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Phytochrome Control of Maize Leaf Inorganic Pyrophosphatase and Adenylate Kinase[1]

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Abstract. **Brief exposure of etiolated maize seedlings to light induces large increases in adenylate kinase and inorganic pyrophosphatase activity of the leaf in the following 48 hr in the dark. Red light is more effective than white or far red light, and far red reverses the effect of red light, indicating phytochrome control. Out of several tested, only these 2 enzymes appear to be coordinately induced, which is consistent with their close functional relationship. For inorganic pyrophosphatase, light treatment induces biosynthesis of a distinctive form of the enzyme characteristic of chloroplasts, readily separable from the enzyme characteristic of etiolated tissue.**

We have previously described (10) a potent and specific inorganic pyrophosphatase in maize leaf chloroplasts, and demonstrated that the level of this readily-solubilized enzyme activity depends upon the amount of light provided for the growing plant. The distribution and other properties of this enzyme are consistent with a role in the C_4 dicarboxylic acid pathway of CO_2 fixation (12) in the tropical grasses (reactions 1 and 2). In these plants the primary CO_2-fixing reaction, phosphoenolpyruvate carboxylase (reaction 1), is supplied

$$PEP + CO_2 \rightarrow \text{Oxalacetate} + P_i \quad \text{(reaction 1)}$$

$$ATP + P_i + \text{pyruvate} \rightleftarrows PEP + PP_i + AMP \quad \text{(reaction 2)}$$

with phosphoenolpyruvate (PEP) by reaction 2 (6), which has also been found in microbial systems (3, 9) and given the trivial name phosphate, pyruvate dikinase (9). The apparent role of the inorganic pyrophosphatase in maize chloroplasts is to drive reaction 2 toward synthesis of phosphoenolpyruvate by removal of the byproduct inorganic pyrophosphate. In this communication we present evidence that the synthesis of maize chloroplast pyrophosphatase is controlled by the photoreversible phytochrome system; that adenylate kinase, which like pyrophosphatase removes a byproduct of phosphoenolpyruvate synthesis (reaction 2), is similarly controlled; and that irradiation of etiolated maize seedlings induces the formation of a distinctive form of pyrophosphatase, readily separable from the form present in the etiolated seedlings.

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Methods

Maize seeds (*Zea mays*), Indiana Hybrid 682, were obtained from the Seed Laboratory of the Indiana State Seed Commissioner, and were used in all experiments for which data is presented. Maize seeds of the cross oh 45 × (Prl × lw_2 B37) F_2, specially selected to produce only albino plants, were obtained through the courtesy of Mr. Richard Ade. To obtain etiolated seedlings, seeds were soaked several hr in water, planted in vermiculite and sprouted in a dark room at 28 ± 4°. In some of the later experiments the seeds were sprouted in moistened rolls of "detoxicated" germination paper (Anchor Paper Company, St. Paul, Minnesota) at 30 ± 2° in light-tight plastic lined canisters. After 6 days growth in the dark, various light treatments were initiated, using approximately 15 seedlings for each experimental point.

At the indicated times shoots were excised at the node, the length was recorded, and the samples were frozen for analysis later. Control experiments indicated no loss of activity due to freezing. The frozen tissue was homogenized, using 1 to 2 ml of cold 50 mM tris acetate buffer (pH 7.5) per seedling, either by grinding in a chilled mortar with an equal weight of Superbrite glass beads (3M Company, St. Paul, Minnesota), or by homogenizing for 1 min in a chilled Waring Blendor. After filtration through nylon tulle, the extract was centrifuged 10 min at 10,000*g* and the supernatant layer was assayed promptly.

Light treatments, except for the experiments depicted in Fig. 1 and 3 which were as previously described (10), were carried out using a light-tight box containing four 300 watt Champion reflector

spot incandescent bulbs cooled by a fan. Irradiated material was placed 0.5 meter below the lamps. Length of irradiation (2, 3, and 4 min for white, red, and far red, respectively) was adjusted so that each treatment was with approximately 1.1 × 10^8 ergs cm^{-2} (Model 65 YSI-Kettering Radiometer, Yellow Springs Instrument Company, Yellow Springs, Ohio). This is approximately equal to the amount of energy in the 10 min white light exposure utilized in the experiments described in Fig. 1 and 3. Filters were Cinemoid, obtained from Kliegel Brothers, Long Island City, New York. For red irradiations 1 layer each of yellow (No. 1), and violet (No. 12) material was utilized; the far red filter consisted of 1 layer each of yellow (No. 1), red (No. 14), and blue (No. 62) material. After irradiation, plant material was returned to darkness at 30°, unless otherwise specified.

