BBA 45634

CHLOROPLAST SHRINKAGE AND INCREASED PHOTOPHOSPHORYLATION *IN VITRO* UPON ILLUMINATING INTACT PLANTS OF *PISUM SATIVUM*

PARK S. NOBEL*

Department of Botany, King's College, University of London, London (Great Britain)

(Received September 5th, 1967)

SUMMARY

1. The effect of the illumination of whole plants of *Pisum sativum* immediately prior to chloroplast isolation on subsequent photophosphorylation *in vitro* was investigated. Using a new technique, chloroplasts were isolated within 2 min of harvesting the plants.

2. Chloroplasts isolated from illuminated pea plants had a rate of endogenous photophosphorylation of 24 μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1}, while those from plants in the dark had a rate of 12. Changes between the two levels had half-times of about 5 min. The addition of ferricyanide and *N*-methylphenazonium methosulfate increased the photophosphorylation rate about twice as much with chloroplasts isolated from plants in the light compared to those isolated from plants in the dark. An action spectrum indicated that light absorbed by chloroplasts *in vivo* was responsible for the increased photophosphorylation *in vitro*.

3. Chloroplasts isolated from illuminated plants had a volume approx. 15% less than those from plants in the dark. The reversal *in vitro* of this shrinkage and of the elevated photophosphorylation rate both had half-times of about 10 min. Light-induced chloroplast shrinkage *in vivo* is discussed in connection with the increased efficiency of photophosphorylation *in vitro* for chloroplasts isolated from illuminated plants.

INTRODUCTION

The activities of chloroplasts *in vitro* may depend on the illumination condition of the plant from which they were isolated. For example, MILLER[1] found that the ability of filtered homogenates of pea leaves to reduce a dye, dichlorophenolindophenol, slowly increased after the plants were illuminated, reached a maximum when the plants had been in the light for 3–4 h, and then declined. Using chloroplasts isolated from young tomato leaves, HOFFMAN AND MILLER[2] have indicated that the

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, *N*-methylphenazonium methosulfate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate.

* Present address: Department of Botanical Sciences and Molecular Biology Institute, University of California, Los Angeles, Calif. 90024, U.S.A.

changes in dye reduction ability have an endogenous rhythm. Analogous experiments on photophosphorylation have not been reported. Furthermore, it is not known whether the observed diurnal variations in chloroplast activity are due to changes within the chloroplast or to changes elsewhere in the plant cell. Finally, the effect of light incident on the plants on chloroplast activities measured *in vitro* is not clear. NOBEL[3] has recently described a technique for gently and rapidly isolating chloroplasts which exhibit a high rate of endogenous photophosphorylation. "Endogenous" means that no magnesium, electron carriers, or protective agents are added to the isolation and incubation media. The high rates of endogenous photophosphorylation under relatively simple incubation conditions may help elucidate the photosynthetic ability of the chloroplasts *in vivo*. The elapsed time from harvesting the plants to obtaining a resuspended chloroplast pellet with the new technique was only 2 min. Hence, certain metabolic changes of chloroplasts which take place in the plant cell may still be evident after the chloroplasts have been isolated. In particular, the effect of illuminating the intact plant on chloroplast volume and endogenous photophosphorylation subsequently measured *in vitro* was determined. The endogenous photophosphorylation rate *in vitro* dramatically increased when the chloroplasts were isolated from peas after they had been in the light for a short time and returned to the lower level when the plants were placed back in the dark. Moreover, upon pre-illuminating the chloroplasts while in the plant cell a shrinkage occurred that could still be measured after the chloroplasts had been isolated.

MATERIALS AND METHODS

The greater repeatability and the higher photophosphorylation rates obtained by using chloroplasts from peas grown under controlled conditions compared with those from commercial spinach[4] prompted the choice of peas as experimental material. *Pisum sativum* 'Laxton's Superb' were grown in moist vermiculite for 14 days[3]. A light intensity of 2000 lux was provided for 12 h each day by daylight fluorescent tubes and the plants were grown at 20 ± 1°. Plants were harvested by cutting immediately below the lowest foliage leaf. 10 g of such leaves and stems were quickly cut with scissors into a bag made of two layers of nylon cloth. The bag was transferred to a chilled mortar containing 10 ml of isolation medium and the material firmly ground for 10 sec. After squeezing through the nylon bag (which retained nearly all whole cells and large debris), the homogenate was centrifuged for 60 sec at 1000 × *g* at 2°, the supernatant fluid was decanted, and the pellet was resuspended by placing the tube on a vortex mixer. Details of the isolation procedure including its timing (only 2 min from harvesting the peas to the obtaining of a resuspended chloroplast pellet) are published elsewhere[3]. The isolation medium was 0.2 M sucrose buffered with 0.02 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES)–NaOH (pH 7.9). In addition, the incubation medium contained 200 μM ADP and 200 μM phosphate (labeled with ^{32}P), both added as sodium salts at pH 7.9 (see ref. 3 for a detailed consideration of the incubation conditions). Incubation at 25° was with 50000 lux provided by tungsten lamps or in the dark. Chloroplasts containing approx. 12 μg chlorophyll were added to the 1 ml of incubation medium. Phosphate esterification was determined for incubation times of up to 1 min. Except where indicated, the elapsed time from harvesting the plants to the termination of incubation by

adding trichloroacetic acid was less than 3 min. The incorporation of phosphate labeled with ^{32}P into ATP was determined by an isobutanol–benzene extraction procedure[3]. Photophosphorylation was defined as ATP formed in the light *minus* that formed in the dark and is expressed in μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1}.

