Effect of Different Carbon Sources on the Ammonium Induction of Different Forms of NADP-Specific Glutamate Dehydrogenase in *Chlorella sorokiniana* Cells Cultured in the Light and Dark$^{1,2}$

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**ABSTRACT**

The ammonium induction of the chloroplast-localized NADP-specific glutamate dehydrogenase (NADP-GDH) was shown not to be a light-dependent process *per se* in *Chlorella sorokiniana*. In the dark without exogenous organic substrates, the cells synthesized low levels of fully active NADP-GDH, provided endogenous starch reserves had not been depleted. When cells were supplied with exogenous acetate, the rate of induction of NADP-GDH activity per milliliter of culture in the dark was equal to or slightly greater than the rate observed under photosynthetic conditions without an organic carbon source. Glucose supported only a low rate of induction of NADP-GDH activity in the dark. Both acetate and glucose inhibited induction of enzyme activity in the light. The NADP-GDH holoenzyme had at least 7 different electrophoretic forms. These forms differed in net charge and/or molecular weight. Their difference in molecular weight was due to the presence of 2 subunits with similar antigenic properties but different molecular weights ($M_1 = 55,500$ and 53,000; $a$- and $b$-subunits, respectively). Depending upon the cultural conditions and length of the induction period, a wide variation was observed in the $a/b$ subunit ratio and in the numbers and sizes of the NADP-GDH holoenzymes.

The ammonium-inducible NADP-GDH$^6$ of *Chlorella sorokiniana* has been reported to be composed of 6 identical subunits with chemical, physical, and immunological properties quite different from those of the constitutive mitochondria-localized 4-subunit NAD-GDH in this organism (8, 15, 16, 26, 30). The active NADP-GDH holoenzyme has been shown to be localized in the chloroplast (18). Moreover, from recent experiments (18), it appears that the 53,000 D subunit in the NADP-GDH holoenzyme in fully induced cells is formed by processing of a 58,500 D precursor-protein that is synthesized in the cytosol on 80S ribosomes. *In vitro* translation of the NADP-GDH mRNA, purified 1,290-fold from fully induced cells by a very specific polysome immunoadsorption procedure, also yielded the 58,500 D putative precursor protein (2).

Molin *et al.* (17) reported that the ammonium induction of NADP-GDH activity was light dependent, whereas accumulation of activity of the NAD-GDH occurred in the light or dark in cells cultured in ammonium or nitrate medium. Although NADP-GDH activity did not accumulate in cells cultured in ammonium medium during a 3 d dark period, the illumination of these cells resulted in immediate accumulation of NADP-GDH activity. Incubation of the cells in the dark in ammonium medium abolished the 30 to 40 min induction lag that is typically seen after addition of ammonium to uninduced cells in continuous light (28). The absence of the induction lag after the dark period suggested that the early events in the induction process had been initiated and some late event was limiting the induction of enzyme activity in cells in the dark.

The chloroplastic localization of the active holoenzyme and the cytosolic site of synthesis of its precursor-protein offered a number of possibilities, regarding the nature of the rate-limiting step in induction of NADP-GDH activity in the dark. As seen for certain chloroplastic enzymes of the photosynthetic carbon reduction cycle, inactive NADP-GDH holoenzyme might accumulate in the chloroplast in the dark and require activation by reduced thioredoxin (4). Since the transport of precursor proteins into isolated chloroplasts has been shown (9, 10) to be light or ATP dependent, the transport of the NADP-GDH precursor protein into the chloroplast *in vivo* might be rate-limiting the dark induction process. Other possibilities unrelated to the cellular localization of the NADP-GDH holoenzyme were also considered. For example, whereas the low rate of cellular protein synthesis in the dark can support the synthesis and accumulation of a stable enzyme, such as the NAD-GDH (11, 12, 17), this low rate of protein synthesis might be insufficient to sustain the accumulation of an unstable enzyme, such as the NADP-GDH, which has been shown to undergo rapid degradation in both induced and uninduced cells (3, 21, 27). Alternatively, because of the low rate of ammonium uptake by these cells in the dark without an exogenous organic substrate (17), the NADP-GDH might undergo rapid inactivation (and subsequent degradation) due to a low endogenous level of a metabolite of ammonium that is proposed (3, 12, 21, 27) to accumulate and inhibit *in vivo* inactivation of the enzyme during its induction in the light. The very low basal level of activity of this inducible enzyme in uninduced cells has been attributed to an inactivation system that becomes operational upon removal of ammonium from the medium of fully induced cells (3, 12, 17, 21, 27).

The research described in this paper had two objectives: (a) to use a Western blot/immunodetection procedure to determine

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6. Abbreviations: NAD-GDH, nicotinamide adenine dinucleotide-specific glutamate dehydrogenase; NADH-GDH, nicotinamide adenine di-nucleotide phosphate-specific glutamate dehydrogenase; IgG, immunoglobulin G.
whether inactive NADP-GDH holoenzyme, precursor protein, or free subunits accumulate in cells cultured in the dark in ammonium medium without an exogenous organic carbon source, and (b) to determine whether ammonium-induced accumulation of NADP-GDH activity/antigen would occur in cells cultured in the dark in medium containing an organic carbon substrate as an exogenous carbon/energy source.

The results described in this paper indicate that cells cultured in the dark in ammonium medium (without an exogenous organic substrate) do not accumulate inactive NADP-GDH holoenzyme, precursor protein, or free subunits. The availability of an endogenous or exogenous organic-carbon source appears to be the rate-determining factor for accumulation of fully active NADP-GDH in cells in the dark. Thus, light per se is not required for induction of the active enzyme.

