were isolated from the low-speed supernatant by centrifugation in a discontinuous sucrose gradient which consisted of 4 ml of 2.5 M sucrose in 25 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, and 0.5 mg/ml sodium heparin, pH 7.4 at 22°, overlaid with 13.9 ml 1 M sucrose in the same buffer in a Beckman SW-27 cellulose nitrate tube. These buffers were similar to those used by Buell et al (37) except that it was necessary, in order to prevent polysome breakdown, to replace NaCl with KCl in all of Chlorella cell breakage and polysome isolation buffers. Equal 20 ml aliquots of the low speed supernatant were carefully layered over the upper 1 M sucrose containing layers, and then centrifuged at 27,000 rpm for 3 h at 4° in an SW-27 rotor. After the centrifugation, the band of polysomes was clearly visible at the 1 M and 2.5 M sucrose interphase. Approximately 6 to 8 ml of polysomes were removed by insertion of an 18-gauge needle approximately 5 mm below the 2.5 M sucrose interphase and extraction of the polysomes into a 10 ml syringe as described by

Palacios et al (75). Approximately 25 A_{260} units of polysomes/g of broken cells were routinely obtained by this procedure.

Determination of Polysome Integrity - The polysomes obtained from the previous step were diluted fivefold with polysome buffer containing 0.5% Triton X-100 and no sucrose, to lower the sucrose concentration. To concentrate the polysomes, 18 ml of diluted polysomes were placed on 20 ml of 1 M sucrose in polysome buffer and centrifuged for 4 h at 100,000 x g at 4° in an SW-27 rotor. The polysomal pellet was then resuspended in polysome isolation buffer containing 0.5% Triton X-100 and 0.2 M sucrose at a final polysome concentration of 10 A_{260} units/ml. In this buffer, polysomes were stable for at least 24 h at 1-2°.

To evaluate polysome integrity, 0.5 ml of resuspended polysomes, containing approximately 5 A_{260} Units was placed on a 12 ml linear sucrose gradient (0.5 M to 1.5 M sucrose) containing 25 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, and 0.5 mg/ml sodium heparin. The gradients were centrifuged in a Beckman SW-40 rotor at 36,000 rpm for 1.5 h at 4°. The absorbance at 254 nm of the fractionated polysomes was measured by piercing the bottom of the tube with an ISCO gradient fractionator and by displacement of the contents upwards into an ISCO UV absorbance monitor (Model No. UA-5) by use of a Pharmacia P-3 peristaltic pump at a setting of 2.3 (approx. 0.75 ml/min).

Polysome Isolation for use in Indirect Immunoadsorption of NADP-GDH-Specific Polysomes - To obtain sufficient quantities of total polysomes, from which to partially purify the NADP-GDH mRNA, a large scale

polysome isolation procedure was employed. As described above, 90 g cells were subjected to the homogenization treatment which resulted in 50% cell breakage. Because of the large quantity of cells, the cells were split up into 3 equal sized aliquots of 30 g each and stored at -20° until cell breakage was performed. Each 30 g of cells required approximately 1.5 h of homogenization which was achieved by repetition of 6 breakage cycles of 5 g cells per cycle in the 75 ml homogenization chamber. All 90 g of cells were subjected to cell breakage, and total polysomes were isolated from the cells on the same day. The combined homogenates obtained from 30 g cells were incubated in a 250 ml centrifuge bottle with 0.1 total volume of freshly prepared 10% sodium deoxycholate and 10% Triton X-100 as described for the small scale polysome isolation procedure. After the beads and unbroken cells were removed by a low-speed centrifugation at 9,750 x g for 5 min in the GSA rotor, the low-speed supernatant was subjected to a second low speed centrifugation at 17,000 rpm for 25 min in an SW-27 rotor. Total cellular polysomes were isolated from the low-speed supernatant by centrifugation in discontinuous sucrose gradients which were modified to contain 4 ml 2.5 M sucrose in 25 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 0.5 mg/ml sodium heparin and 0.2% Triton X-100, pH 7.4 at 22°, overlaid with 10 ml 1.2 M sucrose in the same detergent-containing buffer. Polysomes were separated from the low-speed supernatant by centrifugation for 3 h at 100,000 x g in an SW-27 rotor. Total polysomes were removed from the 2.5 M sucrose interphase with a sterile Pasteur pipet, placed in a 125 ml nalgene bottle, and

frozen in liquid nitrogen. Polysomes which were isolated from the remainder of 60 g cells were combined with the frozen polysomes at 2 separate times later in the day. A total of 1,650 A_{260} units were obtained from 90 g cells with this procedure which corresponded to approximately 36 A_{260} units/g broken cells.

Identification of NADP-GDH Specific Polysomes in Ammonium- and Nitrate-Cultured Cells - Total cellular polysomes were isolated from 3 g each of synchronized ammonium- and nitrate-cultured cells as described above. To lower the sucrose concentration, polysomes were diluted with an equal volume of polysome buffer (25 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA and 1 mg/ml sodium heparin, pH 7.4). Polysomal aggregates were removed by centrifugation at 10,000 rpm for 10 min at 4° in a Sorvall HB-4 rotor. Anti-NADP-GDH IgG-polysome binding reactions were performed by incubation of 10 A_{260} units each of polysomes isolated from induced and uninduced cells with 20 µg of purified ^{125}I-labelled rabbit anti-NADP-GDH IgG in a total volume of 3.0 ml each for 2 h at 1.5°. Because of the possibility that polysome concentration might affect the binding reaction, polysomes (10 A_{260} units) were also incubated in a parallel reaction with 20 µg of ^{125}I-labelled rabbit anti-NADP-GDH IgG at a concentration of 9 A_{260} units/ml, instead of 3.3 A_{260} units/ml. After the antibody-polysome binding reaction, excess unreacted antibodies were removed from the ^{125}I-labelled polysomes by application of the total sample onto 8.0 ml of 1.0 M sucrose in polysomal buffer and centrifugation of the polysomes at 36,000 rpm

for 2.25 h in an SW-41 rotor. The polysomal pellets were partially resuspended in 1.0 ml of polysome buffer containing 0.2% Triton X-100 and gently shaken for approximately 6 h at 1.5° to complete the resuspension. Immediately before sucrose gradient analysis of ^{125}I-labelled polysomes, remaining aggregates were removed by centrifugation at 8,000 rpm for 2 min. Approximately 5.0 A_{260} units of polysomes in 0.5 ml were centrifuged in 11 ml linear sucrose gradients at 36,000 rpm for 1.5 h as described above. The gradients were fractionated into 0.25 ml fractions which were counted directly in the Beckman 4000 Gamma counter. Simultaneously, the absorbance at 254 nm of the fractionated polysomes was continuously monitored with an Isco UV monitor as described above.

Isolation of Total Cellular and Total Polysomal RNA - The isolation of total polysomal RNA from Chlorella polysomes or total cellular RNA from Chlorella homogenates was achieved by extraction with an SDS/phenol/chloroform mixture (23). To obtain total polysomal RNA, total cellular polysomes were isolated from ammonium- or nitrate-cultured cells as described above. Aliquots of 5 ml polysomes containing 50 A_{260} units were pipeted into a 30 ml Corex glass tube on ice which contained 5.0 ml of 10 mM Tris-HCl, 5 mM EDTA, 0.5% SDS, pH 7.4, and 5.0 ml redistilled phenol saturated with 10 mM Tris-HCl, 5 mM EDTA, pH 7.5. The sample was mixed, allowed to stand 5 min on ice, and 5.0 ml ice-cold chloroform was added. After a further 15 min at 4° with occasional mixing, the phases were separated by centrifugation in an HB-4 rotor for 10,000 rpm for 10 min at 2°. The aqueous

phase was carefully removed into a clean Corex glass tube, and was re-extracted twice with equal volumes of phenol and chloroform, exactly as described above, except the time of extraction was 10 min. The aqueous phase was collected by centrifugation as described above. The RNA was precipitated by the additions of 0.1 volume of 2.4 M ammonium acetate and 2.5 volumes of cold 95% redistilled ethanol at -20° for approximately 2 h. The RNA precipitate was pelleted by centrifugation at 2,000 rpm for 2 min in an HB-4 rotor. The ethanol was discarded and the RNA was lyophilized to dryness. At this stage, the RNA was either stored in liquid nitrogen as an aqueous solution or it was subjected to oligo(dT)-cellulose column chromatography (76). However, when total polysomal RNA or non-poly(A)-containing RNA was assayed for [^{35}S]methionine incorporation in rabbit reticulocyte lysates, translational inhibitors such as SDS and heparin were removed from the total polysomal RNA precipitate by extraction with 3M sodium acetate and 5 mM EDTA, pH 7.0 at 22° as described by Buell *et al* (37). The RNA pellet was suspended in 5 ml of the buffer and after 10 min, the RNA was pelleted by centrifugation at 2,000 rpm for 10 min in an HB-4 rotor. The supernatant was discarded. After 3 washes with the sodium acetate buffer, the RNA was centrifuged at 10,000 rpm for 5 min at 2°. The RNA pellet was washed twice in cold 70% ethanol and 0.2 M NaCl, lyophilized to dryness, and resuspended in deionized H_2O at a concentration of 0.5 mg/ml. The RNA was frozen and stored in liquid nitrogen. To obtain total cellular RNA from *Chlorella* cells, ammonium- or nitrate-cultured cells were homogenized

with the Braun cell homogenizer as described above, so that 50% cell breakage was obtained. Twelve milliliters of the homogenate were poured immediately into a 30 ml cold Corex glass tube which contained 0.3 ml of 200 mM EDTA, pH 7.4. Next, 0.6 ml of 20% SDS was added so that the final concentrations of EDTA and SDS were 5 mM and 1%, respectively. The RNA was extracted 3 times by the addition of equal volumes of phenol and chloroform exactly as described above for total polysomal RNA isolation. The RNA was precipitated from the aqueous phase in the same manner. If the 3 M sodium acetate wash was omitted, the RNA was re-precipitated with ethanol and 0.3 M sodium acetate overnight at 20°, or until the RNA was fractionated by oligo(dT)-cellulose column chromatography.

Oligo(dT)-Cellulose Column Chromatography - To fractionate total polysomal RNA or total cellular RNA into poly(A)-containing and non-poly(A)-containing RNA species, oligo(dT)-cellulose column chromatography was employed (9, 76). The RNA pellet obtained after extraction with phenol/chloroform and precipitation with ethanol was resuspended at a concentration of 10-15 A_{260} units/ml in 10 mM Hepes, 0.5% SDS, pH 7.5. To destroy RNA aggregates and secondary structure, the sample was heat-treated in this buffer in a 68° waterbath for 3 min (50). Immediately following the heat treatment, the sample was quick-cooled in an ethanol-ice bath. Sufficient 4 M NaCl was added to the sample so that the final concentration of NaCl was 0.4 M. The sample was then applied to a 0.5 g column of oligo(dT)-cellulose equilibrated in binding buffer (i.e., 10 mM Hepes, 0.5% SDS, 0.4 M NaCl pH 7.5) at

0.8 ml/min by use of a Pharmacia P-3 peristaltic pump at a setting of 2.8. Chromatography was performed at room temperature (at least 23°). The sample was recycled one time through the column at this flow rate. The column was washed with binding buffer at a flow rate of 0.8 ml/min until the absorbance at 254 nm had returned to the baseline value. To remove SDS from the column, the column was washed with binding buffer without SDS until the absorbance at 254 nm was zero. The poly(A)-containing RNA fraction was eluted in a minimum volume of 10 mM Hepes pH 7.5 (i.e. less than 3.5 ml) into a sterilized 12 ml polyallomer tube on ice. The absorbance at 254 nm of the RNA sample was measured and if necessary, calf-liver tRNA (5 mg/ml) was added so that the total concentration of RNA was at least 5 μg/ml. The RNA was precipitated by the additions of either 0.1 volume of 3.0 M sodium acetate, pH 5.5 or 0.1 volume of 2.4 M ammonium acetate, pH unadjusted, and 2.5 volumes of ethanol at -20° overnight. The unbound non-poly(A)-containing RNA fraction was precipitated in the same manner, except that the addition of calf-liver tRNA was not required. The percentage of RNA which was retained by the column was 1% of total cellular RNA and 2.5% of total polysomal RNA. No more than 20% of the poly(A)-containing RNA retained by the column was estimated to be contaminated by non-poly(A)-containing RNA.

Protein Synthesis in Rabbit Reticulocyte Lysates - New Zealand White rabbits were made anaemic by daily, subcutaneous injections of neutralized 2.5% phenylhydrazine-HCl (0.3 ml/kg) over a period of 5 days by Dr. W. Molin from our laboratory. The rabbits were bled

by heart puncture on the seventh day after the initial injection. The blood cells were collected by centrifugation at 600 x g for 10 min at 2°, washed twice in 3 volumes of 0.13 M NaCl, 5 mM KCl, and 7.5 mM $MgCl_2$, and lysed by the addition of an equal volume of deionized H_2O for 5 min at 2°. The cellular debris was removed by centrifugation at 15,000 x g for 10 min at 2°. The lysates were stored in 5 ml aliquots in liquid nitrogen for up to 10 months without an observed loss of translational capacity. The endogenous mRNA present in the lysates was destroyed prior to the addition of Chlorella RNA by the action of a calcium-dependent micrococcal nuclease exactly as described by Pelham and Jackson (52). One milliliter of lysate was thawed and 10 µl of creatine phosphokinase (5 mg/ml) and 25 µl of hemin (1 mM) were added so that the final concentration were 50 µg/ml and 25 µM, respectively. A stock solution was prepared which consisted of 100 µl of 0.2 M creatine phosphate, 100 µl of 2 M KCl and 10 mM $MgCl_2$, and 100 µl of 19 unlabelled amino acids which ranged in concentration from 0.2 to 4.0 mM (23). One hundred and fifty microliters of the stock solution were added to 800 µl of supplemented lysate. To digest the endogenous poly(A)-containing RNA, 9.5 µl of 0.1 M $CaCl_2$ and 9.5 µl of 1 mg/ml micrococcal nuclease were added to the supplemented lysate so that the final concentrations of $CaCl_2$ and micrococcal nuclease were 10 mM and 10 µg/ml, respectively. The lysate was incubated at 20° for 15 min. The nuclease was then inactivated by the addition of 19 µl of 0.1 M EGTA. The micrococcal nuclease-treated lysate was placed on ice and used immediately. In vitro protein synthesis was

carried out essentially as described by Pelham and Jackson (52), except that 25 μCi of ^{35}S-labelled methionine was added to 47.5 μl of nuclease-treated lysate. Poly(A)-containing RNA was added up to a final concentration of 40 μg/ml. Non-poly(A)-containing RNA was added up to a final concentration of 200 μg/ml. Because calf-liver tRNA stimulated the incorporation of ^{35}S-labelled methionine into trichloroacetic acid insoluble products by as much as 50% in 60 min, calf-liver tRNA (5 mg/ml) was routinely added to the lysate mixtures to give a final concentration of 200 μg/ml. Typical *in vitro* protein-synthesis assays consisted of 47.5 μl of nuclease-treated lysate reaction mix, 2.5 μl of ^{35}S-labelled methionine (1169 Ci/mol), up to 4 μl of an aqueous solution of RNA, and 1 μl of an aqueous solution of tRNA. Following incubation for 1 h at 30°, the reaction was terminated by placement of the lysates on ice and by the addition of 0.1 volume of 100 mM NaH_2PO_4, 150 mM NaCl, 10% Triton X-100, 10% sodium deoxycholate, and 150 mM L-methionine, pH 7.2 and by the addition of 0.1 volume of 50 mg/ml BSA. Stimulation of total protein synthesis was estimated by comparison of hot-trichloroacetic acid-insoluble radioactivity in reactions incubated in the presence or absence of exogenous RNA. After protein synthesis was terminated, equal aliquots of lysate (i.e., from 1-5 μl) were spotted onto 1 cm Whatmann No. 3 filter paper discs and placed in a beaker which contained 500 ml of 10% trichloroacetic acid, heated to a rapid boil. After 10 min at this temperature with occasional swirling, the filter paper discs were removed. The precipitated protein on the disc was

washed with 2 volumes of 500 ml each of H_2O, ethanol, and acetone, respectively. The discs were air-dried, placed in a 4 ml scintillation vial, and the radioactivity measured in 2.5 ml of toluene-containing scintillation fluid (27). In initial experiments, Chlorella RNA was translated in a commercial cell-free system derived from nuclease-treated rabbit reticulocytes, exactly as described in the protocol provided (New England Nuclear, Catalog No. NEK-001). Protein synthesis took place at 37° in a 25 µl reaction volume in the presence of 42.5 µCi of ^{35}S-labelled methionine. Incorporation of ^{35}S-labelled methionine into hot trichloracetic acid-precipitable radioactivity was estimated as described above.

Identification of NADP-GDH Synthesized in Vitro - Incorporation of ^{35}S-labelled methionine into NADP-GDH synthesized in vitro was estimated by quantitative direct immunoprecipitation from the lysate with purified rabbit anti-NADP-GDH IgG (27). After the termination of protein synthesis, the total lysate volume was diluted to 110 µl with antibody binding buffer (0.01 M NaH_2PO_4, 0.015 M NaCl, 15 mM methionine, 1% Triton-X-100, 1% sodium deoxycholate, 5 mg/ml BSA, pH 7.2 at 22°). The diluted lysate was carefully layered over 1 M sucrose in antibody binding buffer without BSA in a 12 ml polyallomer tube and subjected to 100,000 x g for 1 h at 4° in an SW-41 rotor. After centrifugation, an aliquot of 35 µl or 70 µl lysate was carefully removed from the supernatant and transferred into a 1.5 ml Eppendorf tube. If the high-speed centrifugation was omitted, the lysates were centrifuged at 15,600 x g for 5 min prior to immunoprecipitation.

Similarly, the antibody binding buffer, carrier NADP-GDH, and rabbit anti-NADP-GDH IgG were pre-centrifuged at 15,600 x g for 5 min, immediately prior to the immunoprecipitation reaction. The lysate supernatant was diluted at 250 µl by the addition of antibody binding buffer. Five micrograms of partially purified NADP-GDH, and 25 µl of purified rabbit anti-NADP-GDH IgG (2.4 mg/ml) were added to the lysate and mixed well. To maximize the probability of quantitative binding of the specific antibody to *in vitro* synthesized NADP-GDH polypeptides, the immunoprecipitation reaction was carried out with a two- to three-fold mass excess of specific antibody. Therefore, the ratio of specific antibody to carrier NADP-GDH was always 12:1, w/w. Each batch of purified rabbit anti-NADP-GDH IgG was titered according to the standard assay procedure described above, before it was used in direct immunoprecipitation reactions. The immunoprecipitation reaction was carried out for 2 h at 4°. By comparison of the amount of radioactivity associated with the immunoprecipitate which was obtained from a lysate incubated for 4 h at 4°, the antibody precipitation reaction was estimated to be complete within 2 h under the conditions described above. After the incubation, the immunoprecipitate was collected by centrifugation of the lysate for 3 min at 15,600 x g at 4°. The supernatant, which contained the vast excess of unincorporated, soluble ^{35}S-labelled methionine and non-antigenic *Chlorella* proteins, was removed and discarded. The immunoprecipitated pellet was vigorously dispersed in 100 µl of antibody binding buffer and transferred onto 200 µl of 0.9 M sucrose in the same buffer in a

400 µl micro-Eppendorf tube (27). To ensure complete transfer of the immunoprecipitate, the 1.5 ml Eppendorf tube was rinsed with an equal volume of antibody binding buffer and combined with the first 100 µl aliquot. The 0.9 M sucrose buffer was precentrifuged at 15,600 x g for 5 min before this washing step (27). The immunoprecipitate was pelleted through the 0.9 M sucrose phase by centrifugation of the micro-Eppendorf tube for 20 min at 15,600 x g at 4°. Under similar conditions, Yeung (70) estimated that 85-90% of the radioactivity in the immunoprecipitate was recovered in the pellet. The supernatant and sucrose layers were removed by aspiration. The pellet was vigorously dispersed in 100 µl PBS, and transferred to a clean 1.5 ml Eppendorf tube. The micro-Eppendorf tube was rinsed with 2 aliquots of 100 µl each of PBS to ensure quantitative recovery of the immunoprecipitate. The washed immunoprecipitate was collected by centrifugation at 15,600 x g for 5 min. The supernatant was discarded. The immunoprecipitate was either frozen at this point and stored at -20°, or it was dissolved in 100 µl of the Laemmli incubation buffer, and heated at 100° for 7 min for SDS gel electrophoresis (77). To obtain an estimate of the radioactivity associated with the immunoprecipitate prior to SDS gel electrophoresis, a 5 µl aliquot of the boiled, solubilized immunoprecipitate was removed, added to 0.4 ml H_2O, and the radioactivity was measured in 4 ml Triton-toluene scintillation fluid as described above.

SDS-Polyacrylamide Gel Electrophoresis of Lysate Products - To fractionate and analyse the products synthesized in vitro, SDS gel electro-

phoresis was performed exactly as described by Laemmli (77). For determination of total protein synthesis *in vitro*, a 5 µl aliquot of the 100,000 x *g* lysate supernatant was mixed with an equal volume of Laemmli incubation buffer. During NADP-GDH mRNA purification experiments, 5 µl aliquots were also removed from the lysate prior to the high speed centrifugation and from the supernatant obtained after the NADP-GDH immunoprecipitate was removed by centrifugation. In all cases, the samples were mixed with an equal volume of Laemmli incubation buffer and heated at 100° for 7 min. The length of incubation was increased by 2 min, since it was observed that samples which were subjected to the longer incubation had lower radioactivity associated with the stacking gel after electrophoresis for approximately 1.5 h at 2 mA/gel. To obtain the position of the authentic NADP-GDH subunit in the gel, *in vivo*-labelled [^{35}S]NADP-GDH (70) was subjected to electrophoresis in a parallel gel. The separating gel was composed of 9% polyacrylamide. After electrophoresis, the gels were soaked for 48 h in 2-liters of a solution of 7% acetic acid and 5% methanol at room temperature. The solution was changed at least twice. The gels were rinsed with H_2O and then frozen in dry ice. In some experiments, the gels were frozen and stored at -20° until being sliced. The frozen gels were sliced into 1 mm slices by a mechanically driven Mickle gel slicer (The Mickle Laboratory Engineering Co., England; distributed in U.S.A. by Brinkmann Instruments). One to two slices of gel were placed in each 7 ml scintillation vial which contained 0.4 ml of 30% H_2O_2. The gel slices were dissolved by incubation of

the vials at 80° for 6 h. After the vials were cooled, the radioactivity was measured in 4 ml Triton-toluene scintillation fluid, exactly as described above.

Estimation of NADP-GDH mRNA Molecular Weight - Sucrose density centrifugation of total Chlorella polysomal poly(A)-containing RNA was performed by a slight modification of the procedure described by Neprokroeff and Porter (25). Eighty micrograms of total polysomal poly(A)-containing RNA were heat-treated for 3 min at 68° in 200 µl of 10 mM Hepes, 1 mM EDTA and 1 mM NaCl, pH 7.5. The RNA was quick-cooled in an ethanol-ice bath. Equal 100 µl aliquots of RNA were applied to two 10.8 ml linear sucrose gradients (10-25% w/vol) prepared in the same buffer. Gradients were centrifuged at 100,000 x g for 19 h at 4° in an SW-41 rotor. Fractions of 0.5 ml were collected from each gradient as described above and corresponding fractions from the two gradients were pooled. The absorbance at 260 nm of each 1 ml pooled fraction was measured with a Gilford spectrophotometer (Model No. 240). According to the A_{260} value obtained, the fractions were combined into 6 larger fractions which represented the overall pattern of sedimentation of RNA in the gradient. Calf-liver tRNA was added to each fraction so that the final concentration of tRNA was 5 µg/ml. The RNA was precipitated and prepared for in vitro translation as described above. One microgram of total poly(A)-containing RNA from each fraction was translated in vitro and the products were immunoprecipitated with purified anti-NADP-GDH IgG exactly as described above. Calf-liver tRNA and Chlorella non-poly(A)-

containing RNA were employed as molecular weight markers and were centrifuged in two parallel gradients. The absorbance at 254 nm of this RNA was measured with an Isco UV absorbance monitor as described above.

Partial Purification of NADP-GDH mRNA by Indirect Immunoadsorption - The NADP-GDH-specific polysomes were selected from total Chlorella polysomes by use of an indirect immunoadsorption procedure (38). Total Chlorella polysomes (1650 A_{260} units) which were frozen in liquid nitrogen, were thawed and placed on ice. To lower the sucrose concentration, the polysomes were diluted with 2.5 volumes of polysome dilution buffer (25 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA and 0.1 mg/ml heparin, pH 7.4). After dilution, the polysome concentration was 24 A_{260} units/ml in a total volume of 68 ml. Three micrograms of purified rabbit anti-NADP-GDH IgG were added per A_{260} unit of polysomes. The antibody-polysome mixture was incubated for 1.5 h at 4° with occasional mixing. The antibody-polysome solution was then pipeted onto discontinuous sucrose gradients which consisted of 2.5 M sucrose in polysome dilution buffer, overlaid with 7 ml 1 M sucrose in the same buffer. Excess unreacted antibodies were separated from total polysomes and antibody-bound polysomes by centrifugation at 26,000 rpm at 4° for 4.25 h in an SW-27 rotor. No detergents were added to the buffers. Following the centrifugation, an opalescant band of polysome was observed at the 2.5 M sucrose interphase. The polysomes were devoid of green pigmentation which remained exclusively in the supernatant after centrifugation. The supernatant and 1 M

sucrose phases were aspirated by suction and discarded. The polysomes were removed with a Pasteur pipet and placed in a 30 ml glass Corex tube. By centrifugation, a 35% recovery of polysomes was obtained, which consisted of 600 A_{260} units polysomes in a 12 ml volume. The sucrose concentration was lowered by the addition of 2.5 volumes of polysome dilution buffer. To prepare the sheep-anti rabbit IgG cellulose for indirect immunoadsorption, 450 mg of anti-rabbit IgG-cellulose were washed with 20 ml of the secondary antibody binding buffer (25 mM Tris-HCl, 140 mM KCl, 5 mM $MgCl_2$ and 0.1 mg/ml sodium heparin, pH 7.4). The sheep anti-rabbit IgG-cellulose was collected by centrifugation at 5,000 rpm for 5 min in an HB-4 rotor. Six hundred A_{260} units of total Chlorella polysomes, which had previously been incubated with rabbit anti-NADP-GDH IgG, were added in a total volume of 30 ml to the sheep anti-rabbit IgG-cellulose. The sheep IgG-cellulose was resuspended in the polysome solution, split into 2 equal 20 ml volumes, and incubated with gentle shaking for 1 h at 2°. After the incubation, the sheep IgG cellulose-antibody-polysome complex was sedimented at 4,000 rpm for 5 min at 4°. Unadsorbed Chlorella polysomes were removed from the cellulose by a series of 5 washes of 200 ml each. The buffer washes consisted of 10 mM Hepes, 5 mM $MgCl_2$ and 0.1 mg/ml sodium heparin, pH 7.4, except that the third wash also contained 1% sodium deoxycholate and 1% Triton X-100. Immunoselected polysomes were released from the cellulose-matrix by the addition of 5 ml of 20 mM EDTA, 10 mM Hepes and 0.1 mg/ml sodium heparin, pH 7.4. Under these conditions,

the ribosomal subunits were dissociated from the mRNA, and the mRNA was released into the supernatant. Complete dissociation of the immunoselected poly(A)-containing RNA and of RNA from the ribosomal subunits was achieved by the addition of 0.05 volumes of 20% SDS and 0.05 volumes of 20% Sarkosyl. The total volume of detergent-containing RNA solution was 5.5 ml. To minimize secondary structure and RNA aggregation, the RNA-containing solution was placed in a 68° waterbath for 2.5 min and rapidly cooled in an ethanol-ice bath. Immunoselected poly(A)-containing RNA was separated from rRNA by oligo(dT)-cellulose column chromatography, essentially as described above. The heat-treated immunoselected RNA was diluted with an equal volume of 10 mM Hepes, 0.8 M NaCl, pH 7.5 at 22°, so that the final concentration of NaCl was 0.4 M. The sample was applied to 1.5 ml (0.3 g) swelled oligo(dT)-cellulose which was equilibrated in binding buffer (10 mM Hepes, 0.4 M NaCl and 0.5% SDS, pH 7.5 at 22°). The flow rate was adjusted and maintained at 32 ml/h by the use of a Pharmacia P-3 pump. The absorbance of the eluate was continuously measured at 254 nm with an LKB Uvicord (type 4701A). Chromatography was performed at 23°. Unbound RNA was collected and recycled once through the column. The unbound, recycled RNA fraction was collected and the RNA was precipitated by the addition of 2.5 volumes of redistilled ethanol at -20° overnight. After the unbound RNA fraction was collected, the column was washed with binding buffer with the A_{254} was returned to the baseline value. To remove SDS from the column, the column was washed with binding buffer without SDS, until

the A_{260} was zero. Poly(A)-containing immunoselected RNA was eluted in a minimum volume (3.3 ml) in the presence of 10 mM Hepes pH 7.5. The RNA was collected into a 12 ml polyallomer tube which was placed in ice. Because of the very small yield of immunoselected polyA-containing RNA, the absorbance at 260 nm was measured on the total sample without dilution. By use of an extinction coefficient for RNA equal to 1 A_{260}U/24 mg RNA, the overall yield of immunoselected mRNA was calculated to be 9 µg. To precipitate the mRNA, calf-liver tRNA was added to the RNA-containing solution so that the final total concentration of RNA was 5 µg/ml (78). The RNA was precipitated by the additions of 0.1 volume of 2.4 M ammonium acetate (78) and 2.5 volumes of redistilled ethanol at -20°. After 36 h at -20°, the RNA precipitate was pelleted by centrifugation at 30,000 rpm for 1 h at -5° in an SW-41 rotor (78). The tube was drained, wiped out carefully with tissues, lyophilized to dryness, and stored in the vapor phase of liquid nitrogen.

