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Light Dependent Increase of Triosephosphate Dehydrogenase in Pea Leaves[1]

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Summary. Data from 3 lines of investigation were presented indicating that chlorophyll is not necessary for the increase in the triphosphopyridine nucleotide-requiring triosephosphate dehydrogenase accompanying the illumination of etiolated pea plants. These include A) the kinetics of the development of chlorophyll and enzyme activity, B) the presence of enzyme activity in leaves grown in the dark on normal plants and C) the high specific enzyme activity in leaves of a chlorophyll-less mutant.

It was also shown that the light-initiated increase of enzyme activity continues for several days after removal from the light and that illumination with far-red light before the dark period inhibited, but did not abolish, this increase. The ability of green plants to continue to produce the enzyme in the dark was eventually lost with time, for after 7 days in the dark a stimulation in leaf protein formation was not accompanied by an increase in enzyme activity.

The action of light on etiolated plants results in the development of proplastids into mature chloroplasts. Associated with this development is an increase in chlorophyll (13), photosynthetic pyridine nucleotide transhydrogenase (8), fructose-1,6-diphosphatase (15), ribulose-1,5-diphosphate carboxylase (5) and the triphosphopyridine nucleotide requiring triosephosphate dehydrogenase (TPN-TPD) (4). Of the 2 triosephosphate dehydrogenases in plant tissues only TPN-TPD is peculiar to photosynthetic tissues (4), associated with the chloroplasts (6) and increased in response to illumination (4).

The development of TPN-TPD activity in *Euglena* parallels the development of chlorophyll (2), with both increases leveling off when the light source is removed. In higher plants it was found that these increases could be seen with red light illumination alone, and that they could be reversed with far-red light. These data have suggested a role for phytochrome in the development of chloroplasts. The investigation reported here was undertaken to gain more information on the role of light in the induction of TPN-TPD.

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Materials and Methods

Plant Culture. Laxton Progress variety of *Pisum sativum* was used throughout the entire investigation. Seeds were soaked for 24 hours in tap water at room temperature and sown between layers of moist paper towels. After 4 days they were removed and placed on coarse (6 mm) wire screen at room temperature with the roots suspended into constant flowing tap water. Plants were allowed to grow to experimental size without the addition of nutrient solution.

Lighting was provided by 4 30-w fluorescent bulbs placed 30 cm above the plants. Etiolated plants and plants returned to the dark were grown in a dark room with constant temperature and humidity. All manipulations in the dark room were carried out using a dim green safety light providing a maximum of 2 ft-c at the source. All light intensities were measured with a Weston meter. Far-red light was provided by passing light from a 250 w incandescent lamp (General Electric Infrared) through a far-red Filter (Carolina Biological Supply Company, Burlington, North Carolina, with a transmittance maximum at 750 mμ).

Analytical Methods. In all experiments samples of leaf material only were taken for assay. Leaves were removed, weighed and blended with 4.0 ml of a solution containing 0.1 M phosphate buffer and 0.03 M EDTA adjusted to pH 8.5. This was ac-

complished using the micro attachment of a Sorvall Omnimixer at 90 % of line voltage for 3 minutes. The homogenates were then centrifuged at 35,000 × *g* in a Sorvall RC-2 refrigerated centrifuge for 20 minutes. Aliquots of this supernatant material were used for enzyme assay and protein determination. In general the results presented represent the average of 4 or 5 determinations and variation is expressed as standard deviation.

The TPN-TPD activity of crude homogenates was measured in a system containing 2.0 ml assay mix (38 mM $Na_4P_2O_7$, 50 mM $Na_2HAsO_4 \cdot 7H_2O$ and 0.3 mM EDTA adjusted to pH 8.5), 0.2 ml of 4.8 mM TPN, 0.2 ml of 10 mM DL-glyceraldehyde-3-P, 0.5 ml H_2O and 0.1 ml of the crude homogenate supernatant fraction. Activity was then determined by measuring the increase in absorbancy at 340 mμ in a Beckman model DU spectrophotometer. Activity is expressed as units per g wet weight or units per leaf. A unit is defined as a change in absorbancy of 0.01 per minute. Initial velocities were measured as the time required for an absorbancy change of 0.05. The DPN-TPD was measured in a similar manner with DPN instead of TPN. DL-glyceraldehyde-3-P was prepared from the diethyl acetal barium salt (Sigma Chemical Company, St. Louis, Missouri) and assayed with an excess of DPN in the above system using crystalline rabbit muscle TPD.

