Plant Physiol. (1969) 44, 655-661

Initial ATP Formation, NADP Reduction, CO_2 Fixation, and Chloroplast Flattening Upon Illuminating Pea Leaves[1]

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Received December 31, 1968.

Abstract. **Chloroplasts in living cells of detached and sectioned leaves of *Pisum sativum* had a thickness of 2.68 ± 0.04 μ in the dark as determined from photographs made using a phase contrast microscope. Upon illumination with 4000 lux for 10 min, the chloroplasts flattened to 2.15 ± 0.04 μ. There was a short lag period of about 11 sec at 1000 lux and 2 sec at 4000 lux before appreciable light-induced flattening occurred. Both ATP and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in detached pea leaves increased upon illumination and then fell during the initial 60 sec. The maximum ATP level was attained in 16 sec at 1000 lux and 10 sec at 4000 lux, while NADPH required about twice as long to reach a maximum. A sustained rate of carbon dioxide fixation occurred after a lag period coinciding in time with the drop in the NADPH level. ATP appeared to be involved not only with carbon dioxide fixation, but also with some reaction beginning sooner, perhaps the light-induced chloroplast flattening. Considering the initial photophosphorylation and the sustained CO_2 fixation rates, the ATP formation rate *in vivo* apparently increased after the leaves had been in the light for a few min.**

Chloroplasts in many plants become reversibly flattened in the light. Such changes were first reported in 1942 by Bunning for chloroplasts in *Nymphaea, Nicotiana,* and *Phaseolus* (3). Light-induced flattening of chloroplasts in *Selaginella* were described by Busch in 1953 also using a light microscope (4). More recently, Kushida, Itoh, Izawa, and Shibata (10) using spinach and Hilgenheger and Menke (7) studying *Nitella* have observed chloroplast flattening in the light *in vivo* by means of electron microscopy. For rapidly isolated pea chloroplasts (11), the associated change occurring in the plant cell is retained during isolation and can be precisely measured (12-14). When such chloroplasts are suspended in sucrose solutions at the same osmolality as the cell sap, the volumes are 29.0 μ^3 for the light and 35.3 μ^3 for chloroplasts from plants in the dark (15). An action spectrum for the light-induced decrease of chloroplast volume *in vivo* has a shoulder at 700 to 715 nm, suggesting that photosystem I alone can support the conformational change. The uncouplers, *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (tri-Fl-CCP) and nigericin, introduced through the cut stems or directly injected into the plants, greatly reduce the volume decrease occurring *in vivo* (14). These results indicate that light energy trapped as ATP or a high-energy state is involved in changing the conformation of the chloroplasts. If this change had no effect on the plant, it would be of only minor interest. However, the light-induced flattening of chloroplasts *in vivo* has been associated with increases in photosynthetic oxygen evolution (20) and in endogenous photophosphorylation by the isolated chloroplasts (12). Not only is light necessary for photosynthesis, but also the light-induced conformational change of chloroplasts apparently increases the rate of certain reactions.

The present experiments focus on metabolic events occurring during the first min of illumination of pea leaves. Using rapidly isolated chloroplasts, the light-induced volume decrease at 2000 lux was found to have a half-time of 3 min (13). However, the "rapid" isolation took 2 min and hence changes occurring in the first few min could not be well defined. Here, the light-induced decrease of chloroplast volume was investigated using phase contrast microscopy of living cells. Since the same chloroplast could be photographed many times at successive intervals, the initial kinetics of chloroplast flattening in the light could be precisely followed. Next, changes in ATP and NADPH in the leaf during the first min at 2 light intensities were determined. Finally, the amount of CO_2 concomitantly fixed was measured. ATP and NADPH increased rapidly in the light, while the light-induced chloroplast flattening had a short lag period and the CO_2-fixation began after an appreciable lag.

[1] This investigation was supported by Public Health Service Research Grant GM 15183 from the National Institute of General Medical Sciences.