^{3}H-labelled cDNA Synthesis - The procedure which was employed for the preparation of ^{3}H-labelled cDNA from either immunoselected or total poly(A)-containing Chlorella RNA was essentially the same as described by Craig et al (80). On the day of cDNA synthesis, 1 ml stock solutions of 10 mM dATP, 10 mM dTTP and 10 mM dGTP were prepared and each solution was adjusted to a pH between 6.5 and 7.5 by the addition of 1 N NaOH. Five microliters of each deoxynucleotide triphosphate stock solution and 120 µl H-labelled dCTP (25.1 Ci/mmol) were pipeted into a sterilized Eppendorf tube. To concentrate the

deoxynucleotide triphosphate-containing solution and to remove the ethanol which was present in ^{3}H-labelled dCTP, the mixture was frozen in liquid nitrogen and lyophilized to dryness. After approximately 90 min of lyophilization, the deoxynucleotide triphosphate-containing mixture was completely dry. The remainder of the cDNA synthesis reagents were pipeted from stock solutions into a clean 1.5 ml Eppendorf tube in the following order: 100 µl of 200 mM Tris-HCl, pH 8.3 at 46°; 20 µl of 120 mM $MgCl_2$; 40 µl of 200 mM dithiothreitol; 50 µl of Actinomycin D (400 µg/ml); 4 µl of oligo$(dT)_{12-18}$ (500 µg/ml), and 20 µl of 1 M KCl. These stock solutions were not usually prepared on the day of cDNA synthesis but were stored at -20°, or at 4°. After mixing, 58.5 µl was placed into the Eppendorf tube which contained the dried deoxynucleotide triphosphates and stored on ice. The immuno-selected poly(A)-containing RNA was prepared as follows: The RNA (2.8 µg), which was stored as a dried pellet in liquid nitrogen, was resuspended in 39.5 µl of sterilized Chelex-treated deionized H_2O. The total volume was pipeted into the Eppendorf tube which contained all of the cDNA synthesis reagents except for reverse transcriptase. To obtain a double stranded sequence at the 3' end of the RNA molecules, the RNA-containing transcription mixture was allowed to stand for 5 min on ice to complete the hybridization of the oligo$(dT)_{12-18}$ to the poly(A) sequences. After the 5 min incubation, 40 units of avian myeloblastosis virus RNA-dependent DNA polymerase (Batch No. G380; 20,200 units/ml) were added in a 2 µl volume so that the total, final volume was 100 µl. The cDNA synthesis reaction was incubated

at 46° for 20 min and terminated by placement of the tube at 4°. For a preliminary estimation of the amount of cold trichloroacetic acid insoluble radioactivity incorporated into DNA, a 1 µl aliquot of the cDNA reaction mixture was removed and added to 50 µl of 1 mg/ml BSA. After the addition of 0.5 ml of cold 10% trichloroacetic acid, the cDNA containing mixture was incubated for 30 min on ice. The precipitated nucleic acid was filtered onto a 2.3 cm glass fiber filter, type C, washed with 3 aliquots of 5 ml each of 5% trichloroacetic acid and 1 wash of 5 ml of 95% ethanol. The incorporation of ^{3}H-labelled dCTP was determined by placement of the disc into a 4 ml scintillation vial which contained 2.5 ml toluene-containing scintillation fluid, and radioactivity measured as described above. To remove the majority of the unincorporated ^{3}H-labelled dCTP, the nucleic acid was extracted from the remainder of the cDNA synthesis mixture by extraction with an SDS/phenol/chloroform mixture (23). Ten microliters of 200 mM EDTA, 5 µl 20% SDS, and 150 µl 0.1 SSC buffer were added to the reaction mixture so that the final concentrations of EDTA and SDS were approximately 7.5 mM and 0.4%, respectively. Next, 130 µl redistilled phenol, saturated with 10 mM Tris-HCl and 5 mM EDTA, pH 7.5, were added to the solution which was mixed vigorously. After a 5 min extraction on ice, 130 µl chloroform were added to the mixture, and the extraction was continued for a further 5 min on ice. The phases were separated by centrifugation at 15,600 x g for 1 min at 22°. The aqueous phase was removed and transferred into a 12 ml polyallomer tube. The organic phase was reextracted by the addition

of 250 µl of 0.1 SSC buffer. After vigorously mixing the phases, the mixture was incubated for 10 min on ice. The aqueous layer was separated by centrifugation as described above, removed and combined with the aqueous samples from the first extraction. To obtain quantitative precipitation of the cDNA, 50 µl of 1 mg/ml calf-liver tRNA was added to the nucleic acid containing solution so that the final concentration of tRNA was 90 µg/ml. Nucleic acid was precipitated by the addition of 0.1 volumes of 3 M sodium acetate, pH 5.5, and 2.0 ml redistilled ethanol, and storage at -20° overnight. On the following day, the nucleic acid precipitate was collected by centrifugation at 30,000 rpm for 30 min at -5° in an SW-41 rotor as described by Osterburg et al (78). The supernatant was discarded, and the cDNA was lyophilized to dryness. To destroy the RNA template, the nucleic acid pellet was resuspended in 0.5 ml of 0.1 M NaOH and 1 mM EDTA and incubated at 70° for 20 min. Following the incubation, the solution was cooled on ice and neutralized by the addition of 0.5 ml of 1 M sodium acetate, pH 4.5. High molecular weight ^{3}H-labelled cDNA was separated from the remainder of unincorporated ^{3}H-labelled dCTP and small oligonucleotide fragments by Sephadex G-50 column chromatography. The sample was applied onto a 10 ml column of G-50 equilibrated with 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.1% SDS, pH 7.4 at 22°. The flow rate was maintained at 1 ml/min with a Pharmacia P-3 pump. The column was developed in the same buffer at room temperature. Sixteen 1 ml fractions were collected into 1.5 ml Eppendorf tubes. To determine the excluded high molecular

weight cDNA-containing fractions, 5 µl aliquots of each fraction were pipeted onto glass fiber filters, type C, and dried in a 110° oven for 5 min. The radioactivity was measured as described above. The void volume of the column was 4 ml. The high molecular weight cDNA-containing fractions were pooled into an 11 ml polyallomer tube and the ^{3}H-labelled cDNA was precipitated by the addition of 100 µg of calf-liver tRNA, 0.1 volume of 3 M sodium acetate, pH 5.5, and at least 2 volumes of ethanol at -20° overnight. The high molecular weight, ^{3}H-labelled cDNA precipitate was pelleted by centrifugation at 30,000 rpm for 30 min at -5° in an SW-41 rotor (78). The supernatant was discarded, and the cDNA pellet was lyophilized to dryness as described above. The dried pellet was redissolved in 100 µl of H_2O and stored in liquid nitrogen in 20 µl aliquots. To determine accurately the specific activity and recovery of the ^{3}H-labelled cDNA, a 1 µl aliquot was subjected to a cold-trichloroacetic acid precipitation, and the radioactivity was measured as described above.

Sedimentation Analysis of ^{3}H-labelled cDNA - DNA was analysed by sedimentation through 11.8 ml linear sucrose gradients (8-18% w/w), prepared in 5 mM EDTA, 0.1 M NaOH and 0.9 M NaCl, essentially as described by Monahan *et al* (79). The ^{3}H-labelled cDNA (20,000 cpm) in 100 µl was layered on top of the gradient in a 12 ml polyallomer tube. The gradients were centrifuged in an SW-41 rotor for 24 h at 39,000 rpm at 5°. The gradient was fractionated by displacement of the contents of the tube upwards by the use of an ISCO gradient fractionater and a Pharmacia P-3 pump. The flow rate was held

constant at 1 ml/min. The DNA-containing samples were neutralized by the addition of an equal volume of 0.15 N acetic acid and the radioactivity in each fraction was measured as described above.

RNA Excess Hybridization to ^{3}H-labelled cDNA - RNA excess hybridizations with ^{3}H-labelled cDNA were carried out in 5 µl graduated micro-capillary tubes sealed at both ends. Hybridization reactions were performed in 3 µl in 10 mM Hepes, 0.6 M NaCl and 1 mM EDTA, pH 7.4 and contained 1,500 cpm of ^{3}H-labelled cDNA per determination, 500 µg/ml heterologous calf-liver tRNA, and quantities of Chlorella poly(A)-containing RNA which varied in amount from 0.3 ng to 600 ng per determination. After the micro-capillary tubes were filled with 3 µl of the hybridization mixture, the tips were heat-sealed and tubes were placed in a boiling water bath for 5 min. The tubes were then immediately transferred into a 68° waterbath. At the appropriate Rot values, pairs of tubes were removed and placed in an ice salt bath to stop the hybridization reaction. The contents of each capillary tube were diluted into 1 ml of buffer containing 100 mM sodium acetate, 50 mM NaCl, 1 mM $ZnSO_4$, 10 µg/ml of denatured salmon sperm DNA and 10 µg/ml of native salmon sperm DNA, frozen in liquid nitrogen, and stored at -20° (80). To determine the radioactivity that was trichloroacetic acid precipitatable before and after treatment with S1-nuclease, each 1 ml sample was thawed and divided into two 450 µl aliquots. Nuclease digestion assays were carried out as described By Vogt (81). One aliquot was digested with 20 units of S1-nuclease (5,500 units/ml, Lot No. 69C-85101) for 2 h at 37° and the other was

incubated identically, but without S1-nuclease. After digestion, the tubes were placed on ice and 50 µl of 1 mg/ml BSA was added. The nucleic acids were precipitated by the addition of 125 µl of 50% trichloroacetic acid followed by 600 µl of 10% trichloroacetic acid and incubation on ice for 30 min. The precipitates were collected on 0.45 µm Millipore filters, washed three times with 5 ml each of 5% trichloroacetic acid, dried, and counted in 2.5 ml toluene-containing scintillation fluid as described above. The data were expressed as the percentage of ^{3}H-labelled cDNA which was double stranded (i.e., the percentage resistant to S1-nuclease treatment versus log Rot).

Cell Cycle Studies - To obtain synchronous cells with a division number of 8, the cells were synchronized and cultured in nitrate medium exactly as described by Turner et al (30). Synchronous daughter cells were selected from continuously lighted cultures by use of isopycnic centrifugation in linear density gradients of Ficoll (30 ml; 26.5 to 31.5% w/w) as described by Hopkins et al (73). The resulting highly synchronous daughter cells were washed free of Ficoll and diluted to 1050 ml with pre-equilibrated nitrate medium so that the initial culture turbidity (A_{550} nm) was 3.6. The culture, which contained 89×10^{6} cells/ml, was transferred into two 1 liter-Plexiglas chambers and placed under continuous illumination (1000 foot candles). The turbidity was held essentially constant by hourly dilutions with fresh pre-equilibrated nitrate medium as described previously (82). The culture pH was maintained between 6.7 and 7.0 with 1 N H_2SO_4 as described by Turner et al (30). To measure the pattern of trans-

latable NADP-GDH mRNA throughout the cell cycle of uninduced cells, 200 ml of cells were harvested at the following times during the cell cycle: 1, 3, 5, 7, 10, 13 and 15 h. The cells were collected by centrifution at 9,750 x g in a GSA rotor and washed twice in 0.01 M Tris-HCl pH 8.25 at 4°. The cells were transferred into a 40 ml sterilized polypropylene centrifuge tube for the last wash. The cell pellets were quick-frozen in an ice-acetone bath and stored at -20°. To measure the level of translatable mRNA during the initial induction phase of the NADP-GDH, the cells in the other 1 liter-Plexiglas chamber were harvested at the third hour of the cell cycle by centrifugation at 9,750 x g for 5 min. The nitrate medium was discarded and replaced with an equal volume of ammonium medium. The culture was returned to the light and synchronous growth was resumed under identical culture conditions. At 30, 60, and 120 min after the addition of ammonium, 200 ml of cells were harvested, washed, and frozen as described above. Partial cell breakage was achieved by use of a slight modification of the procedure described above for asynchronous cell samples. Because of the small quantity of cells in each sample (approximately 0.5 g) the cells were resuspended in 12 ml of cell breakage buffer and mixed with 12 g of 0.1 mm glass beads in the 75 ml homogenization chamber. The cells were subjected to 45 s of homogenization. To determine the percentage of cell breakage in each cell sample, a small aliquot of the cells, before and after the homogenization treatment, was diluted and counted with a haemocytometer (American Optical). Total cellular RNA was

isolated from each cell cycle sample exactly as described above. The poly(A)-containing RNA fraction was obtained by use of oligo(dT)-cellulose column chromatography without modification of the procedure described above. The RNA was stored as an aqueous solution at 250 µg/ml in liquid nitrogen. Total cellular protein was assayed exactly as described by Israel *et al* (31). Cell number per ml of culture was measured with a haemocytometer. During the 120 min induction period, the cells in 10 ml of culture were harvested and processed for determination of NADP-GDH activity exactly as described previously (31).

RESULTS AND DISCUSSION

Cell Breakage Procedure Employed for Isolation of Total Chlorella Polysomes - A necessary requirement for the isolation of the mRNA coding for the ammonium-inducible NADP-GDH was the availability of a high yield of undegraded Chlorella polysomes. To achieve this goal, two major technical difficulties had to be surmounted; the optimization of cell breakage conditions which resulted in minimal polysome degradation due to shearing forces, and the inhibition of RNase activity. Unlike mammalian cells, unicellular, Chlorella cells are surrounded by a multi-component, cellulose-containing cell wall (83). Therefore, it was not possible to use the same mild homogenization techniques which are commonly employed with mammalian cells (84). Consequently, a modified cell breakage was developed to obtain a high percentage of cell breakage and a subsequent good yield of undegraded polysomes. Initial cell breakage studies were performed by use of the Braun cell homogenizer. The use of cell breakage conditions which resulted in 100% cell breakage also resulted in extensive polysome degradation. When the cells were subjected to the homogenization treatment, so that 50% cell breakage was obtained, a higher yield of undegraded polysomes (i.e., 25-30 A_{260} units/g) was subsequently recovered. Under these conditions, it was observed that large or intermediate-sized cells were more susceptible to cell breakage than daughter cells or small intermediate cells. The susceptibility of synchronized large intermediate cells was compared with the susceptibility of asynchronous cells to the same cell breakage conditions.

Results showed that, to obtain 50% cell breakage, the use of synchronous and asynchronous cells required 20 s and 45 s of homogenization, respectively (Fig. 1). A large proportion of the cells which remained intact after 45 s of homogenization consisted of newly released daughter cells. For all polysome and RNA isolation studies described, 20 s and 45 s of homogenization were routinely employed to obtain 50% cell breakage of synchronous and asynchronous cells, respectively.

Analysis and Isolation of Total Chlorella Polysomes - By use of the polysome isolation procedure described in "Materials and Methods", a good yield of intact polysomes (approximately 30 A_{260} units/g of cells) were routinely obtained from Chlorella cells. A typical sedimentation pattern of Chlorella polysomes is shown in Fig. 2. This polysome profile is similar to that found in a wide variety of mammalian tissues (38, 75), higher plant cells (85, 86), the green algae, Chlamydomonas reinhardii (74, 87), and Chlorella fusca (20). As seen in Fig. 2, most of the ribosomes (approximately 75%) migrated in the large polysome region (i.e., greater than 5 ribosomes per polysome), with the highest concentration in the range of polysome sizes composed of 8 to 12 ribosomes per polysome. The peak of monosomes was calculated to constitute approximately 10% of total polysomes. The $A_{260}:A_{280}$ ratio of the resuspended polysome pellet prior to linear sucrose gradient centrifugation varied between 1.6 and 1.75 and is a characteristic associated with undegraded polysomes (86). Since the polysome sedimentation characteristics were unaltered if frozen Chlorella cells were employed, cells were routinely frozen for up to a month at -20° or

Fig. 1. Effect of homogenization time on the percent cell breakage of Chlorella cells. Asynchronous (●) and intermediate sized, synchronous (○) ammonium-cultured cells were subjected to homogenization in a Braun cell homogenizer. The percent cell breakage was measured by a haemocytometer.

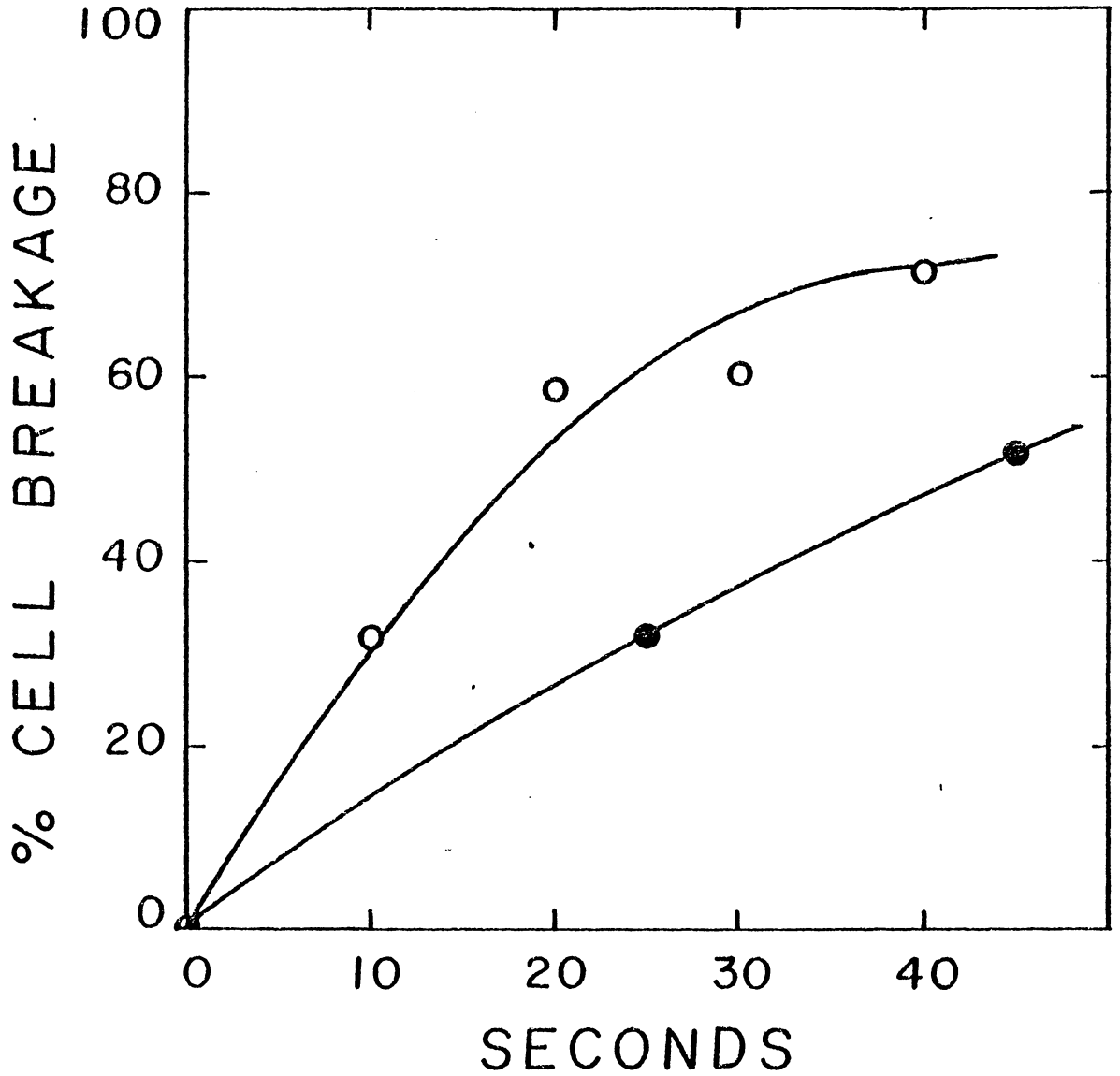
100
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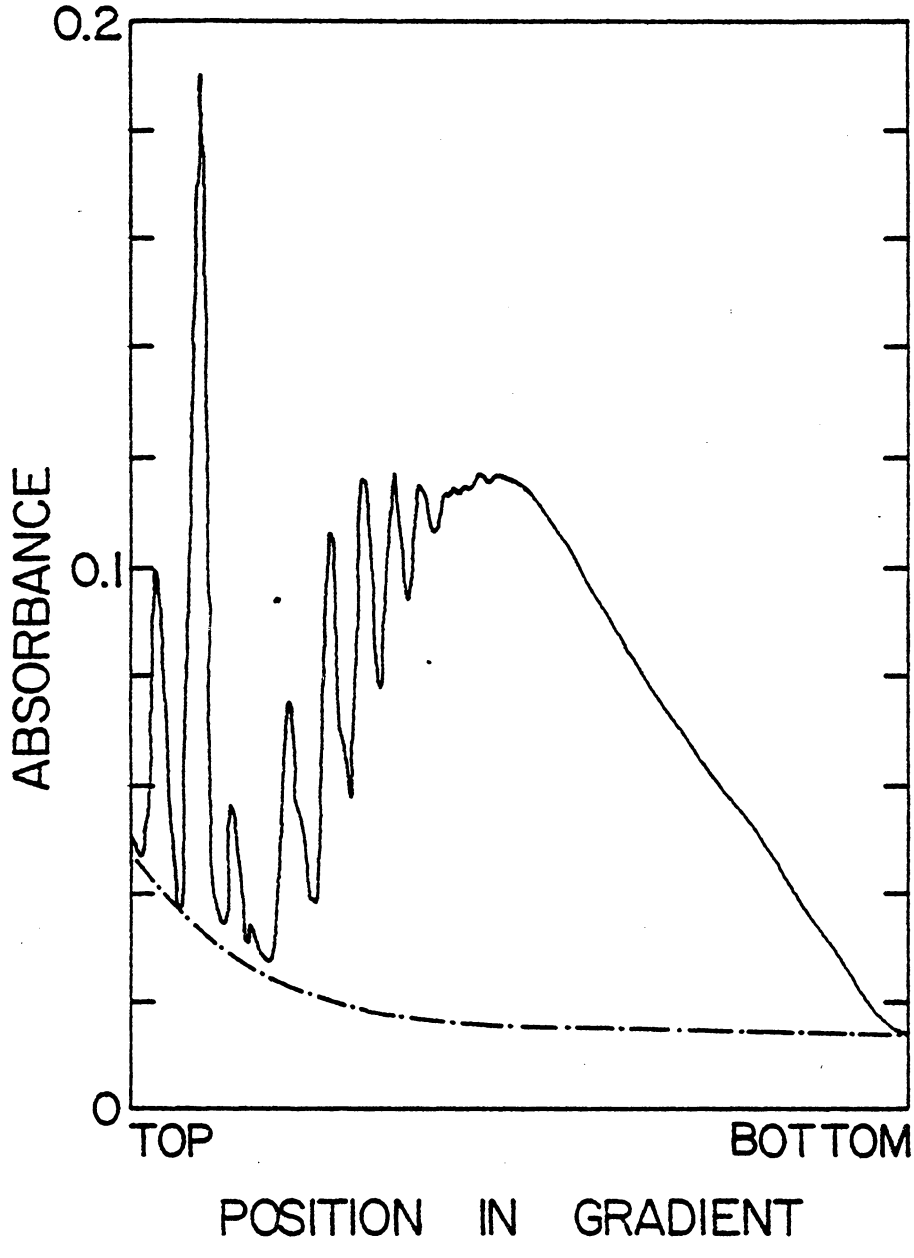
Fig. 2. Profile of Chlorella total polysomes (——). Five hundred microliters of polysomes, containing 10 A_{260} units per ml, were layered over a 12 ml linear sucrose gradient (0.5 to 1.5 M) and centrifuged for 1.5 h at 36,000 rpm in the SW40 rotor (Beckman). The absorbance at 254 nm was measured with a 2 mm path length cell. The absorbance at 254 nm was measured in a parallel gradient which was overlaid with 500 µl of polysome buffer (—•—).


0.2
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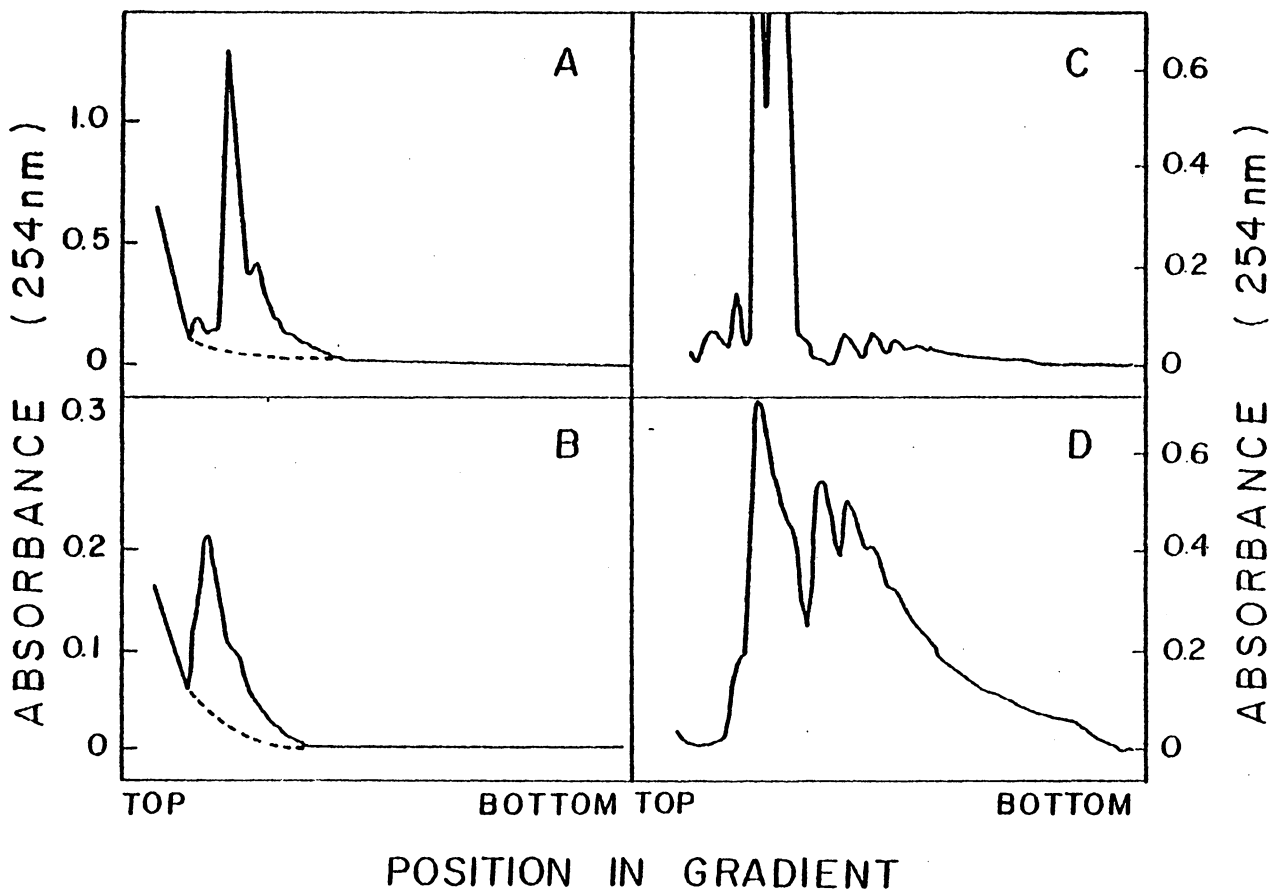
longer in liquid nitrogen. The recovery of total polysomes obtained by this procedure was estimated to be approximately 30%. This value was obtained by comparison of the yield of non-poly(A)-containing RNA obtained by this isolation procedure with that obtained by phenol/chloroform extraction of the whole cell homogenate.

To verify that the polysome profile obtained was composed of polysomes rather than non-specific aggregation of ribosomes (88), resuspended polysomes were incubated with 0.2 µg RNase A for 5 min at 4° prior to sedimentation in a linear sucrose gradient. The treatment with RNase resulted in a complete loss of polysome peaks and a corresponding increase in the size of the monosome peak (Fig. 3A). Since polysomes are extremely susceptible to enzymatic degradation during cell breakage, several precautions described in "Materials and Methods" were routinely employed to minimize RNase activity. Heparin, an effective competitive inhibitor of RNase (84), has been reported to improve greatly the yield of undegraded polysomes and RNA from mammalian cells (84). Consequently, heparin was included in the cell breakage and polysome isolation buffers employed for Chlorella polysome isolation. As another criterion of polysome integrity, incubation of the polysomes with 10 mM EDTA for 5 min at 23°, resulted in a similar loss of absorbance associated with polysomes (Fig. 3B). This result is consistent with the observation that Mg^{++} is required for stabilization of the ribosomal subunits. (89)

The polysome isolation procedure which was employed was a modification of that described by Palacios et al (75). In this study,

Fig. 3A, B, C, D. Profiles of Chlorella polysomes subjected to different treatments prior to linear sucrose gradient centrifugation. Total Chlorella polysomes were treated as follows: A, 0.2 μg RNase A for 5 min at 4°; B, 10 mM EDTA for 5 min at 23°; C, 100,000 x g centrifugation of low-speed supernatant in 1 M sucrose in polysome buffer in the absence of 2.5 M sucrose layer; D, homogenization of cells in 25 mM Tris-HCl, 250 mM NaCl, 25 mM $MgCl_2$, 15 mM EGTA, 250 mM sucrose and 1 mg/ml heparin. After treatments A and B, polysome profiles were determined as described in Fig. 2. After treatment C, the polysome pellet was resuspended in polysome buffer for determination of polysome profile. After treatment D, total polysomes were isolated from the homogenate. The polysome pellet was resuspended in polysome buffer for determination of polysome profile. The polysome profiles, C and D were determined as described in Fig. 2.