RESULTS

The daily rhythm in endogenous photophosphorylation *in vitro* is shown in Fig. 1. Each point refers to isolation of chloroplasts under the indicated illumination condition of the pea plants and subsequent determination of photophosphorylation *in vitro*. For chloroplasts isolated from plants harvested in the dark, the rate of endogenous photophosphorylation was about 12 μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1}.

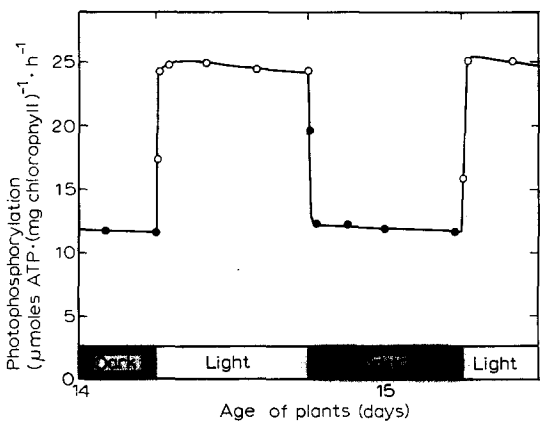

Fig. 1. Daily changes in rates of endogenous photophosphorylation *in vitro*. Chloroplasts were isolated at the times indicated from pea plants harvested during the dark (●) or light (○) on the 14th and 15th days.

For chloroplasts isolated from peas in the light this rate more than doubled. The phosphate esterification rate in the dark, which has been subtracted, tended to be slightly higher for chloroplasts isolated from plants harvested in the light than from those in the dark, but was only 0.2–0.5 μmole ATP·(mg chlorophyll)$^{-1}$·h^{-1}. The diurnal rhythm in endogenous photophosphorylation could result from an endogenous rhythm in the plant or from the daily changes in illumination level. To distinguish between these possibilities, plants were kept in continuous light or dark for 36 h beginning on the 14th day, and ATP formation was determined for chloroplasts isolated every 3 or 4 h. For chloroplasts isolated from plants kept in the dark, the endogenous photophosphorylation rate *in vitro* remained near 12 μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1}. When the plants were kept in continuous light, also no rhythmic changes in the level of endogenous photophosphorylation were observed, the rate being about 24 μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1}. It appears that the change in the illumination condition of the plants triggers the increase or decrease in the rate of endogenous photophosphorylation observed *in vitro*.

The change in the rate of endogenous photophosphorylation of chloroplasts isolated as the plants go from the dark to the light and *vice versa* is rapid compared

to the 12 h duration of the two portions of the daily cycle (Fig. 1). The kinetics of such changes were investigated by isolating chloroplasts from plants whose illumination condition was alternated between dark and light (2000 lux) at 1-h intervals (Fig. 2). Upon illuminating the plants, the half-time for the doubling of the endogenous photophosphorylation rate *in vitro* was 5 min, while the reversal upon putting the plants back in the dark had a half-time of about 6 min.

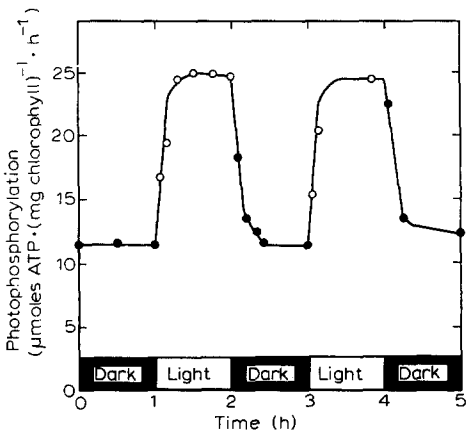

Fig. 2. Changes in the rates of endogenous photophosphorylation upon rapid cycling of the illumination condition of the plants. Where indicated, plants were illuminated with the usual growth light (2000 lux). Chloroplast incubation was terminated within 3 min of harvesting the plants in the dark (●) or light (○).