Materials and Methods

Pisum sativum "Blue bantam" (W. Atlee Burpee Co., Riverside, California) was grown at 20° and 50 % relative humidity in moist vermiculite (11). A light intensity of 2000 lux was provided for 12 hr each day by fluorescent tubes. The plants averaged 10 cm in height when the leaves were removed during the light portion of the daily cycle on the fourteenth day.

For studies on chloroplast flattening, the leaves were infiltrated with water to replace the air in the intercellular spaces, thinly sliced, and placed on a microscope slide. A living mesophyll cell with protoplasmic streaming was brought into focus using a phase contrast Zeiss research microscope, and then the light was extinguished. After 30 min in the dark, the tungsten microscope lamp (previously calibrated in lux) was turned on and used as an actinic as well as photographic light. Pictures were taken of the cell at various intervals using exposure times from one-half sec at 300 lux to one-twenty-fifth sec at 4000 lux. The light intensity was varied by using combinations of neutral density filters. "Dark" refers to photographs taken immediately after the microscope light was turned on. Positives were prepared at a final magnification of about 2500. For calibration, a stage micrometer was photographed. Thickness was directly measured for chloroplasts observed edge-on, while area was determined by tracing the outlines of top views of chloroplasts and weighing the circumscribed paper.

For the ATP and NADPH studies, leaves were stored in the dark for 30 min before use. Then approximately 0.3 g of leaves was incubated for the indicated times at 25° with 1000 or 4000 lux provided by a tungsten reflector flood lamp. Incubation was terminated by immersing the leaves in liquid nitrogen. For ATP determinations, the frozen leaves were ground in a chilled mortar, and then homogenized in 0.6 N $HClO_4$ using a "Virtis" mixer for 2 min at top speed. Following centrifugation at 30,000*g* for 30 min, ATP in the supernatant fluid was determined enzymatically (components obtained from C. F. Boehringer and Soehne GMBH, Mannheim, Germany). The ATP was used to convert 3-phosphoglycerate to 1,3-diphosphoglycerate in the presence of phosphoglycerokinase. This product was converted to 3-phosphoglyceraldehyde *via* phosphoglyceraldehyde dehydrogenase and the concomitant oxidation of exogenous NADH followed at 340 nm using a Cary Model 14 recording spectrophotometer (any endogenous NADH or NADPH was experimentally shown to be oxidized by the $HClO_4$ used for extraction). For NADPH determinations, the ground frozen material was extracted in 0.1 N tris-HCl (pH 8.8), centrifuged as above, and the optical density of the supernatant fluid measured at 340 nm (the extinction coefficient used was 6.22×10^6 cm^2/mole of reduced pyridine nucleotides). A difference spectrum obtained using extracts from leaves in the light *vs.* the dark had a peak near 340 nm and the light-induced change could be reversed by the addition of oxidized N-ethylphenazonium ethosulfate to the extracts (the change at 340 nm reflects a pool of reduced compounds produced in the light and may actually include more than just NADPH). Calibration was checked by adding known amounts of ATP or NADPH to the leaf extract and then performing the respective assays.

For the CO_2 fixation studies, detached pea leaves (0.2–0.3 g) were placed in a chamber and $^{14}CO_2$-labeled air with about 11,000 dpm/ml was passed through at a flow rate of 70 ml/min. The level of CO_2 was 0.030 % as determined with a Beckman infrared gas analyzer (the same level as for the ATP and NADPH studies). The balance of the gas phase was 21 % O_2 and 79 % N_2. After equilibrating for 30 min, the leaves were illuminated for the indicated times and then plunged into liquid nitrogen. Leaves were placed on planchets and radioactivity was determined with a Geiger-Muller counter. Also, some leaves were extracted and the incorporated counts were determined by scintillation techniques using internal standardization.

Data are presented as the light-induced (light minus dark) changes produced, usually expressed in mμmoles/mg chlorophyll. The chlorophyll content was 1.00 mg per 0.800 g of leaves (13). For the plotted data, 3 or more determinations have been averaged under each condition, except for the photographic studies where 10 or more chloroplasts were measured. Results are presented in the form, average ± standard error of the mean.

