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Changes in Enzymatic Activities in Etiolated Bean Seedling Leaves After a Brief Illumination[1, 2]

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Abstract. The phytochrome controlled increase in total protein in the primary leaf pair of etiolated bean (*Phaseolus vulgaris* var. Black Valentine) seedlings, which occurs during growth in the dark subsequent to a brief illumination, was investigated. Enzymes from the chloroplasts, the mitochondria, and the soluble cytoplasm all increase in total activity after the illumination.

The total protein and the ribulose carboxylase increases are not inhibited by FUdR, an inhibitor of DNA synthesis. Cycloheximide, an inhibitor of protein synthesis, applied at a time when the ribulose carboxylase activity increase has already commenced, blocks further increase. It was concluded that the total protein and the enzyme increases in the leaf are the result of increases in the per cell levels.

The initial brief illumination is saturating, but 40 minutes later the seedlings have acquired the ability to respond to a second brief illumination. The rate of increase in ribulose carboxylase activity in seedlings that have been illuminated twice is greater than the rate in seedlings that have been illuminated only once.

Far-red light prevents further increase in enzyme activity 48 hours after the initial illumination. There is a lag period interposed between the time of illumination with far-red light and the time at which the seedlings show the greatest effect of far-red light. It was concluded that the phytochrome influence on protein synthesis is not at the terminal steps.

A brief exposure of dark-grown bean seedlings to white or red light relieves several developmental restrictions characteristic of etiolated growth. Even if seedlings are returned to darkness, their plumular hooks open (9, 10) and leaf development accelerates dramatically (7, 8). Much attention has been focused on the differentiation of the photosynthetic apparatus under these conditions and it was found that chloroplast size and nitrogen content rose (16), and certain photosynthetic enzymes increased in activity (2, 13, 14). But is light-triggered development a chloroplast-centered event or do other cellular compartments participate and if so to what extent? To answer this question, the activity of representative cytoplasmic, chloroplast, and mitochondrial enzymes was determined following a brief illumination of etiolated seedlings.

Because the light activation of the steps here discussed is mediated by phytochrome the simultaneous monitoring of several enzymes promised to provide information on the mechanism of phytochrome control over diverse cellular functions. There exists the possibility that synthesis of all enzyme proteins is equally promoted by photoconversion of phytochrome or that individual organelles, pathways or enzymes respond individually. Of particular interest is the response of enzymes whose synthesis depends on information presumably coded in separate intracellular compartments.

By comparing the effect of selected inhibitors and of light on enzyme changes, the locus of phytochrome control of protein synthesis was sought. Our findings point to a cell-wide effect of phytochrome photoconversion operating at early phases of the synthesis of individual proteins.

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[2] This work represents part of a thesis presented by B. Filner to the faculty of the Graduate School of Brandeis University in partial fulfillment of the requirements for the Ph.D. degree.

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Materials and Methods

Growth and Illumination of the Seedlings. Seeds of *Phaseolus vulgaris* var. Black Valentine purchased from Asgrow Company or the Charter Seed Company were germinated in vermiculite in a dark growth room at 22°. Seven day old seedlings were illuminated for 10 minutes with white light (2000 lux) from GE 150W flood lights above a 6 cm water heat-filter. If far-red light was required, a one-eighth inch thick Rohm and Haas V-58015 Plexiglass Acrylic Plastic filter was placed beneath the same flood lights. The exposure time for far-red illumination was 20 minutes. After illumination, the seedlings were returned to the dark growth room and sampled daily.

Protein. 5% Trichloroacetic acid precipitable protein was determined by the method of Lowry *et al.* (12).

DNA. DNA was determined by the method of Dische (5) on hot 3% perchloric acid extracts of acid and organic solvent washed methanol precipitates.

RNA. Nucleic acids were extracted and RNA separated from DNA using the methods employed by Smillie and Krotkov (18) on similar material. RNA-ribose was determined by the orcinol method of Markham (15).

Preparation of the Enzymes and Analysis of the Assays. A 0.75 g portion of leaves was cut up into a Tenbroeck tissue grinder, and then ground in about 6 ml of appropriate buffer, in the cold room. When the entire 30 leaf pair sample was ground, the homogenates were pooled and centrifuged in a Servall RC2 centrifuge at 4°. The enzyme activity in the supernatant fraction was assayed immediately. In all cases, enzyme activities were substrate dependent and proportional to both assay time and protein concentration. In all cases, extracts of dark grown leaves and extracts of briefly illuminated leaves showed additive activity when mixed together.

Ribulose-1,5-diphosphate Carboxylase. A sample was ground in 0.05 M tris buffer pH 8.0 containing 0.2 mM EDTA, and centrifuged at 12,000 × *g* for 10 minutes. A modification of the method of Weissbach, Horecker, and Hurwitz (20) was used. Into a test tube were placed 0.1 ml of homogenization buffer, 0.1 ml of 0.005 M ribulose-1,5-diP (pH 6), 0.1 ml of extract, 0.1 ml of 0.25 M $NaH^{14}CO_3$ (0.04 $\mu c/\mu mole$). Ten minutes after the addition of the labeled bicarbonate, 0.1 ml of 10% perchloric acid was added. The tube was centrifuged and radioactivity of the supernatant solution determined.

Glyceraldehyde-3-phosphate Dehydrogenase. A sample was ground in 0.05 M tris buffer pH 8.5 containing 1.0 mM EDTA, and centrifuged at 20,000 × *g* for 10 minutes. A modification of the method of Wu and Racker (21) was used. Into a quartz cuvette of 1.0 cm light path were placed 0.1 ml of 0.4 tris buffer pH 8.5, 0.1 ml of 0.1 M $MgCl_2$, 0.1 ml of freshly prepared 0.4 cysteine (pH 8), 0.02 ml of phosphoglyceric phosphokinase (diluted to convert 1.6 μmoles of 1,3-diP-glycerate to glycerate 3-P per minute at pH 6.9 and 25°), 0.03 ml of 0.01 M NADH or NADPH, 0.52 ml of water. The mixture was preincubated with 0.05 ml of appropriately diluted extract for 4 minutes. The reaction was then started by the addition of 0.04 ml of 0.1 M ATP (pH 7) and 0.04 ml of 0.01 M 3-phosphoglyceric acid, and the decrease in optical density at 340 mμ was followed for 5 minutes.