Protein samples were precipitated from a 1.0 ml aliquot of the homogenate supernatant with 10 % trichloroacetic acid, washed once with hot 5 % trichloroacetic acid, dissolved in 0.2 N NaOH and determined by the method of Lowry et al. (10). Chlorophyll was determined by the method of Arnon (1) for leaf samples taken from light grown plants, or by the method of Koski, French and Smith (9) for etiolated and greening plants. The formula of Withrow et al. (16) was used in the latter method.

Results

Assay of the Dehydrogenase. It was found that TPN-TPD could be assayed spectrophotometrically in dilute homogenates of leaf material. There was a linear relationship between the rate of TPN reduction and the amount of the leaf homogenate supernatant fraction if homogenates were prepared within the range of 10 to 40 mg fresh weight of leaves per ml. To further insure valid enzyme activity measurements, the leaf weights were varied within this range so that the amount of enzyme was about the same in all samples of a given experiment. When homogenates were prepared using amounts of leaf material greater than the above range, the linearity between the rate of TPN reduction and the amount of homogenate was lost. Heating these homogenates of higher concentration at 60° for 2 minutes restored linearity under the assay conditions used. This is quite similar to the effects of heating noted by Margulies (12). He suggested that the heat treatment could have inactivated an enzyme which oxidizes TPNH or one which removes glyceraldehyde-3-P.

Effect of Light on the Development of Etiolated Plants. Plants were grown for 14 days in the dark. The top 15 cm of each plant was removed and placed in a test tube containing tap water. The tubes were then placed in racks and positioned under the light source so that all the plants received about 500 ft-c of light. Leaves were removed at 0, 8, 17, 25 and 85 hours, and enzyme activity, protein and chlorophyll were determined (fig 1). There was no increase in protein, presumably because the reserve material in the seed was no longer available to the plant. These results demonstrate a definite increase in enzyme activity without an equivalent rise in protein concentration. Chlorophyll values also increased with time, but there was a lag during the first 17 hours which was not seen with TPN-TPD activity. This differs from the results of Brawerman and Konigsberg (2) with *Euglena* which showed a lag period for both chlorophyll and TPN-TPD.

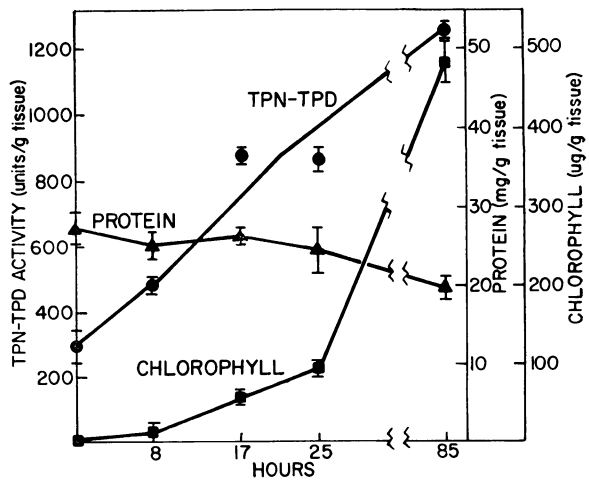

FIG. 1. Development of etiolated plants in the light. Determinations were carried out with leaves from cut tops of etiolated plants which had been placed in water under a 500 ft-c light source.

TPN-TPD Levels in a Chlorophyll-less Mutant. Fuller and Gibbs (3) have reported the presence of TPN-TPD activity in an albino mutant of barley, but the reported activity was several fold less than that found in etiolated plants in this laboratory. The discovery of an albino mutant permitted us to attempt to confirm their results under more favorable assay conditions. The leaves were sampled and compared to wild type, green leaves. The results are shown in table I. When chlorophyll was assayed according to the method of Arnon (1) a slight absorbancy was obtained with extracts of the mutant leaves. The acetone extracts of normal

Table I. *TPN-TPD Activity in Leaves of a Chlorophyll-Less Mutant*

Determination	Mutant leaves	Control leaves
TPN-TPD activity (units/g tissue)	3880	5450
Protein (mg/g tissue)	8.1	21.4
Chlorophyll (mg/g tissue)	<0.1	3.1

green leaves gave a sharp peak at 663 mμ, characteristic of chlorophyll. A similar extract of mutant leaves showed no such peak.