A
B
C
D
ABSORBANCE (254nm)
1.0
0.5
0
0.3
0.2
0.1
0
0.6
0.4
0.2
0
TOP
BOTTOM
TOP
BOTTOM
POSITION IN GRADIENT

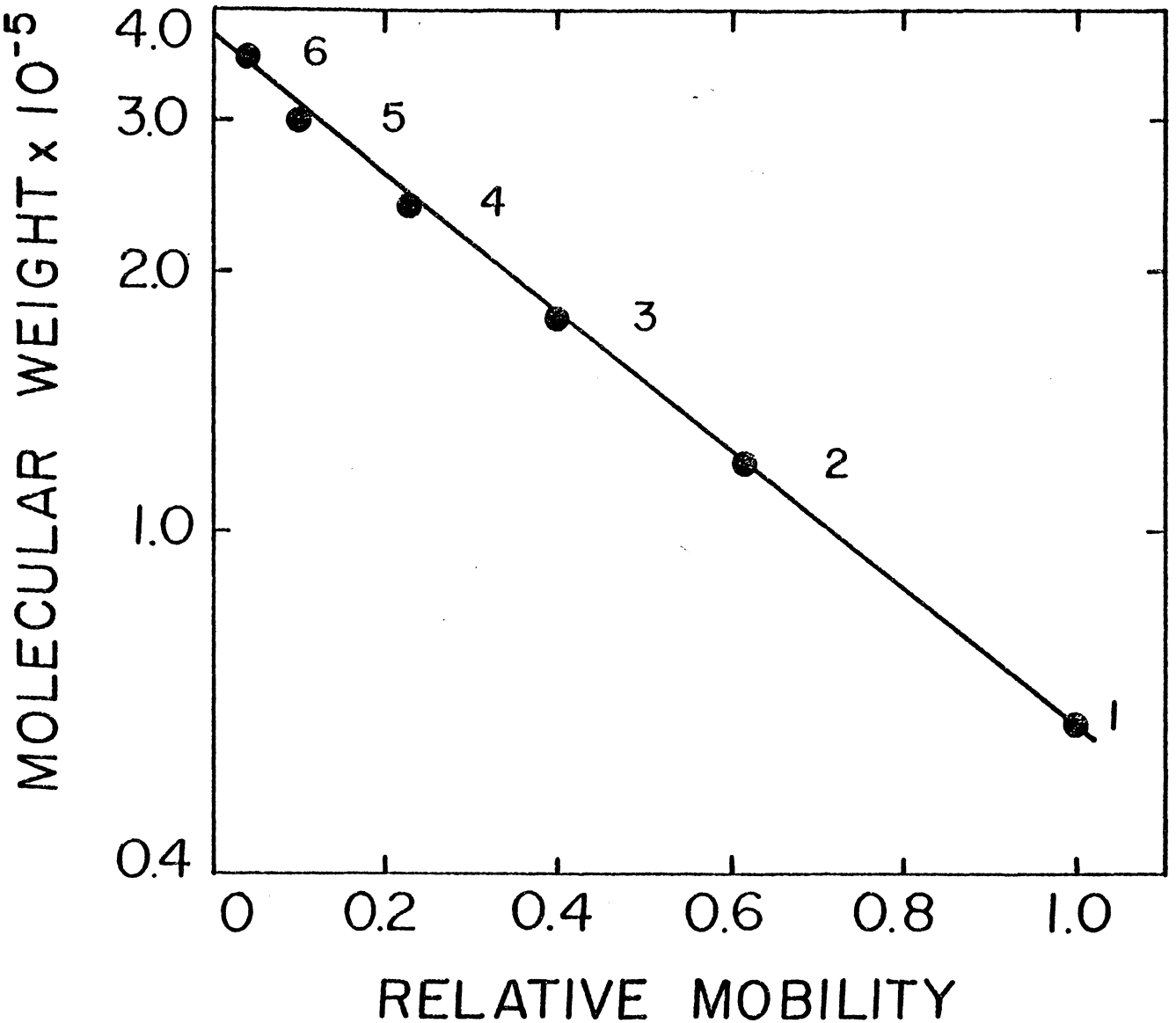
hen oviduct polysomes were isolated by centrifugation onto a 2.5 M sucrose "cushion". If the "cushion" were omitted from the high-speed centrifugation so that Chlorella polysomes were pelleted, the polysomes were observed to be degraded extensively (Fig. 3C). However, if the polysomes isolated from the 2.5 M sucrose cushion, were pelleted by centrifugation through 1 M sucrose, polysome integrity was unaffected. The second high-speed centrifugation was routinely performed to concentrate polysomes for gradient centrifugation analysis. These results can be explained by the presence of RNase in the small, dark green pellet which was present after high-speed centrifugation of the low speed supernatant. If the 2.5 M sucrose "cushion" were omitted, the polysomes in the pellet were presumably degraded rapidly by RNase. Lamellar membrane fragments were often observed to aggregate on top of the 2.5 M sucrose layer. Although complete resuspension of the aggregated material required long periods of time (up to 10 h), the presence of the aggregates did not affect polysome stability or integrity. The addition of 0.2% Triton X-100 to the 1 M and 2.5 M sucrose-containing buffers completely prevented the aggregation phenomenon so that the band of polysomes was not contaminated by the membrane fraction. This modification was included in the large scale polysome isolation procedure employed for the partial purification of the NADP-GDH mRNA. Centrifugation of a small aliquot of total Chlorella polysomes, isolated in the presence of 0.2% Triton X-100, showed that polysome integrity was unaffected.

A major difference in composition of the buffers employed for hen oviduct polysome isolation (75) compared with those employed for

Chlorella polysome isolation, was the requirement for K^+ instead of Na^+ as the monovalent cation. If 25 mM or 250 mM NaCl were included in place of the same concentration of KCl in the cell breakage buffer, the polysome profile which was obtained, indicated extensive degradation (Fig. 3D). This observation has also been reported for Chlamydomonas (74).

The Preparation of a Stable NADP-GDH-Crosslinked-Sepharose-4B Antigen Affinity Column - Initial attempts to purify rabbit anti-NADP-GDH IgG by use of an NADP-GDH-Sepharose-4B column were unsuccessful (70). The results suggested that the NADP-GDH subunits in the holoenzyme, which were noncovalently bound to the Sepharose-4B, coeluted with the specifically-bound antibodies. Therefore, studies were performed to stabilize the noncovalently attached NADP-GDH subunits with the use of the crosslinking agent, DMSl (69). To characterize the effects of the crosslinking agent, the purified enzyme was reacted with DMSl in solution. The resulting crosslinked species were separated by SDS gel electrophoresis and the gel was stained with Coomassie blue. A scan of the separating gel revealed 6 distinct protein bands with mobilities corresponding to the positions for subunit monomer, dimer, trimer, tetramer, pentamer, and hexamer (Fig. 4). The relative quantities of crosslinked species separated in the gel were 18% dimer, 9% trimer, 7% tetramer, 16% pentamer, and 16% hexamer. The uncrosslinked monomer comprised 33% of the total protein applied to the gel. These results strongly suggest that the holoenzyme is composed of 6 subunits and has a molecular weight of 354,000. This molecular weight

Fig. 4. Determination of the number of subunits in the native Chlorella NADP-GDH as shown by Weber and Osborn (68) SDS gel electrophoresis after the subunits of the enzyme were crosslinked with dimethyl suberimidate. The positions in the gel of the monomer subunit and the different crosslinked species were determined by staining the protein in the gel with Coomassie brilliant blue, and then scanning the gel at 650 nm with a Gilford 240 spectrophotometer and the linear transport attachment.

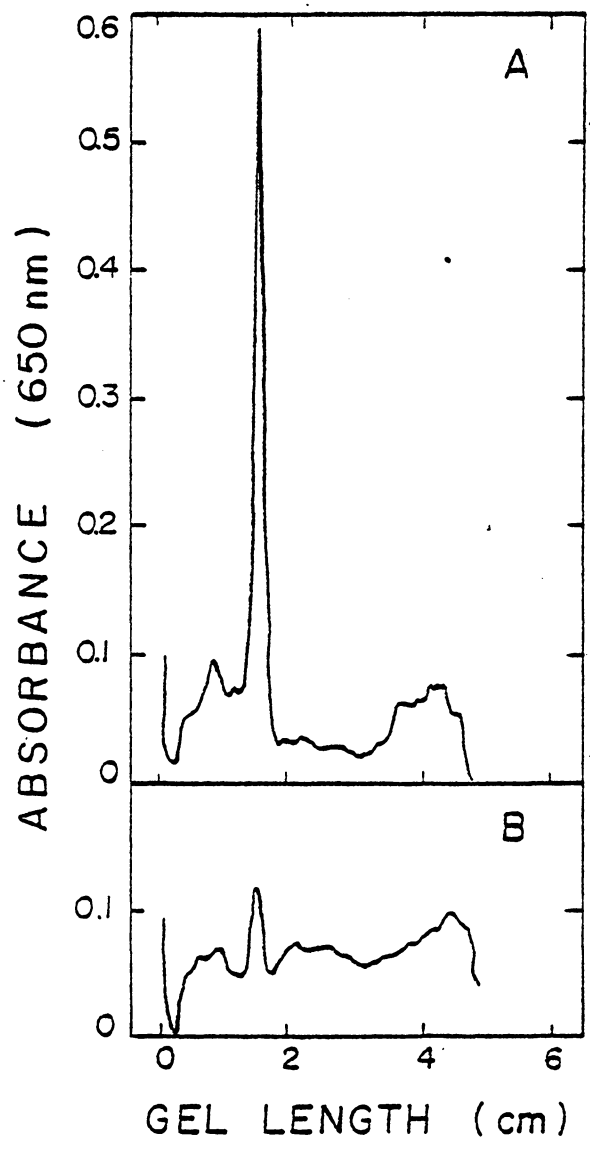

MOLECULAR WEIGHT x 10^{-5}
4.0
3.0
2.0
1.0
0.4
6
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

lies in the range (270,000 to 420,000) which has previously been reported for the holoenzyme by use of different methods of molecular weight determination (26, 29).

To test the effects of DMS1 on NADP-GDH after it was covalently attached to CNBr activated-Sepharose 4B, a comparison was made between the relative stabilities of the NADP-GDH-uncrosslinked-Sepharose-4B gel and the NADP-GDH-crosslinked-Sepharose-4B gel. Before and after the crosslinking reaction, a small aliquot of the NADP-GDH Sepharose-4B gel was incubated with β-mercaptoethanol and SDS to dissociate fully any noncovalently bound NADP-GDH subunits. The supernatants were subjected to SDS polyacrylamide gel electrophoresis and the gels were stained and scanned as described above. As shown in Fig. 5A, before the NADP-GDH-Sepharose-4B was reacted with DMS1, a large NADP-GDH subunit peak eluted from the gel under fully dissociating conditions. However, after the crosslinking reaction, an eightfold decrease in the amount of NADP-GDH subunit dissociated from the gel (Fig. 5B). When a tenfold greater amount of the NADP-GDH-crosslinked-Sepharose-4B was dissociated in the same manner and analysed on an SDS gel, only the monomer and dimer species were detected (82% and 18%, respectively). These data are consistent with crosslinked species larger than the dimer being covalently attached to Sepharose-4B. The use of the crosslinking agent, glutaraldehyde, to stabilize a multi subunit-antigen-Sepharose-4B gel has been reported recently (90).

By use of a stabilized NADP-GDH-crosslinked-Sepharose-4B antigen

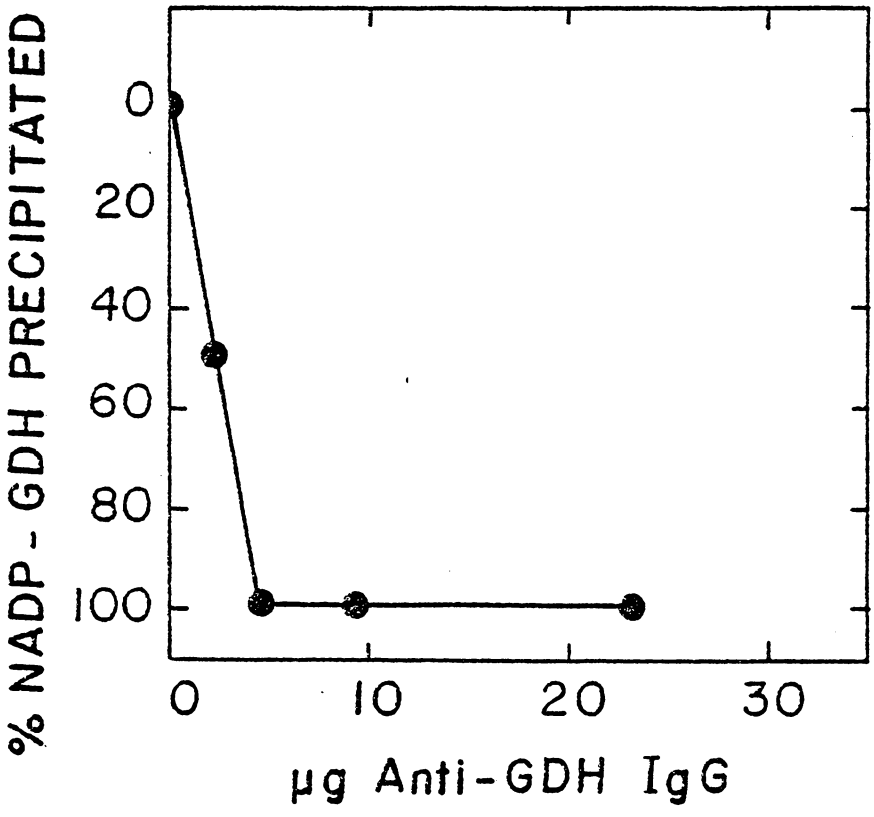
Fig. 5A, B. Effect of dimethyl suberimidate on the stability of the NADP-GDH-Sepharose-4B gel as shown by Weber and Osborn (68) SDS polyacrylamide gel electrophoresis. A, Before the crosslinking reaction with DMS1; B, After the crosslinking reaction with DMS1. The protein eluted from the NADP-GDH-Sepharose-4B gel under denaturing conditions was visualized in the SDS gel by staining the protein with Coomassie brillant blue, and then scanning the gel at 650 nm with a Gilford 240 spectrophotometer and the linear transport attachment.


A
B
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0
0
2
4
6
ABSORBANCE (650 nm)
GEL LENGTH (cm)

affinity column, large quantities of rabbit anti-NADP-GDH IgG were purified for immunological studies of NADP-GDH mRNA. The crosslinked enzyme column, unlike the uncrosslinked enzyme column, was completely stable during antibody binding and elution steps. Less than 1% of radioactive ^{35}S-labelled NADP-GDH which was incorporated in the initial binding of the enzyme to CNBr-activated Sepharose-4B, eluted from the column by buffer washes after the specific antibody was bound and during elution of anti-NADP-GDH IgG by 0.1 M glycine, pH 2.8. The crosslinked enzyme column showed a high anti-NADP-GDH IgG binding capacity (i.e., 14 mg anti-NADP-GDH IgG/ml of affinity gel). Moreover, the same yield of antibodies has been obtained with repeated use of the column. Immunotitration of the purified anti-NADP-GDH-IgG with the enzyme resulted in 100% precipitation of enzyme activity (Fig. 6). This value was obtained with a mass ratio of 3.5:1 (antibody:antigen) which is in the range reported for other purified monospecific antibodies (6, 10). The purified anti-NADP-GDH IgG was determined to be free of RNase as it did not alter the sedimentation characteristics of total Chlorella polysomes.

Binding of ^{125}I-Labelled Anti-NADP-GDH IgG to Chlorella Polysomes - To begin the study of the mechanism which regulates the induction and accumulation of NADP-GDH in induced cells at the transcriptional and post-transcriptional level, it was first necessary to detect the presence of NADP-GDH mRNA in induced cells. The use of antibodies which bind to nascent polypeptides has been used to identify, quantify and isolate polysomes involved in the synthesis of specific proteins

Fig. 6. Immunotitration of purified rabbit anti-NADP-GDH IgG. Rabbit anti-NADP-GDH IgG was purified by use of NADP-GDH-crosslinked-Sepharose-4B column chromatography. Increasing amounts of rabbit anti-NADP-GDH IgG were incubated with 0.8 µg NADP-GDH in a 40 µl reaction volume for 30 min at 23°. Following the immunoprecipitation reaction, the immunoprecipitates were pelleted by centrifugation. The percent NADP-GDH activity remaining in the supernatants were determined by a spectrophotometric assay (31).


% NADP-GDH PRECIPITATED
0
20
40
60
80
100
0
10
20
30
µg Anti-GDH IgG

(9, 75). Purified rabbit anti-NADP-GDH IgG was radioiodinated by an enzymic catalysed reaction. Radioactive rabbit anti-NADP-GDH IgG was observed to elute at the void volume of the G-25 column (Fig. 7). To perform the antibody-polysome binding study, aliquots of the ^{125}I-labelled anti-NADP-GDH IgG were incubated with polysomes isolated from ammonium- (i.e., induced) and nitrate- (i.e., uninduced) cultured cells. After fractionation of the ^{125}I-labelled polysomes on sucrose gradients, a peak of radioactivity was observed to be present in the polysome region of both types of cells (Fig. 8A, B). The peaks of radioactivity were associated with a class of large polysomes (i.e., greater than 15 ribosomes per polysome) which were present in approximately the same region of the two gradients (Fig. 8A, B). No difference in the amount or the position of the radioactive polysomes was observed in the gradients, when a threefold greater concentration of polysomes was employed for the antibody-polysome binding study (data not shown). The position of the radioactive polysomes in the gradient is consistent with the polysomes which would be expected to be engaged in synthesis of a subunit of molecular weight 59,000. For example, by use of this method, ovalbumin (Mr., 48,000) and dihydrofolate reductase (Mr., 20,000) have been shown to be translated by polysomes composed of approximately 12 and 6 ribosomes, respectively (75, 9). The presence of a specific peak of radioactivity in uninduced cells suggested that even though NADP-GDH catalytic activity is not detectable in these cells, the cells contain functional NADP-GDH mRNA which can be translated *in vivo*. These

Fig. 7. Radioactive profile of free ^{125}[I]NaI and ^{125}I-labelled rabbit anti-NADP-GDH IgG from Sephadex G-25 column. After the radioiodination reaction was terminated by centrifugation, 100 µl of the supernatant were applied at 0.5 ml/min to a Sephadex G-25 column. Fractions of 0.5 ml were collected and analysed for radioactivity by a Beckman Gamma 4000 counter.

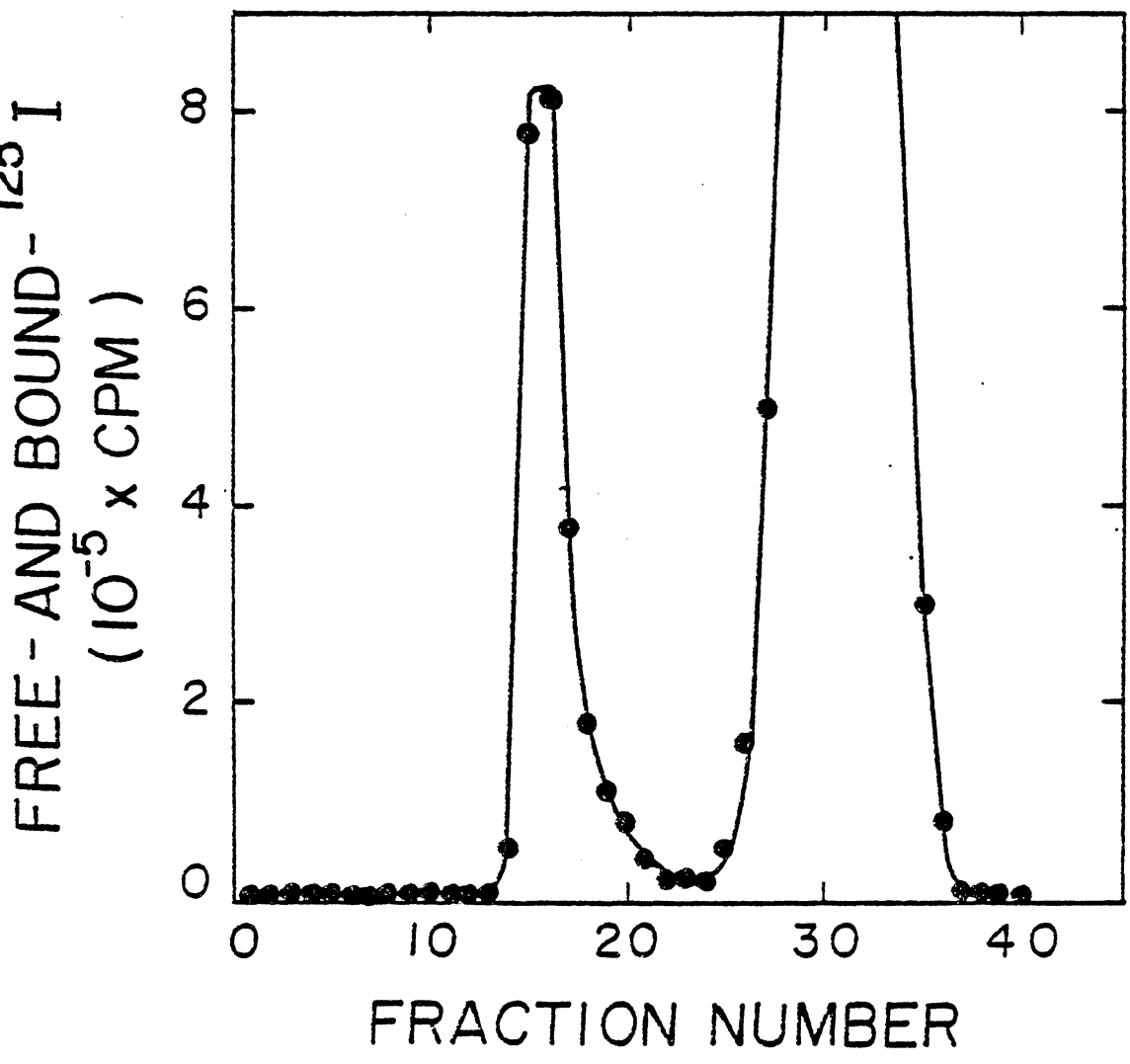
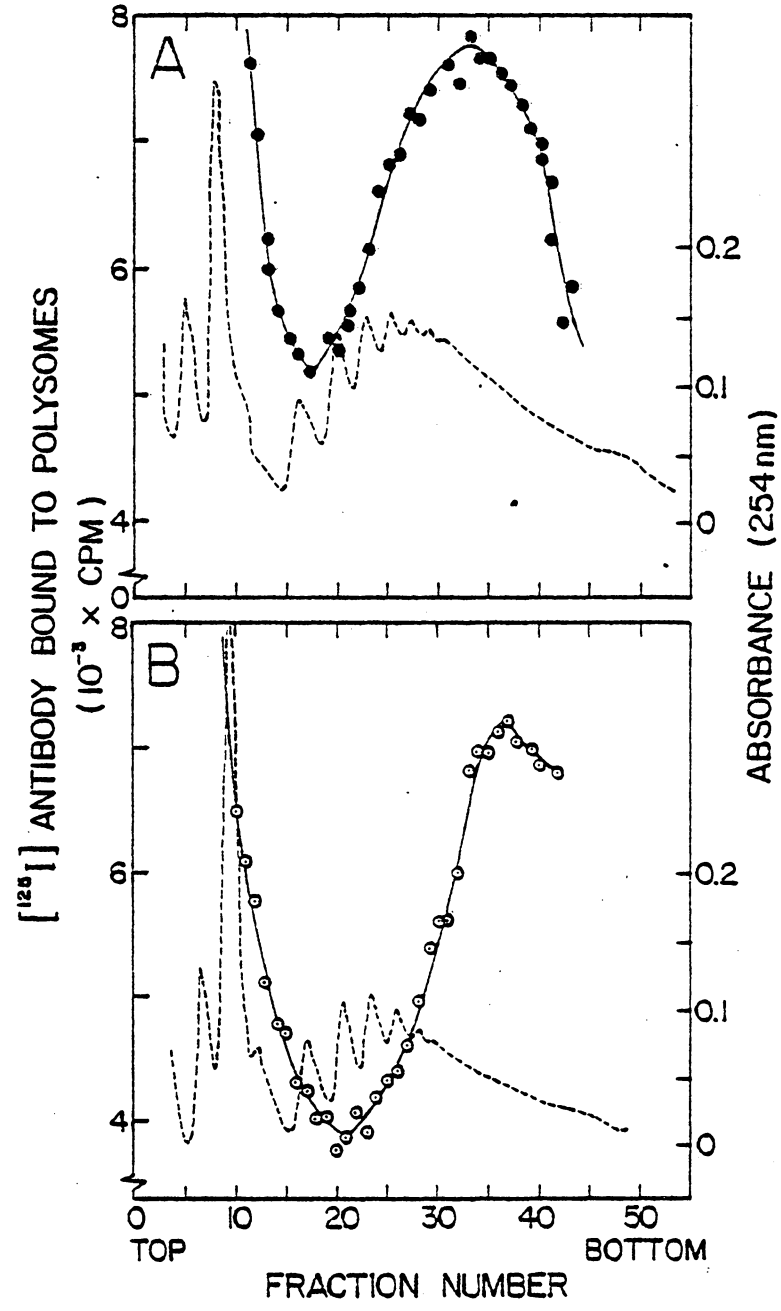

FREE- AND BOUND- ^{125}I
(10^{-5} x CPM)
0
2
4
6
8
0
10
20
30
40
FRACTION NUMBER

Fig. 8A, B. Binding of ^{125}I-labelled rabbit anti-NADP-GDH IgG to total Chlorella polysomes. Polysome preparations were incubated with 20 µg of ^{125}I-labelled rabbit anti-NADP-GDH IgG in a 3.0 ml volume for 2 h at 1.5°. A, 10 A_{260} units of polysomes isolated from induced cells. B, 10 A_{260} units of polysomes isolated from uninduced cells. After the reaction, polysome profiles were determined as described in Fig. 2. The radioactivity in each 0.25 ml fraction was determined as described in Fig. 7. A, radioactivity (●), absorbance (- - -). B, radioactivity (○), absorbance (---).


A
B
8
6
4
0
0.2
0.1
0
[125I] ANTIBODY BOUND TO POLYSOMES
(10-3 x CPM)
ABSORBANCE (254nm)
0
10
20
30
40
50
TOP
BOTTOM
FRACTION NUMBER

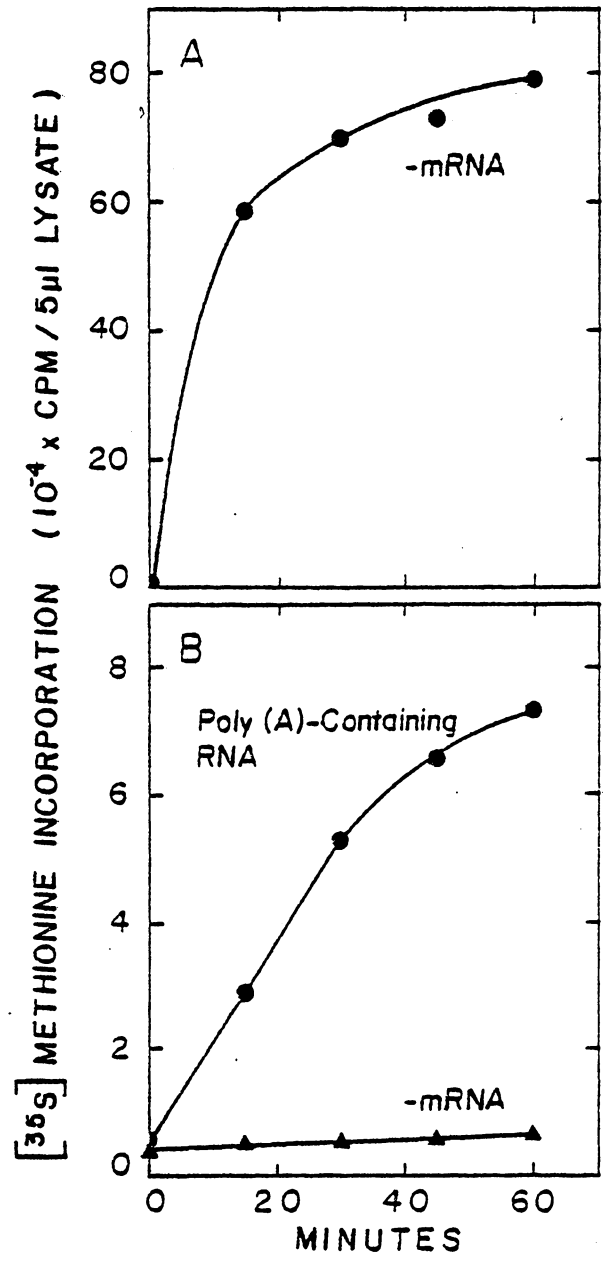
results are entirely in agreement with those of Bascomb (33) who employed immunological techniques, coupled to pulse-chase studies with ^{35}S-sulfate, to demonstrate that uninduced cells rapidly synthesize and degrade NADP-GDH subunits. Moreover, when the inducer was added to uninduced cells, the enzyme-antigen accumulated without an induction-lag. Therefore, the presence of NADP-GDH mRNA in uninduced cells, coupled with the observations that the enzyme is synthesized and rapidly turns over in the absence of inducer, is strong evidence that NADP-GDH induction is regulated at the post-transcriptional level.

Characterization of Protein Synthesis in Rabbit Reticulocyte Lysates - The mRNA dependent cell-free protein synthesizing system has become an important assay for the detection and quantitation of individual mRNA species (84). To characterize further the NADP-GDH mRNA from uninduced and induced cells, an mRNA dependent cell-free protein synthesizing system was used as described by Pelham and Jackson (52). General characteristics of the rabbit reticulocyte lysate protein synthesizing system were examined. Studies were performed to measure ^{35}S-labelled methionine incorporation into total protein in rabbit reticulocyte lysates incubated in the presence and absence of different Chlorella RNA fractions. The initial studies were performed in conjunction with James J. Lynch. The first experiment measured the amount of radioactive methionine which was incorporated by the lysate into total protein, without the addition of heterologous RNA. Total ^{35}S-methionine incorporation was measured before and after treatment

with the micrococcal nuclease (Fig. 9A, B). Before the addition of the nuclease to the control lysate, the lysate was highly active in translation of endogenous mRNA (Fig. 9A). The total ^{35}S-labelled methionine incorporation into total protein was calculated to be 1.7×10^8 cpm/ml of lysate after 60 min of protein synthesis. This value was 85% of the total radioactivity incorporated by a control, untreated lysate, reported by Pelham and Jackson (52). By SDS gel electrophoresis, they identified the predominant protein synthesized in the absence of heterologous mRNA as globin (52). After treatment of the lysate with micrococcal nuclease, the incorporation of ^{35}S-labelled methionine into total protein was decreased by 99% of the control value after 60 min of protein synthesis (Fig. 9B). However, when Chlorella polysomal poly(A)-containing RNA was added to the nuclease-treated lysate, a tenfold stimulation of amino acid incorporation was observed after 60 min. The stimulation was comparable to that reported for poly(A)-containing RNA from other eucaryotic species (9). The addition of Chlorella mRNA to the translation assay mixture resulted in a linear incorporation of ^{35}S-labelled methionine for approximately 30 min. The nuclease-treated lysate was used shortly after the nuclease treatment. Radioactive amino acid incorportion was inhibited by 20% in a treated-lysate which had been frozen and thawed once. Repeated freeze-thaw cycles decreased the translational capacity of the lysate even further (data not shown).

