Light-Dependent Volume Changes and Reactions in Chloroplasts I. Action of Alkenylsuccinic Acids and Phenylmercuric Acetate and Possible Relation to Mechanisms of Stomatal Control[1, 2]

Paul-Andre Siegenthaler and Lester Packer
Department of Physiology, University of California, Berkeley, California

Introduction

Chloroplasts are known to change their volume in vitro by 3 mechanisms. Light and osmotic pressure appear to be the most significant factors for controlling chloroplast volume. The simplest type of volume change shown by isolated chloroplasts has been described by Nishida (8), who found by absorbancy, gravimetric, and volumetric techniques that chloroplasts change their volume in response to exposure to solutions of different tonicity. Hence, chloroplasts in vitro are osmotically sensitive structures like mitochondria and cells. The 2 other mechanisms for bringing about volume changes in chloroplasts require the action of light. One mechanism seems closely geared to the energy transfer reactions that are coupled to electron flow. Packer (12) has shown that suspensions of spinach chloroplasts exhibit light-induced increases of scattered light that are rapid and reversible, occurring in a time interval of 20 to 100 seconds. Substances that interact with the energy transfer pathway, such as ammonium chloride and ADP, inhibit light-scattering increases (2), while ATP under conditions favoring its hydrolysis promotes light-scattering increases (14). These observations are consistent with the view that volume changes are under the control of energy-linked intermediates (2, 14, 15). Itoh et al. (3) have shown that this action of light brings about a low-amplitude shrinkage that results in a 50 to 60 % decrease in volume of whole chloroplasts, as measured by the volume distribution of chloroplasts in the Coulter counter. Chloroplasts isolated from *Euglena* (1) have also been reported to manifest light-dependent volume changes.

Another action of light on chloroplast volume results in high-amplitude swelling (16, 17). Swelling is brought about slowly in the dark but can be accelerated by light, especially if a cofactor such as phenazine methosulfate has been added to the chloroplasts. Light-dependent, high-amplitude swelling of chloroplasts requires 10 to 90 minutes for completion and has not been found to be reversible either in darkness or by the addition of ATP. Moreover, ammonium chloride and ADP do not affect the time course of this process. High-amplitude chloroplast swelling is powerfully inhibited by inorganic phosphate, one of the general requirements for low-amplitude, light-dependent shrinkage.

Under conditions for low-amplitude chloroplast shrinking in the light, an energy-dependent translocation of certain ions, such as calcium, phosphate, and sodium, occurs by a light- and energy-dependent mechanism (9). Since the osmotic and turgid properties of plant cells have been reported to be under the influence of light, it seemed possible that the light-dependent movements of water and ions manifested by chloroplasts in vitro might be involved in such processes within the cell. In particular, it is known that light induces the opening of stomata and that this process is accompanied by the increased turgor of guard cells (5, 24). Since guard cells or their chloroplasts cannot be readily isolated, an indirect approach was undertaken to test the action of certain compounds, such as the alkenylsuccinic acids (22) and phenylmercuric acetate (19), that have been found to be effective agents for the control of stomatal aperture. This approach was suggested by the classical investigation of Zelitch (21–24) that has established the importance of photosynthetic reactions in mechanisms of stomatal control by use of such inhibitors. It has been found that these substances inhibit not only reactions of electron transport and photophosphorylation, but also alter the action of light on chloroplast volume.

Materials and Methods

Chloroplast Isolation. Spinach leaves, purchased commercially, were washed, the midribs removed, and then treated in a Waring blendor for 30 seconds in a medium containing NaCl (175 mM) and Tris (50 mM, pH 8.0). The crude suspension was filtered through 4 layers of cheesecloth and then centrifuged at 200 × *g* for 1 minute. The supernatant fraction was centrifuged for 10 minutes at 200 × *g* and the chloroplast pellet was collected and resuspended in the isolation medium at a chlorophyll concentration of 2.5 to 3.5 mg/ml. The procedures were performed at 4° and chlorophyll was

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determined spectrophotometrically. The chloroplast suspension was diluted in appropriate media for experiments.