Inorganic pyrophosphatase, TPNH diaphorase, protein, and chlorophyll assays were as previously described (10). Acid phosphatase was assayed at pH 5.5 using 1 mM *p*-nitrophenyl phosphate as substrate in a total volume of 1 ml. After incubation for 30 min at 30°, the reaction was terminated by adding 3 ml of 1 M $NaHCO_3$, and the absorption of the solution was read at 410 mμ. Adenylate kinase was assayed by coupling with hexokinase and glucose 6-P dehydrogenase (13).

Results

The response of several different physiological parameters on exposure of etiolated maize seedlings to varied regimens of white light is illustrated in Fig. 1. Responses were measured 48 hr after initiation of the light treatment because previous work indicated maximum response at that time (10), and were compared for dark controls, continuous illumination, and 10 min illumination followed by darkness. The results are indicative of several qualitatively different types of response to light treatment.

As previously described, a brief light exposure stimulates inorganic pyrophosphatase activity several fold, and the effectiveness of the brief light treatment approaches that of continuous illumination. In contrast, non-specific acid phosphatase, a cytoplasmic enzyme (10), is relatively unaffected by light. The level of extractable protein responds less than inorganic pyrophosphatase but more than acid phosphatase; thus the specific activity of the latter enzyme actually decreases on light treatment, while the specific activity of pyrophosphatase increases. The specific activity of inorganic pyrophosphatase in the samples which received 10 min of light was virtually identical with those which were illuminated continuously, indicating the qualitatively similar response of this activity in the dark following a light treatment to the response to continuous light. Distinctively different response patterns are shown by

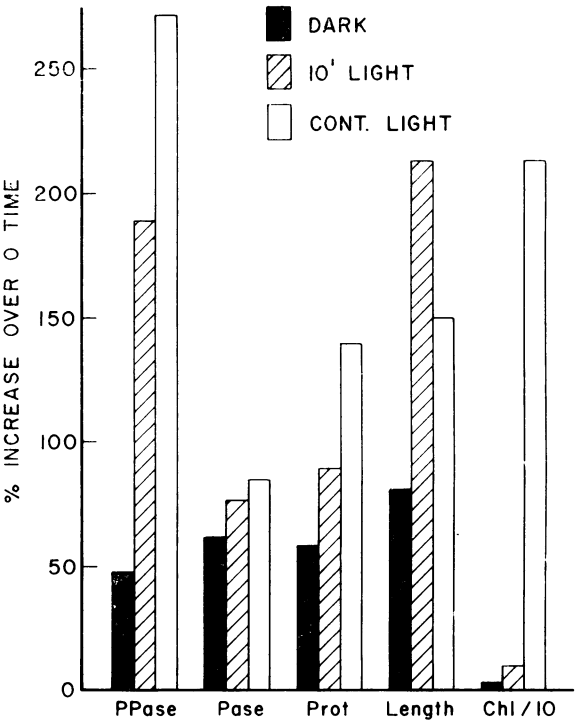

FIG. 1. Comparison of physiological responses to varied light treatments. Six day old etiolated maize seedlings (0 time sample) were given either continuous illumination (white light), 10 min illumination followed by darkness, or continuous darkness, as previously described (10). After 48 hr the various parameters were determined and compared with the 0 time sample. PPase, inorganic pyrophosphatase; Pase, acid phosphatase; Prot, protein; Chl/10, 10 % of the observed value for chlorophyll.

chlorophyll, which requires continuous light, and stem elongation, which is preferentially enhanced by the brief light exposure.