The addition of heterologous calf-liver tRNA stimulated radioactive methionine incorporation into total protein by as much as 50%

Fig. 9A, B. Time course of protein synthesis in rabbit reticulocyte lysates. A, control lysate before micrococcal nuclease treatment with no further additions. B, mRNA dependent lysate after micrococcal nuclease treatment with no further additions (▲), and with 1.5 µg Chlorella total polysomal poly(A)-containing RNA (●). Incorporation of ^{35}S-labelled methionine into hot-trichloroacetic acid insoluble material was assayed by taking samples at various times as shown.


A
B
80
60
40
20
0
8
6
4
2
0
-mRNA
Poly (A)-Containing
RNA
-mRNA
0
20
40
60
MINUTES
[35S] METHIONINE INCORPORATION (10-4 x CPM / 5µl LYSATE)

above the control incorporation after 60 min of protein synthesis (Fig. 10). Although Pelham and Jackson (52) reported that nuclease treatment did not impair the function of the endogenous tRNA, they found that the efficient translation of tobacco mosaic virus and cowpea mosaic virus mRNAs into complete polypeptides required the addition of 58 µg/ml of mouse liver tRNA. When the same concentration of calf-liver tRNA was added to a lysate incubated with Chlorella poly(A)-containing RNA, an enhanced rate of amino acid incorporation into total protein was observed compared to the control rate (Fig. 10). An even higher rate of incorporation was obtained with 175 µg/ml tRNA (Fig. 10). This concentration of tRNA was added to all subsequent translation assays. The total lysate products synthesized in the presence of 175 µg/ml tRNA and in the absence of tRNA (i.e., control) were analysed by SDS gel electrophoresis. The size distribution of the radioactive proteins in the 2 gels showed that the proteins synthesized in the presence of tRNA were distributed in higher molecular weight Chlorella polypeptides compared with the distribution of radioactivity in the control gel (data not shown).

To characterize further the rabbit reticulocyte lysate protein synthesizing system, the effect of different Mg^{++} concentrations on total amino acid incorporation into Chlorella proteins was examined (Fig. 11). Chlorella poly(A)-containing RNA (20 µg/ml) was translated in vitro in the presence of different concentrations of magnesium acetate. The incorporation of ^{35}S-labelled methionine into hot trichloroacetic acid insoluble material was measured after 60 min of

Fig. 10. Effect of calf-liver tRNA on protein synthesis in rabbit reticulocyte lysates. Standard translation assays were performed with the following additions: 20 µg/ml Chlorella total polysomal poly(A)-containing RNA (○), 20 µg/ml Chlorella total polysomal poly(A)-containing RNA plus 58 µg/ml calf-liver tRNA (▲), 20 µg/ml Chlorella total polysomal poly(A)-containing RNA plus 175 µg/ml calf-liver tRNA (△), and mRNA-dependent lysate with no further additions (●). Incorporation of ^{35}S-labelled methionine into hot-trichloroacetic acid insoluble material was determined in 2 µl samples taken at various times as shown.

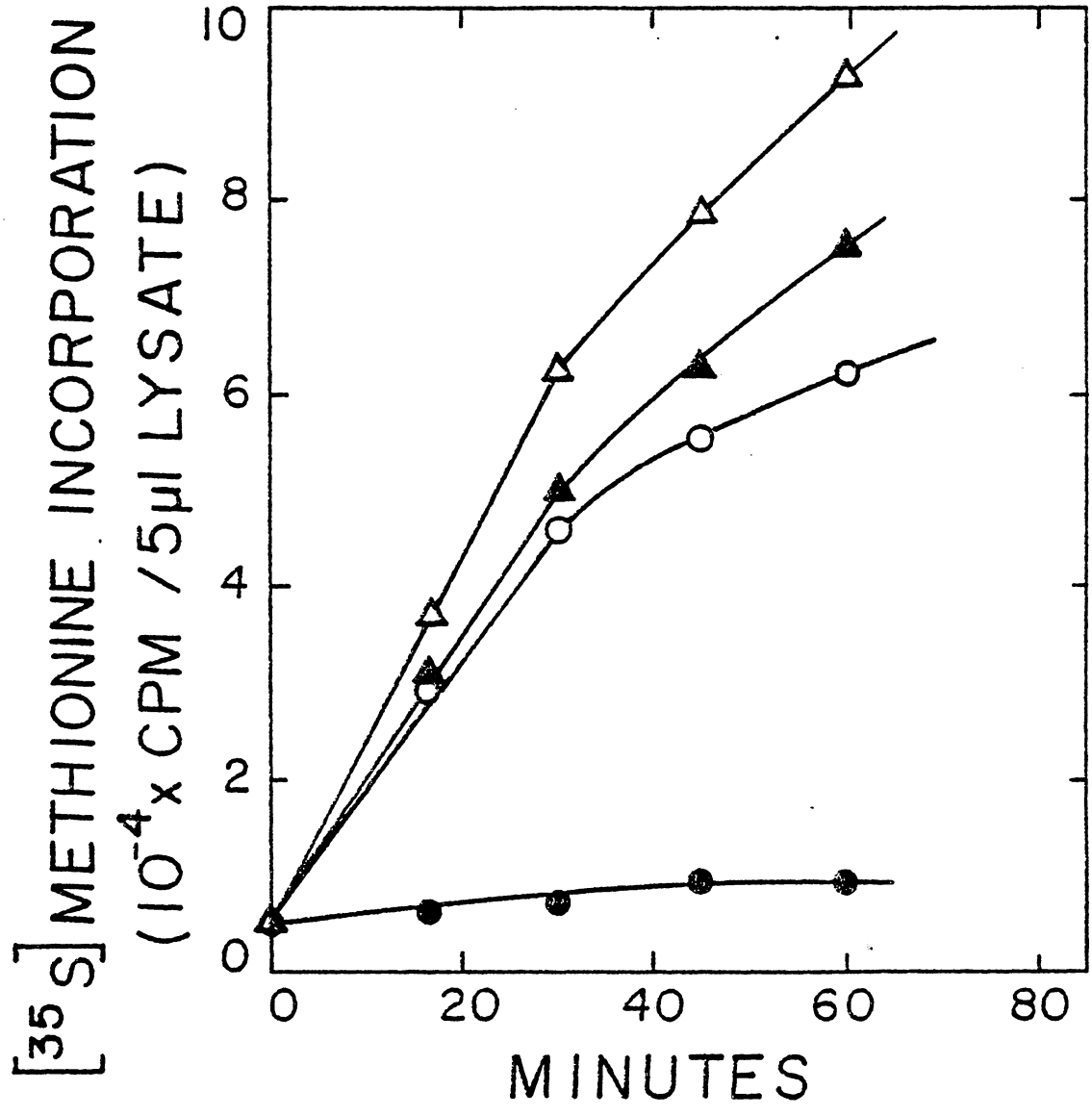
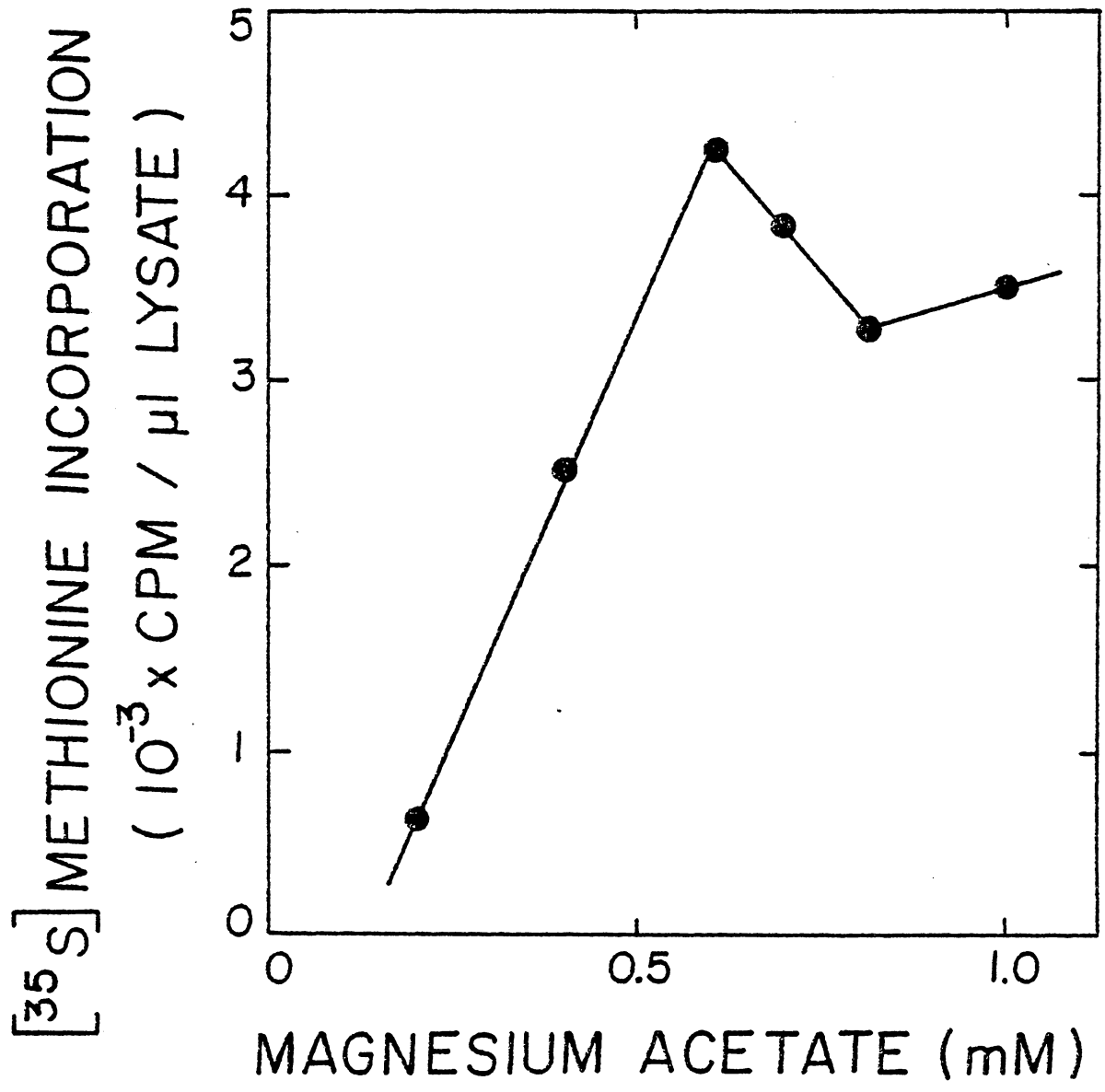
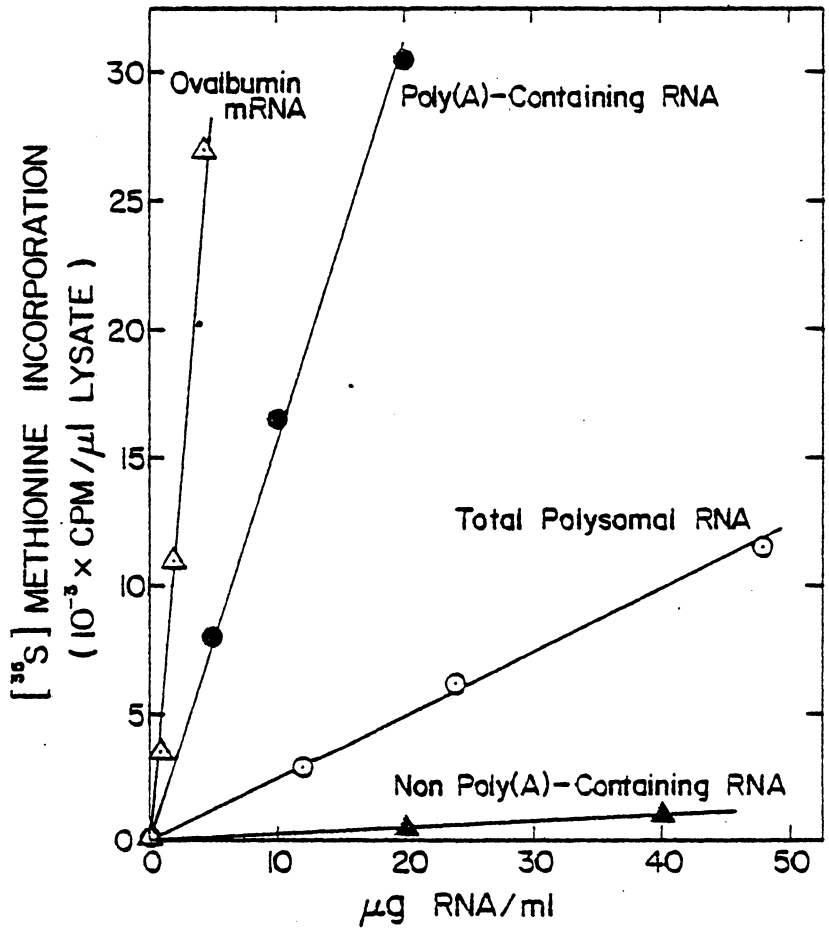

[35S] METHIONINE INCORPORATION
(10-4 x CPM /5µl LYSATE)
0
2
4
6
8
10
0
20
40
60
80
MINUTES

Fig. 11. Effect of magnesium acetate on protein synthesis in rabbit reticulocyte lysates. Standard translation assays were performed with 20 µg/ml of Chlorella total polysome poly(A)-containing RNA and varying concentrations of magnesium acetate. Incorporation of ^{35}S-labelled methionine into hot-trichloroacetic acid insoluble material was measured in 2 µl aliquots.


[35S] METHIONINE INCORPORATION
(10-3 x CPM / µl LYSATE)
5
4
3
2
1
0
0
0.5
1.0
MAGNESIUM ACETATE (mM)

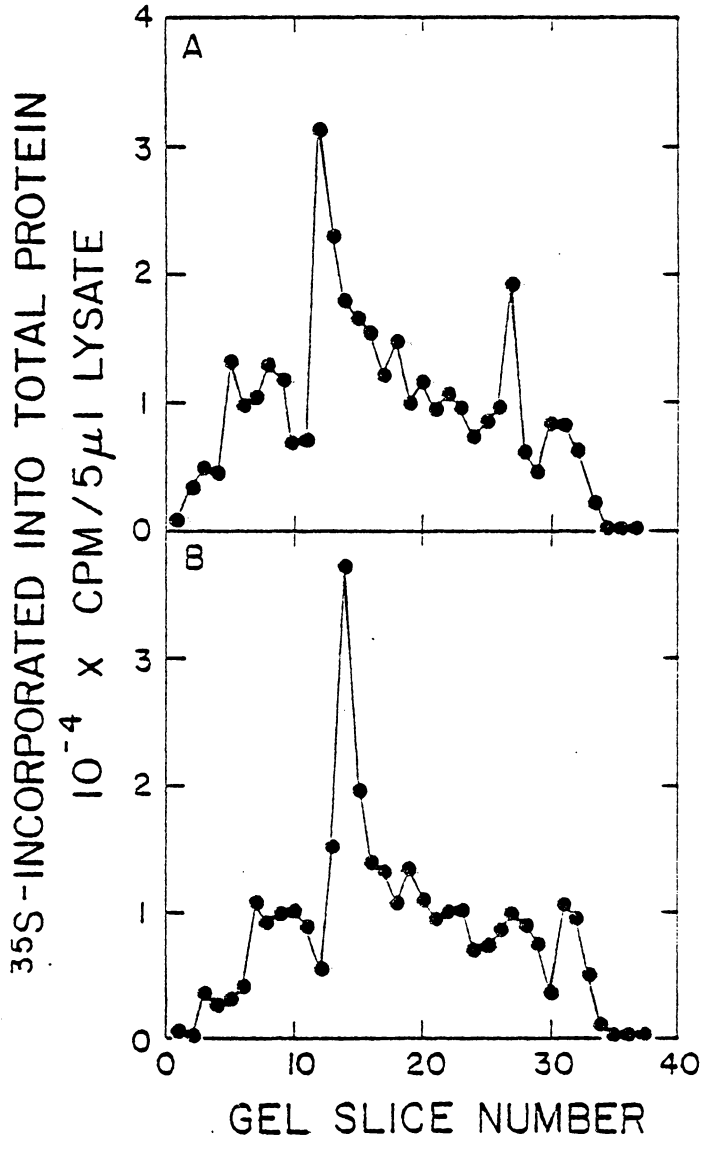
protein synthesis for each concentration of magnesium acetate tested. The maximum incorporation of radioactive methionine into Chlorella proteins was observed to take place in the presence of 0.6 mM magnesium acetate. The results indicated that the function of Mg^{++} in translation is complex. At the lower concentrations tested (i.e. less than 0.4 mM magnesium acetate), the decreased translatability of Chlorella mRNA was presumably caused by the destabilization of the endogenous ribosomal subunits (89). The effects of addition of different concentrations of Chlorella RNA and of purified ovalbumin mRNA were also examined. Ovalbumin mRNA was highly purified from hen oviduct poly(A)-containing RNA by James Lynch and myself for comparison and evaluation of the Chlorella RNA fractions isolated by the procedures described in "Materials and Methods". The details of the purification procedure are described in detail by Lynch (91). Chlorella polysomal RNA was fractionated into non-poly(A)-containing RNA and poly(A)-containing RNA species by use of oligo(dT)-cellulose column chromatography. The translation of all three RNA species was observed to be a linear function of radioactive amino acid incorporation into total protein synthesized in vitro over the concentration range of the RNA species tested (Fig. 12). Under the conditions employed for oligo(dT)-cellulose column chromatography, 97% of total polysomal RNA was comprised of non-poly(A)-containing RNA and 3% was comprised of poly(A)-containing RNA. Non-poly(A)-containing RNA is known to be composed largely of rRNA (34). Therefore, the low amount of translation in the presence of this RNA fraction is in agreement

Fig. 12. Effects of RNA concentration on protein synthesis in reticulocyte lysate reaction mixtures. Standard protein translation assays were performed with the various amounts of RNA indicated. The incorporation of ^{35}S-labelled methionine into total protein was determined in a 2 µl aliquot removed from the lysate reaction mixture after 60 min of protein synthesis. Total protein synthesized from purified ovalbumin mRNA (△), Chlorella total polysomal poly(A)-containing RNA (●), total polysomal RNA (○), and total non-poly(A)-containing RNA (▲). Chlorella total polysomal RNA was separated into poly(A)- and non-poly(A)-containing RNA fractions by oligo(dT)-cellulose chromatography. The binding capacity of the oligo(dT)-cellulose was 46 A_{260} units of poly(rA) per g of cellulose. Poly(A)-containing RNA comprised the RNA fraction which bound to the oligo(dT)-cellulose in high ionic strength buffer (10 mM Hepes, 0.4 M NaCl, and 0.5% SDS) and was specifically eluted by low ionic strength buffer (10 mM Hepes). After one passage of the total polysomal RNA through the oligo(dT)-cellulose, the poly(A)-containing RNA was observed to contain up to 20% contamination with non(poly A)-containing RNA. This result was obtained by subjecting this poly(A)-containing RNA to a second brief heat treatment (68° for 3 min) and by measuring the amount of RNA in the poly(A)-containing RNA fraction which did not readsorb to the oligo(dT)-cellulose in the presence of high ionic strength buffer.


Ovalbumin mRNA
Poly(A)-Containing RNA
Total Polysomal RNA
Non Poly(A)-Containing RNA
[35S] METHIONINE INCORPORATION
(10-3 x CPM/µl LYSATE)
µg RNA/ml
0
5
10
15
20
25
30
0
10
20
30
40
50

with this observation. Poly(A)-containing RNA which is normally very active in translation *in vivo*, was observed to stimulate ^{35}S-labelled methionine incorporation into total protein by fifteenfold compared to the value obtained for non-poly(A)-containing RNA. The addition of total polysomal RNA, up to a final concentration of 50 µg/ml, resulted in an intermediate rate of amino acid incorporation. The same results were obtained for translation of total polysomal, poly(A)-containing, and non-poly(A)-containing RNAs isolated from nitrate-cultured cells. The addition of purified ovalbumin mRNA to the translation assay was observed to be highly stimulatory and resulted in a rate of amino acid incorporation into ovalbumin, which is comparable to that reported by Shapiro *et al* (6) for purified ovalbumin mRNA. Since ovalbumin mRNA has been observed to be preferentially translated *in vitro* protein synthesizing systems compared with other hen oviduct mRNAs (92), the observed increase in the amount of ^{35}S-labelled methionine incorporation into total protein by ovalbumin mRNA compared to total *Chlorella* poly(A)-containing RNA is consistent with these results. In later translation assays, to which calf-liver tRNA was added, the addition of 20 µg/ml of either total polysomal or total cellular poly(A)-containing RNA resulted in total radioactive amino acid incorporation of up to 2×10^5 cpm/µl of lysate. The proteins synthesized *in vitro* were analysed by SDS gel electrophoresis (Fig. 13A, B). The results indicated that the radioactive proteins synthesized *in vitro* from poly(A)-containing RNA, isolated from total polysomal RNA (Fig. 13A) or from total cellular

Fig. 13A, B. SDS polyacrylamide gel electrophoresis (79) of total protein products synthesized in rabbit reticulocyte lysates. A, total proteins synthesized with 20 µg/ml *Chlorella* total polysomal poly(A)-containing RNA. B, total proteins synthesized with 20 µg/ml *Chlorella* total cellular poly(A)-containing RNA. The hemoglobin present in the reticulocyte lysate was observed to migrate with the bromphenol blue marker and was contained in gel slice number 31.


A
B
^{35}S-INCORPORATED INTO TOTAL PROTEIN
10^{-4} x CPM/5μl LYSATE
GEL SLICE NUMBER
0
1
2
3
4
10
20
30
40

RNA (Fig. 13B), were very similar in size and relative number. The proteins comprised a heterogeneous range of polypeptide sizes which would be expected if the RNA was undegraded and the translation assay was synthesizing full length polypeptides. However Palacios et al (54) reported that total Neurospora RNA did not compete effectively with the endogenous globin mRNA which remained after nuclease treatment, so that the predominant species synthesized was globin. In the presence of Chlorella mRNA, globin synthesis comprised a minor fraction of total protein synthesis (Fig. 13A, B).

The [^{35}S]methionine incorporation into total protein was compared for 2 different Chlorella poly(A)-containing RNA concentrations (i.e., 20 µg/ml and 40 µg/ml). A twofold increase in the amount of ^{35}S-labelled methionine incorporation into hot trichloroacetic acid insoluble material was obtained. However, analysis of the radioactive proteins formed by SDS gel electrophoresis showed that at the higher RNA concentration, a larger proportion of apparently incomplete polypeptide chains were synthesized (Fig. 14). Therefore, in all subsequent translation studies, the RNA concentration employed was 20 µg/ml or lower.

Characterization of NADP-GDH Synthesized in Rabbit Reticulocyte Lysates - Incubation of the nuclease-treated rabbit reticulocyte lysate with total polysomal poly(A)-containing RNA, isolated from ammonium-cultured cells, resulted in the synthesis of a protein with the same molecular weight and antigenic properties as the NADP-GDH subunit. The in vitro synthesized subunits were estimated to compose a very small proportion of total protein synthesis in vitro (Table 1). Therefore,

Fig. 14. Effect of RNA concentration on total protein products synthesized in rabbit reticulocyte lysates as shown by SDS polyacrylamide gel electrophoresis (77). Total proteins synthesized from 20 µg/ml Chlorella total polysomal poly(A)-containing RNA (●) and 40 µg/ml Chlorella total polysomal poly(A)-containing RNA (-O-).

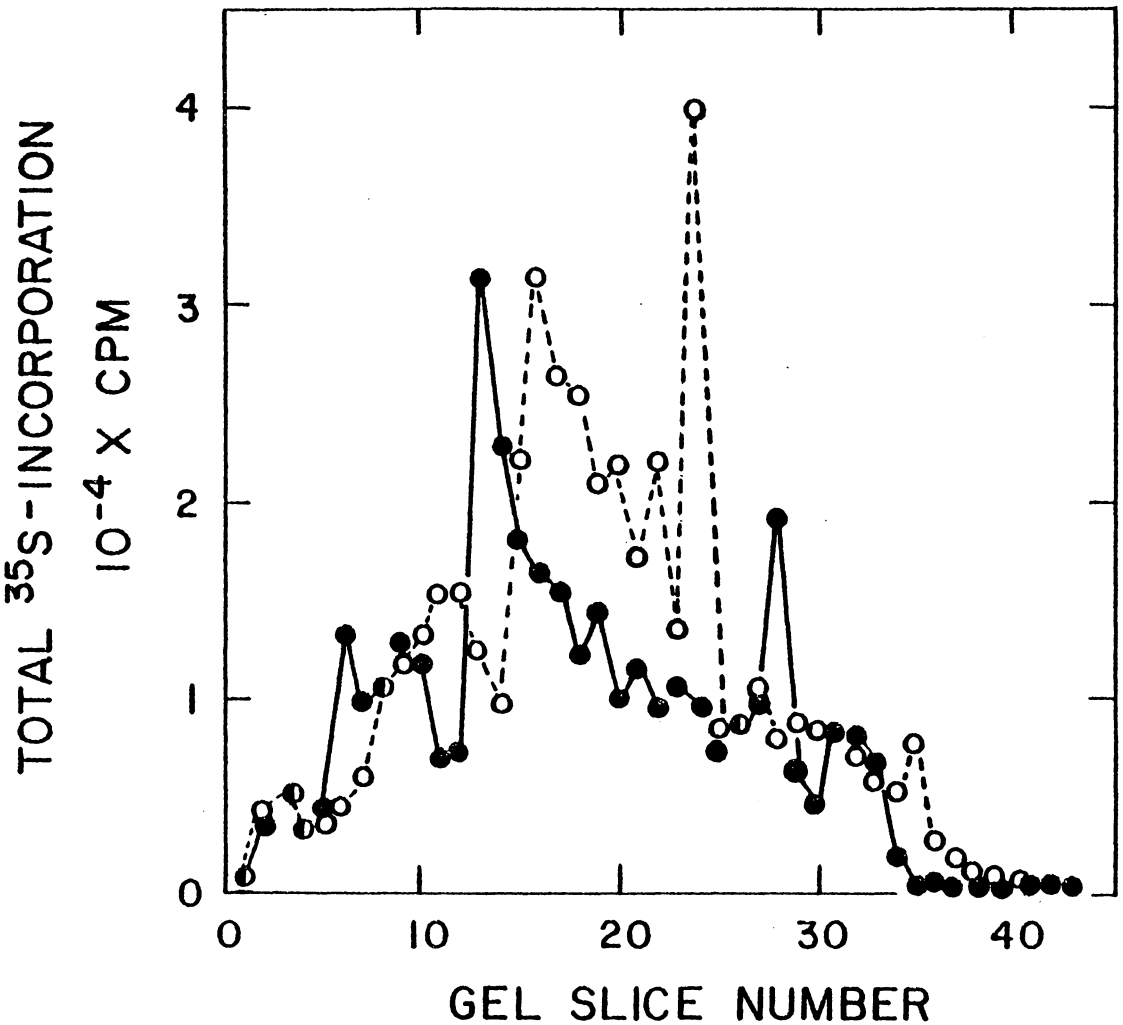

TOTAL ^{35}S-INCORPORATION
10^{-4} X CPM
0
1
2
3
4
0
10
20
30
40
GEL SLICE NUMBER

TABLE I

Comparison of Intracellular Concentrations of NADP-GDH mRNA [a]