The greater endogenous photophosphorylation of chloroplasts isolated from plants harvested in the light *vs.* in the dark may be caused by some critical aspect of the isolation and incubation procedures. To investigate this possibility the following variables were examined: sucrose concentration in the isolation and incubation medium (0.02–0.4 M), type of buffer (TES–NaOH, Tris–HCl, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate–NaOH, *N*-tris(hydroxymethyl)methylglycine–NaOH), pH of the incubation medium (7.0 to 8.5), incubation time (up to 3 min), chlorophyll concentration (up to 50 μg/ml), incubation temperature (15–30°), ADP concentration (10–500 μM), and phosphate concentration (10–500 μM) (see ref. 3 for consideration of these effects for chloroplasts from plants harvested in the light). In other experiments, sucrose was replaced by 80 mM NaCl in all media and also 0.1–3 mM NaCl was added to the usual incubation medium. In all cases, the rate of endogenous photophosphorylation was about twice as high for chloroplasts from peas in the light compared to the dark. The chloroplast isolation and incubation procedures were apparently not the cause of the marked differences in photophosphorylation rate.

The diurnal changes appear to be a physiological response of chloroplasts *in vivo* to the illumination of the plant. To study the cause of the elevated photophosphorylation rate of chloroplasts from plants in the light, attempts were made to reverse the light-induced changes *in vitro*. The rate of endogenous photophosphorylation *in vitro* decreased when chloroplasts were stored for various periods after isolation and before incubation, and this change was greater for chloroplasts isolated from plants in the light compared to the dark. For the first few minutes, this decrease

averaged 3 % per min when the chloroplasts from plants in the light were stored at 0° and 7 % per min when kept at 20°. Since the change was more rapid at the higher temperature, the following experiments employed chloroplasts stored for various times at 20°. Fig. 3 shows the effect of storing chloroplasts isolated from plants both in the light and in the dark on the subsequent rate of endogenous photophosphorylation. For chloroplasts isolated from plants harvested in the dark, the rate of endogenous photophosphorylation decreased about 10 % in the first 5 min of storage and then stayed fairly constant for the next 55 min. On the other hand, the endogenous photophosphorylation rate of chloroplasts from peas harvested in the light continuously decreased and approached the rate obtained with plants in the dark, although initially the rate had been about double. Hence, the increase in endogenous photophosphorylation *in vitro* caused by illuminating the plants can be reversed by storing the isolated chloroplasts.

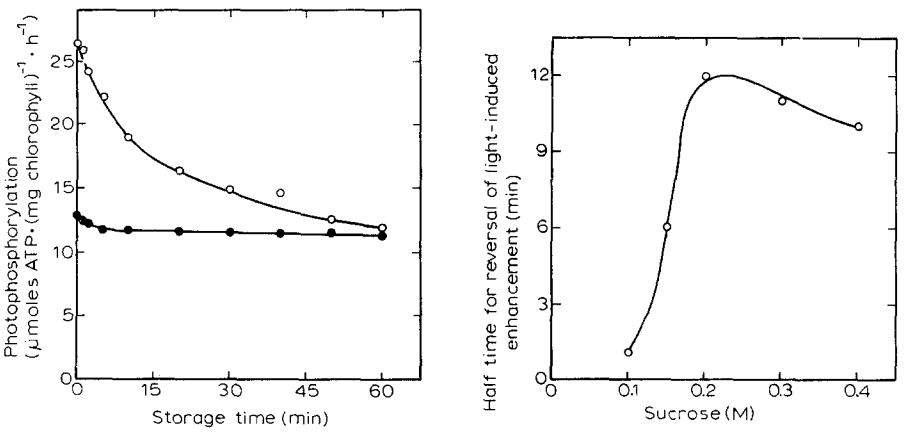

Fig. 3. Changes in the rate of endogenous photophosphorylation upon storage of isolated chloroplasts. Chloroplasts were isolated from plants harvested in the dark (●) or after 1 h in the light (○) and then stored at 20° in the dark for the times indicated. The incubation periods were 15 sec.

Fig. 4. Half-time for the reversal *in vitro* of the light-induced enhancement of endogenous photophosphorylation at various osmolarities. The indicated sucrose concentrations were used in the media employed for chloroplast isolation, storage at 20° in the dark, and incubation for 15 sec (TES–NaOH (pH 7.9) was present at 0.1 times the concentration of sucrose). The endogenous photophosphorylation rate for chloroplasts from plants in the dark or after 1 h in the light was compared for various storage times (*cf.* Fig. 3), and the half-time for reversal determined.

The half-time for the reversal *in vitro* of the elevated photophosphorylation rate characteristic of chloroplasts from illuminated plants was 12 min (Fig. 3). This half-time was influenced by the sucrose concentration (Fig. 4). The changes observed were mainly due to decreases in the rate of endogenous photophosphorylation of stored chloroplasts from plants in the light (*cf.* Fig. 3). At 0.1 M sucrose, the decrease was extremely rapid (half-time of 1.1 min) and approached the limit of time resolution using the present method of isolating chloroplasts. In general, the disappearance of the light-induced enhancement in endogenous photophosphorylation rate for isolated chloroplasts was more rapid at the lower osmolarities of the medium used for storage.