The above effects of a brief light exposure, as well as the previously-reported sensitivity to light of levels of activity in albino maize mutants (10), suggest that in addition to an energy source, light serves as a functional control of pyrophosphatase activity through the phytochrome system. Such systems are characterized by their sensitivity to red light and reversibility by far red light (7). Fig. 2 documents the response of inorganic pyrophosphatase activity of etiolated maize seedlings in the 48 hr period in the dark following treatment with various combinations of white, red, and far red light. Red light is far more effective than the equivalent amount of energy administered as white or far red light. In this experiment the increase in pyrophosphatase activity following a 3 min exposure to red light was approximately 10 fold higher than the corresponding increase in the dark control. Moreover, the effect of red light was completely reversed by far red light

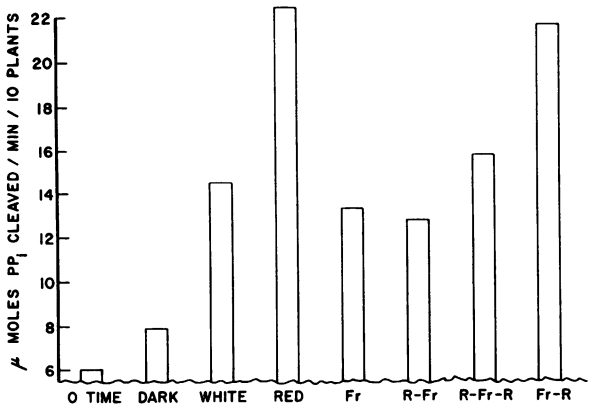

FIG. 2. Response of inorganic pyrophosphatase activity to approximately equivalent amounts of energy as white, red, and far-red light; and antagonism of red and far-red light. Six day old etiolated maize seedlings were irradiated for 2 to 4 min as described in Methods, then kept in darkness for 48 hr at which time the activity was determined and compared with the value obtained from sample taken at the time of irradiation. Multiple exposures of a single sample were performed no more than 10 min apart. R, red; Fr, far-red.

applied immediately afterwards, but far red irradiation before red light treatment did not diminish the red light effect. Although complete photoreversibility of the system was not observed during the second cycle (R-Fr-R), the observed effects are in general consistent with those expected of a system controlled by phytochrome. Similar, but limited, experiments with etiolated albino seedlings also demonstrated considerable stimulation of inorganic pyrophosphatase activity in the dark period following the same brief exposure to red light, suggesting that the phytochrome system is functional in these plants.

The increase in pyrophosphatase activity in the dark following a brief light exposure could be due either to synthesis of new enzyme protein or to activation of a previously existing enzyme. Examples of both types have recently been reported. Rapid activation by light (but not necessarily mediated by phytochrome) of the functionally related enzyme, phosphate, pyruvate dikinase, has been observed in the leaves of *Amaranthus palmeri* (11). Phytochrome-controlled increases in the activity of several enzymes in dark-grown bean seedlings have been demonstrated to require protein synthesis (4), and to occur at rates comparable to those observed here (see Fig. 4).

Several observations reported below support the idea that the photoprocess described here is induction of enzyme synthesis rather than activation. After several abortive attempts to observe effects of inhibitors of protein biosynthesis by floating excised shoots on buffer solutions containing the inhibitors, it was found that injection of chloramphenicol or cycloheximide into the node of etiolated maize seedlings just prior to irradiation almost completely

Table I. *Effect of Inhibitors of Protein Synthesis on Response of Pyrophosphatase Activity to Brief Light Treatment*

Three groups of 6 day old etiolated maize seedlings were injected at the node, with a microliter syringe, with 5 μl of 2 mM chloramphenicol, 2 mM cycloheximide, or water, respectively, and then irradiated 3 min with red light. After 48 hr in darkness, the pyrophosphatase was compared to 0 time and 48 hr dark non-injected controls.

Sample	Units of pyrophosphatase activity per 10 plants: Total	Δ
0 Time	5.38	..
48 Hr dark control	8.98	3.60
48 Hr irradiated, injected with		
Water	11.83	6.45
Chloramphenicol (10 mμmole)	9.18	3.80
Cycloheximide (10 mμmole)	3.17	—2.21

inhibits the response to irradiation, and can even cause loss of activity (table I). Pyrophosphatase activity of etiolated seedlings excised just below the node and floated on 10 mM citrate, pH 5, is not responsive to a brief light exposure, but a normal increase in activity is observed in continuous light (Fig. 3). Thus if the increased activity is due to activation of previously existing enzyme, then some additional factor supplied through the stem is necessary for the activation to occur, and activation can be prevented by inhibitors of protein biosynthesis. More likely the process is induction, with synthesis

