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Light-induced Development of Polyribosomes and the Induction of Nitrate Reductase in Corn Leaves[1]

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ABSTRACT

Nitrate reductase activity was induced by nitrate in green corn (*Zea mays*) leaves in either light or darkness. The induction process required oxygen in darkness but not in light. A light treatment was required before the enzyme could be induced in etiolated leaves.

The capacity for nitrate reductase induction by nitrate was positively correlated with the level of cytoplasmic polyribosomes under a variety of experimental conditions. (*a*) Light-grown leaves contained high levels of polyribosomes (84% of the total population, most of which were of the 80 S type); similarly high levels of nitrate reductase activity were induced. (*b*) The level of polyribosomes and the ability to form nitrate reductase activity rapidly decreased in light-grown leaves following transfer to an anaerobic environment in the dark; both parameters were maintained at a high level when light-grown leaves were kept in the light under anaerobic conditions. (*c*) The ability of light-grown leaves, previously placed in darkness under nitrogen to dissociate polyribosomes to monoribosomes, to form nitrate reductase activity again correlated with the level of reformed polyribosomes following transfer of the leaves back to light. (*d*) Etiolated leaves contained a low level of cytoplasmic polyribosomes (27%), and nitrate reductase activity was induced following exposure to light only after a lag of 2 to 4 hours. During this lag period there was a marked increase in the level of polyribosomes.

The ability of leaves to form nitrate reductase activity and the level of polyribosomes also correlated with the level of *in vitro* incorporation of amino acids into protein by the isolated ribosome preparations. Thus, the apparent requirement of light for nitrate reductase induction in etiolated leaves seems not to be specific. Rather an influence of light upon the development of an active protein-synthesizing apparatus as evidenced by the state of polyribosomes is indicated.

The results also show that energy from photosynthetic phosphorylation can be used to maintain cytoplasmic polyribosomes (and thus to drive cytoplasmic protein synthesis), at least under anaerobic conditions.

The level of nitrate reductase activity in plants is regulated by the availability of nitrate (1). Light appears to be important in the induction process (1, 2, 3, 7, 28); the function of light, however, is a matter of controversy. The enzyme is induced by nitrate in darkness in leaves of light-grown seedlings (2, 3, 28). Increases in leaf nitrate content along with increased nitrate reductase activity in response to light led to the suggestion that light is unnecessary for induction but rather the increased activity results from increased uptake of nitrate (2). Subsequent experiments with dark-grown seedlings suggested a role of light more closely related to the induction process. Dark-grown oat and barley seedlings accumulate large amounts of nitrate in darkness, but nitrate reductase activity does not increase above the low endogenous level until light is supplied (3, 27, 28). However, when dark-grown oat leaves were induced in light for 12 hr and then returned to darkness, the activity continued to increase for another 24 hr (3). A single report of dark induction in dark-grown leaves (2) involved low levels of activity.

The fact that green leaves can form nitrate reductase in darkness whereas dark-grown leaves require light for such induction, suggests that light is necessary for induction, and that the light effects are carried over into a subsequent dark period. Induction in darkness probably utilizes respiratory energy. Mendel and Visser (22) reported that respiratory inhibitors prevented nitrate reduction *per se* in darkness. Their results indicate that photosynthesis also may provide energy for nitrate reduction since reduction was not prevented by respiratory inhibitors in light.

Total leaf protein (5, 20) and a number of other enzymatic activities (8, 18, 26) increase relatively rapidly when etiolated leaves are placed in light. Similarly polyribosome levels (31) and ^{14}C-leucine incorporation (30, 31), respectively, increased in bean leaves and corn shoots.

The role of polyribosomes in protein synthesis is well established, but little is known about leaf polyribosomes. Williams and Novelli (31) and others (4, 21, 23, 25) have reported on polyribosomes isolated from leaves. In most cases, polyribosome preparations from green leaves are largely degraded by endogenous nucleases during isolation, leaving only the smaller polymer and monomer fractions. The preparation of polyribosomes from corn leaves was aided by a recent report that diethyl pyrocarbonate is an effective nuclease inhibitor in plant material (29), and by work of J. M. Anderson and J. L. Key (unpublished data).