TPN-TPD activity in the mutant plant was about 70 % of the activity seen in the wild type, however, the protein was also lower in the mutant plant, so that the specific activity in the mutant plant was higher. These data indicates that TPN-TPD can develop normally in plants containing no detectable chlorophyll. Because mutant plants were detected only when plants were placed in the light, it was not possible to test the levels of TPN-TPD in mutant plants grown in the dark.

Continued Growth of Green Plants in the Dark. In preliminary experiments it was noted that the new leaves formed in the dark on light-grown plants appeared to be similar to etiolated leaves. Since further chlorophyll synthesis does not occur under these circumstances (17), the levels of TPN-TPD in these leaves were determined. Light-grown plants were moved to the dark and after 6 days of growth in the dark, measurements of protein, chlorophyll and TPN-TPD activity were made on leaves which had matured in the light, leaves which grew in the dark on the same plant and etiolated leaves which had no contact with light. These results are shown in table II. TPN-TPD activity measurements of the new leaves grown in the dark are intermediate between fully light- and fully dark-grown leaves. The level of TPN-TPD was significantly higher than that in etiolated leaves with a P value less than 0.01. This may indicate a net formation of enzyme in these leaves in the dark. The level for chlorophyll in these leaves was not significantly different from that in etiolated leaves, and this can be considered as further support for the hypothesis that chlorophyll is not required for the formation of high levels of TPN-TPD in leaves.

Development of Immature, Green Leaves in the Dark. Plants were grown in the light until the second leaf pair had reached a size about one-fourth to one-third that of a mature leaf. These leaves were small enough to allow most of their development to occur in the dark and large enough for analysis. A population of plants was chosen with uniform leaf size and placed in the dark. In some cases the upper leaf bud was removed, because it was found that this stimulated growth of leaves directly below the point of removal. Determinations of TPN-TPD activity, protein and leaf weight were carried out after 0, 2, 4 and 6 days in the dark. The results in table III show that total TPN-TPD activity continued to increase in the dark. Bud removal increased the magnitude and duration of this increase. The increase with bud removal was 3 to 4 fold after 6 days. In other experiments, comparable plants kept in the light showed up to 8 fold increases during the same period of time. Leaves allowed to develop in the dark under the above conditions did not increase in weight after

Table III. *Further Development of Immature, Green Leaves in the Dark, with and without Bud Removal*

Days in dark	Without bud removed	With bud removed
	Wet weight (mg/leaf)	
0 - 12 ± 1		
2	28 ± 4	46 ± 4
4	33 ± 4	62 ± 11
6	36 ± 6	62 ± 11
	Protein (mg/leaf)	
0 - 0.20 ± 0.03		
2	0.46 ± 0.01	0.87 ± 0.14
4	0.46 ± 0.09	1.21 ± 0.12
6	0.60 ± 0.20	1.18 ± 0.23
	TPN-TPD activity (units/leaf)	
0 - 23 ± 5		
2	37 ± 7	46 ± 4
4	45 ± 8	65 ± 9
6	43 ± 5	77 ± 14

Table II. *Development of New Leaves on Plants Moved from Light to Dark*

Determination	Light-grown leaves	New leaves in dark	Completely etiolated leaves
TPN-TPD activity (units/g tissue)	1380 ± 56	863 ± 102	540 ± 59
Protein (mg/g tissue)	22.2 ± 1.6	33.4 ± 1.7	40.0 ± 1.8
Chlorophyll (mg/g tissue)	1.57 ± 0.08	0.087 ± 0.057	0.044 ± 0.009

Table IV. *Leaf Development after Seven Days in the Dark*

Determination	7th day*	9th day	P**
Wet weight (mg/leaf)	89 ± 4	112 ± 9	<0.01
Protein (mg/leaf)	5.7 ± 0.8	8.3 ± 0.8	<0.01
DPN-TPD activity (units/leaf)	133 ± 17	174 ± 7	<0.01
TPN-TPD activity (units/leaf)	108 ± 14	105 ± 6	

* Buds were removed after light-grown plants had been in the dark for 7 days.
** Significance level of the difference between the mean value on the seventh and ninth days.

the sixth day. Further experiments were performed to determine whether or not TPN-TPD continued to increase after longer periods in the dark but under conditions where leafs were still growing. Plants were removed to the dark after the second leaf pair had developed to about one-half maximum size. After 7 days samples were taken for determinations of wet weight, protein, TPN-TPD activity and DPN-TPD activity. The tops of the plants were then removed to stimulate growth of the second leaf pair, and after 2 days the same parameters were measured. The results in table IV indicate that during the 2-day interval there had been significant increases in wet weight, protein and DPN-TPD activity but no increase in TPN-TPD activity. Thus, though TPN-TPD activity increases for several days after light-grown plants are placed in the dark, this increase does stop.