MATERIALS AND METHODS

Materials. YM 10 ultrafiltration membranes were obtained from Amicon; nitrocellulose (BA85), Schleicher & Schuell; [125I]-Protein A, Amer sham; Sephacryl S-300, Pharmacia; plastic boiling bags (Seal-N-Save), Sear s. All other chemicals and materials were of the highest grade available from Calbiochem Corp. or Sigma Chemical Corp. or as described earlier (27, 30).

Organism and Growth Conditions. Chlorella sorokiniana cells were cultured as previously described (20, 28), except that a modified culture medium was used in the studies described herein. When this laboratory was relocated from the Virginia Polytechnic Institute and State University to The University of Florida, difficulty was encountered in culture of C. sorokiniana cells in the nitrate medium (28) used in earlier studies. The cells were observed to be yellowish-green instead of a dark-green color, to accumulate unusually large numbers of starch granules within their chloroplasts, to have a low growth rate (i.e. less than 50% of optimal), and to clump into large aggregates during growth in suspension culture. Moreover, the cells could not be synchron ized by intermittent illumination (28). These characteristics of the organism were shown to be caused by two factors. First, the low growth rate of the cells was shown to be due in part to the absence of certain trace micronutrients in the Florida deionized water (Milli-Q Water Purification System, Millipore Corp.) that were present in the Virginia deionized H,SO, solution. Second, the university steam supply used to autoclave media and glassware at the University of Florida, contained additives (e.g. polyamines, detergents, heavy metals, and acrylates) that inhibited the growth rate of the cells and also induced clumping. The culturing problems were corrected by filter sterilization (0.2 μm Millipore filter) of the culture medium and by the addition of three additional micronutrients (i.e. Ni, V, Sn) to an inorganic salts medium modified from the one described by Sueoka (25). Salts of nickel and vanadium have been reported for optimal metabolism or growth of certain green algae (1, 24), whereas tin had not been reported previously to affect the growth of the green microalga. The composition of the modified medium was (in mM): K,HPO, 18.4; K,SO, 2; MgCl, 1.5; CaCl, 0.34 and (in μM): FeCl, 71.6; EDTA, 72; H,BO, 38.8; MnCl, 10.1; ZnCl, 0.734; CoCl, 0.189; CuCl, 0.352; NiCl, 0.19; SnCl, 0.19; NH,VO, 0.20. The medium contained either 29 mM KNO (nitrate medium) or 29 mM NHCl (ammonium medium). The nitrate medium was adjusted to pH 6.8 and the ammonium medium to pH 7.3. Media flasks, culture tubes, and other glassware were autoclaved with steam from an electric steam generator (model LB-20, American Sterilizer Co.).

The cells were synchronized by three light-dark cycles (9 h: 7 h). The light intensity was adjusted to a level which allowed each cell to divide into four progeny at the end of each cell cycle. Culture turbidity was measured at a wavelength of 640 nm in a Beckman DB spectrophotometer. During synchronous growth in the light or in the dark, the culture turbidity was held constant by continuous dilution with fresh equilibrated (2% CO, air, 38.5°C) culture medium. Before plotting, the data from these experiments were corrected for this dilution by the procedure of Schmidt (20).

Assay of NADP-GDH Activity. The deaminating activity of NADP-GDH was measured spectrophotometrically (27, 30) by the addition of 5 to 20 μl of enzyme sample to a volume of 500 μl of assay mixture, containing 44 mM Tris, 204 mM L-glutamate, and 1.02 mM NADP at pH 8.75 and 38.5°C. 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°C.

After native PAGE, the positions of the active NADP-GDH holoenzyme(s) were determined by use of an activity stain (30) which consisted of the following: assay mixture H,0 (1:1), 0.3 mM nitroblue tetrazolium, and 0.15 mM phenazine methosulfate (pH 8.75, 22°C).

Western Blot/Immunodetection Procedure for NADP-GDH Antigen. Chlorella cells were broken by passage through a French pressure cell at 20,000 p.s.i. The proteins in the whole-cell homogenates were separated by native or SDS-PAGE by the procedures of Davis (6) and Laemmli (14), respectively. After electrophoresis, the gels were equilibrated in transfer buffer (24.8 mM Tris, 192 mM glycine, and 20% methanol, pH 8.4), the proteins were electrophoretically transferred to a nitrocellulose membrane in a Transblot cell (Bethesda Research Laboratories) at 60 V and 170 mamp overnight (5, 29). By use of an immunodetection procedure, modified from the methods of Towbin et al. (29), Renart et al. (19), and Burnett (5), NADP-GDH was detected on the nitrocellulose membrane. The modified procedure had the following steps. To saturate any additional protein binding sites on the membrane, after transfer of proteins from the gel, the membrane was incubated in BSA buffer (10 mM Tris, 3% BSA, and 0.9% NaCl, pH 7.4) in plastic boiling bags for 1 h. All incubation were at 37°C in a shaker incubator (Lab-Line Orbit Environ-Shaker). The membrane was then incubated for 2 h with anti-NADP-GDH IgG (1 mg/ml) (30) diluted 1:200 in ligand buffer (50 mM Tris, 3% BSA, 0.15 mM NaCl, and 0.05% Nonidet P-40, pH 7.4), washed in ligand buffer for 1 h (with several buffer changes), and incubated for 1 h in ligand buffer containing 125-I-labeled Protein A (0.25 μCi/lane). The membrane was washed for 1 h in buffer (10 mM Tris and 0.15 mM NaCl, pH 7.4), blotted dry, and exposed to Kodak X-Omat AR film for 24 to 72 h at -70°C.