Inhibitors. The alkenylsuccinic acids were kindly provided by Dr. I. Zelitch of the Connecticut Agricultural Experiment Station, New Haven, Connecticut. Phenylmercuric acetate was obtained from Distillation Products Industries, Rochester, New York. Other reagents were obtained commercially.

High-Amplitude Volume Changes. Packed volume changes were estimated in chlorocrit tubes as reported earlier (17). The basic reaction mixture (5 ml) contained Tris (20 mM, pH 8), NaCl (350 mM), and chloroplasts (0.2 mg chlorophyll/ml). Two ml aliquots of chloroplast suspensions were transferred to 3 ml graduated protein (or chlorocrit) tubes. The tubes were incubated for 10 minutes in the light (50,000 lux) or in the dark (wrapped in aluminum foil) in a temperature bath maintained at 25°. After the 10-minute preincubation, the tubes were centrifuged at 1000 × *g* at room temperature (approximately 25°) in either light (22,000 lux) or darkness in a clinical centrifuge. Pellet volumes were read from graduations on the tubes.

Low-Amplitude Volume Changes. Light-scattering (546 mμ) was measured at 90° in a Brice-Phoenix Light Scattering Photometer, modified for recording as described by Packer (11). The low intensity of the green light used for the scattering measurements was near the minimum of the photosynthetic action spectrum. Increases in scattering intensity following treatment with actinic light (Wratten No. 26 filter) are expressed as percent changes of the initial scattering level. The reaction mixture (5 ml) contained: Tris (5 mM, pH 8), $MgCl_2$ (5 mM), ascorbate (2.5 mM), phenazine methosulfate (20 μM), and chloroplasts (10 μg chlorophyll/ml). Temperature was maintained at 25° ± 0.1 by circulating liquid around a jacketed 1-cm cuvette.

ATP Synthesis and Hydrolysis. The uptake of P_i was measured in the following reaction mixture: Tris (20 mM, pH 8), NaCl (35 mM), $MgCl_2$ (5 mM), ascorbate (2 mM), phenazine methosulfate (20 μM), ADP (0.5 mM), KH_2PO_4 (0.5 mM), and chloroplasts (50 μg chlorophyll/ml). The assay for adenosine triphosphatase was adapted from Petrack and Lipman (18). The formation of P_i was determined in the following reaction mixture: Tris (20 mM, pH 8), NaCl (35 mM), $MgCl_2$ (5 mM), phenazine methosulfate (20 μM), cysteine (50 mM), ATP (1 mM) and chloroplasts (50 μg chlorophyll/ml). Synthesis and hydrolysis were estimated over a 15-minute period, commencing 60 seconds after the addition of ADP or ATP, respectively, by measuring the change in phosphate concentration by a phosphomolybdic acid method with $SnCl_2$ as reducing agent (14). Assays were performed in test tubes placed 11 cm from a 150-w General Electric reflector flood light (50,000 lux) in a water bath at 25°.

Electron Flow. The reduction of NADP and O_2 evolution by chloroplasts was performed in test tubes under the conditions of illumination and temperature as for ATP synthesis and hydrolysis. NADPH formation was estimated at 340 mμ after 10 minutes incubation in a reaction mixture (3 ml) that contained: Tris (20 mM, pH 8), NaCl (35 mM), $MgCl_2$ (5 mM), NADP (0.33 mM), ferredoxin (98 μg) and chloroplasts (20 μg chlorophyll/ml). In controls NADP was absent.