The effect of sucrose concentration on the reversal of the elevated photophosphorylation rate suggested that chloroplast volume changes may be involved. Hence,

the volumes associated with chloroplasts isolated from plants in the light and dark were compared. After isolating the chloroplasts as usual, the resuspended pellet was immediately centrifuged in the dark for 10 min at 10000 × *g* at 0°, the supernatant fluid was decanted, and the weight of the pellet was determined. The isolation medium had a density of 1.03 g/ml and this number was used to convert pellet weight to volume. For chloroplasts isolated from plants harvested in the dark, the volume was 51.5 μl/mg chlorophyll, while it was 43.7 μl/mg chlorophyll for chloroplasts from plants that had been in the light for 1 h (8 experiments are averaged). Illuminating the chloroplasts while in the plant caused a shrinkage of 15 ± 1 % (S.E.) detectable *in vitro*.

The chloroplast volume change upon storage at 20° was studied by measuring the absorbance of suspensions at 550 mμ (near the minimum of the chloroplast absorption spectrum, see Fig. 7). Initially, the absorbance was about 10 % higher for chloroplasts from plants that had been illuminated for 1 h compared to chloroplasts from plants in the dark (Fig. 5). After storing the chloroplasts for 1 h at 20°, the absorbance difference between the two types had nearly disappeared; the half-time for the change was about 10 min. The higher absorbance for chloroplasts from illuminated plants probably corresponds to the light-induced chloroplast shrinkage observed with the packed volume technique, since absorbance increases are associated with volume decreases. Furthermore, the conformational state of the chloroplast induced by light on the plant was reversed *in vitro* at about the same rate as for the reversal of diurnal changes in photophosphorylation.

Illumination of the plant appears to be responsible for both the elevated photophosphorylation rate and also the chloroplast shrinkage observed *in vitro*. Therefore, the effects of both the quantity and the quality of the light incident on the peas on

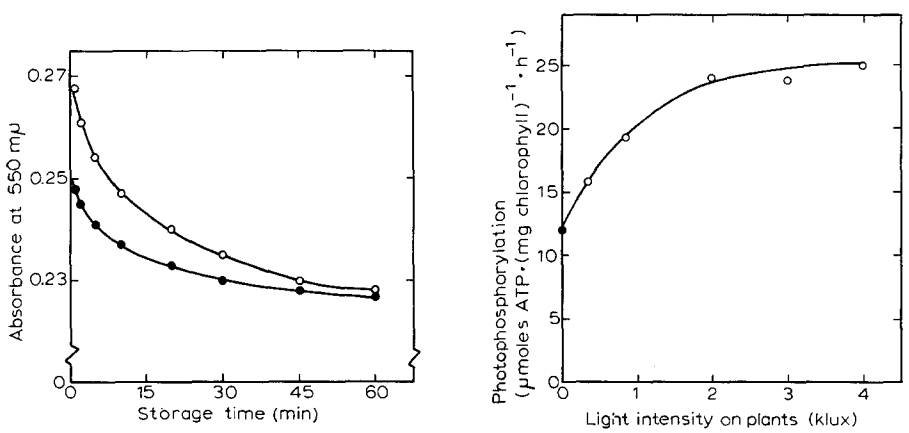

Fig. 5. Absorbance changes upon storage of chloroplasts from plants in the light and dark. Chloroplasts isolated from plants in the dark (●) or after 1 h in the light (○) were stored at 20° in the dark for the times indicated, and the absorbance at 550 mμ was determined using a Unicam SP.800A recording spectrophotometer. The cuvettes containing chloroplasts equivalent to 5 μg chlorophyll/ml of isolation medium were placed at a suitable distance from the photomultiplier so that very little of the scattered light was collected.

Fig. 6. Light intensity incident on the plant *vs.* the rate of endogenous photophosphorylation *in vitro*. The plants were placed for 1 h on the 14th day under the light intensity indicated (provided by daylight fluorescent tubes) and the rate of endogenous photophosphorylation by chloroplasts isolated from the plants was determined.

the 14th day were examined. The rate of endogenous photophosphorylation by chloroplasts *in vitro* increased as the light intensity incident on the plants from which they were isolated was raised (Fig. 6). This increase was half-maximal at only 600 lux, and neared saturation at 2000 lux (the usual light intensity used for growth). The increase of endogenous photophosphorylation from levels characteristic of plants in the dark to those in the light proceeded faster at the higher light intensities. For plants illuminated with 4000 lux, the half-time was 2 min, while for a growth light intensity of 350 lux, the half-time for the change was approx. 5 min. Hence, both the speed and the extent of change of endogenous photophosphorylation *in vitro* can be affected by the illumination condition of the plants.