Separation of NADP-GDH Holoenzyme-Size and Free Subunit-Size Proteins in Cell Homogenates by Gel-Filtration Column Chromatography. To determine whether all of the NADP-GDH antigen was present in holoenzyme-size molecules, cell homogenates were fractionated by gel-filtration chromatography. A 2.5 x 65 cm column was packed with Sephacryl S-300, and buffer (10 mM Tris, 150 mM KCl, and 2 mM DTT, pH 7.4) was pumped through the column at 1 ml/min. The column was calibrated with protein standards. A 1-ml sample of cell homogenate, prepared from 0.5 g fresh weight of Chlorella cells, was applied to the column and 1 ml fractions were collected. The fractions containing proteins with mol wt greater and less than 100,000 were combined separately and each was concentrated to 0.5 ml by use of ultrafiltration on a YM 10 membrane. From these two concentrated preparations, 30 μl aliquots were electrophoresed in a SDS-polyacrylamide gel (10%), and the NADP-GDH antigen(s) were visualized by the Western blot/immunodetection procedure described earlier. The fractionation procedure was further homogenized and prepared from cells containing both α- and β-subunits, such as cells induced for 80 to 120 min in continuous light. NADP-GDH catalytic activity was present only in the high mol wt fraction (>100,000 D) as determined by the spectrophotometric assay of samples taken from the concentrated
preparation before electrophoresis.

RESULTS AND DISCUSSION

Low Rate of Induction of NADP-GDH Activity and Antigen in Cells Cultured in Inorganic Salts Medium in the Dark. A culture of synchronized daughter cells of C. sorokiniana was placed into the dark for 3 h in an ammonium (29 mM) inorganic salts medium in the absence of an organic carbon source, and then the culture was illuminated for a subsequent 2 h period. As a control culture, synchronized daughter cells were maintained in the dark under identical cultural conditions, except ammonium was omitted from the inorganic salts medium. At the end of the 3 h dark period, the control culture was illuminated and ammonium was added simultaneously.

During the 3 h dark period, the turbidity of the cultures did not increase, indicating that the ammonium culture did not exhibit significant growth in the dark (Fig. 1A). However, when the two cultures were illuminated, their turbidities increased rapidly and at the same rate. Thus, the 3 h incubation of cells, in ammonium medium in the dark, did not appear to give them a growth rate advantage over the cells in the control culture, to which ammonium was added at the time of illumination of the cultures.

Although Molin et al. (17) did not detect any accumulation of NADP-GDH activity in cells in ammonium medium in the dark, there appeared to be a low rate of accumulation of NADP-GDH activity in cells cultured under similar conditions in the present study (Fig. 1B). By the end of the 3 h dark period, the cells in ammonium medium contained approximately 3 to 4 milliunits of NADP-GDH activity per ml of culture, whereas the uninduced cells contained less than 1 milliunit of enzyme activity. In the present study, the culture medium and enzyme assay conditions were different from those described by Molin et al. (17). The inorganic salts medium was supplemented with three additional micronutrients (i.e. Ni, Sn, and V), and larger sample volumes of cells were assayed for enzyme activity. As observed previously by Molin et al. (17), when the ammonium culture was illuminated, NADP-GDH catalytic activity increased immediately without an apparent induction lag period (Fig. 1B). The control culture, to which the ammonium was added at the time of illumination, exhibited the normal 30 to 40 min induction lag before NADP-GDH activity began to accumulate (Fig. 1B). Moreover, the rate of accumulation of NADP-GDH activity in the control culture was somewhat slower than in the culture induced in the dark. The low rate of accumulation of NADP-GDH activity in ammonium induced cells in the dark, coupled with the absence of an induction lag and the faster rate of accumulation of enzyme activity after these cells were illuminated, suggested that some component related to the induction process might be accumulating in the induced cells in the dark.

Because the active NADP-GDH holoenzyme had been shown (18) to be chloroplast-localized in C. sorokiniana cells, growing in ammonium medium in the light, it seemed possible that inactive (or slightly active) NADP-GDH might accumulate in the chloroplast in the dark and might require activation by reduced thioredoxin in the light as reported for certain enzymes engaged in CO₂ fixation (4). To determine if a large amount of inactive NADP-GDH antigen was accumulating, in ammonium-induced cells in the dark, cell homogenates were prepared and subjected to native slab gel electrophoresis. After electrophoresis, the proteins were blotted onto nitrocellulose, and probed for NADP-GDH antigen, with anti-NADP-GDH IgG (30) and 125-I-labeled Protein A, in a Western blot/immunodetection procedure. During the 1st h after transfer of the uninduced synchronized cells into ammonium medium in the dark, NADP-GDH antigen could not be detected in homogenates of these cells (Fig. 2A, lane 2). However, after 2 h in ammonium medium in the dark, a single electrophoretic form appeared (lane 3), and was still the only form present at the 3rd h (lane 4). Upon illumination of the culture at the 3rd h, 6 different forms of the NADP-GDH, with lower electrophoretic mobilities than the dark-induced form, began to accumulate. The appearance of these multiple forms corresponded to the onset of rapid accumulation of NADP-GDH activity. During the first 15 min of the light period (lanes 5, 6, and 7), the antigen with the higher electrophoretic mobility remained as the predominant species. After 20 to 30 min, the 7 forms were present at approximately the same level (lanes 9 and 10). Based on a comparison of the electrophoretic mobilities of protein standards, the apparent mol wt of the different forms of NADP-GDH ranged from 280,000 to 400,000. The mol wt of the 6-subunit holoenzyme has been reported (8) to range from 290,000 to 410,000.