Results

Chloroplasts in leaf cells of pea were examined with phase contrast microscopy to see whether light caused any change in conformation. In preliminary experiments using non-infiltrated leaves, a light-induced chloroplast flattening was observed amounting to a decrease of approximately 20 % in thickness for a 10 min illumination with 4000 lux. However, the detail observed in the final photographs was better using leaves infiltrated with water. Furthermore, such infiltration did not affect the active protoplasmic streaming (a sensitive indication of the status of a plant cell) and so the detached leaves were routinely infiltrated with water. In the dark, the chloroplast thickness was 2.68 ± 0.04 μ, while after 10 min at 4000 lux, the chloroplasts flattened to 2.15 ± 0.04 μ. When the top views of chloroplasts were measured, their areas were 19.0 ± 0.2 μ^2 in the dark and 18.7 ± 0.3 μ^2 in the light. Hence, the predominant light-induced change was in chloroplast thickness. Furthermore, the flattening was reversed during 30 min in the dark. The chloroplast flattening for 10 min in the light was about half-maximal near 700 lux and approached saturation above 2000 lux incident on the leaves (Fig. 1).

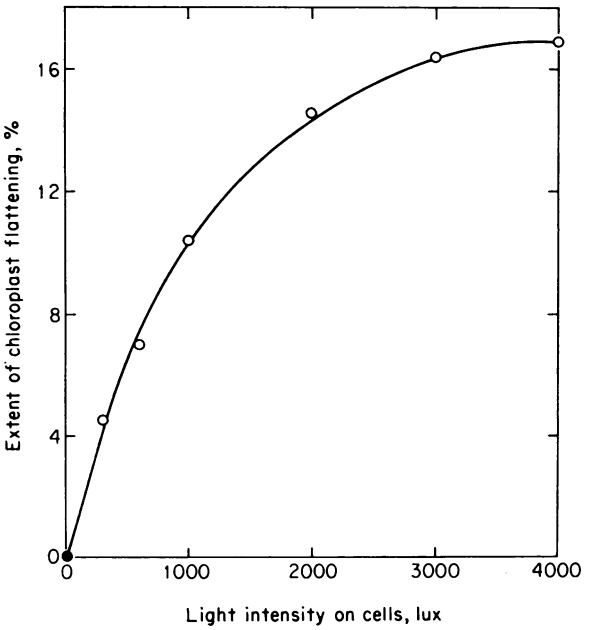

FIG. 1. Chloroplast flattening *in vivo* observed with phase contrast microscopy using detached and sectioned pea leaves illuminated for 10 min with various light intensities.

This effect of light intensity on chloroplast flattening in the plant cell is analogous to that seen when rapidly isolated chloroplasts and a centrifugation technique for estimating volume are used (13). Furthermore, both approaches indicated that the light-induced change was about 20 % in magnitude and was reversible. However, the change seen by the phase contrast microscope was clearly occurring in the plant cell and was specifically shown to be a light-induced flattening of the chloroplasts.

Next, the kinetics of the light-induced chloroplast flattening *in vivo* during a 10 min illumination with 300, 1000, or 4000 lux was determined (Fig. 2). The extent and the rate of flattening were both greater at the higher light intensities. For 4000 lux, the extent of flattening was about 17 %, and the half-maximal time was slightly more than 1 min. At 1000 lux, the asymptote for flattening was about 10 % (including data for a 25 min illumination), and the half-time was 3 min. The initial rate of chloroplast flattening appeared to be rather low. Hence, the change in chloroplast thickness during the first min in the light was examined in detail for 4 light intensities (Fig. 3). The rate of chloroplast