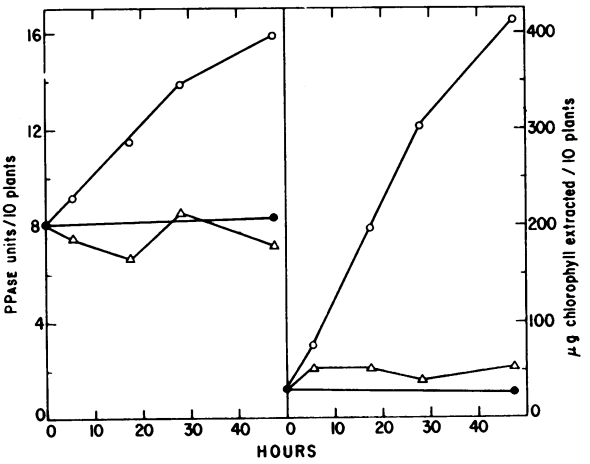

FIG. 3. Induction by light of inorganic pyrophosphatase activity in excised seedlings. Six day old etiolated seedlings were excised in dim light just below the node and floated on 20 ml of 10 mM citrate, pH 5, in a 14 cm Petri dish. Irradiations and assays were done as in Fig. 1. ○, continuous illumination; △, 10 min illumination followed by darkness; and ●, continuous darkness.

of new enzyme requiring an energy source supplied either as metabolite through the stem or by photosynthesis under conditions of continuous illumination.

The increase in pyrophosphatase activity following a brief illumination occurs in the dark over a period of several hr (10), with only a slight lag period (see Fig. 4). There is, however, a definite period beyond which reversal by far red light is no longer possible. Table II summarizes the effect of delaying the far red treatment for various times after treatment with red light. Between one-half hr and 2 hr after irradiation with red light the system has been "committed" to synthesis of new enzyme, and subsequent far red treatments stimulate, rather than oppose, the effect of red light. It has repeatedly been observed that once pyrophosphatase activity has been induced, it is stable indefinitely in the dark, and does not diminish due to reversal of some "activation" process, or to lack of resynthesis in a system in which the enzyme is turning over.

It has been found that other factors, some as yet unknown, can also initiate the induction process, apparently independent of the effect of light. Occasional batches of etiolated seedlings appear to have been induced prior to, or independent of, irradiation. In Fig. 4 is presented an experiment in which heat, in the absence of light, appeared to serve as an effective inducer. Incubation at 37°, rather than 30°, resulted in not only a more rapid response to irradiation than at 30°, but also, after a long lag, increased the pyrophosphatase activity of the dark control to a level equivalent to that observed after a brief exposure to red light. Hendricks and Borthwick have suggested that the primary effect of phytochrome irradiation is on membrane permeability (8), and it seems possible that the selective permeability of highly organized biological membranes might also be altered by other factors including temperature changes. In the present case, an increase in membrane permeability could be conceived of as initiating the induction process by permitting contact between a chemical inducer and the inducible system.

Table II. *Reversal of Effect of Red Light by Far Red Light*

Four groups of 6 day old etiolated seedlings were exposed to red light for 3 min and returned to darkness. At the indicated times single groups were exposed to far red light for 4 min, then returned to darkness. Pyrophosphatase activity was determined 48 hr after the initial (red) irradiation, and compared to control groups which had no light at all or red light only.

Time of far red irradiation (after red irradiation)	Δ Activity (compared to dark control)
Omit far-red	0.75
½ Hr	—0.14
2 Hr	0.96
8 Hr	1.67

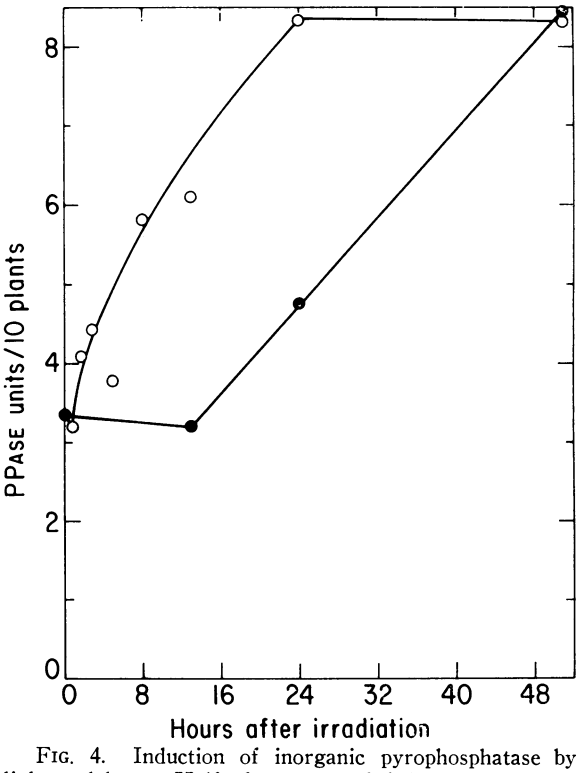

Fig. 4. Induction of inorganic pyrophosphatase by light and heat. Half of a group of 6 day old seedlings grown at 30° were exposed to red light for 3 min, and the entire group was transferred to a 37° room in the dark. Samples were taken at the times indicated. ○, exposed to red light; ●, dark controls.