From the mol wt estimations, it was apparent that holoenzyme-size NADP-GDH antigen accumulated in cells, cultured in ammonium medium, during the 3 h dark period. Moreover, by comparing the intensities of the antigen bands on the Western blot with the milliunits of NADP-GDH activity added to the native gel, it was apparent that the 3 milliunits per ml of culture of NADP-GDH activity that accumulated during the 3 h dark period had the same activity:antigen ratio as the enzyme that accumulated in fully induced cells growing in continuous light. Therefore, after the ammonium-cultured cells were illuminated, the immediate and rapid accumulation of NADP-GDH was not due to the rapid conversion of a large pool of inactive holoen-
wt, two different experiments were performed. In the first experiment, a cell homogenate, containing different electrophoretic forms of NADP-GDH antigen (i.e. same as in Fig. 2A, lane 7), was applied to a DEAE Sephacel ion-exchange column. The proteins were eluted from the column with a 0 to 0.4 M KCl linear gradient. The NADP-GDH antigen(s) in the fractions from the column were subjected to native gel electrophoresis and Western blot/immunodetection. The NADP-GDH forms, with lower electrophoretic mobilities on the native gel, eluted from the column at a lower KCl concentration than did the forms with higher electrophoretic mobilities, indicating that a charge difference existed between the different forms (Fig. 2C). In the second experiment, the cell homogenate was electrophoresed in a native gradient gel, and an activity stain was used to localize the NADP-GDH forms in the gel. Electrophoresis in the native gradient gel was continued until detectable movement of the NADP-GDH antigens in the gel had ceased. When a native gradient gel is run essentially to completion, proteins that have different overall net charges, but have the same mol wt, are predicted to migrate to the same position (i.e. single band) in the gradient gel. Therefore, because several active forms of NADP-GDH were observed in the native gradient gel, ranging in mol wt from 400,000 to 410,000 (Fig. 3, lane 2), the seven different electrophoretic forms of NADP-GDH antigen, observed after

zyme to active enzyme.

In native gel electrophoresis, the mobilities of proteins can be affected by their mol wt, shape, and charge. To determine whether the different electrophoretic forms of NADP-GDH antigen, seen in Figure 2A (lane 7), differed by charge and/or mol wt.

**Fig. 2.** Patterns of accumulation of different electrophoretic forms of NADP-GDH antigen in synchronized daughter cells of *C. sorokiniana* cultured in ammonium medium in the dark and light. Aliquots of cell homogenates, containing approximately equal amounts of total protein from the different harvest times, were electrophoresed in native and SDS-slab gels, and then the proteins were transferred to nitrocellulose. The resulting Western blots were treated with anti-NADP-GDH IgG and 125I-labeled Protein A. A, Native polyacrylamide gel (7.5%). Lane 1, zero time; lane 2, 1 h dark; lane 3, 2 h dark; lane 4, 3 h dark; lane 5, 3 h dark, 6 min light; lane 6, 3 h dark, 10 min light; lane 7, 3 h dark, 15 min light; lane 8, 3 h dark, 20 min light; lane 9, 3 h dark, 30 min light; lane 10, 3 h dark, 40 min light; lane 11, 3 h dark, 1 h light. B, SDS-polyacrylamide gel (10%). Lane 1, zero time; lane 2, 2 h dark; lane 3, 3 h dark; lane 4, 3 h dark, 6 min light; lane 5, 3 h dark, 10 min light; lane 6, 14C-labeled protein standards; lane 7, 3 h dark, 15 min light; lane 8, 3 h dark, 20 min light; lane 9, 3 h dark, 30 min light; lane 10, 3 h dark, 40 min light; lane 11, 3 h dark, 1 h light; lane 12, 3 h dark, 2 h light. The mol wt of the α- and β-subunits were 55,500 and 53,000, respectively. C, Native polyacrylamide gel (7.5%) electrophoresis of NADP-GDH antigen(s) in fractions eluted from a DEAE Sephacel ion-exchange column with a linear gradient of 0 to 0.4 M KCl. Fractions with increasing KCl concentrations were loaded into lanes from left to right. The sample applied to the column was homogenate of cells harvested after 3 h of dark and 15 min of light (see A, lane 7).

**Fig. 3.** Enzyme activity stain of different electrophoretic forms of NADP-GDH in a native gradient polyacrylamide gel (4 to 30%) after electrophoresis of homogenates prepared from *C. sorokiniana* cells. Lane 1, homogenate of cells cultured in ammonium medium, containing 10 mm glucose, for 3 h in the dark; lane 2, homogenate of cells cultured in ammonium medium, in the absence of an organic carbon source, for 2 h in light. The faint band of enzyme activity, detected at 180,000 D, was due to cross-reactivity with the NAD-GDH isoenzyme.
native nongradient gel electrophoresis (Fig. 2A, lanes 6 to 11), probably differ in net charge and mol wt.