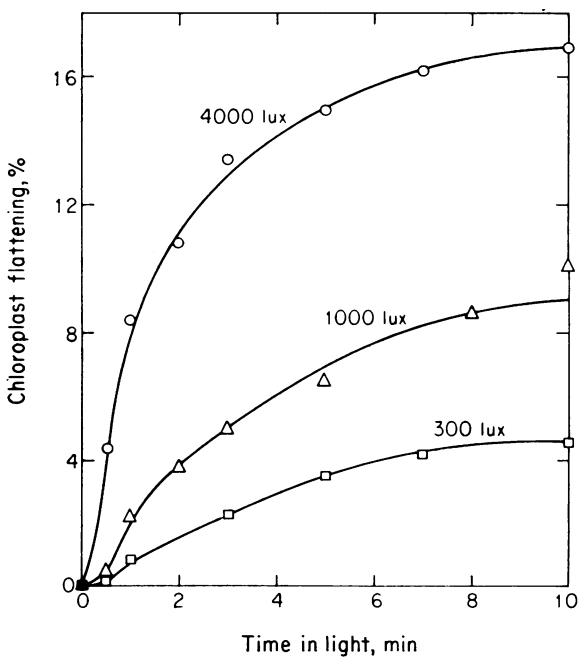

FIG. 2. Time courses of chloroplast flattening *in vivo* during 10 min illuminations with 3 light intensities.

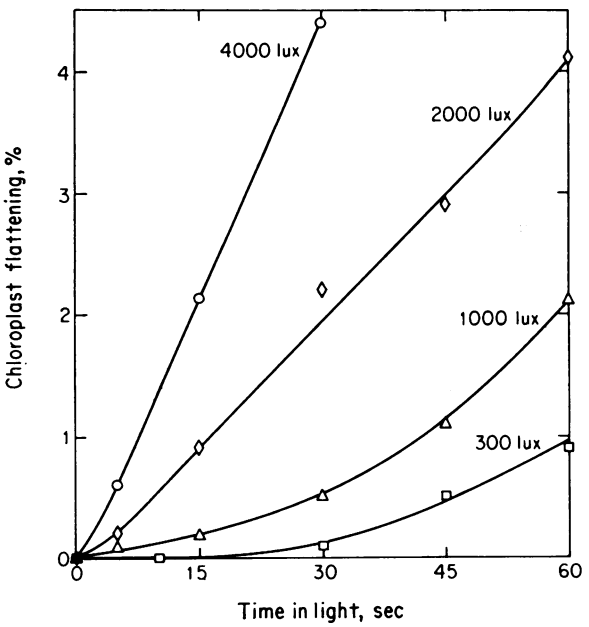

FIG. 3. Initial light-induced chloroplast flattening *in vivo* showing the effect of light intensity on the lag phase occurring in the first minute.

flattening increased during the first min, an effect that was particularly apparent at the lower light intensities. This increase in the rate of flattening after the leaves are in the light may reflect the build-up of some energy pool in the chloroplasts, *e.g.* ATP or the high-energy state created by electron flow. The light-induced chloroplast flattening was also detected *via* electron microscopy of the sliced leaves. However, the initial small changes were unresolvable. This is so because the same chloroplast could not be followed at various times using the electron microscope technique. On the other hand, many pictures of the same chloroplast after various periods of illumination were routinely made

with the light microscope providing high experimental precision for the small conformational changes involved.