Results

High-Amplitude Volume Changes. The observation of light-dependent, high-amplitude swelling of chloroplasts has been made by a number of methods (17). In particular, the chlorocrit or packed volume technique has given the same results in these experiments as the Coulter counter and absorbancy measurements. Figure 1 shows the action of light on the packed volume of spinach chloroplasts, observed at various times during centrifugation. In the dark, pellet volume becomes smaller as the time of centrifugation proceeds; but during this same time interval, chloroplast volume in the light becomes considerably larger. The pellet volume is a measure of chloroplast swelling. After 75 minutes it is 75 % to 100 % larger in the light. At 75 min-

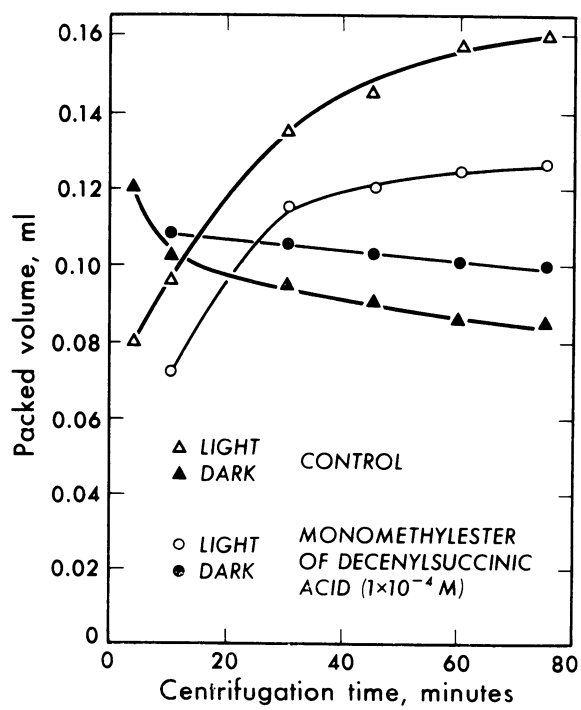

FIG. 1. Influence of the monomethylester of decenylsuccinic acid on the packed volume changes of spinach chloroplasts.

utes the chlorophyll-containing particles in the supernatant fluid have been sedimented (17). The action of 100 μM MMDSA[3] was to cause a 25 % diminution of pellet volume in the light and an inflation of chloroplast volume in the dark (fig 1). The overall action of this compound is to diminish the difference in volume between light and dark.

Using packed volume measurements as the test system, MMDSA and other alkenylsuccinic acid derivatives (DSA and DDSA), and PMA were studied. Figure 2 shows the effect of a wide concentration range of MMDSA and PMA on chloroplast volume; between 1 to 10 μM these compounds do not affect chloroplast volume in light or dark. As the concentration is raised, they bring about a large decrease of packed volume in the light. Furthermore, PMA has no effect in the dark, whereas the MMDSA elicits a marked swelling. Complete inhibition of light-induced swelling is obtained by 1 mM MMDSA. These experiments show that PMA and MMDSA interfere with the light process that causes swelling. MMDSA has an additional action by causing swelling in the dark.

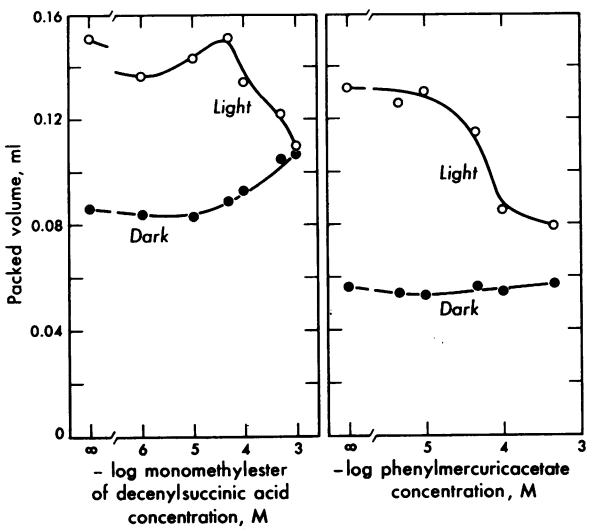

FIG. 2. Influence of the monomethylester of decenylsuccinic acid and phenylmercuric acetate concentration on chloroplast volume in the light and dark. The data given in the figure are from readings made after 75 minutes of centrifugation, at which time sedimentation of the chloroplasts is complete (17).