To determine if the mobilities of the different electrophoretic forms of NADP-GDH antigen(s) in native gels were a result of heterogeneity in the size of the subunits within the holoenzyme(s), cell homogenates were subjected to SDS-slab gel electrophoresis and the positions of the NADP-GDH subunits in the gel were determined by use of the Western blot/immunodetection procedure. Although the NADP-GDH holoholoenzyme, purified from cells cultured for 12 h in ammonium medium, was reported to be composed of 6 identical subunits (8, 30), 2 putative NADP-GDH subunits of different size were observed to accumulate in cells early during the induction period in the present study (Fig. 2B). Based on their mobilities in the SDS gel, the 55,500 D and 53,000 D antigens were designated as being α- and β-subunits, respectively. The β-subunit (M, = 53,000) has the identical mol wt of the NADP-GDH subunit identified in the holoenzyme purified from fully induced cells (18). As discussed by Prunkard et al. (18), the earlier mol wt of 59,000 reported (8) for this subunit was in error.

Because whole cell homogenates were subjected to SDS-slab gel electrophoresis, the α-subunit could have existed as a free subunit rather than being in a holoenzyme in the original cell homogenate. Prunkard et al. (18) recently have obtained experimental evidence that indicates the β-subunit is initially synthesized as a 58,500 D precursor protein. Therefore, it seemed possible that the α-subunit might be a free intermediate in the conversion of the precursor protein to the β-subunit. To determine whether the α-subunit exists only as a free subunit, a homogenate was prepared from cells, containing approximately equal amounts of α- and β-subunits (i.e. Fig. 2B, lanes 8 and 9). By use of gel-filtration chromatography, on a Sephacryl S-300 column in the absence of SDS, the proteins in the fresh cell homogenate were fractionated according to size. The column fractions containing proteins with mol wt greater and less than 100,000 were pooled separately, and then concentrated by ultrafiltration. The proteins in these two preparations were subjected to SDS-gel electrophoresis and the Western blot/immunodetection procedure. The results were very clear. The α- and β-subunit antigens were present only in column fractions containing proteins with mol wt greater than 100,000 (gel not shown). Thus, both subunits appear to exist in holoenzyme-size proteins and not as free subunits in homogenates of cells harvested early during induction of NADP-GDH activity. The existence of the different electrophoretic forms of NADP-GDH, observed in native gels, is probably due in part to the presence of two different types of subunits in the NADP-GDH holoenzymes.

In Figure 2B, the levels of the α- and β-subunits, presumably associated with holoenzyme-size proteins, are shown for cells cultured in the dark and light in the ammonium inorganic salts medium in the dark/light transition experiment. A constant amount of total protein from each harvest time was loaded into the wells of the gel. Therefore, the level (i.e. band intensity) of each subunit is actually being expressed relative to a constant amount of total cellular protein at each harvest time. Before the addition of ammonium to the cells in the dark, neither the α- nor β-subunit could be detected (Fig. 2B, lane 1). However, after the cells had been in the dark in ammonium medium for 2 h, the β-subunit had accumulated to detectable levels (lane 2). After 3 h of darkness, the β-subunit was still the only type of subunit present in the cells (lane 4). However, after the culture was illuminated for 10 min, the α-subunit could be detected (lane 5). From 15 to 40 min after illumination, both the α- and β-subunits increased in concentration (lanes 7–10). After 1 h of illumination, it was apparent that the concentration of the β-subunit increased faster than the α-subunit (lane 11). In fact, after 2 h of illumination, the β-subunit was the predominant subunit species present in the cells (lane 12). In fully induced cells, cultured in the light in ammonium medium for 12 to 48 h, the β-subunit continued to be the major subunit, and the α-subunit was barely detectable in the SDS gels.

Although the reasons for the changing pattern of accumulation of the α- and β-subunits were unknown, by the end of the series of analyses related to the dark-light experiment, it was possible to make two conclusions related to the nature of the rate-limiting step in the induction of NADP-GDH activity. First, a catalytically inactive NADP-GDH holoenzyme, requiring light-dependent processes for activation, does not accumulate in cells cultured in ammonium medium in the dark. Second, under these culture conditions, the cells do not accumulate the 58,500 D precursor protein of the NADP-GDH subunit recently described by Prunkard et al. (18).

For the aforementioned experiments, synchronized daughter cells were employed so that changes in gene dosage would not occur during the induction time course. The cells were synchronized by 3 cycles of intermittent illumination (9 h light:7 h dark), and then taken at the end of the third 7 h dark period for use in the dark-induction experiments. Since Chlorella cells have been observed to utilize their starch reserves during cell division (7) and also during dark periods (13) for cell maintenance in the absence of an exogenous source of organic carbon, it seemed possible that low endogenous carbon/energy reserves might have limited the rate of synthesis or translation of the NADP-GDH mRNA in the earlier dark-induction experiments. Therefore, to determine whether the presence of undepleted endogenous starch reserves would have an effect on the ability of the cells to synthesize active NADP-GDH in the dark, uninduced cells were cultured in continuous light in nitrate medium for 12 h and then transferred immediately to ammonium medium in the dark. Whereas only 3 milliunits of NADP-GDH activity accumulated during the dark period in the earlier experiment (Fig. 1B), the cells accumulated 14 milliunits of NADP-GDH activity per ml of culture during the dark period which was preceded by a 12 h light period instead of a 7 h dark period. Although cells can typically accumulate 130 to 200 milliunits of NADP-GDH activity per ml of culture during a 3 h induction period in the light, the 14 milliunits of enzyme activity per ml of culture, induced during the dark period, was significantly higher than the low basal level of activity (i.e. less than 1 milliunit) observed in uninduced cells in the light. Moreover, the catalytic activity:antigen ratio appeared to be essentially equal for the NADP-GDH holoenzyme(s) that accumulated in cells in the dark and in the light in ammonium medium (Fig. 4, A and B). From this experiment, it was concluded that light per se is not required for the ammonium-induced accumulation of a NADP-GDH holoenzyme with full catalytic activity. Moreover, because cells with larger starch reserves were able to synthesize more NADP-GDH in the dark, it was also concluded that the rate of induction of NADP-GDH activity/antigen in the dark was probably dependent upon the availability of an organic carbon source.