Glycolic Acid Oxidase. A sample was ground in 0.1 M potassium phosphate buffer pH 8.0 and centrifuged at 2000 × *g* for 10 minutes. A modification of the methods of Tolbert and Burris (19) and Zelitch and Ochoa (22) was used. Into the main compartment of a 15 ml Warburg flask were placed 1.5 ml of 0.1 M potassium phosphate buffer pH 8.0, 0.3 ml of water, 0.1 ml of 3.0 mM FMN, 0.1 of 0.3 M KCN (pH 8), 0.5 ml of 0.04 M potassium glycolate (pH 7). Into the side arm was placed 0.5 ml of extract. The flask was equilibrated at 25° for 10 minutes, the contents of the side arm were tipped into the main compartment, shaking at 92 oscillations per minute resumed, and readings taken every 5 minutes for 35 minutes.

Glucose-6-phosphate Dehydrogenase. A sample was ground in 0.05 M tris buffer pH 7.5, and centrifuged at 20,000 × *g* for 10 minutes. A modification of the method of Kornberg and Horecker (11) was used. Into a quartz cuvette of 1.0 cm light path were placed 0.1 ml of 0.5 M tris buffer pH 7.5, 0.1 ml of 0.1 M $MgCl_2$, 0.3 ml of water, 0.2 ml of 0.003 M NADP, 0.2 ml of extract, 0.1 ml of 0.02 M glucose-6-P. The increase in optical density at 340 mμ was followed for 3 minutes.

Glutamic Acid Dehydrogenase. A sample was ground in 0.05 M tris buffer pH 7.5, frozen and thawed 4 times, and then centrifuged at 20,000 × *g* for 10 minutes. The supernatant fluid was passed through a Sephadex G25 column, and then enzyme activity was assayed by the method of Bulen (1). Into a quartz cuvette of 1.0 cm light path were placed 0.5 ml of 1.0 mM NADH, 0.2 of 1.5 M ammonium sulfate, 2.1 ml of extract plus 0.2 M tris buffer pH 8.15, 0.2 ml of 0.2 M potassium α-ketoglutarate. The increase in optical density at 340 mμ was followed for 2 minutes.

Results

The response of fresh weight, protein and RNA content of etiolated leaves to a single light stimulus was recorded to provide a background for the description of changes in particular enzyme activities. Figure 1 shows that all of these parameters increase indicating a general response to the stimulus.

Chloroplast Enzymes. Ribulose-1,5-diP carboxylase (RuC) and NADP-linked glyceraldehyde-3-P dehydrogenase (GAPDH) (6) were assayed at daily intervals following the brief illumination.

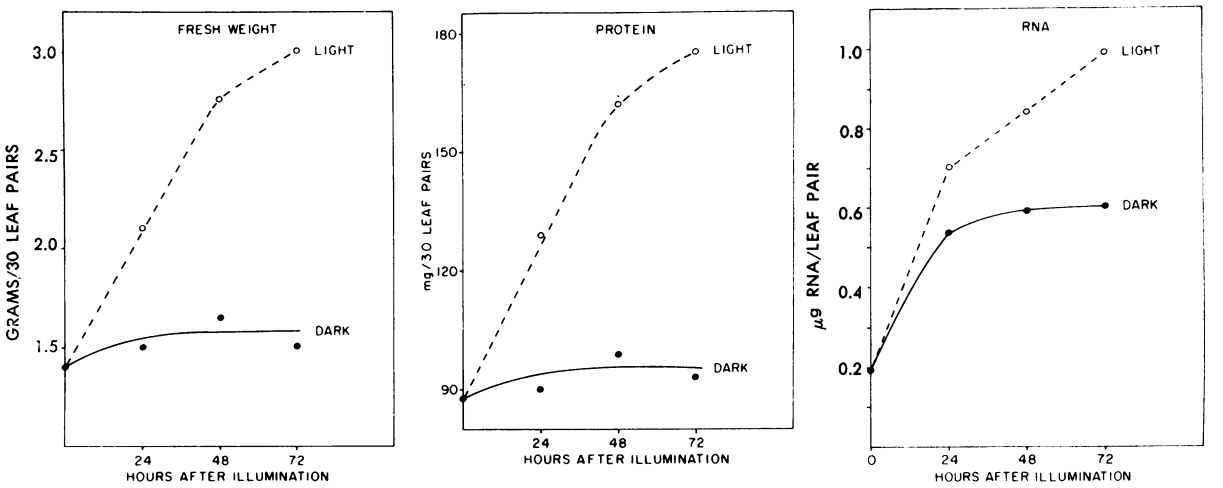


FIG. 1. Fresh weight, protein, and RNA content of leaves grown in the dark for 7 days (fr wt, protein) or 6 days (RNA), and then either kept in the dark (dark) or illuminated for 10 minutes with white light and then returned to the dark (light).

The NADP-GAPDH total activity (fig 2) remained constant in the dark grown seedlings, while the activity in the briefly illuminated seedlings increased steadily for 48 hours to 4 times the initial level. Total RuC activity (fig 3) also increased for 48 hours after the brief illumination. The new level of activity was about 2.5 times that present initially, while the dark control remained unchanged. In the period 48 to 72 hours after the illumination, the RuC activity decreased, but remained higher than the dark control.