This paper reports results of experiments designed to evaluate the role of light, respiration, and photosynthesis in the regulation of nitrate reductase induction. Ribosomal profiles of green and dark-grown corn leaves were prepared after the leaves were subjected to aerobic or anaerobic and light or dark environments. These profiles and amino acid incorporation levels by isolated ribosomes were compared with the level of nitrate reductase

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activity induced in similarly treated leaves. The results indicate that the role of light is associated with the development or maintenance of the protein-synthesizing apparatus rather than being involved specifically in the induction of nitrate reductase activity.

MATERIALS AND METHODS

Plant Materials. *Zea mays* var. PAG SAX-29 or WF9xM14 was grown in vermiculite in 11- × 13-inch plastic pans. For green seedlings, moisture was supplied by cotton wicks connecting the vermiculite with a nutrient solution lacking nitrate but containing the following (in meq per liter): $CaSO_4$, 2; K_2SO_4, 2; $MgSO_4$, 2; $NH_4H_2PO_4$, 2; $(NH_4)_2SO_4$, 4; and (in μmoles per liter): $MnSO_4$, 18.2; $ZnSO_4$, 3.8; H_3BO_3, 9.2; $CuSO_4$, 1.57; $(NH_4)_6Mo_7O_{24}$, 0.10; Fe as Fe-ethylenediamine di-*o*-hydroxyphenylacetic acid, 89.4 (donated by Geigy Agricultural Chemicals, Ardsley, N. Y.). For dark-grown seedlings a nutrient solution containing nitrate was substituted (27). Green seedlings were grown for 8 days in a controlled environment growth chamber at 24 C and 2000 ft-c. Dark-grown seedlings were grown for 9 days in a light-proof box at 24 C.

Enzyme Induction Studies. Ten corn leaves were detached, trimmed to 10 cm, and submerged in 1500 ml of 0.05 M KNO_3 in 5- × 7-inch clear plastic pans by the method of Beevers *et al.* (2). The leaves were maintained submerged by a rectangular piece of Plexiglas 4-mm thick, perforated with 15 holes of 5-mm diameter. Chloramphenicol at 0.03 mg/ml (Parke, Davis and Co.) was added to the treatment solution to prevent bacterial contamination. After the leaves were submerged, the pans were covered with Saran Wrap, and air or nitrogen gas was bubbled through the system. A small opening allowed the gas to escape. When anaerobic conditions were desired, all water was boiled to remove dissolved gases before solutions were prepared. All treatments were conducted at 24 C.

Application of Inhibitors. Ten detached leaves were placed in beakers containing 40 ml of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP,[3] Calbiochem) at 0.02 mg/ml. The samples were placed under a vacuum for 2 min to remove any air bubbles that may have formed in the transpiration stream in the leaves due to cutting. They were then transferred to the growth chamber and allowed to take up the inhibitor in light for 2 hr prior to submergence.

Moisture Contents. Percentage of dry weight of leaves did not change during the 12-hr treatment period, and fresh weights of submerged leaves increased only 3%. Thus, the results were not confounded by changing leaf weights.

Nitrate Reductase Assay and Nitrate and Protein Analyses. Nitrate reductase activity was assayed as previously described (27). Nitrate was determined with a nitrate-ion-activity electrode (Orion Research Inc., Cambridge, Mass.) and an Orion expandable-scale specific-ion meter. Soluble protein was precipitated with trichloroacetic acid (final concentration, 5%) and determined by the method of Lowry *et al.* (13). The standard was bovine serum albumin, fraction V (Sigma Chemical Co.).

Preparation of Polyribosomes. Three grams of fresh or previously submerged leaf material was rinsed in deionized water, blotted dry, diced into approximately 1-cm sections, and immediately frozen on Dry Ice. The leaves were then homogenized in 15 ml of tris-buffer (tris-HCl, pH 7.5, 50 mM; $MgCl_2$, 20 mM; KCl, 50 mM) containing 250 mM sucrose. Homogenization was for 10 sec at high speed in a Willems Polytron PT 20 st. When DEP was used, the concentration of tris-HCl in the homogenizing medium was increased 4-fold so that after homogenization the initial pH was maintained. DEP (0.1%) was always added immediately before homogenizing

The homogenate was centrifuged once at 500*g* for 2 min and twice at 30,000*g* for 15 min. Polyribosomes were prepared by centrifuging the supernatant through 5.0 ml of 1.5 M sucrose in tris-buffer (tris-HCl, pH 7.5, 50 mM; $MgCl_2$, 5 mM; KCl, 15 mM) at 159,000*g* for 120 min (Spinco type 65 rotor). The polyribosome pellet was resuspended in the same buffer, lacking sucrose, to a final concentration of 10 OD 260 nm units per ml. A 0.6- to 0.9-ml sample was layered on a 25-ml, 10 to 34%, linear sucrose gradient and centrifuged at 59,000*g* for 120 min (Spinco SW 25.1, 1- × 3-inch tube). Gradients were fractionated as described previously (11).