A comparison between the relative potency of the alkenylsuccinic acid derivatives on chloroplast volume is shown in figure 3. The results are expressed as the ratio of the light to dark volume.

[3] The following abbreviations are used: MMDSA, monomethylester of decenylsuccinic acid; DSA, decenylsuccinic acid; DDSA, dodecenylsuccinic acid; PMA, phenylmercuric acetate.

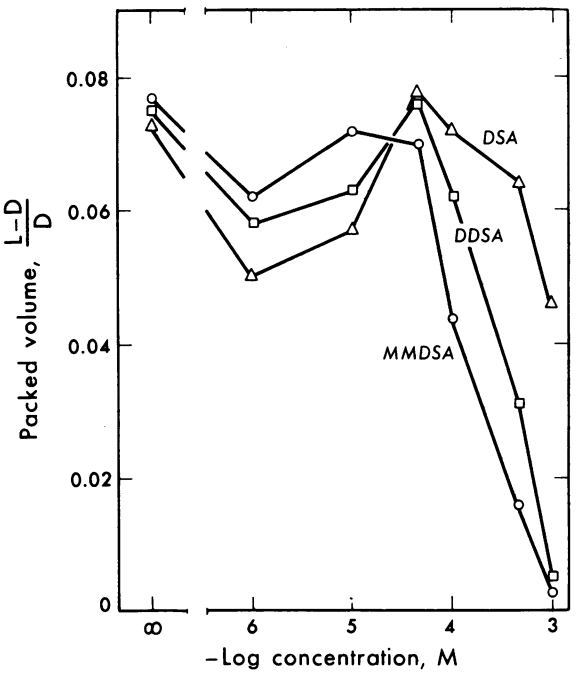

FIG. 3. Action of alkenylsuccinic acids on light-induced high-amplitude swelling measured by packed volume.

MMDSA and DDSA are stronger inhibitors of light-induced swelling than DSA. The alkenylsuccinic acids show, in general, a similar pattern of inhibition on chloroplast swelling.

From the different character of alkenylsuccinic acids, whose action is to increase permeability of cell membranes (6, 22) and of PMA, which has frequently been employed as a relatively specific inhibitor of sulfhydryl groups, it was predicted that if thiols are involved in light-induced swelling, the inhibitory effect of PMA might be reversed by adding back thiols. In fact, cysteine (at a conc of 1 mM)completely reversed the inhibition of light-induced swelling caused by PMA, but did not change the inhibition caused by alkenylsuccinic acids.

Low-Amplitude Volume Changes. It was of interest to examine the action of PMA and alkenylsuccinic acids on low-amplitude volume changes. These energy-dependent volume changes would be expected to be involved in active movements of water and ions in vivo. The results of a typical experiment made by a time recording of light-scattering changes is given in figure 4. Chloroplasts were initially incubated under conditions for photophosphorylation, except that ADP was absent (2, 14). A large light-scattering increase (shrinkage) of approximately 50 % is brought about by illumination of the reaction mixture with actinic red light; this scattering change is reversed in the dark. Continuing cycles of light and darkness yield similar results except that the light-scattering response is

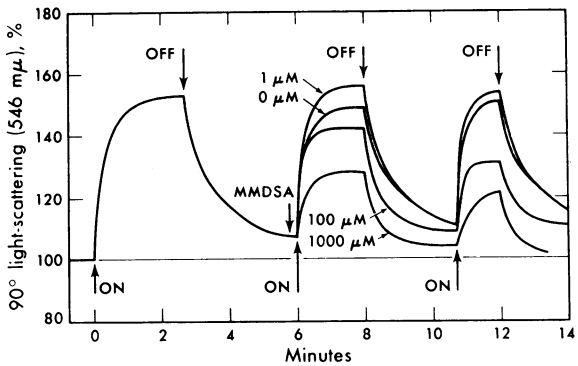

FIG. 4. Action of the monomethylester of decenylsuccinic acid on light-induced low-amplitude shrinkage measured by light scattering.