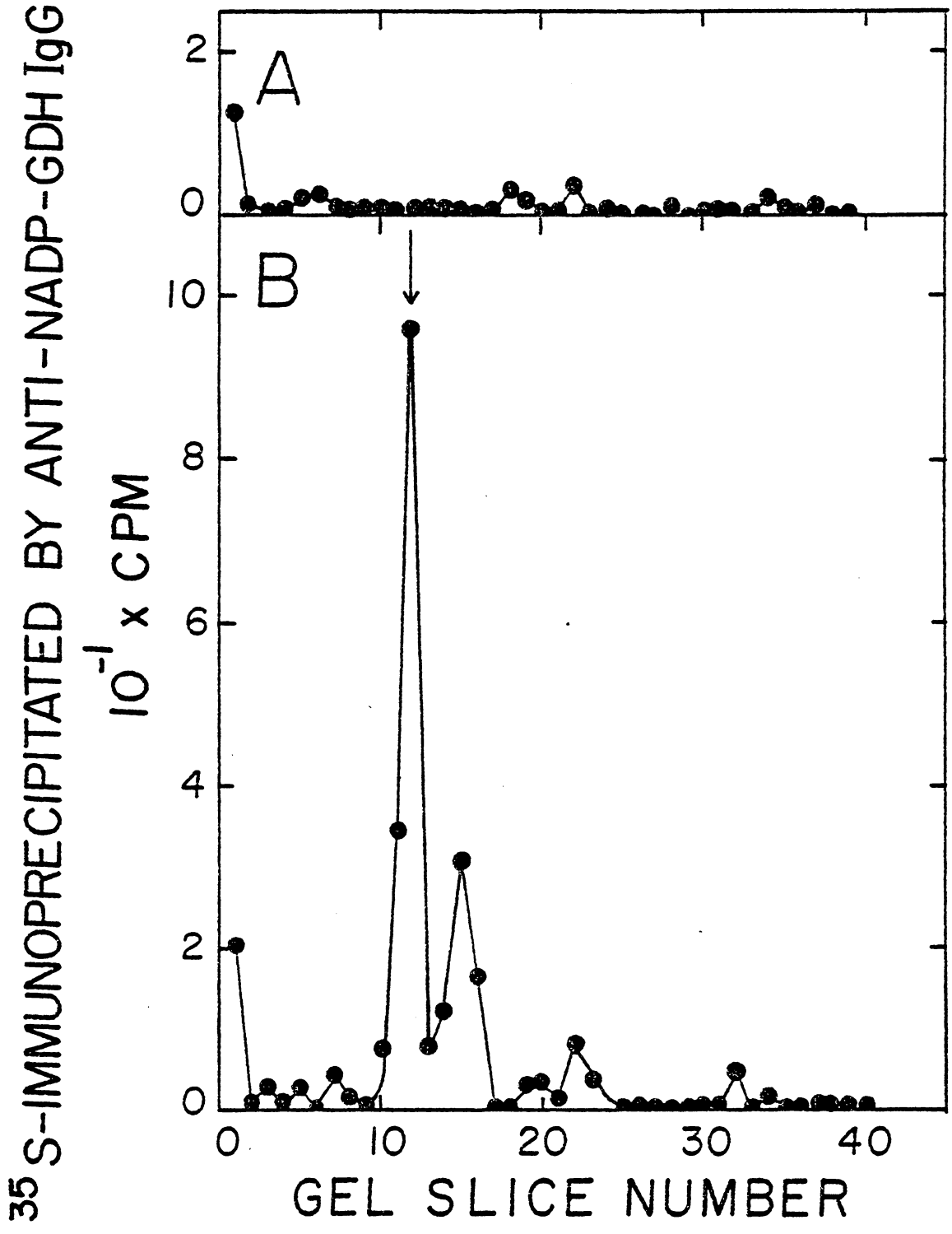
Source of RNA	Amount of RNA assayed µg	CPM incorporated into total protein	CPM in NADP-GDH [b]	NADP-GDH as % of Total Protein
Total polysomal poly(A) RNA	1	1.6×10^6	1530	0.096
Total cellular poly(A) RNA	1	2.2×10^6	1620	0.074

a The data were calculated from a standard in vitro translation assay of 35 µl total volume.

b The radioactivity represents the area of NADP-GDH subunits on SDS polyacrylamide gels.

quantitation of the NADP-GDH synthesized *in vitro* was determined by isolation of immunoprecipitable translation products and by analysis of the distribution of radioactivity in the solubilized immunoprecipitate by SDS gel electrophoresis. Figure 15A, B shows the distribution of radioactivity in the immunoprecipitates which were isolated from lysates incubated with and without *Chlorella* poly(A)-containing RNA. To isolate the antigenic NADP-GDH subunits synthesized *in vitro*, direct immunoprecipitation with purified rabbit anti-NADP-GDH IgG was employed. When the lysate was incubated without added *Chlorella* RNA, no radioactive immunoprecipitable products were detected in the SDS gel (Fig. 15A). However, when 1 μg of polysomal poly(A)-containing RNA, isolated from ammonium-cultured cells, was incubated with the lysate under the same conditions, a major peak of radioactivity was observed to correspond to the position of the authentic NADP-GDH subunit (Fig. 15B). A minor peak of immunoprecipitable material was usually observed to migrate in the gel with a faster mobility than that of the full length NADP-GDH subunit. The percent radioactivity associated with the smaller molecular polypeptide fragment was calculated to be a constant proportion (i.e., 30%) of the major peak of radioactivity associated with the authentic NADP-GDH subunit. This result suggested that the minor peak of radioactivity was not a non-specific contaminant associated with the immunoprecipitate. If it were a contaminant, it would be expected to vary in position and relative proportion from experiment to experiment. A possible explanation for the presence of the smaller molecular weight, antigenic protein

Fig. 15A, B. SDS polyacrylamide gel electrophoresis (79) of anti-NADP-GDH IgG-immunoprecipitated material synthesized *in vitro* from *Chlorella* polysomal poly(A)-containing RNA. A standard translation assay was performed in the absence, A and the presence, B of 20 µg/ml total polysomal poly(A)-containing RNA, isolated from induced cells. Direct immunoprecipitation of the *in vitro* synthesized protein was performed by the addition of 5 µg carrier NADP-GDH and 60 µg of purified rabbit anti-NADP-GDH IgG. The immunoprecipitation reaction was carried out for 2 h at 4°. After the incubation, the immunoprecipitate was pelleted by centrifugation at 15,600 x *g* for 2 min. The immunoprecipitate was washed as described in "Materials and Methods". The arrow in A indicates the position of the authentic NADP-GDH subunit in the gel. After electrophoresis was terminated, the bromphenol blue marker was located in gel slice number 36. The first gel slice contained the radioactivity which was present in the whole stacking gel.


A
B
2
0
10
8
6
4
2
0
0
10
20
30
40
GEL SLICE NUMBER
^{35}S-IMMUNOPRECIPITATED BY ANTI-NADP-GDH IgG
10^{-1} x CPM

will be discussed later.

When equal quantities of total cellular poly(A)-containing RNA and total polysomal poly(A)-containing RNA, isolated from ammonium-cultured cells, were translated *in vitro*, similar amounts of radioactivity were incorporated into immunoprecipitable NADP-GDH subunits (Table 1). This result suggested that a similar concentration of translatable NADP-GDH mRNA was present in the 2 RNA fractions. These data suggest that in fully induced, ammonium-cultured cells, there is not a large pool of functionally active NADP-GDH mRNA which is present in a subcellular location that is unavailable for translation. The incorporation into NADP-GDH in the translation assays, relative to total protein incorporation, ranged from approximately 0.07 to 0.15%. From the specific activity measurement of purified NADP-GDH, Yeung (70) estimated that NADP-GDH comprised 0.5% of the proteins contained in the frozen-thawed low-speed supernatant. Since the NADP-GDH enzyme is unstable *in vivo* (33), it is possible that the estimation of the amount of translatable NADP-GDH mRNA might not be high enough to account for the observed *in vivo* concentration of the enzyme. Three possibilities which are concerned with this issue will be discussed. The first possibility is that the observed concentration of the NADP-GDH mRNA measured by the translation assay is an underestimated value. Although the incorporation of ^{35}S-labelled methionine into total protein was optimized for total poly(A)-containing RNA, no attempt was made to optimize the *in vivo* translation of the NADP-GDH mRNA. If the requirements for translation of the NADP-GDH mRNA differed from those of the bulk of the poly(A)-containing RNA (i.e., mRNA structure, modification of mRNA structure), then the

estimated concentration of the NADP-GDH mRNA might reflect a differential rate of translation of the NADP-GDH mRNA relative to total poly(A)-containing RNA. The synthesis of NADP-GDH *in vitro* was observed to be a linear function of the amount of total poly(A)-containing RNA added to the translation assay (Fig. 16). These data show that the amount of radioactivity incorporated into NADP-GDH *in vitro* was a constant percentage of the total radioactivity incorporated into total protein at the two RNA concentrations tested.

The second possibility for the low estimate of the concentration of the NADP-GDH mRNA was that a portion of the NADP-GDH mRNA sequences did not contain a polyadenylic sequence at the 3' end. To test this possibility, 14 µg of non-poly(A)-containing RNA were translated in 150 µl of rabbit reticulocyte lysate. The translation products were incubated with purified rabbit anti-NADP-GDH IgG as described in "Materials and Methods." The immunoprecipitated material was solubilized and subjected to SDS gel electrophoresis (Fig. 17A, B). The distribution of radioactivity in the gel is shown in Fig. 17A. A small, distinct peak of immunoprecipited material was observed to migrate to the position which corresponded to the authentic NADP-GDH subunit. The total proteins synthesized by the lysate incubated with the non-poly(A)-containing RNA are shown in Fig. 17B. Based on the amount of RNA added to the translation assay and on the proportion of radioactivity incorporated into the GDH subunits relative to total amino acid incorporation, less than 5% of the NADP-GDH mRNA was calculated to be present in the non-poly(A)-containing RNA fraction,

Fig. 16. Synthesis of NADP-GDH as a function of the amount of total poly(A)-containing RNA added to the reticulocyte lysate translation system. The indicated amounts of poly(A)-containing RNA, isolated from ammonium-cultured cells were incubated in the standard translation reaction mixture. After protein synthesis was terminated, incorporation of ^{35}S-labelled methionine into hot-trichloroacetic acid insoluble material was determined in a 5 µl aliquot. Direct immunoprecipitation by rabbit anti-NADP-GDH IgG was performed as described in Fig. 15A, B. The immunoprecipitates were solubilized and subjected to SDS gel electrophoresis. The radioactivity in the gel which corresponded to the position of the authentic NADP-GDH subunit was measured for each RNA concentration tested.

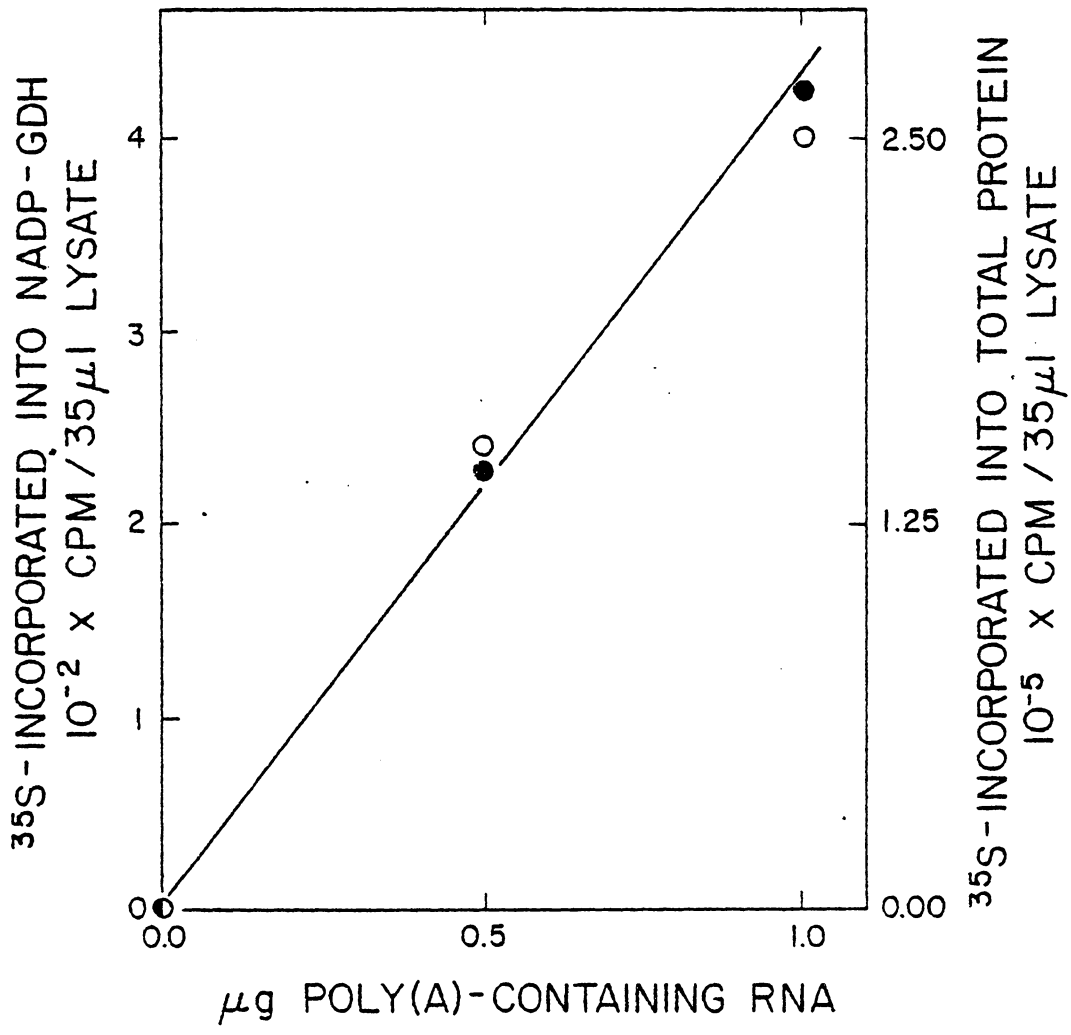
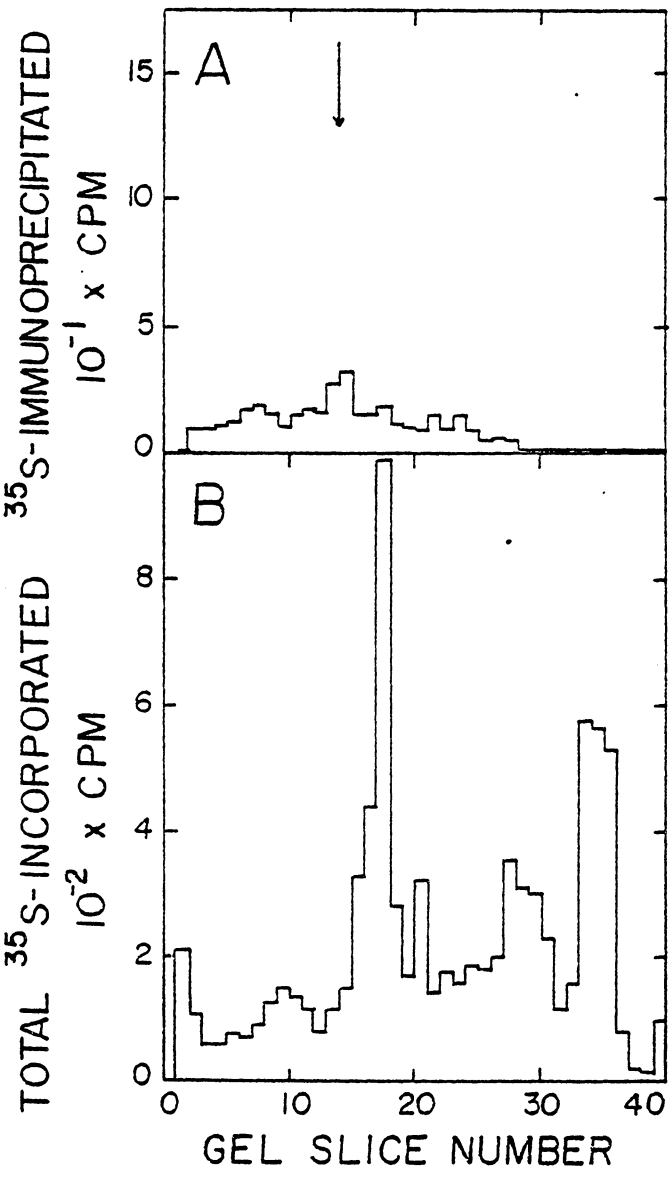

35S-INCORPORATED INTO NADP-GDH
10-2 x CPM / 35µl LYSATE
35S-INCORPORATED INTO TOTAL PROTEIN
10-5 x CPM / 35µl LYSATE
µg POLY(A)-CONTAINING RNA
0
1
2
3
4
0.0
0.5
1.0
0.00
1.25
2.50

Fig. 17A, B. SDS polyacrylamide gel electrophoresis (77) of immunoprecipitated- and total-protein products synthesized *in vitro* from non-poly(A)-containing RNA. Protein synthesis was performed in 150 µl of reticulocyte lysate reaction mixture in the presence of 93 µg/ml of *Chlorella* non-poly(A)-containing RNA. A, material immunoprecipitated by rabbit anti-NADP-GDH IgG. Direct immunoprecipitation by rabbit anti-NADP-GDH IgG was performed as described in Fig. 15A, B. B, Total proteins synthesized *in vitro*. The radioactivity was measured in a 5 µl aliquot of the reticulocyte lysate reaction mixture. The arrow in A indicates the position of the authentic NADP-GDH subunit. After electrophoresis was terminated, the bromphenol blue marker was located in gel slice number 36.


A
B
15
10
5
0
8
6
4
2
0
0
10
20
30
40
GEL SLICE NUMBER
^{35}S-IMMUNOPRECIPITATED
10^{-1} x CPM
TOTAL ^{35}S-INCORPORATED
10^{-2} x CPM

extracted from ammonium-cultured cells. Therefore, this result strongly suggests that the mRNA coding for the NADP-GDH contains a poly(A) sequence at 3' end. Hence, the mRNA is similar to other eucaryotic mRNAs in that it contains a poly(A) region. However, some non-poly(A)-containing mRNAs (e.g., the large subunit of ribulose 1,5-bisphosphate carboxylase) have been found to be translated very inefficiently in the rabbit reticulocyte lysate protein synthesizing system (10, 41).

The final possibility which is considered is that the measurement of the cellular NADP-GDH concentration in ammonium-cultured cells is an overestimate. It is quite reasonable to expect that the specific activity of the NADP-GDH in the crude homogenate is at least 2- to 3 times lower then the concentration of the enzyme in the frozen-thawed low-speed supernatant.

The binding studies with ^{125}I-labelled anti-NADP-GDH IgG and total polysomes isolated from nitrate-cultured cells showed that NADP-GDH was being synthesized on polysomes of uninduced cells. To investigate whether polysomal poly(A)-containing RNA, isolated from uninduced cells could direct the synthesis of NADP-GDH subunits *in vitro*, poly(A)-containing RNA was extracted from these cells and translated in the rabbit reticulocyte protein synthesizing system. As a control experiment, poly(A)-containing RNA, isolated from fully induced cells, was translated *in vitro* in an identical manner. The lysates were incubated with rabbit anti-NADP-GDH IgG as described earlier and the immunoprecipitated products were analysed by SDS gel electro-

phoresis. As shown in Fig. 18, when poly(A)-containing RNA was extracted from polysomes from uninduced cells and translated in vitro, NADP-GDH antigen was synthesized from the RNA. The majority of the immunoprecipitable material migrated further in the gel than that synthesized by poly(A)-containing RNA from induced cells. However when these data were expressed on the basis of a equivalent amount of RNA added to the lysate, uninduced cells were found to contain 75% of the translatable mRNA present in induced cells.

Estimation of the Molecular Weight of the NADP-GDH mRNA - To obtain an estimate of the molecular weight of the NADP-GDH mRNA, total polysomal poly(A)-containing RNA, isolated from ammonium-cultured cells, was fractionated in linear sucrose gradients, and the fractions were translated in vitro and immunoprecipitated by anti-NADP-GDH IgG. The immunoprecipitated material was solubilized and analysed by SDS gel electrophoresis. The radioactivity in the area of the gel which corresponded to the full length NADP-GDH subunit was measured and compared with the total ^{35}S-labelled methionine incorporated into total protein by each RNA fraction (Fig. 19A, B). After centrifugation, the fractionated RNA in the gradient exhibited a normal distribution which was slightly skewed towards smaller RNA molecules (Fig. 19A). The peak of absorbance at 254 nm of the fractionated RNA was located in the region of the gradient which corresponded to a size range of RNA from 10S to 25S. Presumably, the absorbance in the gradient which corresponded to a size of RNA smaller than 10S reflected a combination of partial degradation during isolation and the natural abundance of

Fig. 18. SDS polyacrylamide gel electrophoresis (79) of anti-NADP-GDH IgG-immunoprecipitated material synthesized *in vitro* from *Chlorella* polysomal poly(A)-containing RNA, isolated from synchronized cells cultured in ammonium-(open bars) and nitrate-(hatched bars) containing medium. Protein synthesis was performed in reticulocyte lysate reaction mixtures exactly as described by New England Nuclear (catalog Number NEK-001). The total volume of the translation assay was 25 µl. Protein synthesis was performed in the presence of 20 µg/ml poly(A)-containing RNA isolated from ammonium- and nitrate-cultured cells and in the absence of *Chlorella* RNA. After protein synthesis was terminated, direct immunoprecipitation by rabbit anti-NADP-GDH IgG was performed as described in Fig. 15A, B with the following modifications: The immunoprecipitates were not centrifuged from the lysates prior to centrifugation in 0.9 M sucrose. The immunoprecipitates were pelleted through 0.9 M sucrose by centrifugation at 10,000 rpm in the HB-4 rotor (Sorvall) for 20 min at 4°. The tubes were frozen in liquid nitrogen and the tips were cut off and placed in 100 µl of Laemmli (79) incubation buffer. The distribution of radioactivity in the anti-NADP-GDH-immunoprecipitated material obtained from a reticulocyte lysate mixture incubated in the absence of *Chlorella* RNA is shown (solid bars). The arrow marks the position of the authentic NADP-GDH subunit in the gel.

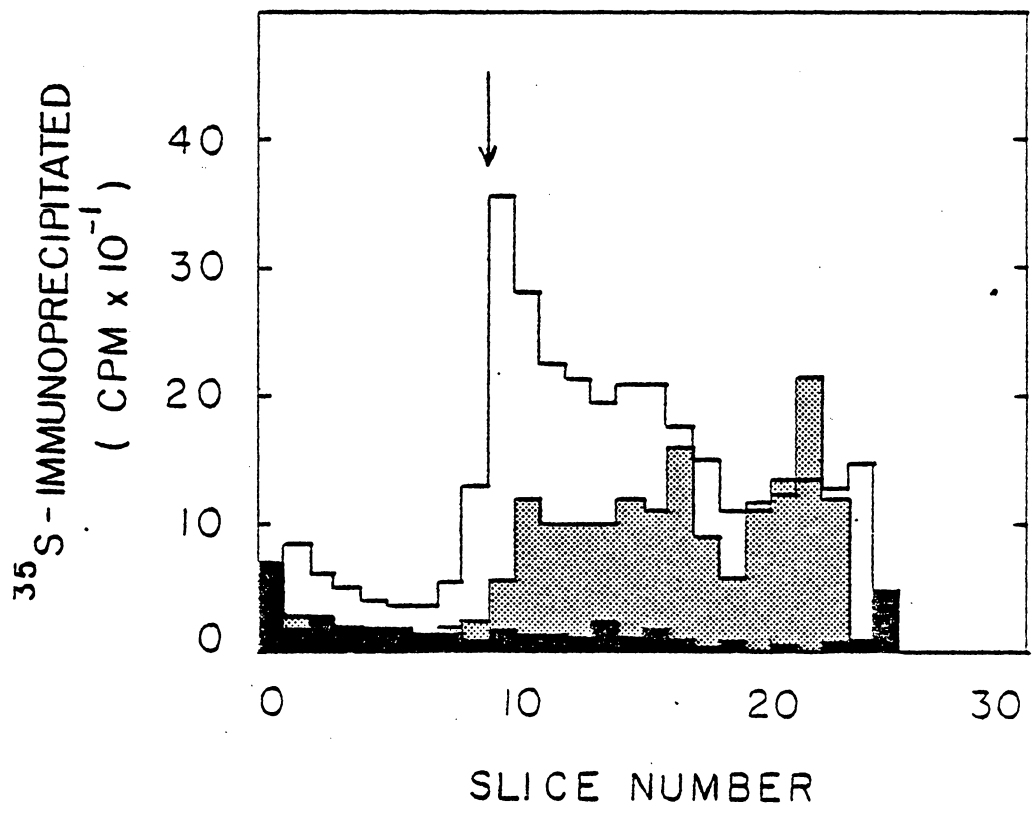
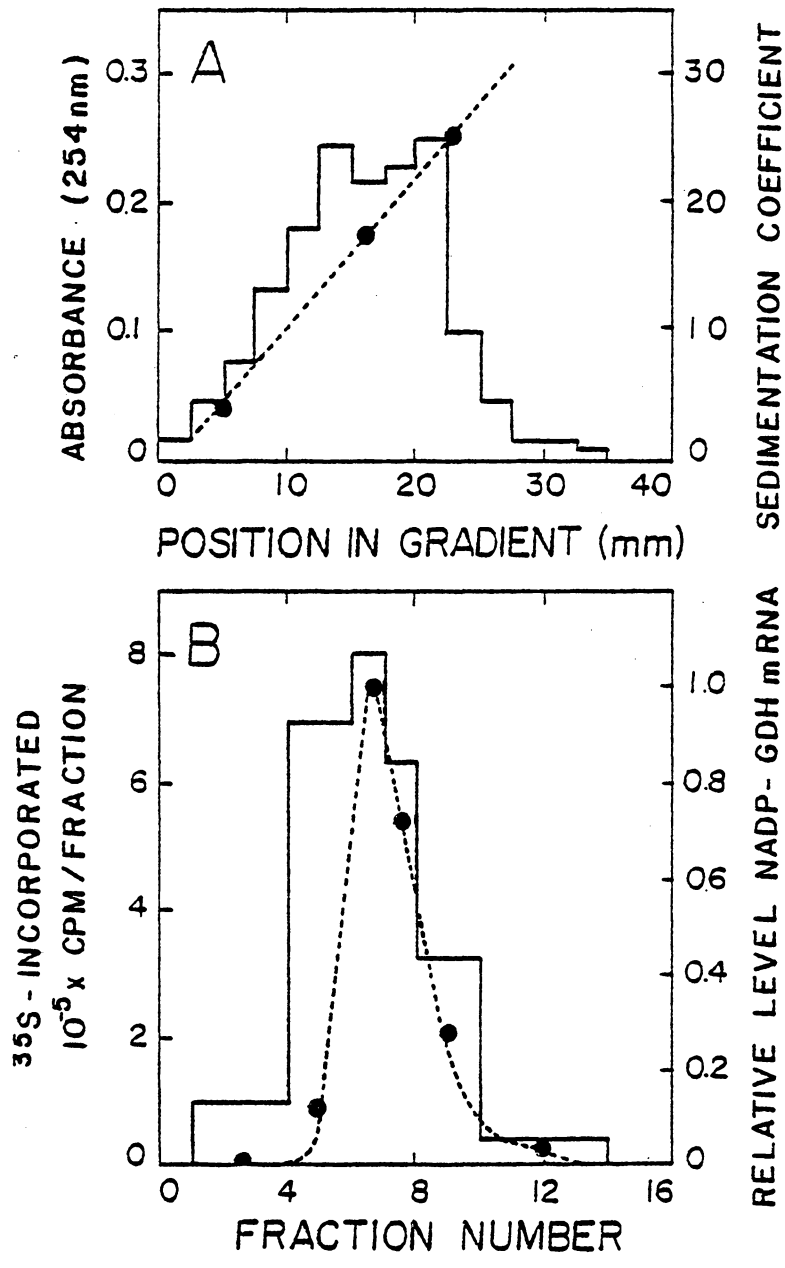

^{35}S - IMMUNOPRECIPITATED
(CPM x 10^{-1})
40
30
20
10
0
0
10
20
30
SLICE NUMBER

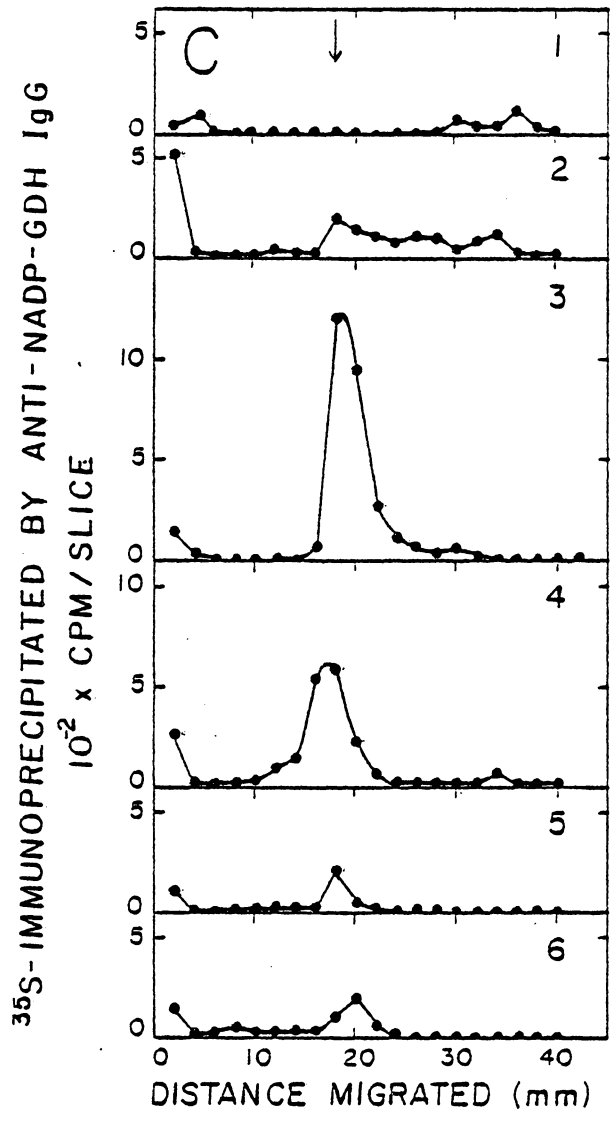
Fig. 19A, B. Estimation of molecular weight of NADP-GDH mRNA. Heat denatured, Chlorella polysomal poly(A)-containing RNA (80 µg) was sedimented in a 10 to 25% linear sucrose density gradient. Fractions were collected and A_{260} values were determined. A, Sedimentation profile of total polysomal poly(A)-containing RNA. The molecular weight marker RNA was centrifuged in a parallel gradient. The RNA-containing fractions were combined into 6 larger fractions which were translated in a reticulocyte lysate protein synthesizing system. B, Incorporation of ^{35}S-labelled methionine into total protein in each fraction (open bars); mRNA activity for NADP-GDH as determined by direct immunoprecipitation with rabbit anti-NADP-GDH IgG from protein products after protein synthesis was terminated(-●-).


A
ABSORBANCE (254nm)
SEDIMENTATION COEFFICIENT
POSITION IN GRADIENT (mm)
B
35S - INCORPORATED
10-5 x CPM/FRACTION
RELATIVE LEVEL NADP-GDH mRNA
FRACTION NUMBER

small mRNA molecules in Chlorella. The distribution of NADP-GDH mRNA is shown in Fig. 19B. The NADP-GDH mRNA was observed to sediment as a symmetrical peak in the gradient. The maximum NADP-GDH mRNA template activity was located in the gradient which corresponded to a sedimentation coefficient of 18S (Fig. 19B). This value corresponds to a molecular weight of 810,000 (93). The large size of this mRNA is in agreement with the calculated size of 14S for a template RNA that would be the minimum size to code for the amino acid sequence of the NADP-GDH subunit (Mr., 59,000). Therefore, by use of this method, the NADP-GDH mRNA was estimated to be 28% larger than the minimum RNA size required. Because it is difficult to completely eliminate secondary structure in the poly(A)-containing RNA and in the rRNA molecular weight markers, this estimate is considered to be an approximation and should be verified by another method (25). However this result is in complete agreement with the observation that the molecular weight of eucaryotic mRNA is substantially larger than required to code for the amino acids of the protein. The analysis of RNA sequence of several mRNAs (e.g., ovalbumin mRNA, globin mRNA) has revealed the presence of several non-coding regions interspersed within the coding region which would partially account for the large size (34). These data are consistent with the possibility that the NADP-GDH mRNA might also contain a complex structure.