In an attempt to identify the pigment system in the plant responsible for the changes in endogenous photophosphorylation *in vitro*, various wavelength bands were selected for illuminating the plants. Preliminary experiments indicated that blue light (460 mμ) was the most effective. The increase in endogenous photophosphorylation caused by this light was linear up to $3 \cdot 10^{14}$ quanta$\cdot$cm$^{-2}\cdot$sec^{-1}, a light intensity which caused an increase of 9 μmoles ATP$\cdot$(mg chlorophyll)$^{-1}\cdot$h^{-1}. An intensity of blue light of $2 \cdot 10^{14}$ quanta$\cdot$cm$^{-2}\cdot$sec^{-1} led to approx. 50 % of the increase in the rate of endogenous photophosphorylation caused by the usual transition of the plants from dark to light (see Fig. 1); the response of the plants at each of 5 different wavelengths was measured using this photon intensity (Fig. 7). Red light (665 mμ) was about half as effective as blue light in leading to increases in the rate of endogenous photophosphorylation. Green (525 mμ) and orange (590 mμ) light had only a small effect while far red (724 mμ) caused no detectable increase in the rate of endogenous photophosphorylation over the rate observed for chloroplasts from plants

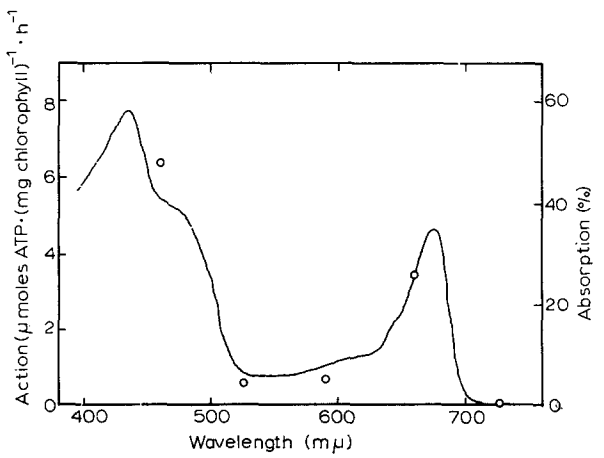

Fig. 7. An action spectrum for the diurnal changes in endogenous photophosphorylation. On the 14th day, the plants were placed for 30 min under light selected by appropriate filter combinations (bandwidths at half-maximum of 11 to 18 mμ) in conjunction with a tungsten lamp. The "action" was the increase in the rate of endogenous photophosphorylation by chloroplasts from the illuminated plants over that for dark plants (about 12 μmoles ATP$\cdot$(mg chlorophyll)$^{-1}\cdot$h^{-1}). 2 or 3 experiments using a photon intensity of $2 \cdot 10^{14}$ quanta$\cdot$cm$^{-2}\cdot$sec^{-1} are averaged for each condition. The number of incident quanta was calculated from the measured transmission characteristics of the various filters and the known spectral properties of tungsten lamps[5–7]. The absorption spectrum was determined with a Unicam SP.800A recording spectrophotometer; the cuvette was mounted near the photomultiplier to collect the radiation scattered over a wide solid angle; the chlorophyll concentration was 5 μg/ml.

in the dark. For comparison, an absorption spectrum for pea chloroplasts is also plotted in Fig. 7. The close correspondence between the chloroplast absorption curve and the action spectrum for the diurnal changes strongly suggests that light absorbed by the chloroplasts *in vivo* leads to the increase in endogenous photophosphorylation measured *in vitro*.

Light absorbed by the chloroplasts *in vivo* may establish conditions favoring one type of photosynthetic electron flow *in vitro* and this may underlie the enhanced photophosphorylation rate of chloroplasts from illuminated plants. To investigate this point, the fraction of endogenous photophosphorylation supported by cyclic, noncyclic, and pseudocyclic electron flow was determined for chloroplasts isolated from plants in the light and dark (Table I). The three types of electron flow interact with each other and so a number of admitted oversimplifications were introduced to facilitate their experimental separation. Photophosphorylation dependent on cyclic electron flow was measured in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (10 μM), which was considered to block noncyclic and pseudocyclic electron flow. To estimate noncyclic electron flow, the reduction rate of endogenous $NADP^+$ (see ref.3) was determined and 1 ATP was assumed to be formed per $NADP^+$ reduced. Pseudocyclic electron flow uses molecular oxygen as the terminal electron acceptor and should not occur when argon replaces oxygen in all solutions and gas phases. Pseudocyclic electron flow was previously found to account for most of the endogenous photophosphorylation of chloroplasts from illuminated plants[3]. In the present experiments with chloroplasts isolated from plants both in the light and also in the dark, about 85 % of the endogenous photophosphorylation was supported by photosynthetic electron flow of the pseudocyclic type. Although the rate was 2-fold higher for chloroplasts from plants harvested in the light than in the dark, the percentage of endogenous photophosphorylation supported by the three types of electron flow was essentially the same for the two cases (Table I). Therefore, the higher rate of endogenous photophosphorylation for chloroplasts isolated from illuminated com-

TABLE I

PERCENTAGE OF ENDOGENOUS PHOTOPHOSPHORYLATION SUPPORTED BY VARIOUS TYPES OF PHOTOSYNTHETIC ELECTRON FLOW FOR CHLOROPLASTS FROM PLANTS HARVESTED IN THE DARK AND LIGHT

Chloroplasts were isolated from plants in the dark ("dark plants") or after 1 h in the light ("light plants"). To measure endogenous $NADP^+$ reduction, the chloroplasts were incubated in the light or dark, centrifuged at 40000 × *g* for 1 h at 0°, and the initial rate of light-induced change at 340 mμ determined for the supernatant fluid. The endogenous photophosphorylation rate of isolated chloroplasts averaged 12 μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1} for plants in the dark and 24 in the light. Five determinations under each condition are averaged.