slightly diminished after the first cycle. To this test system an addition of MMDSA was made at various concentrations in the dark. This treatment does not result in a change of the light-scattering level. However, on illumination with actinic light, 1 μM MMDSA elicits a slight activation of the light-scattering response, whereas 100 and 1000 μM levels are decidedly inhibitory. After 3 cycles (alternating periods of light and darkness), 1 mM MMDSA completely inhibited the response. In general, the inhibition of MMDSA was progressively more effective with each successive light-dark cycle. Hence, the inhibition is time dependent and may reflect reaction or translocation times.

Employing the test system for light-scattering described above, a comparison between the alkenylsuccinic acid derivatives was made as shown in figure 5. MMDSA and DDSA inhibit light-scattering increases of chloroplasts on illumination, and complete inhibition occurs at a concentration of 1 mM. DSA first stimulates the scattering change up to a concentration of 0.1 mM and then becomes inhibitory; i.e., at a concentration of 1 mM 20 % inhibition is observed.

The effect of PMA on light-scattering is completely different from alkenylsuccinic acid derivatives (fig 6A). When PMA is added in the dark, no change in the scattering level is observed, but upon illumination the scattering response is enormously enhanced. The maximum effect was reached between 2 and 10 μM. At 100 μM the stimulation effect by PMA on light-scattering was unchanged even if PMA was preincubated 10 minutes in the dark with chloroplasts. Also, the stimulation persists if PMA is preincubated with chloroplasts in the light. It is significant that these responses are reversible and reproducible. In fact, alternative periods of light and darkness result in progressively larger light-scattering responses (fig 6B). Thus PMA stimulates rather than inhibits low-amplitude volume changes in chloroplasts.

Photosynthetic Reactions: ATP Synthesis and Hydrolysis. Since chloroplast volume is dependent

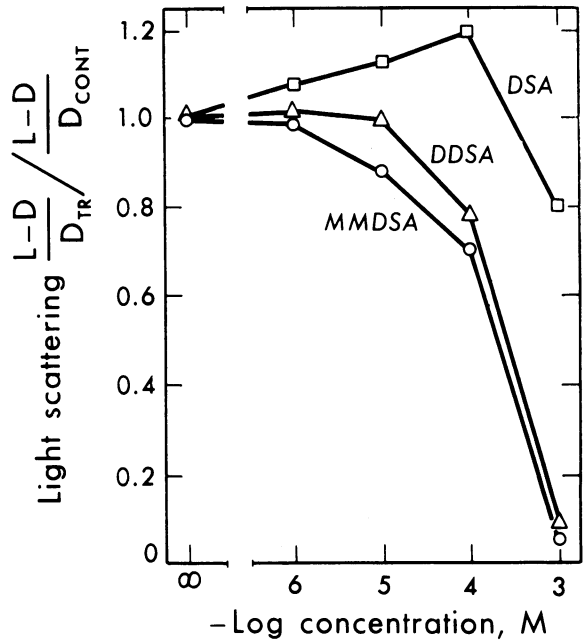

FIG. 5. Comparison of various concentrations of alkenylsuccinic acids on low-amplitude shrinkage measured by light scattering. Results are expressed as the treated light-dark (L-D/D) changes divided by the light-dark changes of the control; each point represents the average of 3 complete light-dark cycles.

on light (electron flow) for swelling, and on both light and energy transfer for shrinkage (12), it was important to investigate the action of stomatal inhibitors on these reactions. Previous studies have established that conditions of cyclic photophosphorylation support scattering changes in chloroplasts in response to actinic light and that ATP, as a result of its light-activated hydrolysis, increases the magnitude of scattering responses (13). The uptake of