Induction of NADP-GDH Activity and Antigen in Cells Cultured in Presence of Acetate or Glucose in the Dark. To determine whether an organic carbon source, supplied along with ammonium in the dark, could substitute for light in the induction of NADP-GDH catalytic activity, synchronized daughter cells were cultured in the dark in ammonium medium in the presence of either 20 mM acetate or 10 mM glucose. Before the addition of ammonium and acetate or glucose to the cultures, the synchronized daughter cells already had been in the dark for 7 h as part of the synchronization procedure. Two control cultures were placed in ammonium medium without an organic carbon source; one culture was illuminated, whereas the other was kept in the dark.
After addition of ammonium and either acetate or glucose to uninduced cells in the dark, the turbidities of the cultures increased continuously in essentially a linear manner (Fig. 5A). By the end of the 3 h dark period, the turbidities of the acetate and glucose cultures had increased 1.7-fold and 2.7-fold, respectively. The turbidity of the control culture, in ammonium medium in the light, increased 2.2-fold during the 3 h light period. The control culture, maintained in the dark in ammonium medium without an organic carbon source, showed no increase in turbidity.

After an induction lag, NADP-GDH activity accumulated in cells cultured in either acetate or glucose in the dark (Fig. 5B). At the end of the 3 h dark period, the acetate and glucose cultures contained 96 and 21 milliunits per ml of NADP-GDH activity, respectively. The control culture in the light accumulated 82 milliunits per ml of enzyme activity during the 3 h induction period. Although the turbidity of the glucose culture increased at the highest rate, this culture had the lowest rate of accumulation of NADP-GDH activity. With the exception of a shorter induction lag in the acetate culture, the accumulation patterns for NADP-GDH activity per ml were almost parallel in the 

**Fig. 5.** Patterns of accumulation of culture turbidity and NADP-GDH activity in synchronized daughter cells of *C. sorokiniana* cultured in ammonium medium in the dark with either 20 mM acetate or 10 mM glucose, or in the light in the absence of an organic carbon source. Ammonium chloride was added to the cultures at zero time. A, Turbidities of cultures growing in the dark with acetate (Δ) or glucose (○), or in the light in absence of organic carbon source (●). B, NADP-GDH activities in cultures growing in the dark with acetate (Δ) or glucose (○), or in the light in absence of organic carbon source (●).
acetate and light-control cultures. However, the rate of accumulation of cell mass (i.e. equivalent to culture turbidity) was higher in the control culture than in the acetate culture (Fig. 5A).

Therefore, when the kinetics of enzyme induction were expressed on a differential rate basis (i.e. enzyme activity/cell mass versus induction-time), it was obvious that the acetate culture had the highest differential rate (i.e. 2-fold greater than light-control culture) of accumulation of NADP-GDH activity (plot not shown). It was surprising to observe that a chloroplast-localized enzyme, such as the NADP-GDH, would be induced at a higher rate in cells cultured in the dark with an organic carbon source than in cells rapidly growing under photosynthetic conditions with CO2 as their carbon source.

The patterns of accumulation of the forms of NADP-GDH, in the different cultures, were examined by use of the Western blot/immunodetection procedure. Although somewhat difficult to see in photographs of the autoradiograms of the Western blots shown in Figure 6, A and B, NADP-GDH antigen began to accumulate within 40 min after addition of ammonium to the control culture in the light. In this control culture, as demonstrated by native gel electrophoresis, the antigen associated with NADP-GDH holoenzyme(s) first appeared as a series of 4 to 5 protein bands (Fig. 6A, lanes 3 and 4). By 1 h 20 min into the induction period, 7 electrophoretic forms of holoenzyme antigen were visible in the native gel, ranging in apparent mol wt from 280,000 to 400,000 (lane 5). By 3 h, there was a decrease in concentration of the holoenzyme forms with lower electrophoretic mobilities (lane 9). To examine the patterns of accumulation of the α- and β-subunits, associated with the NADP-GDH holoenzymes in the control culture, the same cell homogenates were electrophoresed in a SDS-gel (Fig. 6B). Within 40 min after addition of ammonium to the control culture, a faint band of NADP-GDH antigen, corresponding to the position of the β-subunit (Mr = 53,000) could be detected in the gel (Fig. 6B, lanes 2 and 3). By 1 h, the α-subunit (Mr = 55,500) was also visible in the gel (lane 4). The concentration of both the α- and β-subunits increased until approximately 2 h into the induction period (lane 7), thereafter the α-subunit decreased in concentration while the β-subunit continued to increase. By 3 h, the α-subunit was barely detectable, and the β-subunit was the predominant species of subunit (lane 10).