It has been suggested that adenylate kinase and inorganic pyrophosphatase serve to drive reaction 2 toward synthesis of phosphoenolpyruvate by removal of by-product AMP and pyrophosphate (5). If this process represents a major metabolic role of these enzymes, their close functional relationship might be reflected in similarities in distribution, intracellular location in the chloroplasts, and control by the phytochrome system. The distribution of a potent adenylate kinase activity parallels that of inorganic pyrophosphate (5), and both are located in the chloroplasts (6).

The sensitivity of adenylate kinase activity of etiolated maize seedlings to a brief red light treatment is compared to that of pyrophosphatase, unrelated enzymes, and other physiological parameters in Fig. 5. The activity of adenylate kinase is stimulated almost as greatly as that of pyrophosphatase, and to a far greater extent than the cytoplasmic enzyme, acid phosphatase, or the chloroplast enzyme (10), TPNH diaphorase. These observations are entirely consistent with phytochrome control of adehylate kinase, and emphasize the specific nature of the photoresponse. Continuous white light has recently been reported to elicit a similar selectivity of response (6).

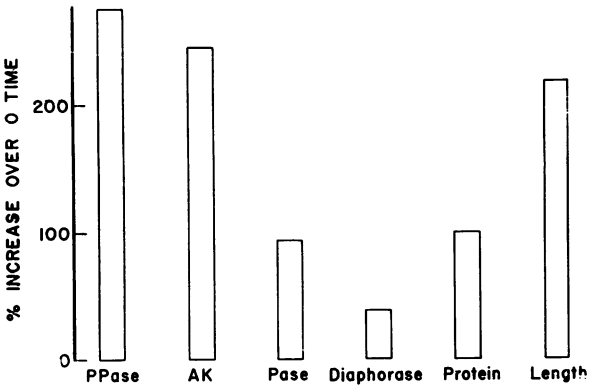

FIG. 5. Alteration in levels of several parameters in response to red light. Six day old etiolated seedlings were irradiated for 3 min with red light, returned to darkness, and assayed 48 hr later as described in Methods. Values reported are compared with values obtained from samples taken at time of irradiation. PPase, inorganic pyrophosphatase; AK, adenylate kinase; Pase, acid phosphatase.

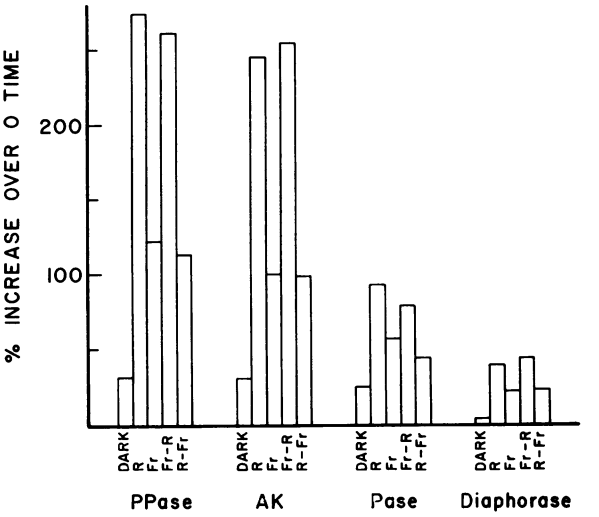

FIG. 6. Patterns of response of several enzymes from maize leaf chloroplasts and cytoplasm to varied light treatments. Irradiation conditions are described in Methods; multiple irradiations were performed within 10 min. All other conditions as in Fig. 5. Symbols are as defined in Fig. 2 and Fig. 5.