Experimental condition	*Predominant type of electron flow*	*Percentage of endogenous photophosphorylation*	
		Dark plants	*Light plants*
Addition of 10 μM DCMU to the incubation medium	Cyclic	3.2	3.6
Reduction rate of endogenous $NADP^+$ (assumed 1 ATP formed per $NADP^+$ reduced)	Noncyclic	8.1	7.0
Photophosphorylation in air (usual condition) *minus* that using argon as gas phase	Pseudocyclic	86	84

pared to dark plants is apparently due to a more efficient use of each of the types of electron flow and not to a switching from one type to another.

Chloroplasts isolated by the present technique can give rates of 2300 μmoles ATP formed·(mg chlorophyll)$^{-1}$·h^{-1} after being osmotically broken and supplemented with high concentrations of ADP and phosphate as well as with photophosphorylation cofactors such as *N*-methylphenazonium methosulfate (PMS) and magnesium[3]. Under the conditions of the present experiments which lead to high rates of endogenous photophosphorylation (see ref. 3), the effect of adding single cofactors to these fairly intact chloroplasts was examined to help understand the effect of illuminating the plants on chloroplast activities *in vitro*. The addition of such photophosphorylation cofactors might eliminate the enhancement of the endogenous rate *in vitro* caused by light incident on the plant. Secondly, these cofactors might cause the same stimulation in rate for both types of chloroplasts with the result that the rate would remain about 12 μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1} higher for chloroplasts from plants in the light. Thirdly, chloroplasts isolated from plants in the light may in some way actually be more efficient for photophosphorylation; in this case, the rate would be stimulated more for chloroplasts from illuminated plants than for chloroplasts from plants in the dark. The addition of 100 μM magnesium to the incubation medium increased the rate of photophosphorylation 60 to 65 % for chloroplasts isolated from plants harvested both in the light and the dark. This was an increase of 15.3 μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1} for chloroplasts isolated from plants in the light and of 7.9 for the dark (5 experiments are averaged). The capacity for photophosphorylation appears to be higher when the chloroplasts are isolated from illuminated plants.

Electron flow cofactors were added to the usual incubation medium to investigate further the effect of the illumination of the plant on photophosphorylation *in vitro*. Ferricyanide (1 mM), a cofactor for noncyclic electron flow, increased the endogenous photophosphorylation rate about 3-fold for chloroplasts from plants in the light or dark (Table II); PMS (20 μM), a cofactor for cyclic electron flow, stimulated both about 5-fold (Table II). For chloroplasts isolated from plants in the dark, this was an increase in photophosphorylation rate of 27 and 52 μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1} for ferricyanide and PMS, respectively. For chloroplasts isolated from plants in the light, the rate was increased much more, *viz.* 52 μmoles ATP·(mg

TABLE II

EFFECT OF ELECTRON FLOW COFACTORS ON PHOTOPHOSPHORYLATION RATES FOR CHLOROPLASTS ISOLATED FROM PLANTS HARVESTED IN THE DARK AND LIGHT

Chloroplasts were isolated from plants in the dark ("dark plants") or after 1 h in the light ("light plants"). 5 determinations are averaged.

Addition to usual incubation medium	*Photophosphorylation (μmoles ATP·(mg chlorophyll)$^{-1}$·h^{-1})*	
	Dark plants	*Light plants*
None	12.0	24.2
1 mM $K_3Fe(CN)_6$	38.7	76.1
20 μM PMS	63.9	109.8

chlorophyll)$^{-1} \cdot h^{-1}$ by ferricyanide and 86 by PMS. Chloroplasts isolated from illuminated plants by the present technique seemingly have a greater capacity or efficiency for photophosphorylation than chloroplasts isolated from plants in the dark.

DISCUSSION

These experiments with rapidly isolated chloroplasts indicate that light absorbed by chloroplasts *in vivo* can enhance the photophosphorylation rate measured *in vitro*. The contraction of chloroplasts caused by illuminating the plant may be responsible for the increase in photophosphorylation rate *in vitro*; alternatively, both the chloroplast shrinkage and the increase in photophosphorylation may reflect some more primary light reaction. Some light-induced chloroplast changes observed *in vivo* will first be discussed, then relevant light-induced activities of chloroplasts *in vitro* will be summarized, and finally an attempt will be made to relate the changes in photophosphorylation observed here with the known properties of chloroplasts.