Amino Acid Incorporation Studies. Amino acid incorporation was studied with modifications of the methods of Mans and Novelli (16, 17) and Williams and Novelli (30). The reaction mixture of 0.5 ml contained: tris-HCl, pH 7.5, 100 mM; $MgCl_2$, 10 mM; KCl, 16 mM; ATP, 1 mM; GTP, 0.3 mM; phosphoenolpyruvate, 12.8 mM; pyruvic kinase, 6.25 e.u./ml; ^{14}C-leucine, (178 mc/mmole) 0.5 μc; polyribosomal RNA, 0.4 mg; enzyme protein, 0.5 to 1.0 mg (prepared from 2-day-old etiolated corn shoots).

RNA Analysis. RNA was extracted from the polyribosome preparations as previously described (9). The RNA was fractionated on polyacrylamide gels by the method of Loening (12).

RESULTS AND DISCUSSION

Isolation and Properties of Leaf Polyribosomes. The sucrose density gradient profile for cytoplasmic ribosomes in freshly excised green leaves, isolated in the presence of DEP (Fig. 1A), indicated a high percentage of large polyribosomes with a relatively low monoribosome level. In the absence of DEP, the larger polyribosomes were greatly degraded with increased levels of the smaller polyribosome and monoribosome forms (Fig. 1B).

^{14}C-Leucine incorporation studies showed that polyribosomes from fresh leaves were capable of directing amino acid *in vitro* incorporation (Fig. 2). Polyribosomes isolated in the absence of DEP maintained a higher level of amino acid incorporation during the first 15 min of the assay period than did polyribosomes isolated in the presence of DEP. After 20 min the two systems differed little in level of incorporation. Incorporation is apparently limited to some extent by DEP when it is used, but may also be limited by poor polyribosome preparations when DEP is not used.

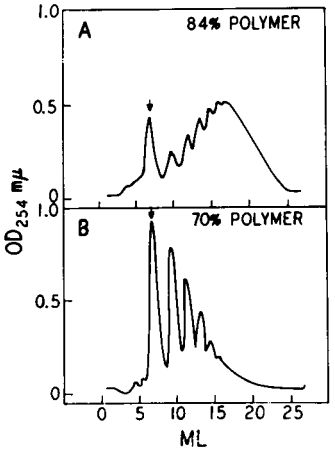

FIG. 1. The influence of diethyl pyrocarbonate on the distribution of ribosomes isolated from light-grown leaves. A: Plus DEP in extraction medium; B: minus DEP in extraction medium. Arrows denote monoribosome peaks.

[3] Abbreviations: CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; DEP: diethyl pyrocarbonate.

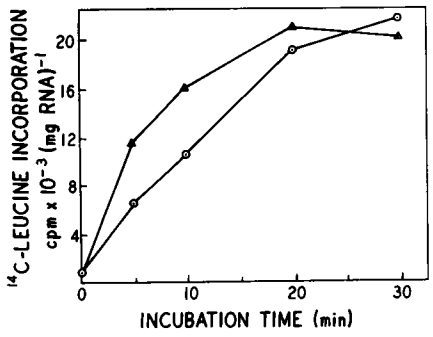

FIG. 2. ^{14}C-Leucine incorporation by ribosomes isolated from light-grown leaves. ⊙: plus DEP in extraction medium; △: minus DEP in extraction medium.

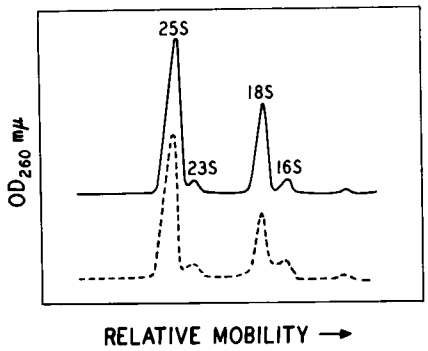

FIG. 3. Polyacrylamide gel fractionation of RNA extracted from ribosomes of light grown leaves. ——: plus DEP in extraction medium; -----: minus DEP in extraction medium. Electrophoresis was for 3 hr at 5 ma/gel.