Chloroplast associated enzymes thus showed an early response to illumination. However, the activities of the 2 enzymes which were assayed did not increase between 48 and 72 hours, even though the increase in overall protein content continued. Mitochondrial and soluble cytoplasmic enzymes were therefore also examined.

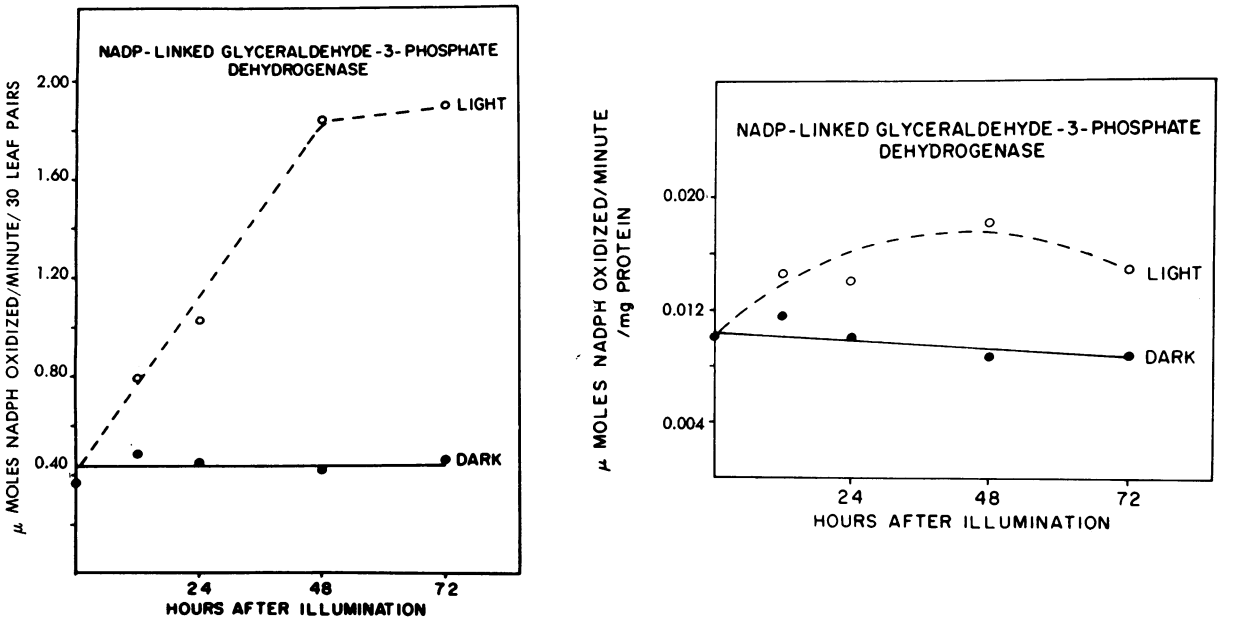


FIG. 2. Total activity (left) and specific activity (right) of NADP-linked glyceraldehyde-3-P dehydrogenase. Light treatment as in figure 1. (Reaction mixture contained in 0.1 ml 0.4 tris buffer pH 8.5, 0.1 ml 0.1 M $MgCl_2$, 0.1 ml 0.4 M cysteine, 0.02 ml phosphoglyceric acid phosphokinase, 0.03 ml 0.01 M NADPH, 0.52 ml water, and 0.05 ml extract. Following a 4 minute preincubation the reaction was started by the addition of 0.04 ml 0.1 M ATP and 0.04 ml 0.01 M 3-PGA. Optical density changes at 340 mµ were recorded for 5 minutes.) Values are the averages of 4 to 6 determinations.