The lag in chloroplast flattening upon illumination of the leaves may be correlated with the build-up of the immediate energy source for the conformational change. Hence, attempts were made to measure ATP and NADPH using non-aqueously isolated chloroplasts (2, 5, 18) to minimize the loss of soluble species. Metabolic activity was arrested with liquid nitrogen. The leaves were ground and then lyophilized for 72 hr at −30°. The powder was suspended in a hexane-CCl_4 mixture of density 1.34 g/ml and centrifuged for 10 min at 20,000*g*. Hexane was added to the supernatant fluid lowering its density to 1.30 g/ml. This fluid was recentrifuged (10 min at 20,000*g*) and a chloroplast-containing pellet obtained. This pellet had 35 ± 2 μg chlorophyll per mg dry weight (the extraction and/or destruction of chlorophyll was less than 10 % based on the final recovery). However, other observations (chloroplast volume *vs.* the reciprocal of the sucrose concentration) have indicated that the non-osmotic volume of pea chloroplasts is 16.2 μl/mg chlorophyll (15). This non-osmotically responding volume corresponds to a solid phase not penetrated by water and comprises 15.4 mg of non-aqueous chloroplast material/mg chlorophyll. In other words, pure chloroplasts have 65 μg chlorophyll per mg dry weight. Therefore, the non-aqueously isolated particles (35 μg chlorophyll/mg dry wt) were only slightly over half chloroplasts by weight (no significant increase in chlorophyll per mg dry weight was obtained by varying the freezing procedure, solution densities, and centrifugation times or by using continuous density gradients). As another check on the purity of the chloroplast fraction isolated non-aqueously, the distribution of ribosomes was determined. The 70S ribosomes would be expected only in the chloroplasts, while 80S ribosomes are in the cytoplasm. Since such large particles would not be expected to diffuse into or out of chloroplasts, evidence of 80S ribosomes in the chloroplast fraction would further substantiate the suspected cytoplasmic contamination. Ribosomes were prepared from both the chloroplast fraction and a sample of lyophilized leaf and analyzed by density gradient centrifugation (19). The ratio of 80S to 70S ribosomes was 2.45 for the whole leaf and 0.93 for the chloroplast fraction. Thus the chloroplast fraction contained 38 % as many cytoplasmic (80S) ribosomes as did the leaf extract when expressed per chloroplastic (70S) ribosome. Next, a technique (1) involving no liquids, but rather a spinning of the lyophilized powder, was tried. As described (1), a green chloroplast-containing spot collected on the top of the chamber, but its chlorophyll content was only 22 μg per mg dry weight, *i.e.* only about one-third chloroplasts by weight. Observation of the "chloroplast" fractions with a phase contrast microscope indicated the absence of whole cells and leaf fragments, but cell wall debris and cytoplasmic contamination were evident. Hence, neither technique yielded a pure fraction of pea chloroplasts. Therefore, following the arresting of metabolic activity with liquid nitrogen, but without any lyophilization, the ground leaves were extracted and the ATP and NADPH were directly determined in the extract.

The effect of light on ATP in detached pea leaves during the initial 60 sec was determined (Fig. 4). Upon illumination, the ATP level quickly rose, reached a maximum, and then fell. For 4000 lux, the maximum was reached in only 10 sec, while for 1000 lux it took about 16 sec (Fig. 4). After the initial peak for 4000 lux, the ATP level declined 34 % to reach a minimum at 30 sec and then increased again. To cause the decrease, some reaction or reactions having short lag periods must have used the ATP or its precursor (the high-energy state). ATP is obviously required for CO_2 fixation. Also, the light-induced chloroplast flattening is an energy-requiring process which may be using ATP or its precursor.

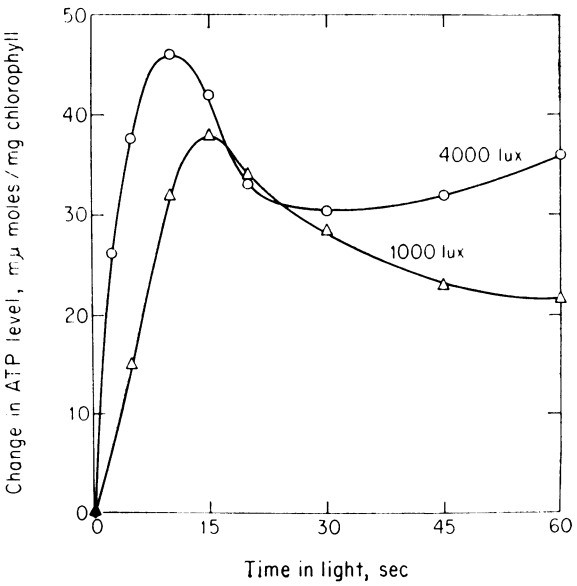

FIG. 4. Rapid light-induced changes in ATP caused by 1000 or 4000 lux incident on detached pea leaves.