As shown by both native and SDS-gel electrophoresis of cell homogenates, cells cultured in ammonium medium in the dark, with acetate as the organic carbon source, had essentially identical patterns of accumulation of the same NADP-GDH holoenzyme electrophoretic forms and subunit species as the control culture in the light (photographs of gels not shown). In contrast, the cells that were induced in the dark with glucose as their carbon source, accumulated additional electrophoretic forms of NADP-GDH antigen that were undetected in either the control or acetate cultures (Fig. 7, A and B). Although the previously observed NADP-GDH holoenzyme forms, with apparent mol wt ranging from 280,000 to 330,000, were observed to accumulate in the glucose culture, a new form of NADP-GDH holoenzyme was observed with an electrophoretic mobility corresponding to a protein with a mol wt of approximately 670,000 (Fig. 7A). The electrophoretic mobility of this new holoenzyme form was equal to or slightly greater than the protein standard, thyroglobin (Mr = 669,000). Because the mol wt of the new electrophoretic form of NADP-GDH holoenzyme (Mr = 670,000) was approximately twice that of the holoenzyme forms with higher electrophoretic mobilities (i.e. apparent Mr = 280,000-330,000), it seemed possible that this new form might be an oligomer (i.e. dimer) of the NADP-GDH holoenzyme. GDHs from other eukaryotic organisms have been shown to polymerize under various metabolic conditions (23).

An attempt was made to determine whether the low electrophoretic mobility of the new form(s) of NADP-GDH holoenzyme, observed in the native nongradient gels, was due to an increase in mol wt or decrease in net negative charge of the holoenzyme(s). A cell homogenate from the glucose culture (Fig. 7A, lane 10) was electrophoresed for 16 h in a native gradient gel. When the standard amount of cell homogenate was electrophoresed in the gradient gel, NADP-GDH antigen was detected only in the 330,000 to 360,000 mol wt region of the gel (Fig. 8C, lane 2). Moreover, an activity stain for NADP-GDH revealed a series of bands of active enzyme only in the lower mol wt range of the native gradient gel (Fig. 3, lane 1). These native gradient gel data were consistent with a change in net charge, rather than an increase in mol wt of the NADP-GDH holoenzyme, being responsible for the appearance of the new form of the enzyme in homogenates of glucose-cultured cells. However, when the
FIG. 7. Patterns of accumulation of different electrophoretic-forms of NADP-GDH antigen in synchronized daughter cells of *C. sorokiniana* induced in the presence of 10 mM glucose in ammonium medium in the dark. At zero time, glucose and ammonium were added to the uninduced cells. Aliquots of cell homogenates, containing approximately equal amounts of total protein from the different harvest times, were electrophoresed in native and SDS gels, and then transferred to nitrocellulose. The resulting Western blots were treated with anti-NADP-GDH IgG and 125I-labeled Protein A. A, Native polyacrylamide gel (7.5%). Lane 1, zero time; lane 2, 20 min; lane 3, 40 min; lane 4, 1 h; lane 5, 1 h 20 min; lane 6, 1 h 40 min; lane 7, 2 h; lane 8, 2 h 20 min; lane 9, 2 h 40 min; lane 10, 3 h; lane 11, cell homogenate from control culture induced in ammonium medium in continuous light in absence of glucose. B, SDS-polyacrylamide gel (10%). Lane 1, zero time; lane 2, 20 min; lane 3, 40 min; lane 4, 1 h; lane 5, 1 h 20 min; lane 6, 1 h 40 min; lane 7, 2 h; lane 8, 2 h 20 min; lane 9, 14C-labeled protein standards; lane 10, 2 h 40 min; lane 11, 3 h. The mol wt of the β-subunit was 53,000.

amount of cell homogenate, electrophoresed in the native gradient gel was increased 8-fold, a smear of NADP-GDH antigen was seen between the 670,000 and 360,000 mol wt region of the gel (Fig. 8C, lane 1). Because of this later observation, it seems possible that a dimer of the NADP-GDH holoenzyme(s) might have existed in the original homogenate, but dissociated during the 16 h electrophoresis period in the native gradient gel. Thus, at the present time, the physical nature of the NADP-GDH holoenzyme form with the low electrophoretic mobility is uncertain.

When the cell homogenates, from the glucose culture, were analyzed by SDS gel electrophoresis, an unexpected pattern of accumulation of NADP-GDH subunit(s) was obtained. Within 20 min after the addition of ammonium to the cells, the β-subunit was detected (Fig. 7B, lane 2). The α-subunit was absent from the cell homogenates for the entire 3 h induction period.

FIG. 8. Comparison of different electrophoretic-forms of NADP-GDH antigen from synchronized daughter cells of *C. sorokiniana* induced for 3 h in ammonium medium in the dark or light with 10 mM glucose and in ammonium medium in the light with CO2 as the sole carbon source. After electrophoresis, the proteins were transferred to nitrocellulose, and the resulting Western blot was probed with anti-NADP-GDH IgG and 125I-labeled Protein A. A and B, Electrophoresis in SDS-polyacrylamide gels (10%). A, Cell homogenates from induced cultures in 10 mM glucose in the dark (lane 1) or in the absence of an organic carbon source in the light (lane 2). B, Cell homogenate from an induced culture in 10 mM glucose in the light (lane 1) or in the dark (lane 2). C, Electrophoresis for 16 h in a native gradient polyacrylamide gel (4-30%). Lanes 1 and 2 contain the same homogenate from cells induced in the presence of 10 mM glucose in the dark, except in lane 1 the sample was concentrated 8-fold. The mol wt of the α- and β-subunits were 55,500 and 53,000.

However, by 1 h 20 min, a second antigen band with a mol wt of 45,000 was seen in the gel (lane 5). This antigen was present in cell homogenates at all the harvest times thereafter. Because this 45,000 D antigen is barely detectable in some experiments, it is unclear as to whether this antigen is another type of subunit found in the NADP-GDH holoenzyme or is a degradation product of α- or β-subunits in cells induced in the dark with glucose as the carbon source.