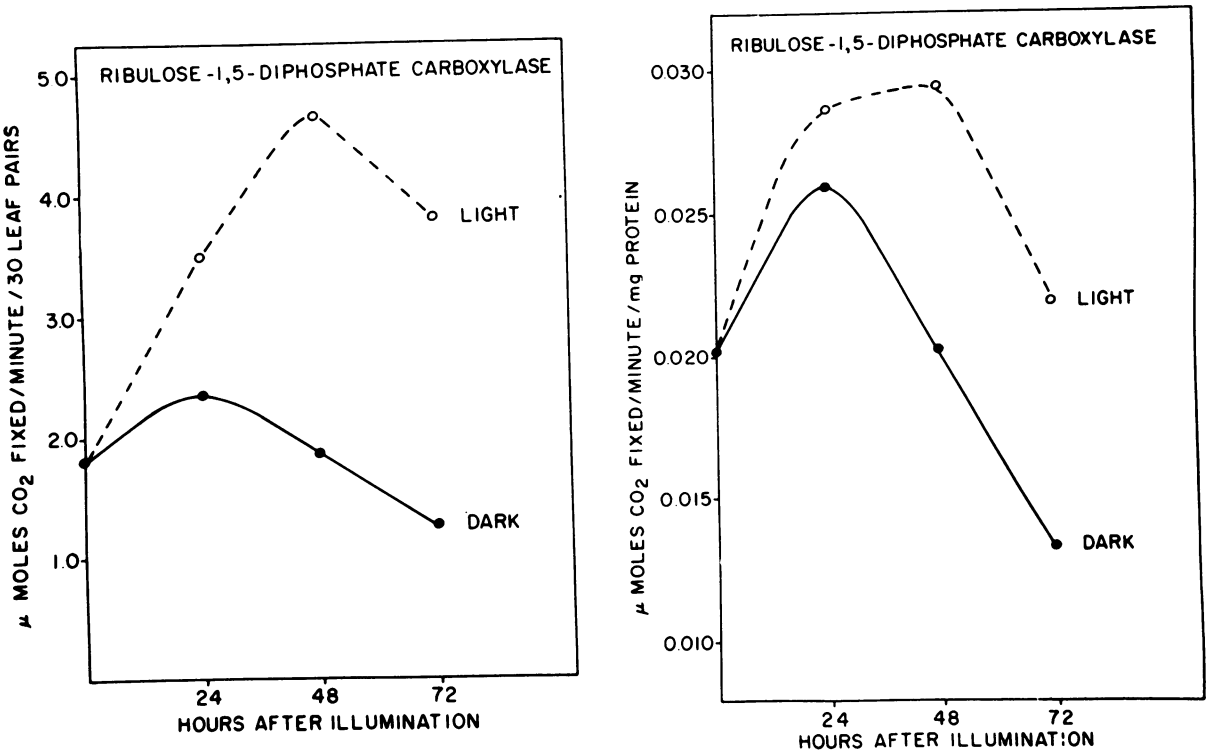


FIG. 3. Total activity (left) and specific activity (right) of ribulose-1-5-diphosphate carboxylase. Light treatment as in figure 1. [Reaction mixture contained 0.1 ml homogenization buffer, 0.1 ml 0.005 M ribulose-1,5-diP, 0.1 ml extract and 0.1 ml 0.25 M NaH $^{14}CO_3$ (0.04 $\mu c/\mu$mole). After 10 minutes 0.1 ml 10% perchloric acid was added, the mixture centrifuged and radioactivity of the supernatant solution determined.] Values are the averages of 5 to 8 determinations.

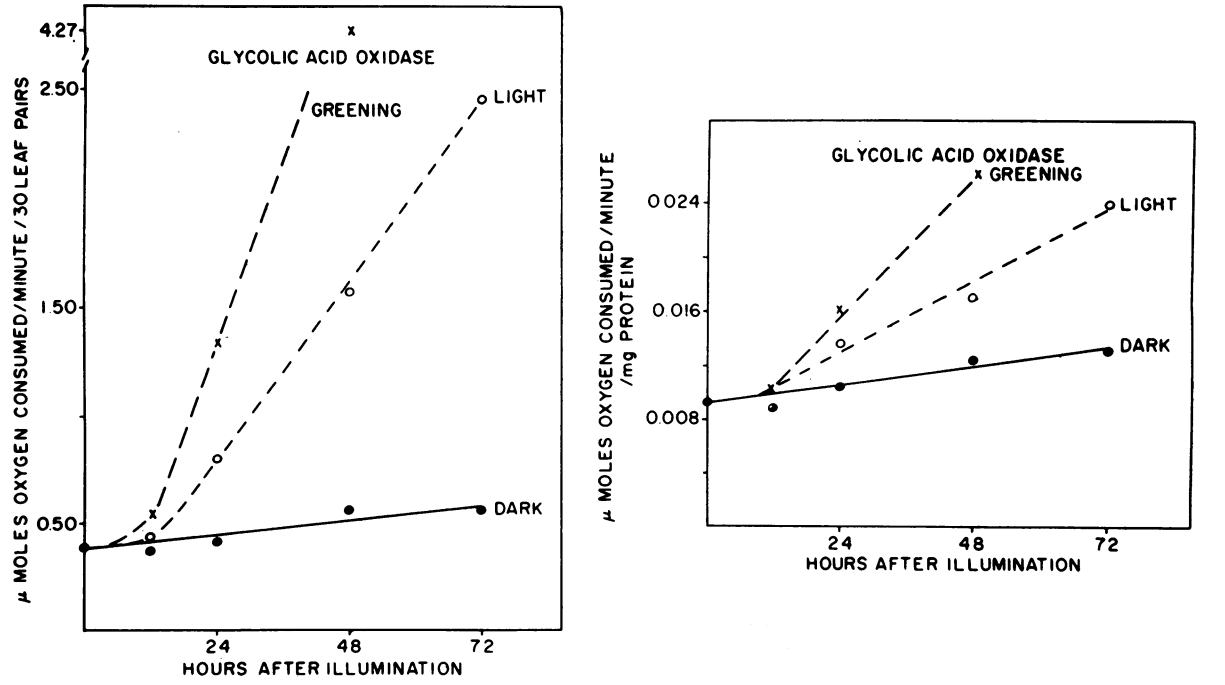


FIG. 4. Total activity (left) and specific activity (right) of glycolic acid oxidase. Light treatment as in figure 1, with the addition of a sample (greening) which was exposed to continuous white light 7 days after sowing. (Oxygen uptake was determined manometrically. Reaction mixture contained 1.5 ml 0.1 M phosphate buffer pH 8.0, 0.3 ml water, 0.1 ml 0.003 M FMN, 0.1 ml 0.3 M KCN, 0.5 ml 0.04 M potassium glycolate, 0.5 ml extract. Volume changes were observed at 25° for 30 min). Values are the averages of 2 determinations.