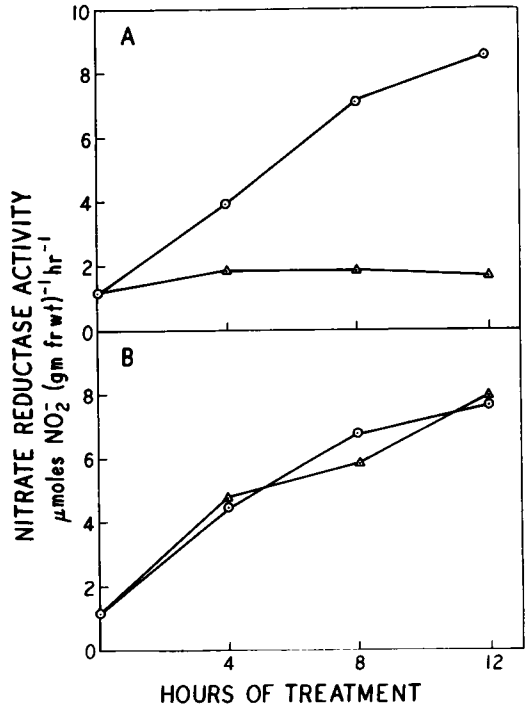

FIG. 4. Activity of nitrate reductase in excised light-grown leaves incubated in 0.05 M KNO_3 in light or darkness. A: ⊙, darkness + O_2; △, darkness + N_2. B: ⊙, light + O_2; △, light + N_2. Light was supplied at 2000 ft-c.

Values for polyribosome level and ^{14}C-leucine incorporation rate by polyribosomes from freshly excised green leaves served as controls for subsequent experiments. DEP was routinely used in polyribosome isolation.

RNA was extracted from ribosomes prepared from green leaves to determine the extent of chloroplast ribosome contamination (Fig. 3). The low level of 23 S and 16 S rRNA compared to 25 S and 18 S rRNA indicates a low level of chloroplast ribosome contamination of the "cytoplasmic" ribosome preparation.

Nitrate Reductase Activity and Polyribosome Level in Green Leaves Under Aerobic and Anaerobic Environments. Detached green leaves were placed in solutions of nitrate under air or N_2 to determine the importance of respiratory energy in the induction of nitrate reductase in darkness. Enzymatic activity increased nearly 8-fold under air during the treatment period (Fig. 4A). Oxygen was apparently required for the dark induction, since very little induction occurred above the low endogenous level under N_2. Nitrate accumulation was approximately half as much under N_2 as under air (Table I). Its concentration should not be limiting for induction, however, since Beevers *et al.* (2) reported significant dark induction of nitrate reductase in green corn leaves, submerged under aerated nitrate solutions, that contained only 400 μg nitrate per gm fresh weight. Even though nitrate content increased to nearly 700 μg/gm fresh wt under N_2 (Table I), the low endogenous level of enzymatic activity increased but little. One set of leaves was removed from the nitrate solution after 12 hr, cut into 1-cm sections, and placed in aerated deionized water for an additional 12 hr. Since very little nitrate was desorbed from the tissue (Table I), the nitrate taken up during the dark period was probably absorbed into the leaf cells and available for reduction.

Ribosome profiles (Fig. 5, A and B) and amino acid incorporation studies (Table II) suggest that the lack of enzymatic induction under N_2 relates to a rapid dissociation of polyribosomes under the energy-depleting anaerobic environment. The dissociation of polyribosomes and the decreased ability of anaerobic ribosomes to synthesize protein, as measured by ^{14}C-leucine *in vitro* incorporation, were reported earlier by Lin and Key (10) utilizing soybean root preparations.