The molecular weight estimate of the NADP-GDH mRNA (Fig. 19B) was obtained from the data shown in Fig. 20. Each panel represents the distribution of radioactivity in the solubilized immunoprecipi-

Fig. 20. SDS polyacrylamide gel electrophoresis (77) of anti-NADP-GDH IgG-immunoprecipitated material synthesized *in vitro* from RNA-containing sucrose gradient fractions. Each panel represents the material immunoprecipitated by anti-NADP-GDH IgG which as synthesized from 1 μg of total poly(A)-containing RNA in a rabbit reticulocyte protein synthesizing system. The 6 RNA-containing fractions were obtained by sedimentation of heat denatured poly(A)-containing RNA in a sucrose density gradient (data from Fig. 19B).


C
1
2
3
4
5
6
^{35}S-IMMUNOPRECIPITATED BY ANTI-NADP-GDH IgG
10^{-2} x CPM/SLICE
DISTANCE MIGRATED (mm)
0
10
20
30
40

tates after SDS gel electrophoresis which was obtained from translation of the 6 RNA fractions. As shown in Fig. 20, panel 2, the translation of small mRNA molecules (i.e., S value less than 15S) resulted in products which were specifically immunoprecipitated by anti-NADP GDH IgG. The immunoprecipitated material was distributed as a broad peak of radioactivity in the gel with higher average mobility than the mobility of the authentic NADP-GDH subunit. These data suggest that the immunoprecipitable material was derived from translation of partially degraded NADP-GDH mRNA. The antigenic fragments synthesized *in vitro* were recognized and precipitated by the specific antibody. Translation of RNA fractions containing higher molecular weight RNA produced no immunoprecipitable material *in vitro* which was smaller than the full length polypeptide (i.e., Fig. 20, panels 5 and 6). Under these conditions, there was no evidence for abnormal protein synthesis (i.e., RNA hydrolysis during protein synthesis, pretermination of polypeptide synthesis).

Partial Purification of NADP-GDH mRNA by Indirect Immunoadsorption and Oligo(dT)-Cellulose - Because NADP-GDH mRNA was estimated to comprise a small percentage of total *Chlorella* poly(A)-containing RNA (i.e., approximately 0.1%), and was indistinguishable from the bulk of the poly(A)-containing RNA on the basis of size (Fig. 19B), an immunological isolation procedure was the method chosen for the purification of the NADP-GDH mRNA.

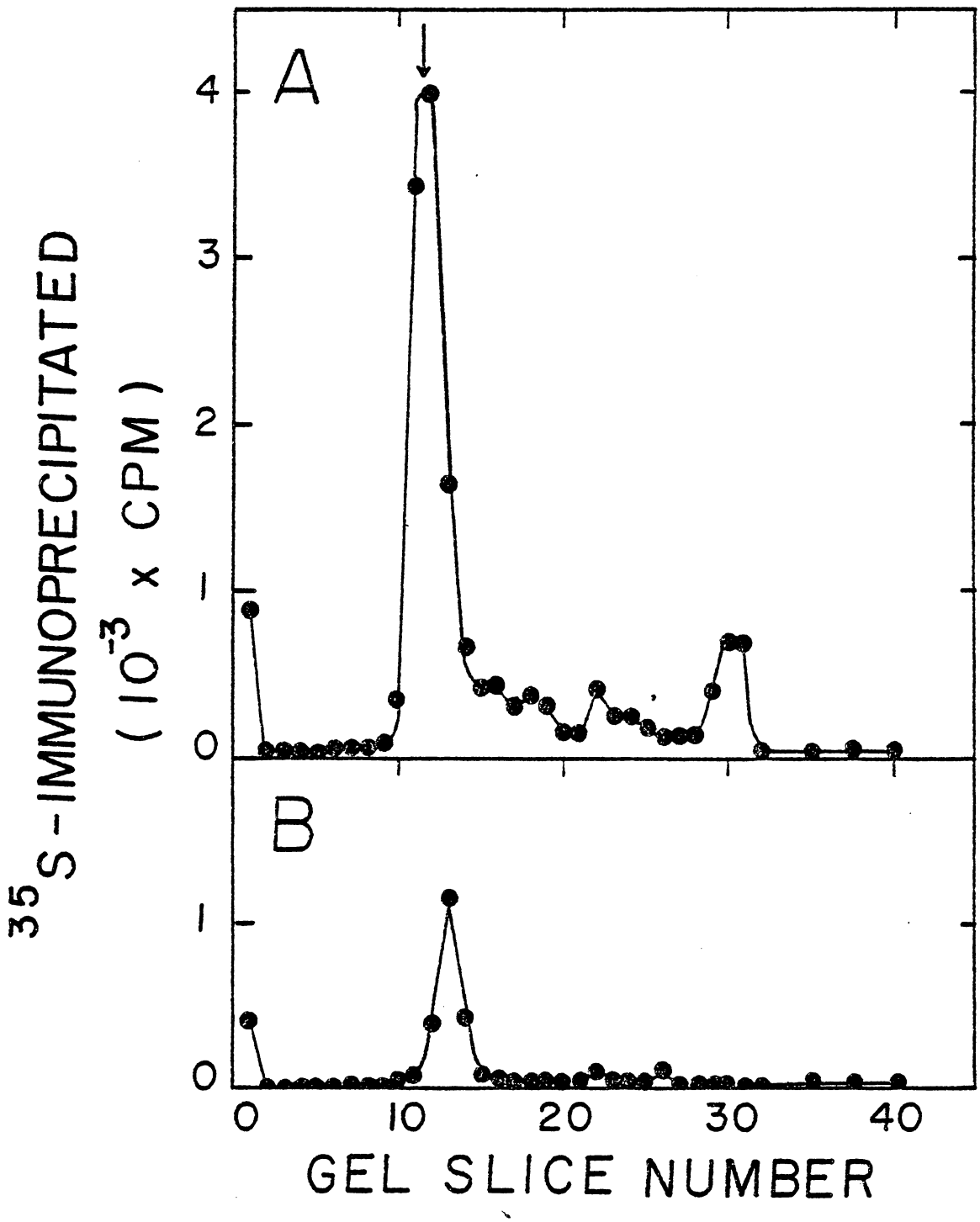
An indirect immunoadsorption procedure, developed by Schutz *et al* (38) was employed for the partial purification of the NADP-GDH-

specific polysomes. Rabbit anti-NADP-GDH IgG was incubated with total Chlorella polysomes. The antibody-polysome complex was next specifically adsorbed to sheep anti-rabbit IgG-cellulose. After the immunoselected polysomes were dissociated from the cellulose, the RNA was fractionated by oligo(dT)-cellulose column chromatography. To measure the fold purification of the immunoselected poly(A)-containing RNA fraction, 1 µg aliquots of poly(A)-containing RNA, extracted from total Chlorella polysomes, and immunoselected poly(A)-containing RNA were translated in rabbit reticulocyte lysates. The amount of radioactivity which was immunoprecipitated by rabbit anti-NADP-GDH IgG was compared for the 2 RNA species.

When immunoselected poly(A)-containing RNA was translated in vitro, and the products immunoprecipitated by rabbit anti-NADP-GDH IgG, a large peak of radioactivity was observed in the SDS polyacrylamide gel which corresponded to the mobility of the NADP-GDH subunit (Fig. 21A, B).

To obtain an estimate of the amount of immunoprecipitable material which was removed by the 100,000 x g centrifugation of the lysate, immunoprecipitation by anti-NADP-GDH IgG was performed before 100,000 x g (Fig. 21A) and after the high-speed centrifugation (Fig. 21B). After the high-speed centrifugation, the recovery of full length NADP-GDH subunits was calculated to be 22% of the radioactivity in the NADP-GDH subunits before 100,000 x g. By centrifugation, the antigenic fragments, with a faster sedimentation rate, were removed. It is unclear whether the majority of the full length polypeptides are

Fig. 21A, B. SDS polyacrylamide gel electrophoresis of anti-NADP-GDH IgG-immunoprecipitated material synthesized *in vitro* from partially purified poly(A)-containing RNA obtained by indirect immunoadsorption of NADP-GDH-specific polysomes. One microgram of immunoselected poly(A)-containing RNA was translated in a standard reticulocyte lysate protein synthesizing system. Direct immunoprecipitation by rabbit anti-NADP-GDH IgG was performed before, A and after, B the 100,000 x *g* centrifugation of the lysate mixture. Immunoprecipitation was carried out as described in Fig. 15A, B. The arrow in A indicates the position of the authentic NADP-GDH subunit.


A
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10
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30
40
GEL SLICE NUMBER
35S-IMMUNOPRECIPITATED
(10-3 x CPM)

released from the ribosomes during protein synthesis. The percentage recovery of the NADP-GDH subunit was the same value as that obtained for the total ^{35}S-labelled immunoselected proteins after the high-speed centrifugation (i.e., 24%) and agreed favorably with several reported estimates (24, 53).

The NADP-GDH mRNA was estimated to be purified 5.6-fold in the immunoselected poly(A)-containing RNA fraction compared with the initial poly(A)-containing RNA fraction (Table II). If the difference between the total ^{35}S-labelled methionine incorporation into total protein is equalized for the 2 RNA fractions the total fold purification was estimated to be sevenfold. The overall recovery of the NADP-GDH mRNA in the immunoselected poly(A)-containing RNA was 2.8%. Since poly(A)-containing RNA was approximately 2.7% of total polysomal RNA fraction, the overall purification of NADP-GDH mRNA from total Chlorella polysomal RNA was two hundred-fold. The NADP-GDH synthesized by the immunoselected poly(A)-containing RNA was calculated to be 0.71% of the total protein synthesized by the RNA fraction (data from Table II).

To characterize further the immunoselected poly(A)-containing RNA, aliquots of the lysate were removed after protein synthesis was terminated and subjected to SDS gel electrophoresis. As a comparison, proteins in the lysate were analysed on SDS polyacrylamide gels before and after immunoprecipitation (Fig. 22A, B). The distribution of radioactivity synthesized by the immunoselected RNA isolated from Chlorella polysomes was also analysed before 100,000 x g (Fig. 22A)

TABLE II

Partial Purification of NADP-GDH mRNA by Indirect Immunoadsorption and oligo(dT)-cellulose [a]

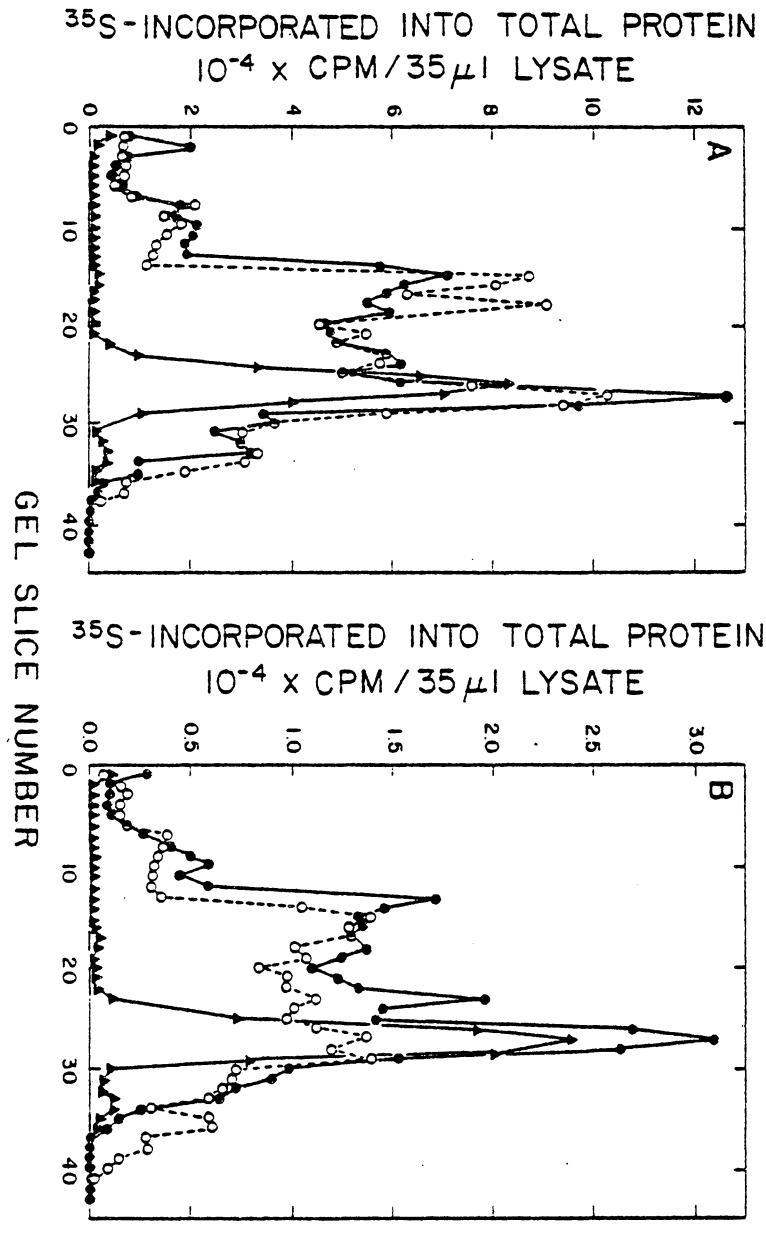
Fraction	RNA µg	cpm incorporated into Total protein 10^{-6} x cpm/ µg RNA	Specific [b] Activity cpm in NADP-GDH/ µg RNA	10^{-3} x cpm Total Translation Activity	Fold Purification	Recovery %
Initial *Chlorella* poly(A)-containing RNA [c]	1800	1.60	1530	2,750	1	100
Immunoselected poly(A)-containing RNA	9	1.20	8500	76.5	5.6	2.8

a The data were calculated from a standard *in vitro* translation assay of 35 µl total volume.

b The radioactivity represents the area of NADP-GDH subunits on SDS polyacrylamide gels.

c The initial amount of poly(A)-containing RNA was calculated to be 3 µg poly(A)-containing RNA/ A_{260} units of polysomes.

Fig. 22A, B. SDS polyacrylamide gel electrophoresis (79) of total protein products synthesized *in vitro* from partially purified poly(A)-containing RNA obtained by Indirect Immunoadsorption of NADP-GDH-specific polysomes. The immunoselected RNA was translated *in vitro* as described in Fig. 21A, B. Incorporation of ^{35}S-labelled methionine into total protein was determined in a 5 µl aliquot. A, before 100,00 x *g* of the reticulocyte lysate reaction mixture; Incorporation of ^{35}S-labelled methionine into total portein, before (●) and after (-○-) direct immunoprecipitation by rabbit anti-NADP-GDH IgG; Incorporation of ^{35}S-labelled methionine into total protein synthesized in a reticulocyte lysate reaction mixture in the absence of *Chlorella* RNA (▲) B, Same as A, except immunoprecipitation reaction was performed after 100,000 x *g* centrifugation of the lysate reaction mixture. The major radioactive protein synthesized in the reticulocyte lysate reaction mixture incubated without *Chlorella* RNA was observed to have the mobility of hemoglobin present in the lysate (▲).

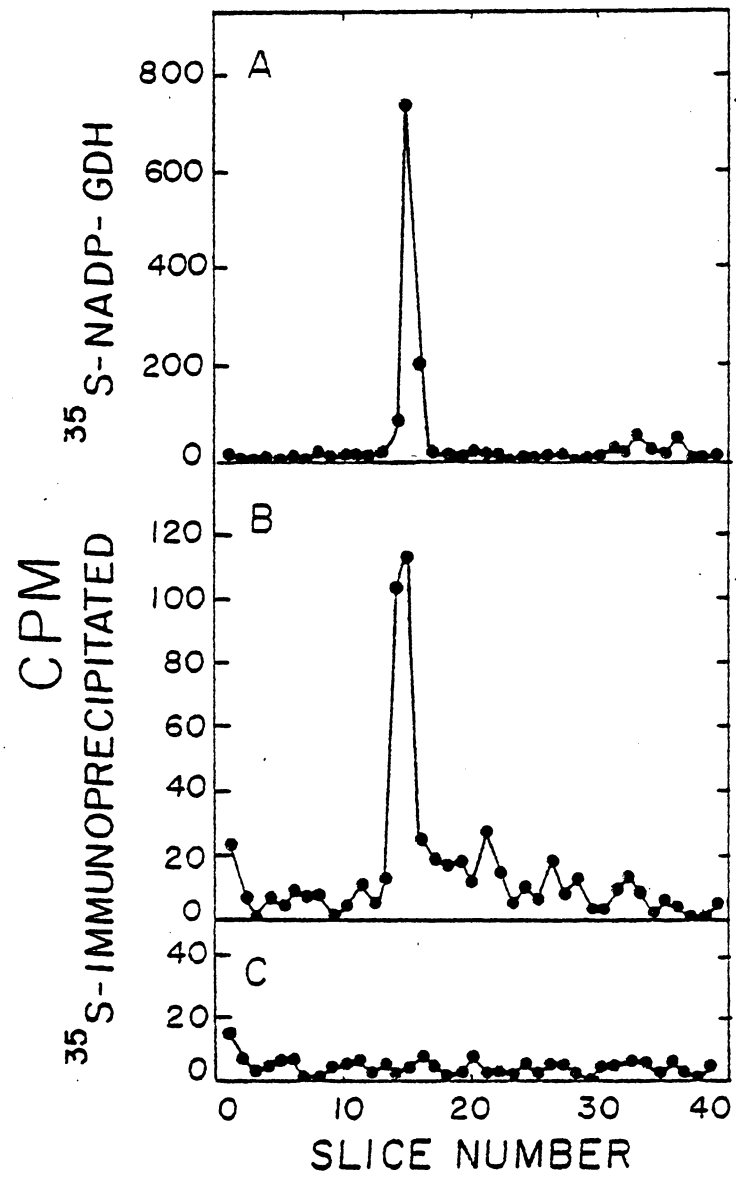

^{35}S-INCORPORATED INTO TOTAL PROTEIN
10^{-4} x CPM/35μl LYSATE
A
^{35}S-INCORPORATED INTO TOTAL PROTEIN
10^{-4} x CPM/35μl LYSATE
B
GEL SLICE NUMBER

and after 100,000 x g (Fig. 22B). As shown in Fig. 22A, the pattern of radioactive proteins which remained in the supernatant after the immunoprecipitation reaction, closely resembled the pattern of radioactive proteins which were translated in the lysate. A large peak of radioactive protein was observed to migrate to the position which corresponded to the major radioactive protein synthesized in the lysate without added RNA. This protein is presumably globin. Globin been reported to be synthesized by the remaining endogenous mRNA in the treated-lysate (52). After the 100,000 x g centrifugation (Fig. 22B), a similar distribution of radioactivity was observed in the gel. However, after the immunoprecipitation reaction, one radioactive protein peak, which corresponded to the mobility of the NADP-GDH subunit, was observed to be decreased in amount. Although the pattern of radioactive proteins synthesized from the immunoselected RNA did not resemble the distribution of radioactive proteins synthesized from total polysomal poly(A)-containing RNA (Fig. 13A), proteins synthesized from the immunoselected RNA comprised a heterogeneous range of polypeptide sizes. Therefore, these results are consistent with the low estimate of the NADP-GDH mRNA purity.

The procedure for the partial purification of the NADP-GDH mRNA, described in "Materials and Methods" included a modification that significantly improved the yield and the purity of the NADP-GDH mRNA in the immunoselected poly(A)-containing RNA. The first attempt to purify the NADP-GDH mRNA by indirect immunoadsorption did not employ Triton X-100 in the buffers which were used for the 100,000 x g centri-

fugation of the low speed supernatant. In the absence of Triton X-100, the polysomes were heavily contaminated by a dark green membrane fraction. When the antibody-polysome complex was incubated with sheep anti-rabbit IgG-cellulose, a large proportion of the green pigment adsorbed to the cellulose and was not removed until the cellulose was washed with a buffer containing 1% Triton-X100 and 1% sodium deoxycholate. By the unmodified procedure, 6 µg of immunoselected poly(A)-containing RNA was obtained from 120 A_{260} units of anti NADP-GDH IgG-bound polysomes. In contrast, 9 µg of immunoselected poly(A)-containing RNA was isolated from 600 A_{260} units of polysomes by the modified procedure. The overall yield of immunoselected RNA was threefold greater by use of the unmodified procedure. To obtain an estimate of the purity of the NADP-GDH mRNA in this immunoselected poly(A)-containing RNA, 0.2 µg of the immunoselected RNA was translated in vitro and the products were subjected to direct immunoprecipitation by rabbit anti-NADP-GDH IgG exactly as described above. As seen in Fig. 23A, B, C the unmodified procedure also resulted in the retention of the NADP-GDH mRNA throughout each purification step. Compared to the mobility of the authentic in vivo-labelled ^{35}S-NADP-GDH subunit (Fig. 23A), a substantial peak of radioactive protein migrated to the position expected for the NADP-GDH subunit (Fig. 23B). To verify the specificity of the purified antibody, the products of a lysate incubated without added RNA were immunoprecipitated by rabbit anti-NADP-GDH IgG under the same conditions. No radioactive proteins were immunoprecipitated from the lysate in the absence of Chlorella

Fig. 23A, B, C. SDS polyacrylamide gel electrophoresis (79) of anti-NADP-GDH IgG immunoprecipitated material synthesized *in vitro* from partially purified poly(A)-containing RNA obtained by indirect immunoadsorption of NADP-GDH-specific polysomes. The unmodified direct immunoadsorption procedure was employed. Immunoselected poly(A)-containing RNA (0.2 µg) was translated in a reticulocyte protein synthesizing system. Direct immunoprecipitation by rabbit anti-NADP-GDH IgG was performed as described in Fig. 15A, B. A, *in vivo* ^{35}S-labelled NADP-GDH; B, *in vitro* synthesized anti-NADP-GDH IgG-immunoprecipitated material; C, *in vitro* synthesized anti-NADP-GDH IgG-immunoprecipitated material obtained from a lysate reaction mixture incubated without *Chlorella* RNA.


A
B
C
800
600
400
200
0
120
100
80
60
40
20
0
40
20
0
0
10
20
30
40
SLICE NUMBER
CPM
35S-NADP-GDH
35S-IMMUNOPRECIPITATED

RNA (Fig. 23C). By comparison of the amounts of radioactivity in the NADP-GDH subunits synthesized *in vitro* by immunoselected poly(A)-containing RNA and total polysomal poly(A)-containing RNA, a small enrichment of the NADP-GDH mRNA in the immunoselected RNA was obtained by use of the unmodified purification procedure. The amount of NADP-GDH synthesized *in vitro* was compared for the immunoselected poly(A)-containing RNA isolated by the modified and unmodified procedures. The specific activities of the NADP-GDH synthesized *in vitro* from the two immunoselected RNA fractions were 8,500 cpm and 1200 cpm per μg of RNA, isolated by the modified and unmodified procedures, respectively. Therefore, the addition of Triton X-100 in the modified procedure significantly lowered the amount of non-specifically adsorbed polysomes which were bound to the cellulose.

In the development of the indirect immunoadsorption procedure, Schutz *et al* (38) found that several insoluble matrices (e.g., polyacrylamide, polystyrene) displayed high levels of nonspecific adsorption of polysomes. Since PAB-cellulose showed no detectable non-specific affinity for polysomes (i.e., less than 1%), they concluded that PAB-cellulose was the most suitable matrix (38). However if this matrix is to be successfully employed for the purification of plant mRNAs, in the opinion of this author, the complete elimination of the chlorophyll-containing pigments from the polysomes, prior to the addition of PAB-cellulose, would be a necessary requirement.

The purity of the NADP-GDH mRNA in the immunoselected poly(A)-containing RNA was also examined by RNA excess-cDNA hybridization

analysis of the immunoselected and total polysomal poly(A)-containing RNA fractions. Complementary DNA was transcribed from both RNA fractions with the use of reverse transcriptase (80). When immunoselected poly(A)-containing RNA was transcribed by the procedure described in "Materials and Methods", high molecular weight cDNA (i.e., immunoselected cDNA) eluted in the void volume of the Sephadex G-50 column (Fig. 24). Approximately 37% of the total radioactivity applied to the gel filtration column was excluded in the fractions which contained the high molecular weight cDNA. The yield of immunoselected cDNA was approximately 8 x 10^5 cpm of ^{3}H-labelled cDNA per microgram of added RNA. Based on the specific activity of the ^{3}H-labelled dCTP and on the assumption that all 4 bases were represented in equal concentration in each cDNA molecule, the calculated yield of cDNA corresponded to 1.0 ng of cDNA per 1.0 µg of immunoselected poly(A)-containing RNA. The size distribution of immunoselected cDNA was analysed by sedimentation in linear alkaline sucrose gradients (Fig. 25). The immunoselected cDNA sedimented as a broad symmetrical peak of radioactivity in the gradient. The distribution of radioactivity in the peak was similar to that obtained for cDNA transcribed from the small subunit of ribulose 1,5-bisphosphate carboxylase mRNA which was partially purified by the indirect immunoadsorption procedure by James Lynch (91). Molecular weight DNA markers were unavailable for this study so the average molecular weight of the immunoselected cDNA was not determined. However, for RNA-cDNA hybridizations, a full length cDNA sequence is not required (94). It is more important that

Fig. 24. Radioactive profile of immunoselected cDNA from Sephadex G-50 column. Immunoselected ^{3}H-labelled cDNA was synthesized from immunoselected poly(A)-containing RNA, isolated by indirect immunoadsorption of NADP-GDH-specific polysomes. The cDNA containing sample (1 ml) was applied onto a 10 ml column of Sephadex G-50 at 1 ml/min. Radioactivity in each 1 ml fraction was determined as described in "Materials and Methods". The high molecular weight cDNA-containing fractions which eluted at the void volume were pooled.