Variations in chloroplast shape *in vivo* are well known. BÜNNING[8] in 1942 described diurnal rhythms in the conformation of chloroplasts in Nymphaea, Nicotiana, and Phaseolus. In 1953, BUSCH[9] described similar changes for the single chloroplasts in young epidermal leaf cells of *Selaginella serpens*. During the day the chloroplasts were flat, while at night they contracted and became spherical. These changes followed an endogenous diurnal rhythm, *i.e.* the conformation altered periodically even when the illumination conditions were kept constant. Infiltrating the leaf with various buffers and other non-physiological treatments caused a transition of the chloroplasts from the flat to the spherical form, but not *vice versa*. From this, BUSCH[9] conjectured that the flat chloroplasts represented a higher energy state and that the transition from the flat to the spherical form was passive. The first electron-microscopic evidence for light-induced shrinkage of chloroplasts *in vivo* was presented by KUSHIDA *et al.*[10] in 1964. Chloroplasts in spinach leaves in the dark were more or less round. Upon illumination, they became flattened in a direction perpendicular to the cell wall. HILGENHEGER AND MENKE[11] using both light and electron microscopy have also found that chloroplasts in *Nitella flexilis* reversibly shrink upon illumination. For young internodal cells, a light intensity of 7000 lux caused a decrease in chloroplast thickness of 15–20 % and the contraction had a half-time of 2 min. At lower light intensities, the decrease in thickness was less and occurred more slowly. PACKER, BARNARD AND DEAMER[12] observed little change in the overall conformation of spinach chloroplasts *in vivo* upon illumination using non-infiltrated leaves, although the grana appeared more tightly layered for plants in the light. Upon vacuum infiltration with various solutions, light-scattering and electron-microscopic changes were observed that are consistent with the light-induced chloroplast shrinkage *in vitro*. ZURZYCKI[13,14] found that the light-induced flattening of chloroplasts in intact leaf cells of *Mnium undulatum* and of chloroplasts isolated from this moss occurred for all intensities of red light (668 mμ) and for the lower intensities of blue light (483 mμ). For isolated chloroplasts, the flattening (observed as an increase in area) was half-maximal at an intensity of red light of about 400 ergs$\cdot$cm$^{-2}\cdot$sec^{-1} (approx. $1.3 \cdot 10^{14}$ quanta$\cdot$cm$^{-2}\cdot$sec^{-1}), while a somewhat higher intensity was necessary *in vivo*. For this photon intensity, the increase in chloroplast area *in vitro* with blue light was about 80 % greater than for red light. In summary, chloroplasts *in vivo* decrease in thickness by a light-

induced reversible process. This flattening may be due to light absorbed by the chloroplasts leading to a high energy state.

A light-induced irreversible swelling of isolated chloroplasts was described in 1965 by PACKER, SIEGENTHALER AND NOBEL[15]. NOBEL, MURAKAMI AND TAKAMIYA[16] demonstrated that the outermost thylakoids of the grana stack swelled most easily and the swelling proceeded from the outside toward the inside of the grana stack. After about 15 min illumination, the chloroplasts were completely ruptured and only fragmented lamellae remained[16]. This type of swelling has not been found *in vivo* and also would not be expected *in vitro* for the incubation conditions of the present experiments[3,17].

A light-induced chloroplast shrinkage occurs *in vitro* and this appears to be related to the chloroplast flattening observed *in vivo*. In 1962, PACKER[18] described a light-scattering response of illuminated spinach chloroplasts *in vitro* which indicated some conformational change. ITOH, IZAWA AND SHIBATA[19] found that chloroplasts *in vitro* reversibly shrink in the light. Electron micrographs showed that the isolated chloroplasts became flattened upon illumination and calculations from their data indicate a decrease in thickness of about 20 %. IZAWA, ITOH AND SHIBATA[20] obtained an action spectrum for the light-induced chloroplast shrinkage *in vitro* which indicated that light absorbed by chlorophyll was responsible for the chloroplast shrinkage. Red light (680 mμ) at an intensity of about 150 ergs$\cdot$cm$^{-2}\cdot$sec^{-1} (approx. 0.5$\cdot$10^{14} quanta$\cdot$cm$^{-2}\cdot$sec^{-1}) led to half the rate of shrinkage as saturating white light. PACKER AND SIEGENTHALER[21] found that various anions of weak organic and inorganic acids greatly stimulated the light-induced shrinkage of isolated chloroplasts. Recently, CROFTS, DEAMER AND PACKER[22] have proposed that the light-induced hydrogen uptake may cause the formation of an undissociated acid which then diffuses out of the grana interior, osmotically leading to the chloroplast shrinkage. The mechanism for the light-induced chloroplast shrinkage *in vivo* is unknown, but may prove to be similar to this explanation since analogous anions occur *in vivo*.