As additional controls, cells were also induced in the light in ammonium medium, containing either acetate or glucose. After 3 h of growth in the light, the turbidities of the acetate and glucose cultures had increased 2.5-fold and 3.3-fold, respectively (plot not shown). After 2 h of induction in the light, the cells
cultured in acetate, glucose, and no organic carbon source contained 32, 8, and 55 milliunits of NADP-GDH activity per ml of culture, respectively (plot not shown). From these results, it appears that acetate and glucose actually inhibited the rate of accumulation of NADP-GDH activity in cells cultured under photosynthetic conditions.

A comparison was made of the numbers/sizes of active forms of NADP-GDH in cells cultured in ammonium medium in the presence and absence of glucose for 3 h in the dark or light (Fig. 9). By use of native nongradient gel electrophoresis, it was shown that cells, induced in the presence of glucose for 3 h in the dark (Fig. 9, lane 1), contained a major active form of NADP-GDH with a low electrophoretic mobility (apparent Mr = 670,000) and also active forms with higher mobilities (Mr = 280,000-330,000). Shatilov and Kretovich (22) also observed the accumulation of a similar high mol wt catalytically active form of NADP-GDH in C. pyrenoidosa cells cultured under similar conditions. In C. sorokiniana cells induced in glucose medium for 3 h in the light, the predominant active forms of the NADP-GDH were in the mol wt range of 280,000 to 360,000 (Fig. 9, lane 2). Although these cells also contained the form with the low electrophoretic mobility (Fig. 9, lane 2), this form was at a much lower concentration than in cells cultured in the same medium in the dark (Fig. 9, lane 1). This form was present in cells induced in the absence of glucose in the light (Fig. 9, lanes 3 and 4) or in the dark (Fig. 2A, lane 3). The cell homogenates, prepared from cells harvested after 3 h of induction in glucose medium in the dark or light, were also electrophoresed in SDS gels. The cells induced in the dark, in the presence of glucose, contained only the β-subunit (Fig. 8A, lane 1). Cells induced in glucose medium in the light contained both the α- and β-subunits (Fig. 8B, lane 1).

From the results obtained from the dark/light induction experiments in the absence and presence of organic carbon compounds, it can be concluded that the induction of fully active NADP-GDH is not a light-dependent process per se. In the dark in the absence of exogenous organic substrates, the cells can synthesize low levels of fully active NADP-GDH, provided endogenous starch reserves have not been depleted. When the cells are provided with an exogenous organic substrate, such as acetate, the rate of induction of NADP-GDH activity/antigen per ml of culture in the dark can be equal or slightly greater than the rate of induction of the enzyme under photosynthetic conditions with CO₂ as the carbon source. In fact, the differential rate of induced accumulation of the enzyme in cells cultured in acetate in the dark is twice the rate observed in cells cultured autotrophically in the light. However, the presence of acetate or glucose in the culture medium of cells significantly inhibits the accumulation of NADP-GDH activity in cells growing under photosynthetic conditions. Although the NADP-GDH is chloroplast localized, it appears to be involved in ammonium assimilation in cells cultured in the dark in media containing high levels of ammonium (i.e. 29 mm).

By use of a Western blot/immunodetection procedure, it was discovered that subunits of two different sizes (i.e. Mr = 55,500 and 53,000; α- and β-subunits, respectively) can be associated with the NADP-GDH holoenzyme(s). During induction of NADP-GDH activity in the light, both subunits accumulate during the 1st 2 h of a typical induction period; however, thereafter accumulation of the α-subunit ceases, and by 3 h, it is barely detectable in the cells. Because the NADP-GDH was previously purified (and characterized) from cells that had been cultured for 12 to 48 h in ammonium medium, the α-subunit type holoenzyme(s) was not detected. Of particular interest is the finding that purified antibodies, produced against the NADP-GDH holoenzyme containing only β-subunits, cross-reacts with the α-subunit on Western blots of SDS gels. The purified NADP-GDH that was used to immunize the rabbits was examined by SDS-gel electrophoresis and was found to contain no detectable contamination by the α-subunit. Moreover, the antibodies against the β-subunit holoenzyme were purified on an antigen-affinity column to which the β-subunit holoenzyme was covalently coupled. Thus, the α- and β-subunits appear to be immunologically related. These two subunits could be encoded by different genes with a high degree of sequence homology or by the same gene with transcriptional or post-transcriptional events regulating the size of the NADP-GDH mRNA transcript. Alternatively, the two subunits could be produced by differential processing of a common precursor-protein.

Although the different electrophoretic forms of the NADP-GDH holoenzyme(s) are probably due in part to the presence of either α- or β-subunits (or possibly both) in the holoenzyme(s), the difference in electrophoretic mobilities of the β-subunit holoenzyme(s) is too great to be due to subunits that differ in mol wt by only 2,500 D. There is likely some type of covalent modification that alters the net charge on the NADP-GDH holoenzyme(s). To gain some insight into the catalytic role that these different forms of NADP-GDH might have in ammonium assimilation in cells cultured under various environmental conditions, research is currently in progress to purify and characterize these NADP-GDH isoforms and/or isoenzymes.

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Fig. 9. Comparison of the numbers/sizes of active forms of NADP-GDH holoenzyme(s) from synchronized daughter cells of C. sorokiniana cultured in ammonium medium in the light and in ammonium medium with 10 mM glucose in the dark or light. The native nongradient polyacrylamide gel (7.5%) was stained for NADP-GDH activity. Lane 1, glucose and 3 h dark; lane 2, glucose and 3 h light; lanes 3 and 4, no organic carbon source and 3 h light (homogenates from two separate experiments).
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