In light, enzymatic activity was induced under external anaerobic conditions equal to that under air (Fig. 4B). Apparently neither externally supplied carbon dioxide, as reported previously by Perilla (7) nor oxygen were required for induction in light. Nevertheless, CO_2 and oxygen produced internally may possibly affect induction in some way. The level of polyribosomes in light-

Table I. *Nitrate Levels in Light-grown Corn Leaves Submerged in 0.05 M KNO_3 in Light or Darkness*

Treatment Time	Level of Nitrate			
	Dark		Dark	
	Air	N_2	Air	N_2
hr	*μg/g fresh wt*			
0	170	170	170	170
4	920	500	440	500
8	1110	700	740	870
12	1560	850	930	1110
24[1]		760		
	Between treatments	Within treatment	Between treatments	Within treatment
$LSD_{0.01}$	100	130	80	125

[1] Submerged in nitrate 12 hr, then desorbed in water for 12 hr.

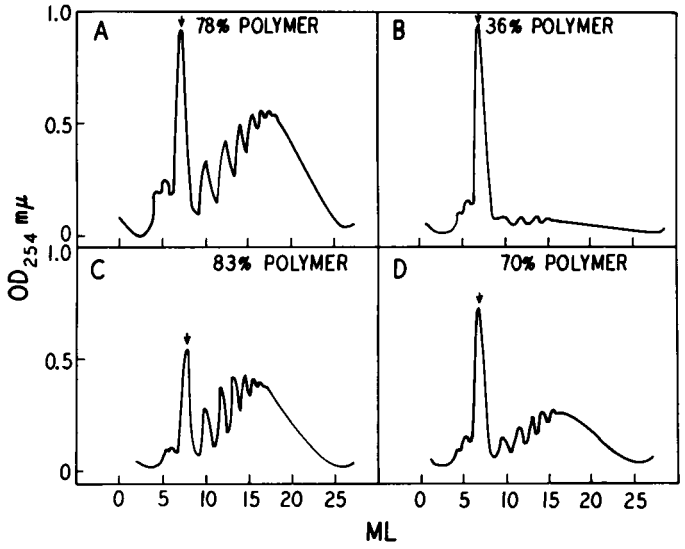

FIG. 5. Ribosome profiles from excised light-grown leaves after 2 hr of incubation in deionized water in light or darkness. A: Darkness + O_2; B: darkness + N_2; C: light + O_2; D: light + N_2. Ribosomes from leaves submerged 4 hr have similar profiles. Related assays of nitrate reductase are shown in Figure 4. Arrows denote monoribosome peaks.

treated leaves (Fig. 5, C and D) and their amino acid *in vitro* incorporating activity (Table II) again correlated well with the ability of the leaves to form an active nitrate reductase.

The concentration of protein decreased slightly in leaves in darkness under N_2 (Table III), but did not decrease under air in darkness or under N_2 or air in light. Experiments carried out for 24 hr showed even larger losses in soluble protein under N_2 in darkness, while no significant decreases were detected under any of the other conditions. Thus, the development of an active nitrate reductase in the leaves appeared related to their ability to maintain a relatively constant level of protein. In addition, protein maintenance was clearly dependent upon the presence of a relatively high level of polyribosomes in the tissue.

Induction under air and N_2 separated the process into two phases. In darkness, the dependence of polyribosome maintenance and enzymatic induction on oxygen suggested that respiration was providing the necessary energy for each. However, polyribosome maintenance and enzymatic induction occurred in light under anaerobic conditions, indicating that photophosphorylation contributed the required energy.

In the absence of CO_2, noncyclic photophosphorylation should decrease but cyclic photophosphorylation should proceed (15). ATP thus formed is available for active potassium fluxes (14, 24). It is not unreasonable to assume that such ATP may also be available for polyribosome maintenance. This concept was further tested by treating the leaves with CCCP, an uncoupler of photosynthetic phosphorylation (24), under anaerobic conditions in light. When freshly excised leaves were submerged in nitrate solutions in light under N_2, CCCP prevented the appearance of nitrate reductase activity (Fig. 6A). The enzyme was induced under anaerobic conditions in leaves that were depleted of polyribosomes before the light treatment. Activity in the pretreated leaves was, however, only 35% of that attained in leaves not given the dark pretreatment (Fig. 6B). Again CCCP prevented the appearance of enzymatic activity. Other experiments indicate that CCCP does not affect the level of nitrate uptake in detached leaves (R. C. Huffaker, unpublished data). Thus, in CCCP-treated leaves nitrate reductase induction was not prevented by the lack of nitrate.