The patterns of response of these enzymes to varied light regimens are presented in Fig. 6. Under a variety of conditions inorganic pyrophosphatase and adenylate kinase show virtually identical responses, distinctively different from other chloroplast or cytoplasmic enzymes. The similarity of the responses of the 2 enzymes, which is consistent with, but not proof for, their proposed functional relationship, suggests that they share common cellular controls; *i.e.*, are subject to coordinate induction. In contrast, several maize leaf enzymes involved in the classical Calvin pathway of CO_2 fixation, which may be less important than the phosphoenolpyruvate carboxylase pathway in maize (12), appear not to be coordinately induced by light (1).

Aqueous extracts of maize leaves subjected to gel elcctrophoresis or DEAE-cellulose column chromatography contain 2 major components of inorganic pyrophosphatase activity. Column chromatography of extracts of both etiolated and light-grown plants of the same age (Fig. 7) indicates that only 1 of these forms of the enzyme is subject to control by light. The results suggest that the form eluted first from the column is the chloroplast enzyme, induced by light, and that the other, non-inducible or constitutive form, is a cytoplasmic enzyme found in both etiolated and normal plants. Previous studies of maize leaf pyrophosphatase (10) were done on extracts of somewhat older light-grown plants in which the great bulk of the activity is due to the inducible form. Comparison of the properties of the 2 forms of the enzyme are currently underway in our laboratory.

Comparison of the effects of chloramphenicol and cycloheximide on the induction of inorganic pyrophosphatase are of interest with regard to the mode of action of these antibiotics. Cytoplasmic ribosomes are insensitive to chloramphenicol but sensitive to

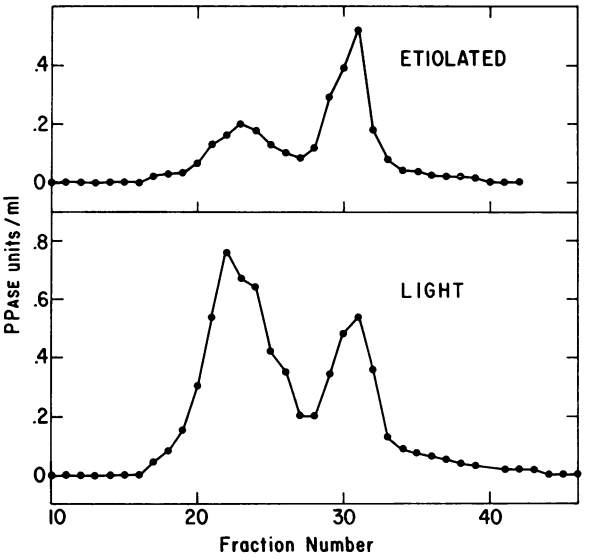

FIG. 7. Column chromatography of extracts from etiolated and light-grown plants. Five grams of tissue from 7 day old maize seedlings, either etiolated plants excised at the node (upper curve) or leaf tissues from plants grown in the light as previously described (10) (lower curve), were homogenized for 1 min in a Waring Blendor with 25 ml of cold 50 mM tris acetate, pH 7.5, which contained 2 mM PP_i. After centrifugation for 10 min at 10,000*g*, 20 ml of the supernatant layer was applied to a 1 × 14 inch column of DEAE cellulose which had been equilibrated with 50 mM tris acetate, pH 7.65. The column was eluted with a linear gradient of 0.02 M $MgCl_2$ to 0.12 M $MgCl_2$, each dissolved in 500 ml of 50 mM tris acetate, pH 7.65. Fraction size was 10 ml, and flow rate was 2 ml/min. Recovery was 90 to 100 % of applied pyrophosphatase activity.

cycloheximide, and chloroplast ribosomes have the opposite sensitivity (2). Thus the almost complete inhibition of the response to light after injection of chloramphenicol (table I) suggests that the light-induced inorganic pyrophosphatase is located in the chloroplasts, in agreement with earlier studies on the intracellular distribution of the enzyme (10), and that the cytoplasmic enzyme is unaffected by this antibiotic. The smaller increase in pyrophosphatase activity which occurs during the same 48 hr period in plants not exposed to light is probably due to continued synthesis of the cytoplasmic enzyme, which is effectively inhibited by cycloheximide (table I). The residual activity 48 hr after cycloheximide treatment and irradiation is nearly equivalent to the increment of increased activity in the irradiated, water-injected controls. This suggests that the cytoplasmic enzyme "turns over", and that because its resynthesis is inhibited by cycloheximide it has been completely lost after 48 hr, whereas normal amounts of chloroplast enzyme are induced by light and synthesized in the presence of cycloheximide. This hypothesis is currently under investigation in our laboratory.

Acknowledgments

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