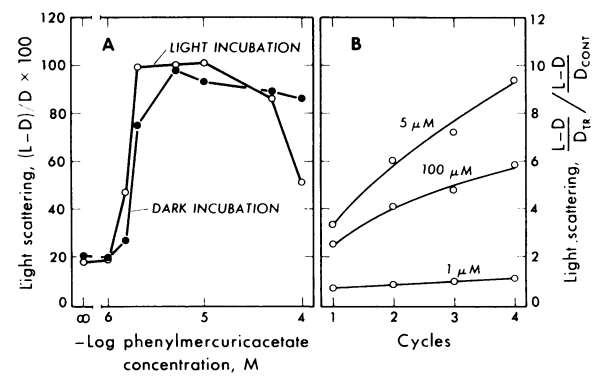

FIG. 6. Action of phenylmercuric acetate on low-amplitude volume changes measured by light scattering. Conditions as in Methods but the complete reaction mixture is incubated for 10 minutes in the light or in the dark with phenylmercuric acetate at various concentrations (A) and as a function of the number of light-dark cycles (B).

P_i under conditions of cyclic photophosphorylation was studied as a function of the concentration of alkenylsuccinic acids and PMA. All the compounds stimulate ATP synthesis at low concentrations and then begin to inhibit P_i uptake at higher concentrations. PMA, MMDSA, DDSA and DSA caused 50 % inhibition of photophosphorylation with 0.01 mM, 0.2 mM, 2.0 and 4.0 mM, respectively. As with light-induced swelling (packed volume) and shrinkage (light-scattering), MMDSA is found to be a stronger inhibitor than DSA. MMDSA at 1 mM completely inhibits photophosphorylation. PMA is a more effective inhibitor of photophosphorylation on a concentration basis than the alkenylsuccinic acids.

Under light-triggered adenosine triphosphatase conditions (14), e.g. in the presence of a thiol (50 mM cysteine), the action of the alkenylsuccinic acids and of phenylmercuric acetate on the liberation of P_i from ATP is quite different (fig 7). At concentrations which elicited a 50 % inhibition of the photophosphorylation reaction, these compounds stimulate ATP hydrolysis. At 1 mM, MMDSA caused 40 % inhibition of this activity. At a higher concentration, 5 mM DSA remains a strong activator. PMA and MMDSA have a similar effect on ATP synthesis and hydrolysis. The discrepancy between the action of PMA and alkenylsuccinic acids on ATP synthesis and hydrolysis might be caused by the protective effect of cysteine.

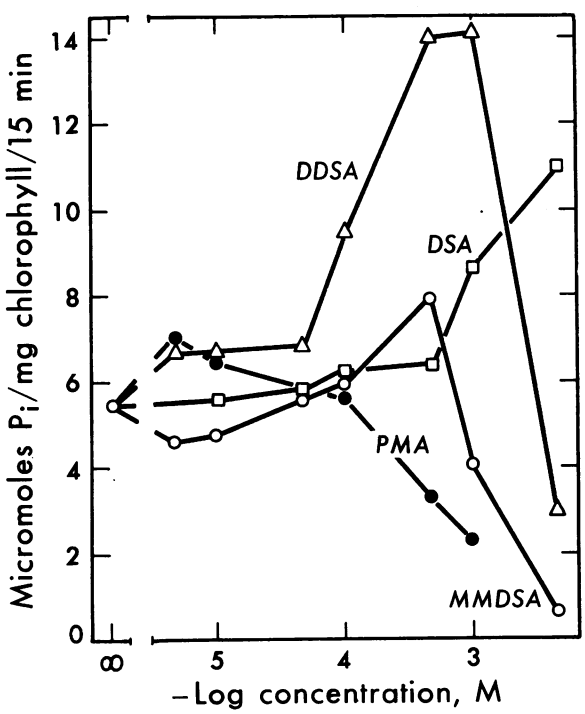

FIG. 7. Effect of alkenylsuccinic acids and phenylmercuric acetate on light-triggered ATP hydrolysis.