The light-induced changes in NADPH for 1000 and 4000 lux were also determined during the initial 60 sec (Fig. 5). Upon illuminating the leaf, NADPH rose to a maximum and then decreased, similar to the responses of ATP. However, NADPH attained its peak later than did ATP. At 4000 lux, the maximum in NADPH was reached at about 20 sec, while at 1000 lux it took slightly more than 30 sec to reach a maximum (Fig. 5). In this regard, although CO_2 fixation requires NADPH, the light-induced chloroplast shrinkage apparently does not (10). Hence, the gradual dip in NADPH

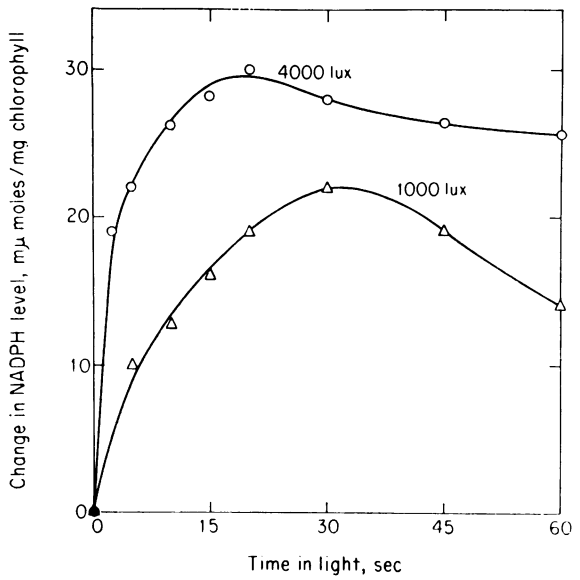

FIG. 5. Changes in NADPH level in leaves during a 1 min illumination with 1000 or 4000 lux.

level may reflect CO_2 fixation, while the larger and more rapid ATP changes may represent the sum of CO_2 fixation plus other processes.

Finally, the $^{14}CO_2$ fixation for illumination periods up to 1 min was determined (Fig. 6). For 1000 lux incident on the leaves, very little CO_2 was fixed in the initial 30 sec, after which a sustained rate of fixation was established. For 4000 lux, the lag period was shortened and also the on-going rate was higher. By extrapolation from the early sustained rate of CO_2 fixation, the lag times were approximately 28 sec at 1000 lux and 19 sec at 4000 lux (Fig. 6). These are estimates for the times at which ATP and NADPH would be used in substantial amounts in the dark reactions of photosynthesis. The $^{14}CO_2$ fixation was also investigated for incubation periods greater than 60 sec. For example, the amount of CO_2 fixed for 120 sec at 4000 lux was 325 mμmoles/mg chlorophyll (4 determinations averaged). The rate at 120 sec was 19.4 μmoles CO_2 fixed (mg chlorophyll)$^{-1}$ (hr)$^{-1}$. This implies a minimum rate for ATP consumption as will be discussed below.

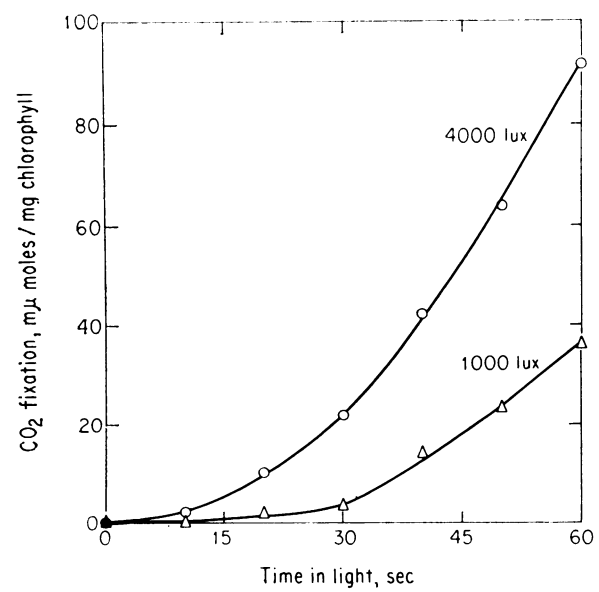

FIG. 6. Initial light-induced $^{14}CO_2$ incorporation into leaves indicating a lag period during the first minute before appreciable CO_2 fixation occurs.