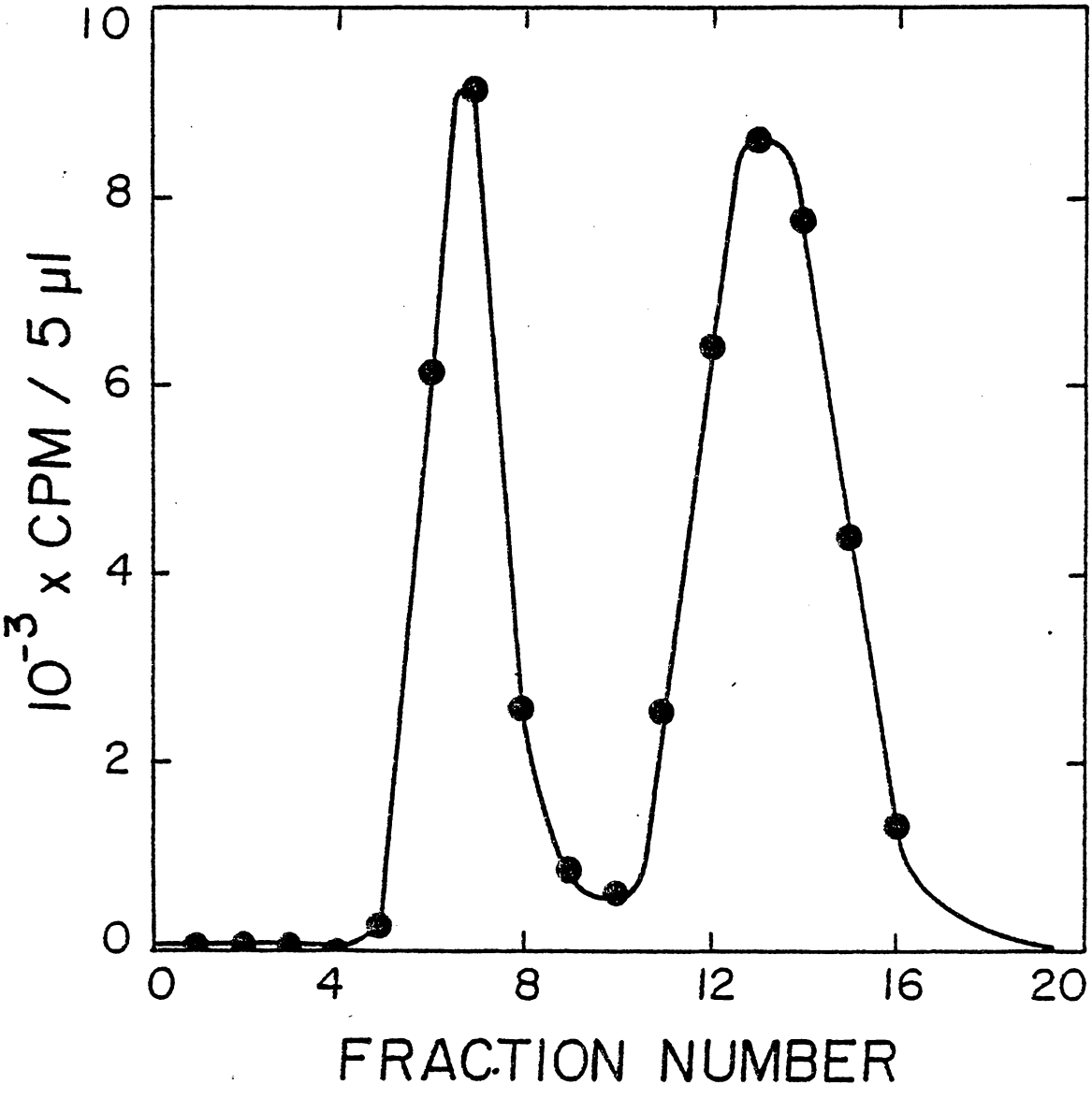
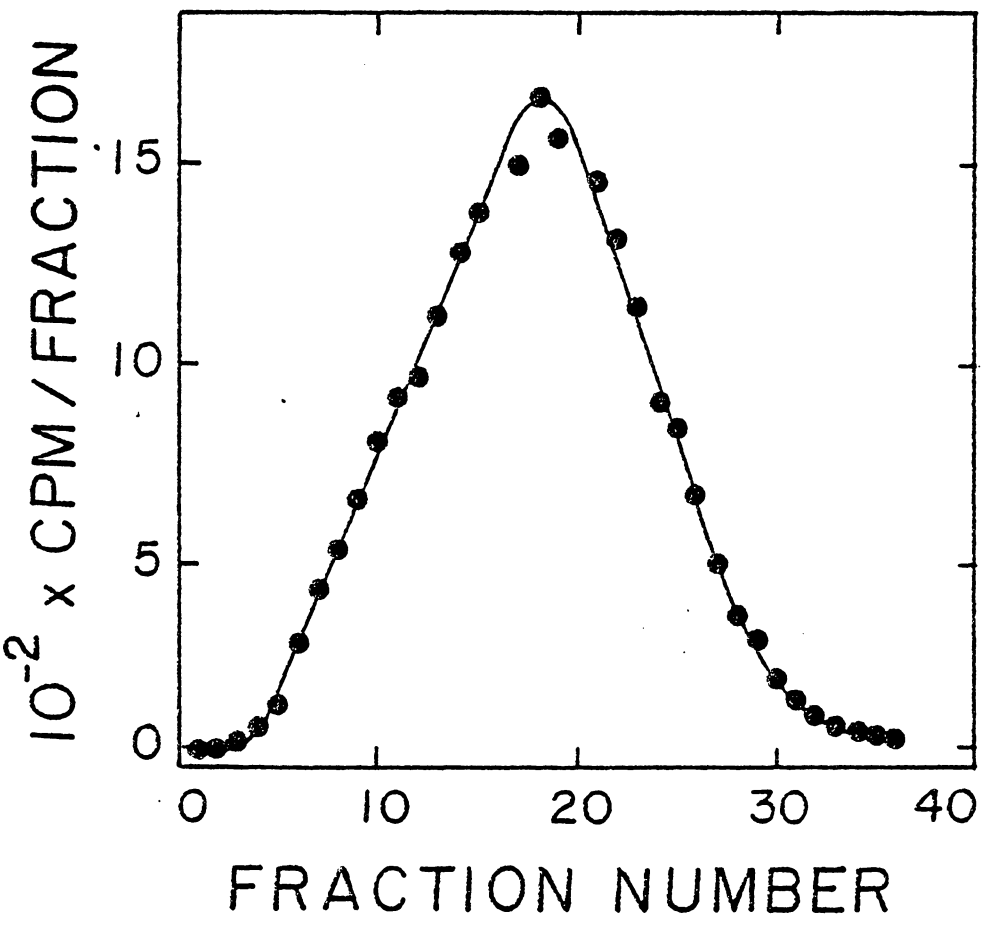

10
8
6
4
2
0
10^{-3} x CPM / 5 µl
0
4
8
12
16
20
FRACTION NUMBER

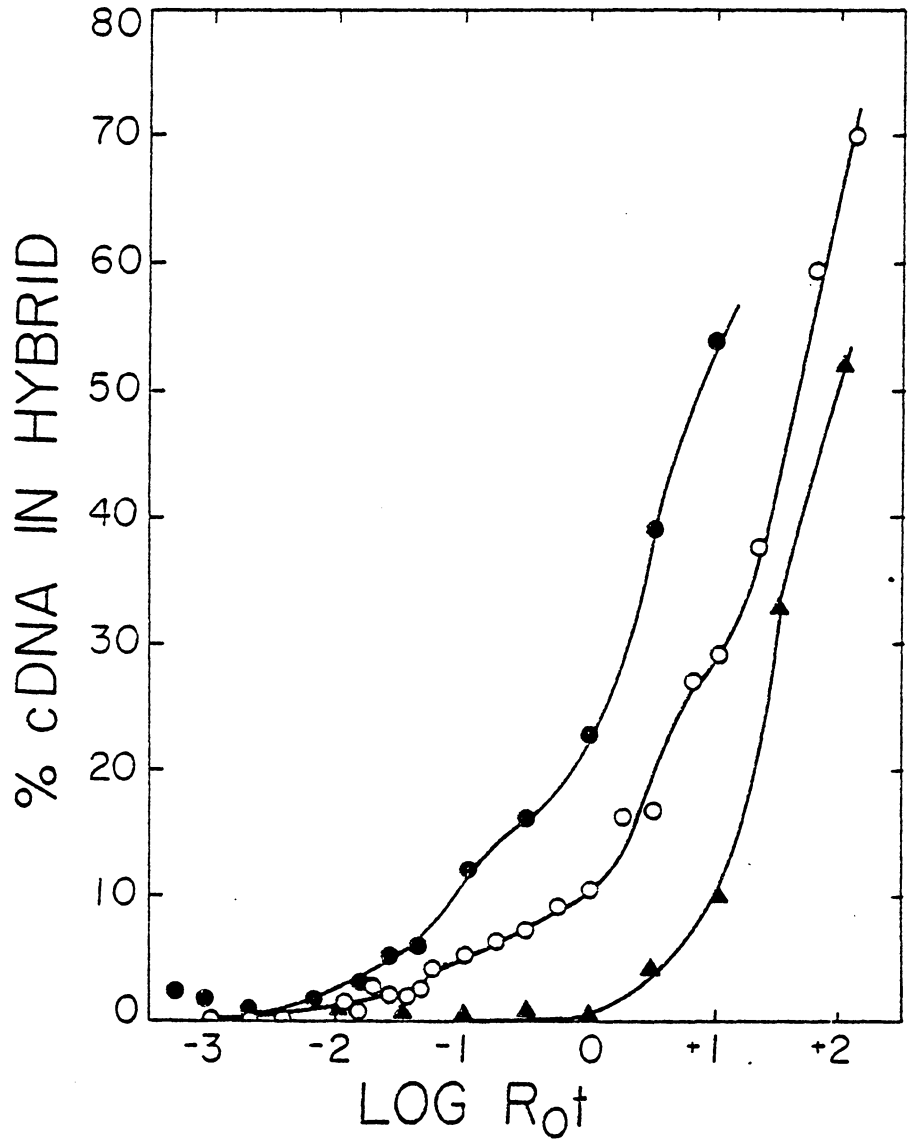
Fig. 25. Radioactive profile of immunoselected cDNA in an alkaline sucrose gradient. Immunoselected ^{3}H-labelled cDNA was synthesized from immunoselected poly(A)-containing RNA, isolated by indirect immunoadsorption of NADP-GDH-specific polysomes. Immunoselected cDNA (20,000 cpm) was sedimented in an alkaline sucrose gradient in the SW-41 rotor (Beckman) for 24 h at 39,000 rpm at 5°. The radioactivity in each 0.3 ml fraction was determined. Molecular weight DNA markers were unavailable for use in this study. Therefore, it is not possible to estimate the molecular weight distribution of the immunoselected cDNA in the gradient.


10^{-2} x CPM/FRACTION
0
5
10
15
0
10
20
30
40
FRACTION NUMBER

the cDNA is of a uniform size so that the kinetics of hybridization to excess mRNA obey a pseudo first order rate law (44).

The immunoselected cDNA was hybridized to an excess of immunoselected poly(A)-containing RNA and total polysomal poly(A)-containing RNA (Fig. 26). Because of the small yield of immunoselected poly(A)-containing RNA, the hybridization reaction could not be performed at log Rot values greater than + 1.0. For comparison, total polysomal poly(A)-containing RNA was hybridized in excess concentration to cDNA transcribed from the same total RNA fraction. The synthesis of cDNA complementary to total polysomal poly(A)-containing RNA, (i.e., total cDNA) and the homologous hybridization of the two species was performed by Dr. C. F. Thurston in our laboratory. Analysis of the hybridization kinetics revealed that at log Rot +1.0, 55% of the immunoselected cDNA was hybridized to the immunoselected poly(A)-containing RNA. At the same log Rot value, only 29% of total cDNA was in hybrid form with the total polysomal poly(A)-containing RNA. This result indicated that the sequence complexity of the immunoselected poly(A)-containing RNA was much less than that in the total polysomal poly(A)-containing RNA fraction. Further evidence for this difference between the two RNA fractions was obtained by the comparison of the kinetics of hybridization of immunoselected cDNA and total cDNA to total poly(A)-containing RNA. Whereas the homologous hybridization of total cDNA to total polysomal poly(A)-containing RNA exhibited complex kinetics consistent with the presence of 3 major classes of sequence abundance, the hybridiza-

Fig. 26. Hybridization of cDNA synthesized from immunoselected poly(A)-containing RNA to excess immunoselected- and total-poly(A)-containing RNA. Immunoselected poly(A)-containing RNA was reacted with 750 cpm of ^{3}H-labelled cDNA synthesized from immunoselected RNA (●). Total poly(A)-containing RNA isolated from induced cells was reacted with 750 cpm of ^{3}H-labelled immunoselected cDNA (▲), and 750 cpm of ^{3}H-labelled cDNA synthesized from total poly(A)-containing RNA (○). Quantities of RNA ranging from 0.3 ng to 600 ng were used to drive the hybridizations that were stopped at the corresponding Rot values. Endogenous resistance of the cDNA preparations to the S1-nuclease treatment was approximately 5%.


80
70
60
50
40
30
20
10
0
% cDNA IN HYBRID
-3
-2
-1
0
+1
+2
LOG R_0t

tion of immunoselected cDNA to the same RNA fraction exhibited simple kinetics of hybridization. Two of the most abundant sequence classes in poly(A)-containing RNA were observed to be undetectable in the immunoselected RNA. The majority of the species in the immunoselected mRNA (i.e., greater than 80%) were observed to be present in the least abundant class of sequences present in unfractionated total poly(A)-containing RNA. In conclusion, these results agree with those obtained from the translation assay in several ways. First NADP-GDH mRNA was shown to be present in low concentration in the cell. Second, indirect immunoadsorption resulted in a purification of the NADP-GDH mRNA. However, the NADP-GDH mRNA was not in high enough concentration in immunoselected poly(A)-containing RNA that the immunoselected cDNA could be used as a specific hybridization probe to quantify the NADP-GDH mRNA sequences in the cell.

Mechanism of Induction of NADP-GDH by Ammonium - To determine if the increase in NADP-GDH enzyme activity, upon addition of ammonium to previously uninduced cells, was accompanied by an increase in the amount of translatable NADP-GDH mRNA, the amount of NADP-GDH mRNA was measured by the translation assay at several different times during the initial induction phase. Total cellular poly(A)-containing RNA was extracted from cell cycle samples which were harvested at 0, 30, 60, and 120 min after the addition of ammonium to uninduced synchronous cells. The level of translatable NADP-GDH mRNA was measured in each sample by *in vitro* translation and direct immunoprecipitation as described in "Materials and Methods". The distribution of the radioactivity in the

solubilized immunoprecipitates after SDS gel electrophoresis is shown in Fig. 27. Each panel represents the immunoprecipitable material obtained from translation of 1 μg of total cellular poly(A)-containing RNA which was extracted from cells harvested at the aforementioned times. These data show that the NADP-GDH mRNA was present at each time tested during the initial induction phase. The percentage of total ^{35}S-labelled methionine incorporation into total protein which comprised the NADP-GDH was calculated to be 0.055, 0.077 and 0.071% at 30, 60, and 120 min of induction, respectively. Therefore, after 60 min of the induction period, the level of NADP-GDH mRNA was approximately 75% of the level of NADP-GDH mRNA in fully induced cells. To obtain the pattern of NADP-GDH mRNA accumulation during the induction phase, the amount of NADP-GDH mRNA measured by these data was corrected for the percentage of cell breakage in each cell cycle sample and for the dilution of the culture over the 2 h induction period. As seen in Fig. 28, the NADP-GDH mRNA was observed to increase in a linear fashion from 30 min after the addition of inducer to the culture. The NADP-GDH enzyme activity accumulated in a linear fashion with a 35 min induction lag, exactly as reported earlier (29). Bascomb (33) showed, by use of rocket immunoelectrophoresis, that the accumulation of NADP-GDH catalytic activity was accompanied by a parallel increase in enzyme-antigen This observation suggested that when the inducer was added to uninduced cells, NADP-GDH was synthesized de novo (33). He also demonstrated that, during the initial induction phase, the NADP-GDH enzyme was unstable and turned over with a calculated t 1/2 of 1.5 h

Fig. 27. SDS polyacrylamide gel electrophoresis (79) of anti-NADP-GDH IgG-immunoprecipitated material synthesized *in vitro* from poly(A)-containing RNA, isolated from synchronous *Chlorella* cells during the initial NADP-GDH induction period. Ammonium was added to a previously uninduced synchronous cell culture at the 3rd h of the cell cycle. Total cellular poly(A)-containing RNA (1 µg), isolated from cell cycle samples, harvested at the times indicated in each panel, was translated in the reticulocyte lysate protein-synthesizing system. Direct immunoprecipitation by rabbit anti-NADP-GDH IgG was performed as described in Fig. 15A, B. The arrow in the 1st panel indicates the position of the authentic NADP-GDH subunit in the gel.

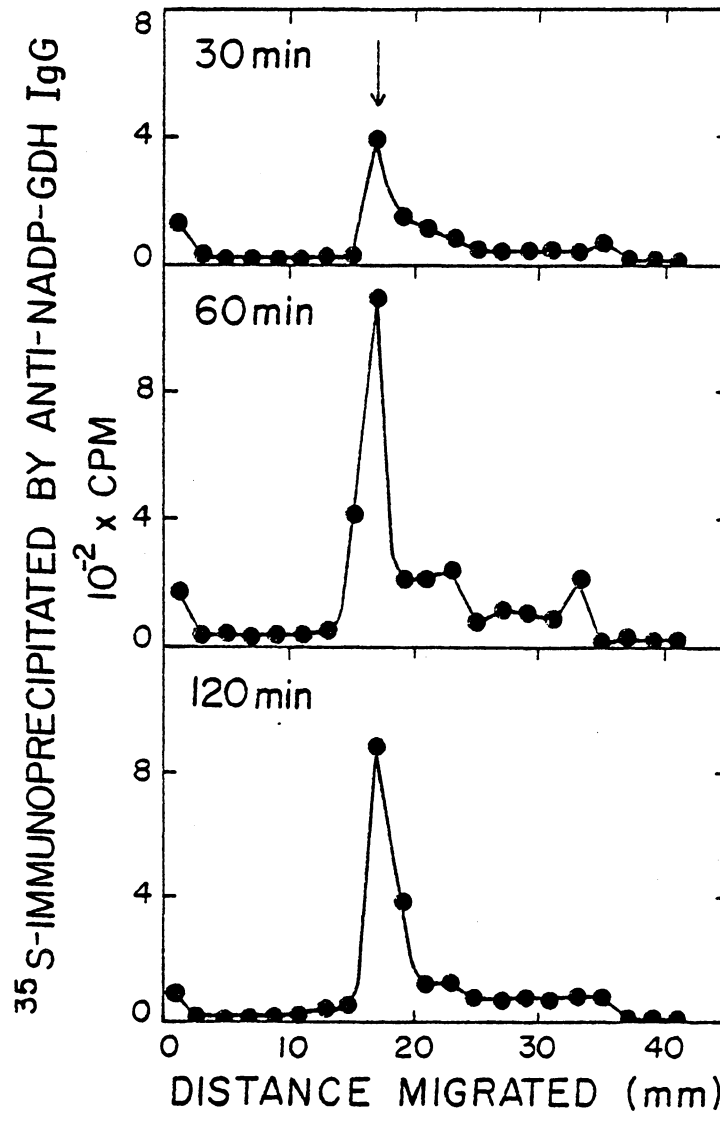
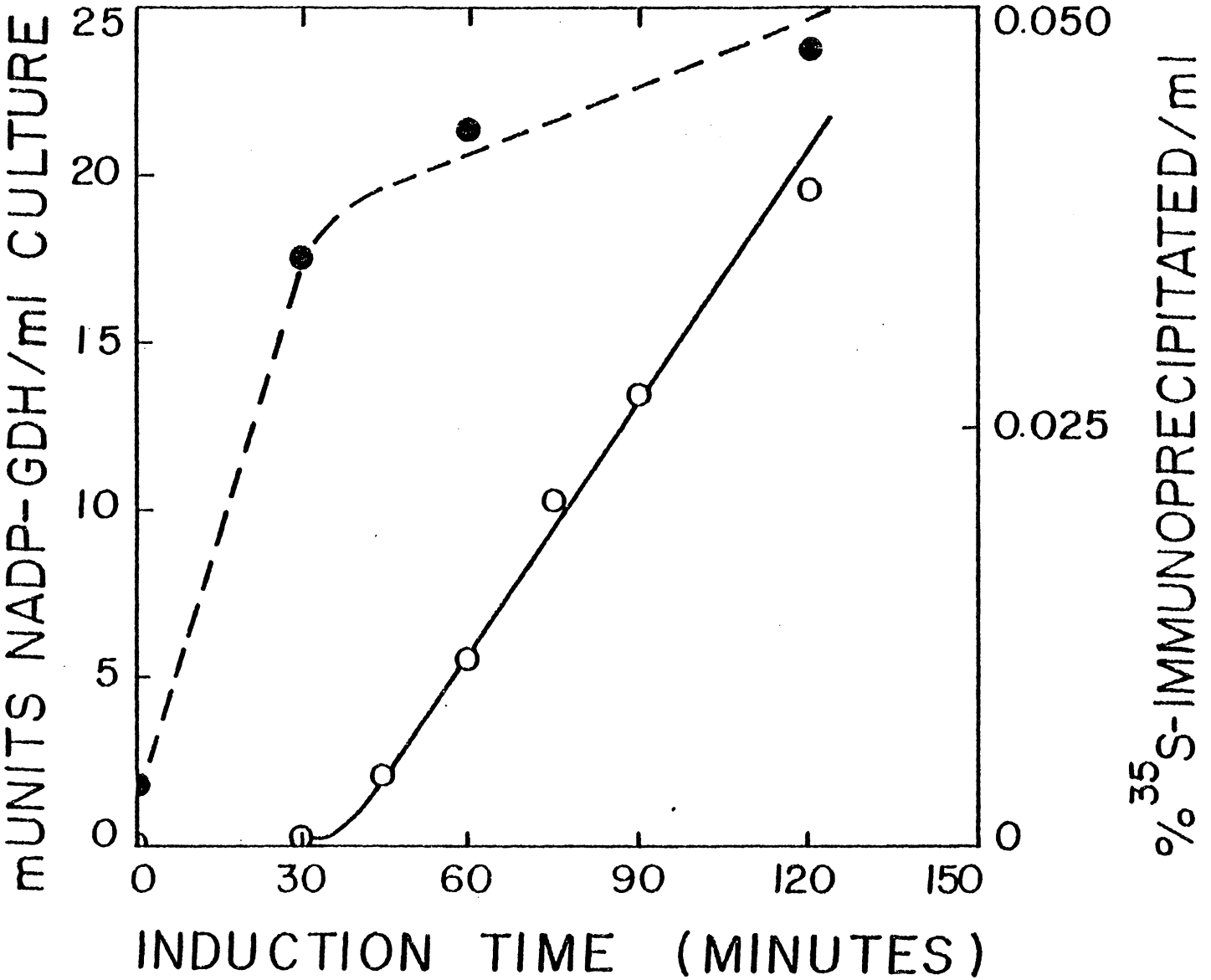

30 min
60 min
120 min
8
4
0
0
10
20
30
40
DISTANCE MIGRATED (mm)
^{35}S-IMMUNOPRECIPITATED BY ANTI-NADP-GDH IgG
10^{-2} x CPM

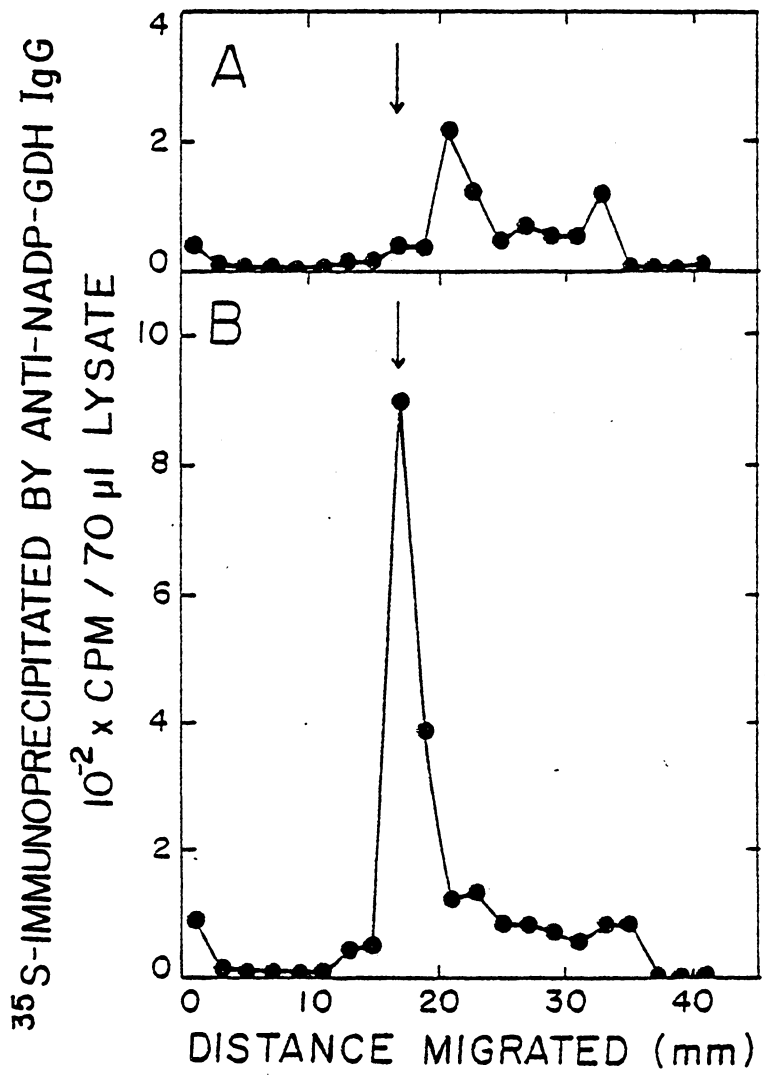
Fig. 28. Initial induction kinetics of the ammonium-inducible NADP-GDH and of NADP-GDH mRNA at the 3rd h of the cell cycle of Chlorella growing at an average rate of 18% per h in nitrate-containing medium. The deaminating activity of NADP-GDH was measured by a spectrophotometric assay (31). The anti-NADP-GDH IgG immunoprecipitated material synthesized in vitro from 1 µg of total cellular poly(A)-containing RNA, isolated from Chlorella cells at the various times indicated (data from Fig. 27 and Fig. 29A), was corrected for the percent cell breakage and the amount of poly(A)-containing RNA per ml of culture (-●-). Direct immunoprecipitation by rabbit anti-NADP-GDH IgG was performed as described in Fig. 15A, B. Catalytic activity of NADP-GDH (○).


mUNITS NADP-GDH/ml CULTURE
25
20
15
10
5
0
0.050
0.025
0
% 35S-IMMUNOPRECIPITATED/ml
0
30
60
90
120
150
INDUCTION TIME (MINUTES)

(33). Therefore, since the enzyme is very instable in vivo, its mRNA would be predicted to accumulate during periods of NADP-GDH accumulation. The results shown in Fig. 28 are consistent with this prediction. The relative difference in the slopes of mRNA accumulation after 30 min and enzyme accumulation suggest that there is a difference in stability of the two species. Since eucaryotic mRNA has been shown to be relatively stable in comparison to procaryotic mRNA (2), these data support the inference that the NADP-GDH mRNA is more stable than the NADP-GDH enzyme.

An observed change in rate of accumulation of translatable NADP-GDH mRNA occurred at 30 min after addition of the inducer. The timing of the rate change coincided with the onset of catalytic activity. Poly(A)-containing RNA, isolated from uninduced cells prior to the addition of inducer and from cells cultured in the presence of inducer for two hours, was translated in vitro. When the immunoprecipitated material was analysed by SDS gel electrophoresis, a striking result was obtained (Fig. 29A, B). In the absence of inducer (Fig. 29A) very little full length NADP-GDH subunit was synthesized in vitro. The majority of the immunoprecipitated material synthesized from this RNA migrated in the gel faster than the mobility of the authentic NADP-GDH subunit. In contrast, the poly(A)-containing RNA isolated from the induced cells directed the synthesis of a polypeptide with the mobility of the authentic enzyme subunit (Fig. 29B). The most plausible explanations for these results are (a) that NADP-GDH mRNA is subject to post-transcriptional modification in the presence or absence of

Fig. 29A, B. Comparison of anti-NADP-GDH IgG-immunoprecipitated material synthesized *in vitro* from *Chlorella* cellular poly(A)-containing RNA isolated from nitrate- and ammonium-cultured synchronous cells. A, Immunoprecipitated material synthesized from 1 µg total cellular poly(A)-containing RNA isolated at the 5th h of the cell cycle. B, same as in A except that total cellular poly(A)-containing RNA was isolated from cells which were cultured for 3 h in nitrate-containing medium and a further 2 h period in ammonium-containing medium. Direct immunoprecipitation by rabbit anti-NADP-GDH IgG was performed as described in Fig. 15A, B. The arrow indicates the position of the authentic NADP-GDH subunit in the gel.


A
B
^{35}S-IMMUNOPRECIPITATED BY ANTI-NADP-GDH IgG
10^{-2} x CPM / 70 µl LYSATE
DISTANCE MIGRATED (mm)
0
2
4
6
8
10
20
30
40

inducer, or (b) that two mRNAs code for the NADP-GDH in Chlorella and that the nitrogen source determines which mRNA is expressed. Since Palacios et al (94) have recently reported that two mRNAs, transcribed from different genes, code for glutamine synthetase in Neurospora crassa, the latter explanation cannot be ruled out. They observed that, depending on the nitrogen source (i.e., glutamate or glutamine), differential expression of the two genes resulted in a noncoordinate accumulation of both mRNAs in the cytoplasm (94). Therefore, it is possible that the immunoprecipitable material obtained from translation of poly(A)-containing RNA from uninduced cells might reflect the presence of a second mRNA species which codes for NADP-GDH antigen in the presence of the inducer. However, the rapid burst of translatable NADP-GDH mRNA upon addition of inducer is more consistent with there being only one mRNA species which is subject to post-transcriptional modification by the inducer. Addition of ammonium to uninduced cells would be proposed to alter the translational capacity of the NADP-GDH mRNA (i.e., by methylation, demethylation, structural modification of secondary structure) so that full length NADP-GDH subunit was synthesized in vitro. Post-transcriptional modification of mRNA structure has been shown to alter the specificity of translation in vitro (59).

In the absence of inducer, only the full length NADP-GDH subunit is presumably translated in vivo (33). The synthesis of smaller antigenic polypeptide sizes in vitro from RNA isolated from uninduced cells might reflect differences in control of translation in vivo versus

translation *in vitro*. For example, the modified NADP-GDH mRNA might require *Chlorella*-specific proteins or non-poly(A)-containing RNA sequences which would regulate the synthesis of the full length subunit *in vivo*. However, in the absence of such regulatory elements, a shorter polypeptide sequence might be translated *in vitro*.

Both of the explanations concerning the induction mechanism by ammonium would also account for the results shown in Fig. 15 which were obtained by translation and immunoprecipitation of total poly(A)-containing RNA from induced cells. The presence of the minor peak of radioactive protein in the SDS gel, which has a faster mobility than the authentic NADP-GDH subunit might result from either (a) translation of a single, partially modified mRNA species or (b) translation of a second mRNA which codes for NADP-GDH antigen which is present in low concentration in fully induced cells.

In contrast to the observed rate change in accumulation of NADP-GDH mRNA during the initial induction phase, Bascomb (33) observed that, when inducer was added to previously uninduced cells, the NADP-GDH antigen accumulated in a linear fashion without a rate change or an induction lag. In the present study, because of the small quantity of cells available for measurement of NADP-GDH mRNA levels, total cellular poly(A)-containing RNA was employed instead of polysomal poly(A)-containing RNA. Therefore, until the pattern of translatable NADP-GDH mRNA during initial induction is obtained by *in vitro* translation of RNA isolated from polysomes, it is possible that the concentration of translatable mRNA on polysomes might differ from that present

in the total cellular poly(A)-containing RNA during the initial induction phase. There may be a rapid modification or de-modification of NADP-GDH mRNA not on polysomes (e.g., ribonucleoprotein particles in cytoplasm or nucleus) during the initial induction period (first 30 min). This rapid conversion might occur faster than the rate of initiation of translation (i.e., loading of mRNAs with ribosomes). A difference in translatability of mRNA in polysomal RNA versus cellular RNA has been observed by Palacios et al (54). When polysomal RNA, isolated from Neurospora crassa was translated in vitro, a tenfold difference in the amounts of glutamine synthetase mRNA was found between cultures grown on glutamate or glutamine as the sole nitrogen source. However, only a four- to five-fold difference in glutamine synthetase mRNA specific activity was observed if total cellular RNA was employed for translation. They concluded that the twofold discrepency in the amount of glutamine synthetase mRNA could be due to differential degradation of glutamine synthetase mRNA due to technical manipulation, to different proportions of glutamine synthetase mRNA versus other cellular RNA in the 2 nitrogen cultures, or to the presence of glutamine synthetase in ribonucleoprotein particles outside of polysomes (54).