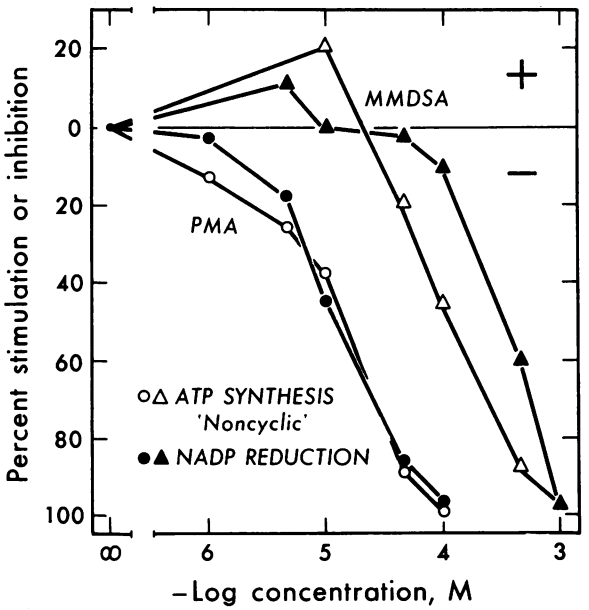

FIG. 8. Effect of the monomethylester of decenylsuccinic acid and phenylmercuric acetate on noncyclic photophosphorylation and NADP reduction. The rates of ATP synthesis and NADP reduction were 90 and 111 μmoles/mg chlorophyll per hour, respectively, when calculated from initial rates (2 min).

Electron Flow. Since the concentration-dependent effect of PMA and MMDSA on the rate of cyclic photophosphorylation and the extent of volume changes were rather different, it seemed worthwhile to investigate if their action was on electron flow or photophosphorylation. This test was made with a noncyclic system established with NADP in the presence of ferredoxin (fig 8). Electron flow was measured by following reduction of NADP. Inhibition by PMA begins at 1 μM and is complete at 100 μM for both NADP reduction and the accompanying noncyclic photophosphorylation. The concentration range of PMA that is inhibitory on cyclic and noncyclic photophosphorylation are similar.

MMDSA is less effective as an inhibitor than PMA and inhibits cyclic and noncyclic photophosphorylation and NADP reduction in a similar manner based on concentration.

When 2, 6-dichlorophenolindophenol was used as the electron acceptor, the inhibition of electron flow by PMA and MMDSA was the same as with NADP plus ferredoxin. Also measurements of O_2 evolution instead of NADP reduction result in the same inhibition by the stomatal reagents.

Discussion

Mechanisms are now known by which light, the electron and energy-transfer reactions initiated, and the products formed can control the movement of

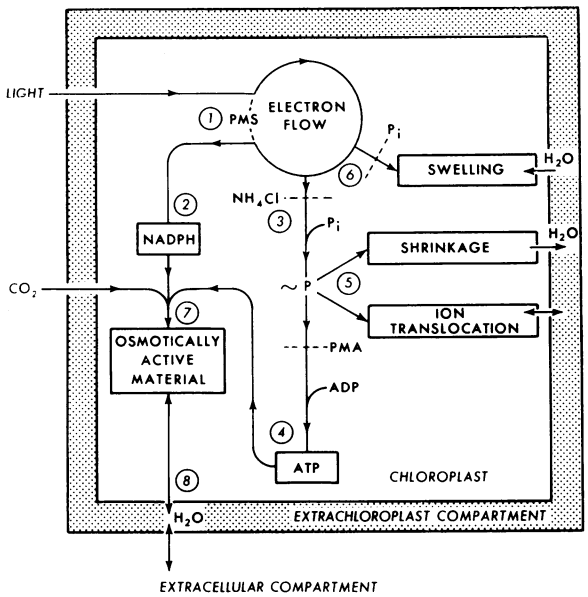

FIG. 9. Scheme illustrating possible relation of light-dependent volume changes and reactions in chloroplasts to mechanisms of stomatal control. Explanation in text (also cf. 17).

water and ions in spinach chloroplasts in vitro (17, 9). As illustrated in figure 9, light initiates electron flow (reactions 1, 2) that is coupled to energy transfer (reaction 3). Energy transfer leads to ATP synthesis (reaction 4), and/or shrinkage and the translocation of ions (reaction 5). The energy-dependent processes (reactions 3–5) are inhibited by uncoupling agents such as ammonium chloride. Light-induced swelling, dependent upon electron flow only (reaction 6), is inhibited by phosphate and unaffected by ammonium chloride (16). Reaction 2 is coupled to CO_2 fixation; photosynthesis results in accumulation of osmotically active material (reaction 7). Accumulation or withdrawal of osmotically active material (reaction 8) should induce water movements in chloroplasts by a passive mechanism (8).