The appearance of nitrate reductase activity correlated with the ability of the leaves to reform polyribosomes following transfer from dark to light. When dark-pretreated leaves (see Fig. 5B and Table II for state of ribosomes and amino acid incorporation level) were returned to light for 2 hr, the polyribosome level increased from 36 to 56%, and CCCP prevented the increase in polyribosomes. Increasing the light period up to 6 hr gave no further increase in polyribosome level. In the absence of CCCP, ^{14}C-leucine incorporation increased to nearly 70% of the control level under the same conditions (Table IV). The inhibitor prevented the increase in amino acid-incorporating activity during the subsequent light period.

RNA was extracted from the monoribosome and polyribosome regions of the CCCP-treated and control ribosome preparations to ensure that the observed recovery in polyribosomes was the result of reformation of 80 S cytoplasmic polyribosomes and not by contaminating chloroplast ribosomes. The level of 23 S and 16 S RNA did not change in either the polyribosome or monoribosome fraction indicating that recovery was the result of reformation of cytoplasmic polyribosomes (see Fig. 3 for proportion of cytoplasmic and chloroplast ribosomal RNAs in the preparation).

Photosynthetic energy can be utilized to maintain cytoplasmic polyribosomes intact and functional (*i.e.*, to drive cytoplasmic protein synthesis) under anaerobic conditions. Once polyribo-

Table II. *^{14}C-Leucine Incorporation by Ribosomes Extracted from Light-grown Leaves*

Leaves were incubated in deionized water in light or darkness and under O_2 or N_2 for 2 hr.

Assay Time	Leucine Incorporation			
	Light		Dark	
	O_2	N_2	O_2	N_2
min	*% of control*			
5	105	102	102	28
10	98	93	104	26
20	106	100	98	27
30	93	88	105	26

[1] The control ribosomes for these experiments were isolated from freshly excised light-grown leaves in the presence of DEP. See Figure 2 for actual values of amino acid incorporation over the 30-min time course.

Table III. *Soluble Protein Levels in Light-grown Corn Leaves Submerged in 0.05 M KNO_3 in Light or Darkness*

Treatment Time	Level of Protein			
	Dark		Light	
	Air	N_2	Air	N_2
hr	*mg/g fresh wt*			
0	12.9	13.9	12.9	12.8
4	13.0	14.4	14.1	13.6
8	13.0	13.1	14.3	12.3
12	13.0	12.7	13.0	12.2
24	12.1	11.6	13.1	12.6
	Between treatments	Within treatment	Between treatments	Within treatment
$LSD_{0.01}$	0.70	0.90	0.50	0.65

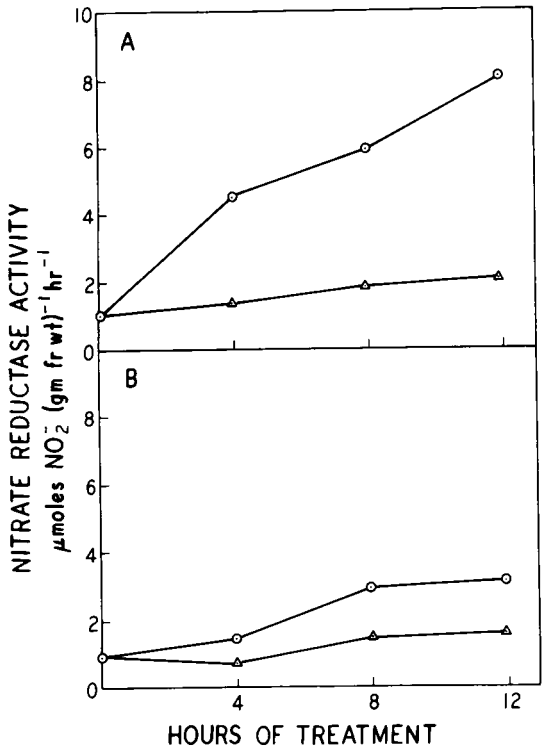

FIG. 6. Effect of CCCP on activity of nitrate reductase in light-grown leaves. Leaves were incubated in 0.05 M KNO_3 in light + N_2. A: No pretreatment; B: 2 hr of pretreatment in darkness under N_2 to dissociate polyribosomes (Fig. 5B). ⊙: control; △: CCCP.