The light-induced chloroplast shrinkage *in vivo* has many properties in common with the changes in endogenous photophosphorylation observed *in vitro* in the present series of experiments. For example, the shrinkage of Nitella chloroplasts upon illumination of the cells was also more rapid and greater in extent as the light intensity was increased[11], and both processes had fairly similar half-times. The chloroplasts in Mnium flattened more in response to blue than to red light[13,14], similar to the greater increase in endogenous photophosphorylation *in vitro* for blue compared to red light incident on the pea plants, and the incident photon intensities for half-maximal effects were similar. Moreover, illumination of the plants caused a chloroplast contraction that could still be measured after a rapid isolation of the chloroplasts. The reversal of the light-induced contraction and of the changes in endogenous photophosphorylation followed a similar time course when the chloroplasts were stored *in vitro*. Also, when the isolated chloroplasts were stored in media of lower osmotic strength which was expected to cause a more rapid reversal of the light-induced shrinkage, the rate of decrease of the light-induced photophosphorylation changes *in vitro* was much greater. Apparently, the changes in level of endogenous photophosphorylation *in vitro* induced by illuminating the plant are accompanied by conformational changes of the chloroplasts.

The diurnal changes in photophosphorylation observed here did not exhibit

an endogenous rhythm. This is in marked contrast to the changes in dye reduction ability reported by HOFFMAN AND MILLER[2] for isolated tomato chloroplasts. In addition, some conformational changes of chloroplasts *in vivo* have been shown to follow an endogenous rhythm[8,9]. Also, VANDEN DRIESSCHE[23,24] has found that both changes in the shape of chloroplasts in *Acetabularia mediterranea* and changes in the ability to evolve oxygen exhibit an endogenous diurnal rhythm which depends on nuclear DNA; these daily oscillations are possible only in the presence of some unknown light-dependent substance. Perhaps for the changes in endogenous photophosphorylation observed here only the light-dependent substance or condition is involved, but in both cases a detailed molecular explanation remains to be presented.

Light absorbed by the chloroplast *in vivo* does not cause the electron flow leading to endogenous photophosphorylation *in vitro*. Rather, the light absorbed by the chloroplasts *in vivo* makes them more efficient for photophosphorylation *in vitro*. The transport of certain ions may be involved. However, although magnesium and chloride are important cofactors for photophosphorylation and electron flow *in vitro*, illuminating the plants apparently does not change their concentration in the chloroplasts sufficiently to account for the elevated rate of photophosphorylation. For example, when these ions were added to the incubation medium (also to the isolation medium in the case of chloride), the photophosphorylation rate remained about twice as high for chloroplasts from plants in the light compared to chloroplasts from plants in the dark. These experiments *in vitro* must be interpreted with caution, since the location, local concentration, binding, *etc.*, of the ions may be different *in vivo*. Furthermore, the effect of light was different for chloroplasts in the plant and those *in vitro*. The amount of ATP formed increased linearly with time for incubation periods of up to 3 min for chloroplasts from plants in the light or dark. When plants in the dark were illuminated with 4000 lux for 1 min, the rate of endogenous photophosphorylation for chloroplasts *in vitro* increased 50 %. This suggests that light (50000 lux) *in vitro* under the conditions employed does not induce the same changes as light absorbed by the chloroplasts *in vivo*.

The light-induced chloroplast shrinkage *in vivo* may lead to the higher rates for photophosphorylation observed *in vitro* for these rapidly isolated chloroplasts. For example, the structural modifications may facilitate energy transfer from absorbing pigments to the active center by slightly changing critical distances, may affect enzyme–substrate complexes or have an allosteric effect, and may change the transport or exchange properties of the chloroplast membranes. VANDEN DRIESSCHE[23] has also suggested that the structural deformation of the chloroplasts *in vivo* may be responsible for variations in photosynthetic capacity. On the other hand, light absorbed by chloroplasts *in vivo* may lead to ion movements, perhaps involving the alteration of the electrochemical gradient of hydrogen ions. This might then osmotically lead to the light-induced shrinkage of chloroplasts and at the same time establish conditions favorable for ATP formation. In addition, intermediate and end products of photosynthesis may influence the ongoing rate of photophosphorylation and the conformation of the chloroplast. Any explanation must account for the reversal of the light-induced enhancement of the endogenous photophosphorylation rate during storage of the chloroplasts, kinetically similar to the reversal *in vitro* of the shrinkage, while the photophosphorylation rate for chloroplasts from plants in the dark was little affected by storage. An understanding of this correlation between

structure and function may have to await a detailed analysis of the inorganic and organic contents of chloroplasts under various illumination conditions.

The present experiments have demonstrated that the photophosphorylation rate *in vitro* can be doubled by illuminating the plants from which the chloroplasts are rapidly isolated. Photophosphorylation and related photosynthetic activities themselves require light; in addition, it appears that light absorbed by the chloroplasts within the plant cell may also lead to changes actually rendering the chloroplasts more efficient for photosynthesis.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the invaluable assistance provided by many people at the Department of Botany, King's College, University of London, during the course of this research, which was undertaken on a U.S. National Science Foundation Postdoctoral Fellowship.

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