Discussion

The present experiments emphasize changes in chloroplast reactions during the initial 60 sec of illumination of detached pea leaves (table I). The lag before CO_2 fixation proceeds at an appreciable rate probably reflects the build-up of intermediate sugars involved with the carbon fixation pathway.

Table I. *Illumination Times to Reach Various Metabolic States in Detached Pea Leaves*

Condition	Time 1000 lux	4000 lux
	sec	*sec*
Sustained rate of CO_2 fixation	28	19
Maximum NADPH level	32	20
Maximum ATP level	16	10
Sustained rate of chloroplast flattening	11	2
10 mμmoles increase in ATP/mg chlorophyll	10	3

At the time when appreciable CO_2 fixation commences, a drop in ATP and NADPH should take place. As seen in table I, NADPH reaches its maximum level about when CO_2 fixation begins, and hence the subsequent drop in NADPH is probably due to its use in the photosynthetic dark reactions. However, ATP reaches its maximum level in about half of the time required for the analogous change in NADPH. ATP or its precursor apparently is used by some process or processes beginning prior to the known ATP consumption in the CO_2 fixation reactions. Although the chloroplast flattening was observed under a rather different experimental arrangement than for the ATP, NADPH, and CO_2 studies, the early drop in the ATP level may be due to processes associated with the light-induced chloroplast conformational changes. The chloroplast flattening *in vivo* in the light depends on ATP or a high-energy intermediate produced by electron flow (14). Furthermore, the efflux of potassium out of chloroplasts in illuminated plant cells can be abolished

by the uncouplers tri-Fl-CCP and nigericin (15) indicating a similar energetic basis. Such ion movements and accompanying decreases in chloroplast volume could underlie the decrease in ATP observed prior to appreciable CO_2 fixation. Moreover, the lag in the chloroplast flattening may reflect the build-up of some energy pool in the light. For example, the time required for ATP to increase by 10 mμmoles/mg chlorophyll is about the same as the lag in chloroplast flattening (table I). If the chloroplast flattening used either ATP or a precursor (the high-energy state), this could account for the early drop in the ATP level in the light and also would be consistent with the short lag before appreciable chloroplast flattening occurred.

The chloroplast volume *in vivo* can be roughly estimated from the thickness and area. Representative side views of a chloroplast in the light and in the dark were constructed based on the measured dimensions. These profiles were then rotated and the volumes delineated by the resulting surfaces of revolution were determined by numerical integration. This procedure is superior to assuming that chloroplasts are flat cylinders uniformly and exactly 0.5 μ thick (8). For pea leaves in the dark, the chloroplast volume was 32.4 μ^3 which decreased 21 % to 25.4 μ^3 in the light. As determined by packing volume and with a Coulter counter, isolated pea chloroplasts suspended at the same osmolality as the cell sap are 35.3 μ^3 from plants in the dark and 29.0 μ^3 in the light (15). Although the isolated chloroplasts were slightly larger than the estimated volume *in vivo*, the light-induced volume decrease of 18 % occurring in the plant cell and retained by the rapidly isolated chloroplasts (15) agrees quite closely with the volume change estimated from the photomicrographs of living cells.