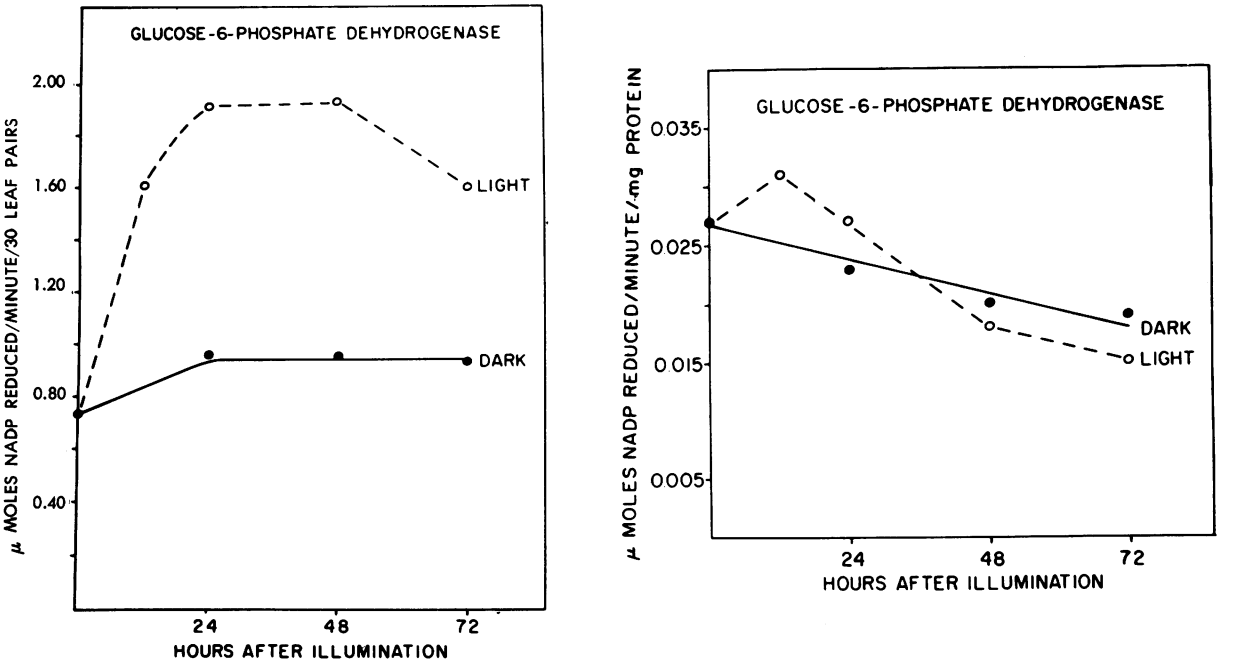


FIG. 5. Total activity (left) and specific activity (right) of glucose-6-P dehydrogenase. Light treatment as in figure 1. (Reaction mixture contained 0.1 ml 0.5 tris buffer pH 7.5, 0.1 ml 0.1 M $MgCl_2$, 0.3 ml water, 0.2 ml 0.003 M NADP, 0.2 ml extract and 0.1 ml 0.02 M glucose-6-P. Optical density changes at 340 mμ were recorded for 3 min). Values are the averages of 2 determinations.

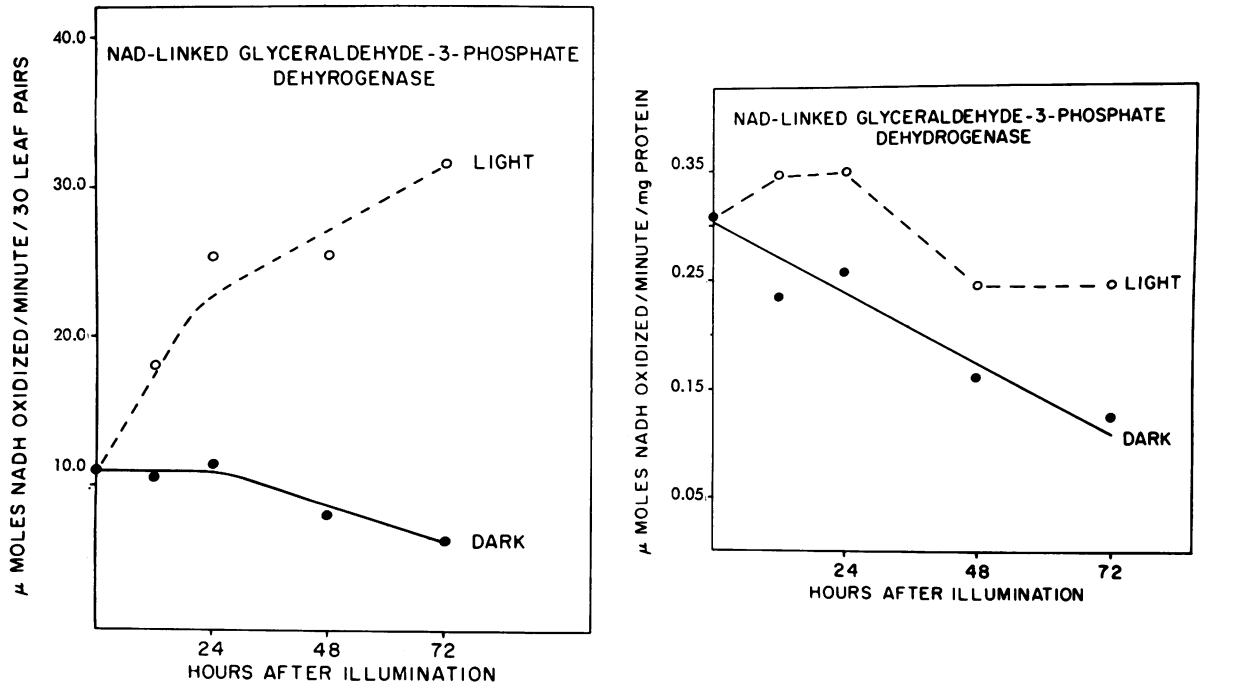


FIG. 6. Total activity (left) and specific activity (right) of NAD-linked glyceraldehyde-3-P dehydrogenase. Light treatment as in figure 1. (Assay as in figure 2 but with NAD instead of NADP in the reaction mixture.) Values are the averages of 4 determinations.

Soluble Cytoplasmic Enzymes. Glycolic acid oxidase (GAO), glucose-6-P dehydrogenase (G-6-PDH) and NAD-linked glyceraldehyde-3-P dehydrogenase (GAPDH) were assayed at daily intervals following a brief illumination.

The total activity of GAO (fig 4) increased only slightly during 3 days growth in the dark while the total activity of briefly illuminated seedlings increased more than 6 fold. The increase in activity began between 12 and 24 hours after the brief illumination. The changes in the specific activity (fig 4) paralleled the changes in total activity. A comparison of GAO activity of continuously illuminated (greening) leaves with activity of leaves given only the brief illumination is also presented in figure 4. Continuous light results in a greater increase in the activity of this enzyme.