Evidence for Post-Transcriptional Regulation of NADP-GDH Induction by Accumulation of Its mRNA in Uninduced Cells - By use of the in vitro translation assay, the level of NADP-GDH mRNA was measured at several times during the cell cycle of synchronous cells growing in the absence of inducer. The patterns of culture turbidity and of cell number

throughout the cell cycle are shown in Fig. 30. As previously determined for highly synchronous Chlorella, the culture turbidity increased in a linear fashion with a rate change during the cell division period of the cell cycle (30). The increase in cell number was approximately sevenfold. To isolate total cellular RNA from each cell cycle sample, the cells were subjected to 45 s of homogenization with the Braun cell homogenizer. The range in percent cell breakage varied from a minimum of 55% to a maximum of 82%, at the first and fifth hours of the cell cycle, respectively. When the amounts of total cellular RNA and total cellular poly(A)-containing RNA, isolated from the cell homogenates, were corrected for the percentage of cell breakage obtained for each cell cycle sample, and for the dilution of the culture, they were observed to increase throughout the cell cycle (Fig. 31). The patterns of accumulation of total cellular RNA, total poly(A)-containing RNA, and total protein throughout the cell cycle were superimposable (Fig. 31). The coordinate increase in total cellular RNA and total protein in synchronous cells has been described previously by Schmidt (95). However, the pattern of accumulation of total poly(A)-containing RNA throughout the cell cycle has not been measured previously. The percentage of total cellular RNA which bound to oligo(dT)-cellulose was 1.02 ± 0.05%. To determine if the poly(A)-containing RNA isolated from uninduced synchronous cells was undegraded, total ^{35}S-labelled proteins synthesized *in vitro* from RNA isolated from three different times during the cell cycle, were analysed by SDS gel electrophoresis (Fig. 32 A, B, C). At each time in the cell cycle

Fig. 30. Patterns of accumulation of cell number (●) and of culture turbidity (○) during the cell cycle of Chlorella growing at an average rate of 18% per h in nitrate-containing medium.

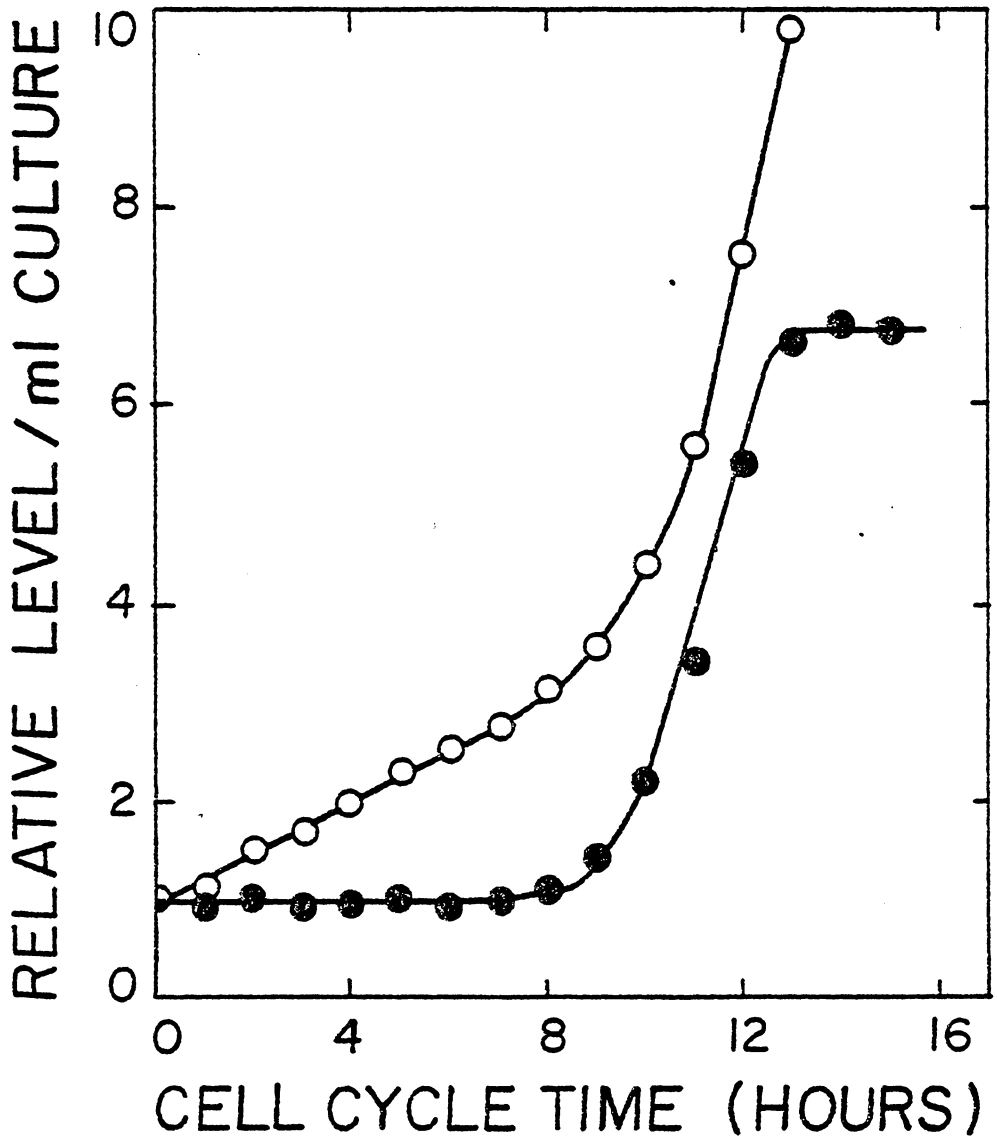

RELATIVE LEVEL / ml CULTURE
0
2
4
6
8
10
0
4
8
12
16
CELL CYCLE TIME (HOURS)

Fig. 31. Patterns of accumulation of total cellular protein (●), of total cellular RNA (○), and of total cellular poly(A)-containing RNA (▲) during the cell cycle of Chlorella growing at an average rate of 18% per h in nitrate-containing medium.

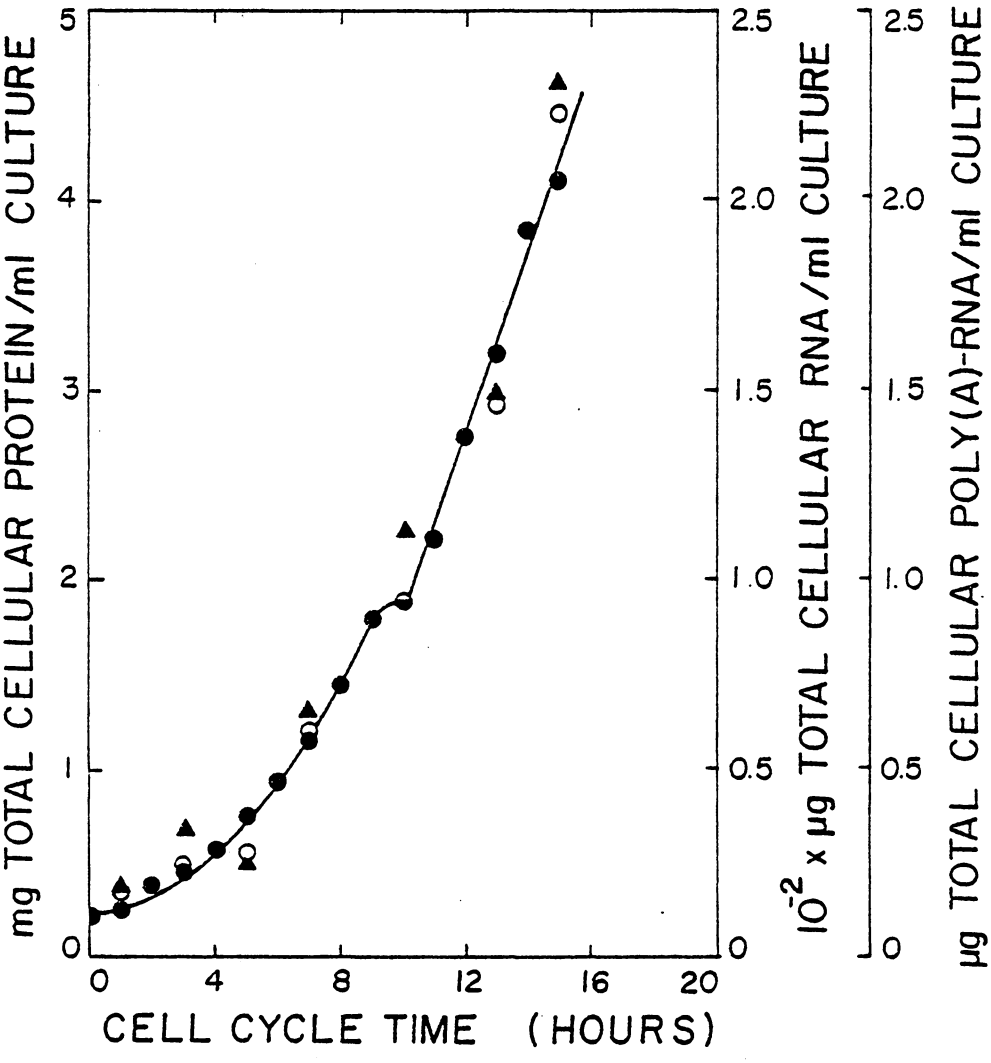
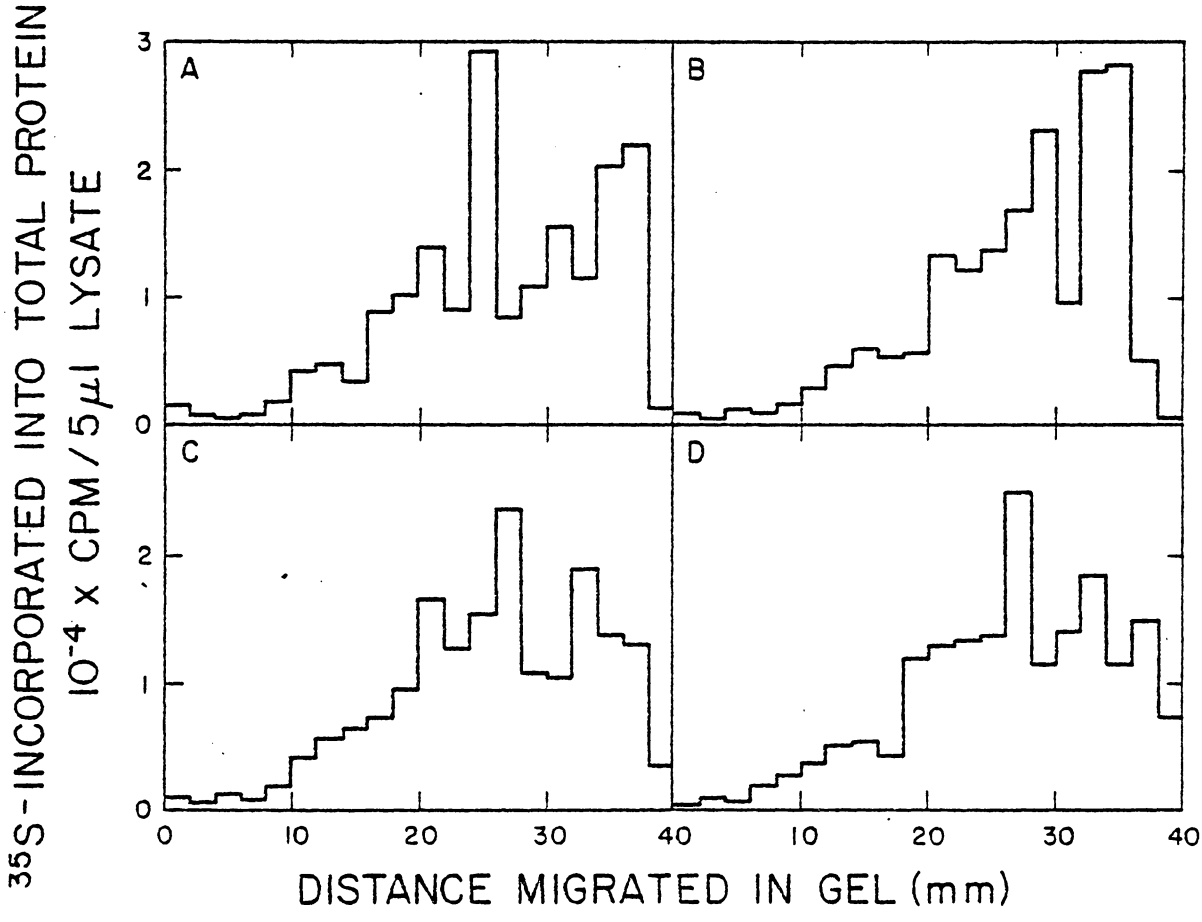

mg TOTAL CELLULAR PROTEIN/ml CULTURE
10^{-2} x µg TOTAL CELLULAR RNA/ml CULTURE
µg TOTAL CELLULAR POLY(A)-RNA/ml CULTURE
CELL CYCLE TIME (HOURS)
0
4
8
12
16
20
0
1
2
3
4
5
0
0.5
1.0
1.5
2.0
2.5

Fig. 32A, B, C, D. SDS polyacrylamide gel electrophoresis (79) of total proteins synthesized *in vitro* from total cellular poly(A)-containing RNA isolated from synchronous cells at several times during the cell cycle of *Chlorella* cells growing at an average rate of 18% per h in nitrate-containing medium. Total cellular poly(A)-containing RNA (1 µg) was incubated in the reticulocyte lysate reaction mixture. Incorporation of ^{35}S-labelled methionine into total protein, was determined in a 5 µl aliquot of the reticulocyte lysate mixture, incubated with RNA isolated at the following times during the cell cycle: A, 1st h, B, 5th h, and C, 10th h. D, same as in B, except that the cells were harvested at the 3rd h of the cell cycle and cultured in ammonium-containing medium for an extra 2 h period.

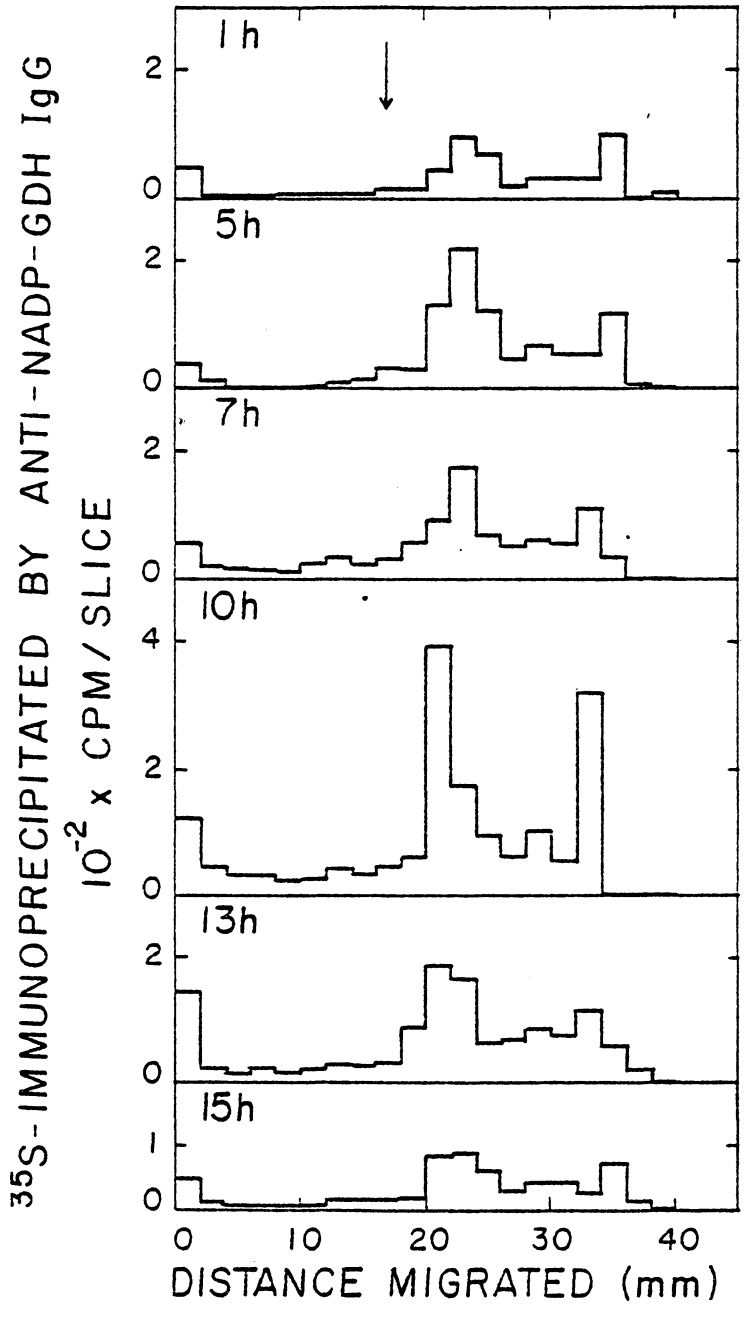

^{35}S-INCORPORATED INTO TOTAL PROTEIN
10^{-4} x CPM / 5μl LYSATE
A
B
C
D
0
1
2
3
0
10
20
30
40
DISTANCE MIGRATED IN GEL (mm)

examined, the total proteins synthesized in vitro were observed to comprise a heterogeneous range of polypeptide sizes. The overall pattern of radioactivity in each gel exhibited many similarities which suggest that the poly(A)-containing RNA was relatively undegraded in each sample. When the patterns of radioactive proteins synthesized by poly(A)-containing RNA, isolated from uninduced synchronous cells (Fig. 32B) and from the induced synchronous cells (Fig. 32D) were compared at an equivalent time in the cell cycle (i.e., 5 h), a slight shift in the average size distribution towards larger polypeptides was obtained from translation of RNA from induced cells. However, this difference is probably not significant as the total amount of ^{35}S-labelled methionine incorporated into total protein was 5.3×10^6 cpm and 4.2×10^6 cpm (per 35 µl lysate) for the poly(A)-containing RNA isolated from uninduced and induced cells, respectively.

When 1 µg of total poly(A)-containing RNA, extracted at several times during the cell cycle of uninduced cells, was translated in vitro and immunoprecipitated with rabbit anti-NADP-GDH IgG, the amount of immunoprecipitable material increased throughout the cell cycle until the tenth hour (Fig. 33). The major peak of radioactive protein was observed to increase until the cell division period of the cell cycle (i.e. tenth hour) and then decreased in cells at the beginning of the next cell cycle. At each hour of the cell cycle examined, there was no evidence for full length NADP-GDH synthesis in vitro (Fig. 33).

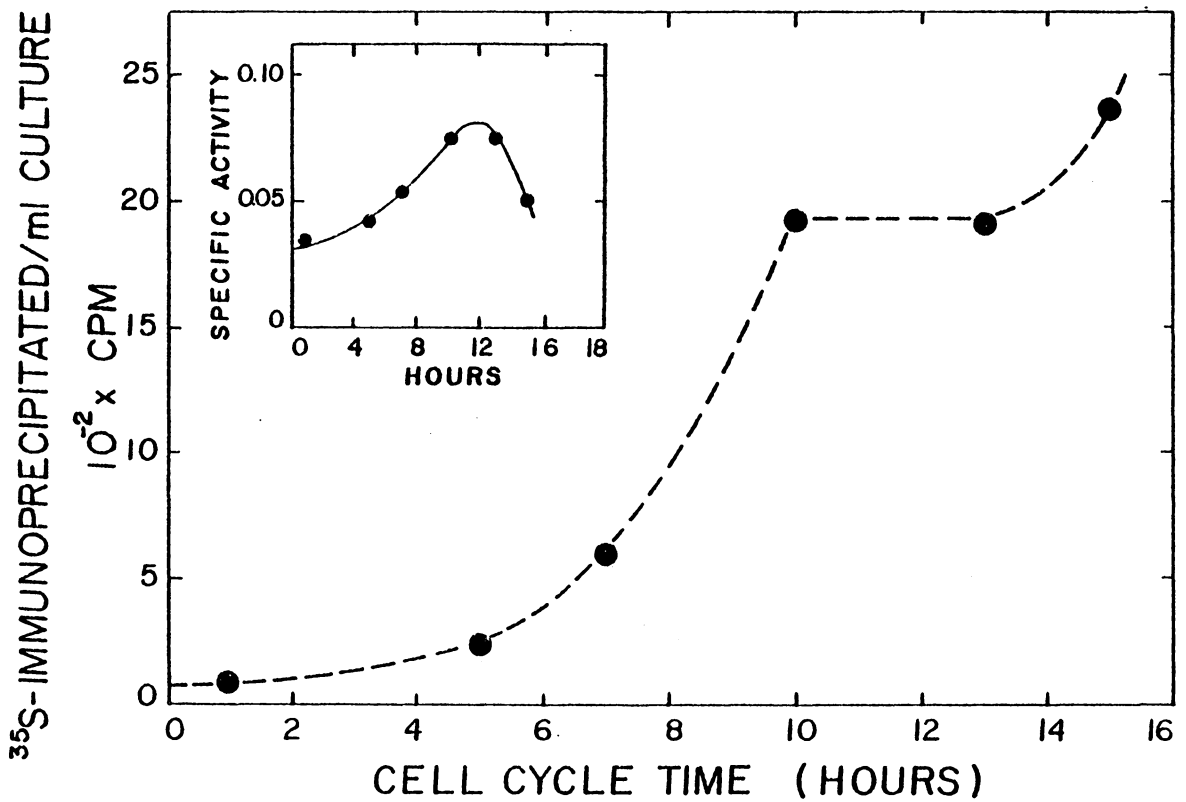
When the amount of immunoprecipitable NADP-GDH material in the SDS gels, obtained from translation of the poly(A)-containing RNA from

Fig. 33. SDS polyacrylamide gel electrophoresis (79) of anti-NADP-GDH IgG-immunoprecipitated material synthesized *in vitro* from total cellular poly(A)-containing RNA isolated from synchronous *Chlorella* cells at several times during the cell cycle of cells growing at 18% per h in nitrate-containing medium. At the times indicated in each panel, 1 µg of total cellular poly(A)-containing RNA was translated in the reticulocyte lysate reaction mixture. The protein products were subjected to direct immunoprecipitation by rabbit anti-NADP-GDH IgG as described in Fig. 15A, B. The arrow in panel 1 indicates the position of the authentic NADP-GDH subunit in the gel.


1h
5h
7h
10h
13h
15h
2
0
2
0
2
0
4
2
0
2
0
1
0
0
10
20
30
40
DISTANCE MIGRATED (mm)
35S-IMMUNOPRECIPITATED BY ANTI-NADP-GDH IgG
10-2 x CPM/SLICE

uninduced synchronous cells, was corrected for the percent cell breakage and the number of micrograms of poly(A)-containing RNA per ml of culture, the amount of translatable NADP-GDH mRNA was observed to increase throughout the cell cycle (Fig. 34). The specific activity of the NADP-GDH mRNA per microgram of poly(A)-containing RNA translated in vitro is shown in the inset of Fig. 34. These data were obtained from the amount of immunoprecipitable material shown in each panel of Fig. 33. As shown in Fig. 34, the amount of translatable NADP-GDH mRNA increased slowly until the period of the cell cycle corresponding to the S-phase. During the period of DNA replication, the rate of NADP-GDH mRNA accumulation increased rapidly until the beginning of the next cell cycle, whereupon the NADP-GDH mRNA continued to accumulate at a decreased rate. To explain the observed pattern of accumulation of translatable NADP-GDH mRNA throughout the cell cycle, it is assumed that the rate of translation of the NADP-GDH mRNA in vitro is constant for all RNA samples isolated from synchronous cells at several times during the cell cycle (e.g., no cell cycle dependent changes in initiation or elongation of the NADP-GDH mRNA in vitro). This pattern of accumulation of translatable NADP-GDH mRNA is entirely consistent with the hypothesis proposed in the "Introduction" of this dissertation concerning the regulation of NADP-GDH potential in synchronous cells. One possible explanation for the observed pattern of enzyme potential was that the NADP-GDH mRNA accumulated in uninduced cells at a rate proportional to gene dosage. The observed pattern of accumulation of translatable NADP-GDH mRNA sequences is entirely in agreement with this hypothesis.

Fig. 34. Pattern of accumulation of NADP-GDH mRNA throughout the cell cycle of synchronous Chlorella cells growing at an average rate of 18% per h in nitrate-containing medium. The data obtained from Fig. 33 were corrected for the percent cell breakage and the amount of poly(A)-containing RNA per ml of culture (—●—). Inset: The amount of anti-NADP-GDH IgG-immunoprecipitated material from translation in vitro of 1 µg of total cellular poly(A)-containing RNA isolated from synchronous cells at the various times indicated (data from Fig. 33).


^{35}S-IMMUNOPRECIPITATED/ml CULTURE
10^{-2} x CPM
0
5
10
15
20
25
0
2
4
6
8
10
12
14
16
CELL CYCLE TIME (HOURS)
SPECIFIC ACTIVITY
0
0.05
0.10
0
4
8
12
16
18
HOURS

SUMMARY

The mRNA coding for the ammonium-inducible NADP-GDH from Chlorella was studied in induced and uninduced cells to determine the molecular mechanisms which regulate the cellular levels of this enzyme. A procedure for isolation of a high yield of total undegraded cellular polysomes was developed. A crosslinking reagent was employed to prepare a stable NADP-GDH-crosslinked-Sepharose-4B antigen affinity column for the purification of rabbit anti-NADP-GDH IgG. Binding studies with ^{125}I-labelled antibody and total polysomes, isolated from induced and uninduced cells, showed that the NADP-GDH was being synthesized on polysomes from both types of cells. When poly(A)-containing RNA was extracted from polysomes isolated from induced and uninduced cells, and translated in an mRNA-dependent in vitro translation system, NADP-GDH antigen was synthesized from the RNA from both sources. Based on sucrose density gradient analysis, Chlorella NADP-GDH mRNA has a sedimentation coefficient of 18S. Comparison of the amounts of NADP-GDH synthesized in vitro from poly(A)-containing RNA and non-poly(A)-containing RNA showed the NADP-GDH mRNA contained a 3' poly-adenylic acid sequence. By use of an indirect immunoadsorption procedure, the NADP-GDH mRNA was purified five- to sevenfold from total poly(A)-containing RNA. The overall purification of the NADP-GDH mRNA from total polysomal RNA was approximately two hundred-fold. Complementary DNA was synthesized from the partially purified RNA with reverse transcriptase. The cDNA sequences hybridized to the least abundant class of mRNA sequences present in total poly(A)-containing

RNA. In vitro translation of total poly(A)-containing RNA showed that NADP-GDH synthesis was 0.1% of total protein synthesis. Upon addition of inducer to previously uninduced, synchronous cells, the amount of translatable NADP-GDH mRNA increased in a linear fashion after 30 min of the induction period. A change in rate of NADP-GDH mRNA accumulation was observed after 30 min of the induction period. The results support the prediction that since the NADP-GDH enzyme is unstable in vivo, during periods of NADP-GDH accumulation, the NADP-GDH mRNA accumulates. When poly(A)-containing RNA, isolated from uninduced synchronous cells was translated in vitro, NADP-GDH antigen was synthesized at each time in the cell cycle examined. The amount of translatable NADP-GDH mRNA increased throughout the cell cycle with a rate change occuring during the S-phase. This pattern of NADP-GDH mRNA accumulation is consistent with the hypothesis that NADP-GDH mRNA accumulates in uninduced cells at a rate proportional to gene dosage. These results provided one explanation for the observed pattern of enzyme potential in synchronous cells cultured in the absence of inducer. The data are consistent with the possibility that a single mRNA, which is subject to post-transcriptional modification by the inducer, codes for NADP-GDH.

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EVIDENCE FOR POST-TRANSCRIPTIONAL REGULATION OF INDUCTION OF NADP-SPECIFIC GLUTAMATE DEHYDROGENASE BY ACCUMULATION OF ITS mRNA IN UNINDUCED SYNCHRONOUS *CHLORELLA* CELLS

by

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(ABSTRACT)

The mRNA coding for the ammonium inducible NADP-specific glutamate dehydrogenase (NADP-GDH) from *Chlorella* was studied in induced and uninduced cells to determine the molecular mechanisms which regulate the cellular levels of this enzyme. A procedure for isolation of a high yield of total undegraded cellular polysomes was developed. The crosslinking reagent, dimethyl suberimidate, was employed to prepare a stable NADP-GDH-crosslinked-Sepharose-4B antigen affinity column for the purification of rabbit anti-NADP-GDH IgG. Binding studies with ^{125}I-labelled antibody and total polysomes, isolated from induced and uninduced cells, showed that the NADP-GDH was being synthesized on polysomes from both types of cells. When poly(A)-containing RNA was extracted from polysomes isolated from induced and uninduced cells, and translated in an mRNA-dependent *in vitro* translation system, NADP-GDH antigen was synthesized from the RNA from both sources. Based on sucrose density gradient analysis, *Chlorella* NADP-GDH mRNA has a sedimentation coefficient of 18 Comparison of the amounts of NADP-GDH synthesized *in vitro* from poly(A)-containing RNA

and non-poly(A)-containing RNA showed the NADP-GDH mRNA contained polyadenylic acid sequence. By use of an indirect immunoadsorption procedure, the NADP-GDH mRNA was purified five- to sevenfold from total poly(A)-containing RNA. The overall purification of the NADP-GDH mRNA from total polysomal RNA was approximately two hundred-fold. Complementary DNA was synthesized from the partially purified RNA with reverse transcriptase. The cDNA sequences hybridized to the least abundant class of mRNA sequences present in total poly(A)-containing RNA. In vitro translation of total poly(A)-containing RNA showed that NADP-GDH synthesis was 0.1% of total protein synthesis. Upon addition of inducer to previously uninduced, synchronous cells, the amount of translatable NADP-GDH mRNA increased in a linear fashion after 30 min of the induction period. A change in rate of NADP-GDH mRNA accumulation was observed after 30 min of the induction period. The results support the prediction that since the NADP-GDH enzyme is unstable in vivo, during periods of NADP-GDH accumulation, the NADP-GDH mRNA accumulates. When poly(A)-containing RNA, isolated from uninduced synchronous cells was translated in vitro, NADP-GDH antigen was synthesized at each time in the cell cycle examined. The amount of translatable NADP-GDH mRNA increased throughout the cell cycle with a rate change occuring during the S-phase. This pattern of NADP-GDH mRNA accumulation is consistent with the hypothesis that NADP-GDH mRNA accumulates in uninduced cells at a rate proportional to gene dosage. These results provide one explanation for the observed pattern of enzyme potential in synchronous cells cultured in the absence of

inducer. The data are consistent with the possibility that a single mRNA, which is subject to post-transcriptional modification by the inducer, codes for NADP-GDH.