Rapidly attained maxima in the levels of ATP and NADPH in the light confirm previous observations (6, 16, 17). Santarius and Heber (17) found that ATP reached a maximum in 15 to 30 sec for about 40,000 lux incident on *Beta vulgaris*, *Coleus shirensis*, *Elodea densa*, and *Spinacia oleracea*. When leaves of *Beta vulgaris* or *Spinacia oleracea* were illuminated with 40,000 lux, NADPH in the chloroplasts increased for about 30 sec and then fell (6) (times for the maxima in ATP and NADPH are approximate since the data were at 0, 7, 15, 30, and 60 sec of illumination). The maximum light-induced increase in ATP was about 30 mμmoles/mg chlorophyll (17). For 30 sec illumination of tobacco with 20,000 lux, Keys (9) reported an ATP increase amounting to approximately 32 mμmoles/mg chlorophyll. Similarly, ATP in pea leaves increased 46 mμmoles/mg chlorophyll (10 sec at 4000 lux, Fig. 4). The time for ATP to increase 30 mμmoles/mg chlorophyll was less than 4 sec at 4000 lux. For light intensities of 20,000 to 40,000 lux, ATP could have reached its maximum level in a fraction of a sec. To investigate the initial kinetics of NADPH and ATP formation *in vivo*, either low light intensities or techniques for measuring changes occurring during the first sec of illumination must be used.

The amount of ATP and NADPH in pea chloroplasts isolated non-aqueously (2, 5, 18) could not be precisely determined. As indicated in Results, a pure pea chloroplast fraction should have 65 μg chlorophyll per mg dry weight, while the "chloroplasts" isolated non-aqueously had 35 μg chlorophyll/mg dry weight and those isolated from the dry powder without the use of any liquids (1) had only 22. Furthermore, the chloroplast fraction isolated non-aqueously from lyophilized material contained many 80S (cytoplasmic) ribosomes. Some possible causes of the contamination are briefly considered. When leaf material is placed in liquid nitrogen, the ensuing rapid freezing may cause the local formation of microscopic ice crystals where water molecules are preferentially crystallized. Solutes, including large molecules like proteins, may be pushed up against organelles and increase the size of the subsequently isolated chloroplasts. Secondly, pea chloroplasts *in vivo* are imbedded in a gel matrix whose properties are largely unknown. This matrix may adhere to the chloroplasts during lyophilization. Finally, the grinding and extracting of the lyophilized powder probably would not always cause fracture at the chloroplast surface. Therefore, a dehydrated chloroplast may not break away from the dehydrated matrix and cytoplasm precisely at the chloroplast limiting membrane. In any case, caution should be exercised in applying the non-aqueous and non-liquid methods for isolating chloroplasts.

Chloroplasts isolated from pea plants in the light have double the rate of endogenous ATP formation compared with those from plants in the dark (12). Endogenous refers to the light-induced photophosphorylation obtained when only 0.2 mM ADP and 0.2 mM phosphate (labeled with ^{32}P) are added to chloroplasts suspended in an osmoticum (sucrose) buffered at pH 7.9. The doubled rate of photophosphorylation for chloroplasts from illuminated *vs.* dark plants also occurs when N-methylphenazonium methosulfate, ferricyanide, or magnesium are added (12). To evaluate whether an analogous increase in rate occurs *in vivo* it is assumed that 1) all ATP formed in the light was extracted without loss by hydrolysis, 2) the determined rate of $^{14}CO_2$ incorporation into the leaves is the rate of CO_2 fixation, and 3) 3 ATP molecules are required per molecule of CO_2 fixed. If the steady state CO_2 fixation rate were more than one-third of the initial ATP formation rate, this would indicate that the photophosphorylation rate increased. From Fig. 4, the initial rate of ATP increase at 4000 lux was 29 μmoles (mg chlorophyll)$^{-1}$ (hr)$^{-1}$. From Fig. 6 and data mentioned in Results, the CO_2 fixation rate for 4000 lux was 13 μmoles (mg chlorophyll)$^{-1}$ (hr)$^{-1}$ at 60 sec and 19 at 120 sec. Both of these CO_2 fixation rates are greater than 29/3, implying that

the rate of ATP formation in the plant cell increased. Such an increase in photophosphorylation rate caused by light incident on the plants has previously been correlated with the light-induced chloroplast flattening (12). Here, the chloroplast flattening at 4000 lux has a half-time near 1 min and is two-thirds complete at 120 sec (Fig. 2 and 3). Apparently light causes chloroplasts to decrease in thickness and concomitantly to increase their photosynthetic efficiency in the plant cell.

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