Total G-6-PDH activity (fig 5) increased for only 24 hours. The specific activity (fig 5) showed an early peak at 12 hours, and dropped to a level below that of the dark controls.

NAD-GAPDH total activity (fig 6) decreased in the dark grown seedlings during the 3 day period examined, while the activity in the briefly illuminated seedlings increased 3 fold. The specific activity (fig 6) of these illuminated seedlings increased only slightly, but was about 2 times the specific activity of the control which had been decreasing.

Mitochondrial Enzyme. The mitochondrial enzyme chosen for assay was glutamic dehydrogenase (GDH) (17). After the brief illumination the total activity (fig 7) increased steadily during the 72 hour period investigated. However, the specific activity (fig 7) decreased steadily.

Requirement for Cell Division. A sampling of enzymes from the various cell compartments shows that the response to light is a general one encompassing the activation of many synthetic capacities. It is also clear that various enzymes are produced at different rates and after differing periods. This general but individually characteristic response makes questions about control mechanisms more intriguing.

The most general tissue response resulting in the increase of all cellular components is cell division. RuC was selected to investigate the degree of dependence of the increase in enzyme activity upon an increase in cell number (DNA). New cell formation would presumably be blocked with 5-fluoro-

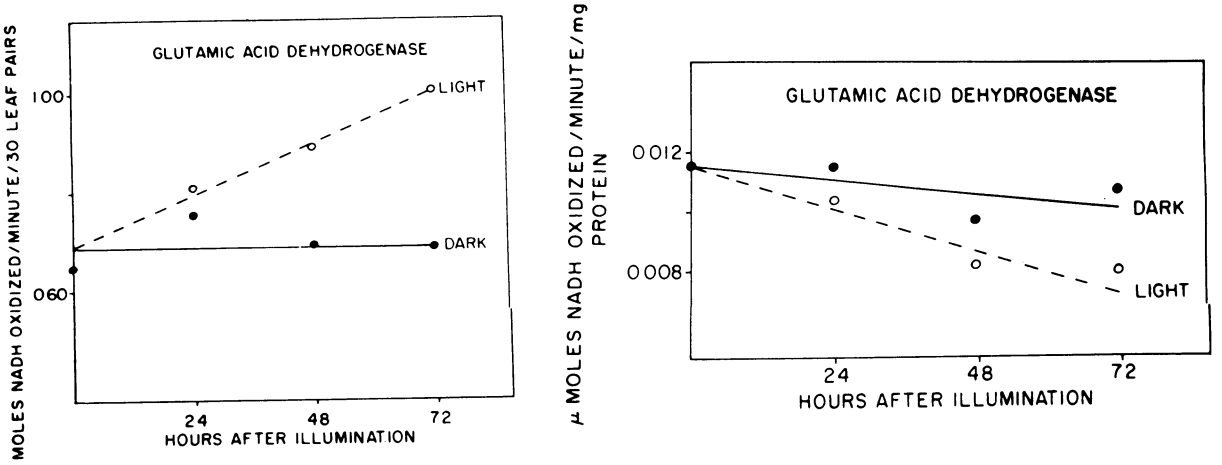


FIG. 7. Total activity (left) and specific activity (right) of glutamic dehydrogenase. Light treatment as in figure 1. (Reaction mixture contained 0.5 ml 0.001 M NADH, 0.2 ml 1.5 M ammonium sulfate, 2.1 ml of extract and 0.2 M tris buffer pH 8.15, 0.2 ml of 0.2 M potassium α-ketoglutarate. Optical density changes at 340 mμ were recorded for 2 min). Values are the averages of 3 determinations.

Table I. *Effect of FUdR on Increase in Ribulose-1,5-diphosphate Carboxylase Activity*

Treatment	Sample Age	RuC	DNA	Protein
	days	*μmoles CO_2 fixed/min 30 leaf pairs*	*μg*	*μg*
Dark	7	0.94	1470	106
Dark	9	1.17	1670	106
Illuminated on day 7[1]	9	2.60	2400	186
Illuminated[1] + FUdR on day 7[2]	9	2.30	1740	192

[1] Ten minutes light then returned to dark.
[2] Sprayed with 1.0 μM FUdR.

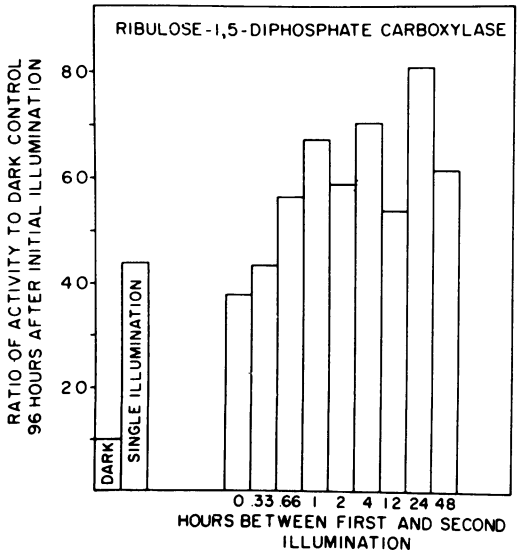
deoxyuridine (FUdR), an inhibitor of DNA synthesis (3). Seedlings were sprayed with one micromolar FUdR immediately after they were briefly illuminated. RuC activity was assayed 48 hours later (table I). The treatment did not block the light-triggered increase in enzyme activity, while it did inhibit DNA synthesis. This confirmed the conclusion drawn from the magnitude of the increase in DNA content (about 50 %), and from the similar estimated increase in cell number (4), that the higher level of enzyme activity represents a new level of the enzyme on a per cell basis.