Table IV. *Effect of CCCP Pretreatment on ^{14}C-Leucine Incorporation by Reassociated Polyribosomes Extracted from Light-grown Leaves after 2 hr of Incubation in Light under N_2*

Leaves were pretreated 2 hr (water or CCCP, see "Materials and Methods") followed by 2 hr of dark under N_2 to dissociate polyribosomes. The control ribosomes for these experiments were isolated from freshly excised light-grown leaves in the presence of DEP. See Figure 2 for actual values of amino acid incorporation over the 30-min time course. The ribosome preparations were 36% polyribosome when CCCP was included in the pretreatment incubation medium and 56% when CCCP was omitted.

Assay time	CCCP	Water
min	*% of control*	
5	30	69
10	34	65
20	29	62
30	27	71

somes were dissociated, photosynthetic energy was not sufficient for complete recovery, though some recovery occurred with a correlated increase in nitrate reductase activity.

Nitrate Reductase Induction and Ribosome State in Dark-grown Leaves. Dark-grown corn (unpublished data) and barley leaves (6, 27, 28) have a low endogenous level of nitrate reductase activity. In light, in the presence of adequate nitrate, nitrate reductase activity increases rapidly after a 2- to 6-hr lag period. A steady state level occurs between 24 and 48 hr and is intensity dependent.

Figure 7 shows the time course of polyribosome development

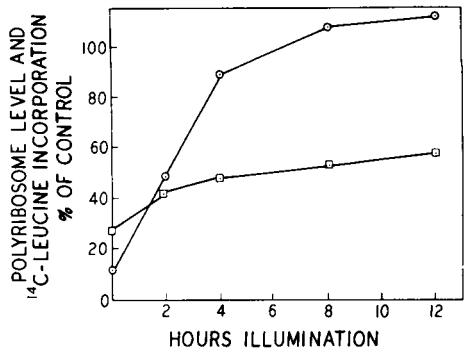

FIG. 7. Time course of polyribosome formation in intact dark-grown leaves following illumination and ^{14}C-leucine incorporation by ribosomes extracted from similarly treated leaves. ⊡: Polyribosome level; ⊙: ^{14}C-leucine incorporation. Kinetics of incorporation is the same for all treatments over the 30-min assay periods. Ribosomes used for control in kinetics study were extracted from fresh leaves in the presence of DEP. Actual values for controls range from 350 to 550 for 0 min to 20,000 to 27,000 cpm/mg for 30-min assay periods. (See Fig. 2 for kinetics).

over a 12-hr light period in dark-grown leaves and related levels of ^{14}C-leucine incorporation by the polyribosome preparations. The polyribosome level increased from 27 to 42% during the first 2 hr of light, continued to increase gradually throughout the 12-hr light period, and would probably approach that of green leaves (Fig. 1) if the light treatment were continued for an extended period. Amino acid incorporation reached a maximal level after 8 to 12 hr of light. Newly formed polyribosomes are more active, per unit of ribosomes, than are polyribosomes from light-grown leaves. After 4 hr of light, polyribosomes made up only 48% of the total ribosome population (*versus* 84% in light-grown leaves) whereas amino acid incorporation was nearly 85% of that of polyribosomes extracted from light-grown leaves. Williams and Novelli (31) reported similar findings with leaves of dark-grown beans. One hour of light led to an increase in the level of polyribosomes from 33 to 48% of the total population while ^{14}C-leucine *in vitro* incorporation increased by 150 to 200%. Marks *et al.* (19) reported similar results with reassociated rabbit reticulocyte polyribosomes.

The ability of corn leaves to produce an active nitrate reductase apparently depends on the presence of polyribosomes as an indication of an active protein synthesizing apparatus. Induction will proceed in green leaves once nitrate is made available if polyribosomes are present at the onset of the induction treatment. If the polyribosomes are previously dissociated, however, induction will not occur until they are reformed. In dark-grown leaves the polyribosome level is quite low, and nitrate reductase activity cannot be induced in darkness, above the low endogenous level, regardless of nitrate availability. Polyribosomes are formed during the initial stages of a light treatment, and after 2 to 4 hr nitrate reductase activity can be induced. Apparently then, the requirement for light for the formation of nitrate reductase is for development of an active protein-synthesizing apparatus as evidenced by polyribosome formation and not specifically for induction of the enzyme. The specific effect of light on the protein-synthesizing apparatus may be related to the control of messenger RNA synthesis and the monoribosome to polyribosome transformation, as suggested by data of Williams and Novelli (31) and some of the present results. None of the results, however, rule out other possible effects of light on the general regulatory activation of protein synthesis.

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