Effect of Far-Red Light on Leaf Development in the Dark. Plants were grown in the light and placed in the dark when the second leaf pairs were only a fraction of their mature size. One group was placed directly in the dark, while the other was given an 8 minute exposure to far-red light before the dark period. This was done by placing the plants in a light-sealed box covered by a far-red filter. A comparison of the increase in wet weight, protein and TPN-TPD activity in the far-red-treated and control plants is given in table V. The far-red treatment caused an inhibition of all 3 parameters. More interesting, however, is the fact that the increase in TPN-TPD activity is still 70 % that of control plants after the far-red-treatment. This suggests that an active phytochrome pigment is not required for the continued increase in TPN-TPD activity in the dark. Phytochrome does not appear to be specific for the development of TPN-TPD activity, for all parameters are inhibited to nearly the same extent by the far-red treatment.

Table V. *Effect of Far-Red Light on Leaf Development in the Dark*

Days in dark	Control	Far-red treatment
	Wet weight (mg/leaf)	
0 - 17 ± 2		
1	28 ± 3	26 ± 1
2	40 ± 5	30 ± 1
3	38 ± 4	30 ± 3
	Protein (mg/leaf)	
0 - 0.59 ± 0.07		
1	0.95 ± 0.11	0.83 ± 0.02
2	1.14 ± 0.12	0.95 ± 0.11
3	1.10 ± 0.07	1.04 ± 0.35
	TPN-TPD activity (units/leaf)	
0 - 26 ± 5		
1	46 ± 4	37 ± 2
2	62 ± 4	51 ± 4
3	66 ± 5	56 ± 7

Discussion

Because the action of chlorophyll in the light reaction leads to the production of TPNH and TPNH is a substrate of TPN-TPD, an increase of chlorophyll might be necessary for the subsequent induction of TPN-TPD. Results reported here do not support this idea. When etiolated plants were placed in the light, increases in chlorophyll lagged considerable behind increases in TPN-TPD. This is inconsistent with the idea that chlorophyll is required for the induction of TPN-TPD. Also, when light-grown plants were allowed to continue growth in the dark, the new leaves had a significant amount of TPN-TPD activity, but chlorophyll levels were not different from those in etiolated plants. Finally, when an albino mutant was assayed for TPN-TPD activity and chlorophyll, it was found that the enzyme was present at induced amounts but chlorophyll was absent.

When the reversibility of the light-induced increases in TPN-TPD activity was studied it was found that removal of the light source did not prevent a continued increase in activity for several days. After 7 days in the dark TPN-TPD activity was not increased under conditions where both protein and DPN-TPD rose significantly.

Interpreted in the light of the Jacob and Monod (7) theory this would suggest this light triggered

the synthesis of an inducer molecule which continued to exert its influence for several days after the light had been removed. After 7 days the supply of this effector molecule was depleted, and the repressor was free to block further synthesis of the enzyme. An alternate explanation requires that a messenger-RNA produced in the light be stable for several days in the dark promoting the synthesis of TPN-TPD.

The experiments of others (11, 14) have shown that increases in chlorophyll and TPN-TPD can be obtained with red light and inhibited with subsequent far-red light. This has been interpreted as indicating that phytochrome is the receptor pigment for light in these inductions. It was found here that the depletion of the active form of phytochrome by far-red light did not prevent the continued synthesis of TPN-TPD in the dark. If this continued synthesis is the reflection of an active structural gene for TPN-TPD, then phytochrome could not be the inducer. The far-red illumination did cause an inhibition of TPN-TPD development, but protein synthesis and leaf weight were inhibited to the same extent, so the specific activity of TPN-TPD remained at the induced level. Therefore, if phytochrome is the light-sensitive molecule, it must in turn bring about the formation of still another molecule which acts as the inducer for TPN-TPD.

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