Additional Illumination. To obtain information about where the limits of the controlling and/or responding capacities of the system lie, a second 10 minute illumination was given after various intervals. The initial 10 minute illumination was saturating with respect to the increase in RuC activity (fig 8), since illumination for 20 minutes had the same effect. If there was an interval of 40 minutes or more between 2 illuminations the seedlings had acquired the ability to respond to the second light treatment, as reflected in the appearance of additional RuC activity. The additional response was obtained with dark intervals ranging from 40 minutes to 48 hours between the 2 light treatments (fig 8). The magnitude of the response cannot be precisely determined from these data.

The relative rates of appearance of the additional activity is shown in figure 9. The interpretation of these experiments is complicated by the fact that the total RuC activity is decreasing in the dark (fig 3). The ratios increase while the total activities decrease. It is possible that the higher activity in the sample illuminated for a second time 24 hours after the first time represents a sustained higher rate of synthesis or that it represents a longer period of synthesis at the same rate as the once illuminated sample. The decrease in activity 48 to 72 hours after illumination is not due to complete cessation of enzyme synthesis because this decrease is not as fast as the decay of enzyme activity in the presence of cycloheximide, an inhibitor of protein synthesis (fig 11). It is probable that the higher activity of the doubly illuminated seedlings represents a higher rate of synthesis rather than a prolongation of the period of synthesis.

Far-red Effects. The requirement for the continued presence of the far-red absorbing form of phytochrome was investigated. The effect of far-red light, administered at various times after the initial illumination, on protein content and on RuC activity is shown in figure 10. Far-red light prevented some of the expected increase in protein content when it was given 4, 8, 12, and even 24 and 48 hours after the initial brief illumination with white light. Thus at no time within the first 48 hours does the newly acquired ability to synthesize more protein become stabilized in the briefly illuminated seedlings.

The kinetics of inhibition by far-red light was compared with the kinetics of inhibition by cycloheximide. Seedlings were illuminated with white light for 10 minutes when they were 7 days old. One day later 1 set of seedlings was illuminated with far-red light. At the same time another set of seedlings was sprayed with cycloheximide (200 μg/ml). This latter sample was resprayed 24 hours later. Protein increase and RuC activity were assayed 48 and 72 hours after the initial brief illumination (fig 11).



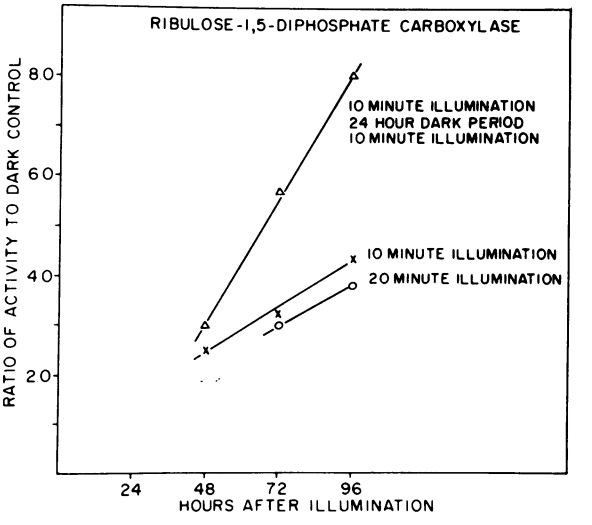


FIG. 8. (Left) Effect of the interval between the first and second 10 minute illumination on ribulose-1,5-diP carboxylase activity measured 96 hours after initial illumination.

FIG. 9. (Right) Effect of additional illumination on the increase of ribulose-1,5-diP carboxylase activity. In addition to the standard 10 minute illumination on day 7, a second 10 minute illumination was given either immediately (20 min illumination) or 24 hours later.

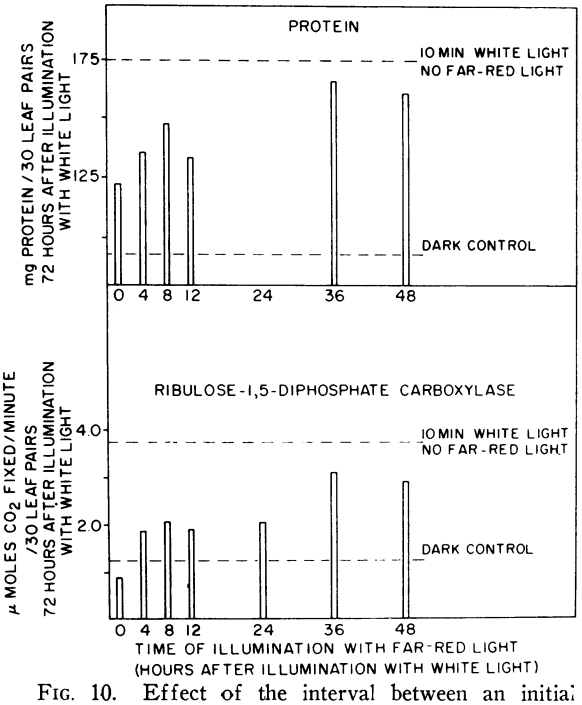


FIG. 10. Effect of the interval between an initial white illumination and subsequent far-red illumination on protein (top) and ribulose-1,5-diP carboxylase activity (bottom) determined 72 hours after initial illumination.

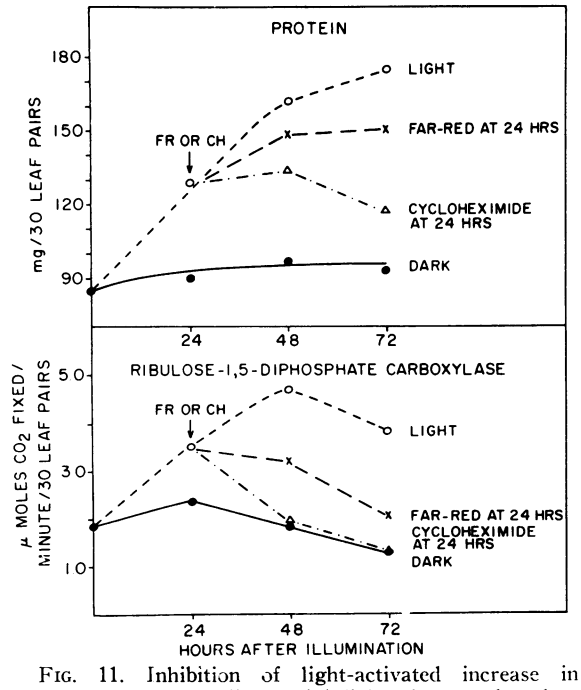


FIG. 11. Inhibition of light-activated increase in protein content and ribulose-1,5-diphosphate carboxylase activity by far-red illumination and cycloheximide treatment. Seven-day plants were illuminated for 10 minutes with white light and then returned to darkness (light). One sample was irradiated with far-red light (FR), another sprayed with a solution of cycloheximide (CH) 200 μg/ml.

Upon illumination with far-red light, net protein increase proceeded more slowly than in the control, and then it stopped. RuC activity decreased slowly for 24 hours, and then decreased rapidly in the next 24 hours In the presence of cycloheximide protein content increased slightly for 24 hours and then decreased. RuC activity decreased in the 48 hour period after the administration of the cycloheximide. The rate of loss of the activity was higher in the first 24 hours.

These experiments indicate that considerable net protein increase can occur for a definite but limited period after far-red illumination. Cycloheximide stops protein synthesis almost immediately. RuC activity falls as soon as protein synthesis stops. Apparently it is an unstable enzyme and the drop in total activity gives us a rough measure of this instability. The RuC activity of the far-red treated seedlings does not decline as precipitiously as that of the cycloheximide treated seedlings in the 24 hours after treatment. This indicates that there is synthesis of the enzyme for a while after far-red illumination. There is then a period of decline of activity at a rate comparable to that caused by the cessation of protein synthesis. The loss of the ability to synthesize RuC might be a function of the decay of the messenger RNA present at the time of far-red treatment.

Table II. *Inhibition of Increase in Glucose-6-phosphate Dehydrogenase Activity by Exposure to Far-red Light*

Sample	24 hr	48 hr
	μmoles NADP reduced per min per 30 leaf pairs	
Dark	1.55	1.44
10 min of white light	2.24	2.53
10 min of white light; far-red at 12 hr		2.16
10 min of white light; far-red at 24 hr		2.36

An effect of far-red light on enzymatic response of the seedlings to brief exposure to white light was also shown with G-6-PDH. The seedlings were illuminated with far-red light 12 or 24 hours after the initial illumination. The enzyme was assayed 24 and 48 hours after the initial illumination (table II). (These seedlings were grown from a different lot of seeds than those used previously. In contrast to the first seedlings, the G-6-PDH total activity continued to increase from 24 to 48 hours after the initial 10 minute illumination.) These results are similar to those with RuC if the enzyme is assumed to be more stable. Far-red light blocks the increase in activity, but not immediately.

Discussion

The primary leaf pair of etiolated bean seedlings is one of the sites in which a short period of illumi-

nation has morphogenic consequences. In response to light, there is renewed growth of the immature leaf. A doubling of fresh weight and protein occurs in the 72 hour period subsequent to the illumination.

The total activities of 6 enzymes, each of which was already present to some extent in the etiolated leaf, increased during growth in the dark subsequent to a 10 minute illumination. The range of the increases in total activity was from 1.5 fold (GDH) to 6.3 fold (GAO), with the other values intermediate at 2.6 fold (G-6-PDH, RuC), 3 fold (NAD-GAPDH) and 4.1 fold (NADP-GAPDH). Since the increase in activity was specific for each enzyme, and since the extent of the DNA increase (1.5 fold) was considerably less than that for 5 of the 6 enzymes which were assayed, it may be postulated that the changes were indicative of higher levels of enzyme activity on a per cell basis, rather than of an increase in the number of cells. A small population of new cells, with very high activities for these particular enzymes is an unlikely explanation since concentrations of FUdR which stopped 70 % of the expected increase in DNA content did not prevent either the net increase in protein content or the net increase in RuC activity following the brief illumination.

The mechanism by which light alters the synthetic machinery of leaf cells remains an intriguing problem. The generality of the response (increased cell division, increase in total protein) makes it safe to assume that most cellular regulatory mechanisms, including hormonal ones, become affected. These experiments demonstrate, however, that even after the signal for accelerated development has been partially implemented the entire process remains under phytochrome control. Increase in RuC activity remains reversible by far-red light even 48 hours after the initiation of changes. It is interesting to compare the longevity of physiological reversibility with the relatively rapid decay of spectrophotometrically demonstrable phytochrome (P730) reported by Klein *et al.* (10) in bean hypocotyl.

The difference in the rate of decline in enzyme activity after far-red and after cycloheximide treatment would suggest that the conversion of phytochrome acts not simply as a switch shutting off a terminal step of protein synthesis. Light reversal may instead affect an early step in the pathway leading to enzyme production allowing the slow exhaustion of already formed intermediates. Alternately the slow deceleration of synthesis following light reversal may be the reflection of a complex and indirect regulatory mechanism which requires time for its activation. Experiments designed to distinguish between these alternatives are in progress.

The significance of increased RuC activity following repeated illumination is not clear. Again two possibilities occur. Either the synthesis of red-sensitive phytochrome requires at least 40 minutes. or the initial illumination produces an increased capacity in the overall synthetic machinery to respond to additional stimulation.

A comparison of the level of GAO in briefly and in continuously illuminated seedlings shows that under both conditions the early response to light is quite similar, and therefore our experiments provide conditions which serve as a simplified model for several components of true greening.

Acknowledgments

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