The mechanisms regulating the water movements of chloroplasts are delicately controlled, both by photosynthetic reactions and concentrations of reactants and products. The introduction of a compound that interferes with reactions 1 to 8 (fig 9) would be expected to influence chloroplast volume by altering water relations in the plant cell. Hence, PMA and the alkenylsuccinic acids, which have been found to inhibit electron and energy transfer reactions, affect the regulation of chloroplast volume. Earlier studies by Smith and Baltscheffsky (20) and Nozaki, et al. (10) have already established that PMA inhibits electron flow and photophosphorylation (cf. fig 8). Moreover, Shimshi (19) reported that PMA sprayed on the leaves decreases photosynthesis in *Zea mays* plants as reflected by CO_2 incorporation because of stomatal closure and is also dependent upon soil moisture. Although PMA is found to be an inhibitor of photosynthetic reactions, it is interesting that at concentrations inhibitory for photophosphorylation, it stimulates light-triggered ATP hydrolysis (fig 7).

Since PMA was an inhibitor of electron flow, it was expected that it would inhibit volume changes of chloroplasts, and indeed, this was the case for light-induced swelling (reaction 6). Thiol groups may be required for this type of swelling since cysteine was found to exert a protective action. But the surprising finding made with PMA was its remarkable enhancement effect on light-induced shrinkage (reaction 5) that is dependent upon energy transfer (reaction 3). Since shrinkage is related to the formation of high energy intermediates preceding ATP synthesis (13), this finding suggests that PMA inhibits ATP synthesis at a step near the termination of the energy transfer pathway (reaction 4); its action resembles that of quinacrine reported by Dilley and Vernon (2). Inhibition of reaction 4 would cause accumulation of high energy intermediates formed in reaction 3 and therefore promote shrinkage (reaction 5).

Zelitch (22) has described the action of alkenylsuccinic acids as inhibitors of light-induced opening of stomata or as agents that induced stomatal closure. Kuiper (6) has suggested that these substances are incorporated into lipid layers of cytoplasmic membranes; e.g., root cells, to increase their permeability to water. Similar effects have been observed by Itoh et al. (4) and others who have noted chloroplast swelling in the presence of anionic detergents like dodecyl benzene sulfonate. Thus it was not surprising to observe that alkenylsuccinic acids induce chloroplast swelling in the dark and that they abolish light-induced volume changes. Contrary to PMA, these compounds inhibit light-induced shrinkage.

Because of the indirect approach employed in this type of investigation (see also ref. 7), it cannot be proven that the influence of alkenylsuccinic acids and phenylmercuric acetate on spinach chloroplast volume changes and on photosynthetic reactions exactly reflect the known effects of these substances on stomatal control (cf. 24). However, since the photosynthetic reactions that occur in spinach (and other) chloroplasts promote a series of energy-dependent processes such as volume changes, water movement, translocation of ions and presumably of other osmotically active material, it is assumed that guard cell chloroplasts also possess these properties. Our view is that the occurrence of such light-dependent phenomena in chloroplasts is involved in the maintenance of turgor in mesophyll and guard cells, the difference being that in guard cells, turgor controls stomatal aperture. Hence, a study of the action of inhibitors and environmental factors, both chemical

and physical, on such phenomena in chloroplasts may be helpful in elucidating mechanisms of stomatal control.

Summary

Light (electron flow) and energy-dependent mechanisms that control the movement of water and ions in spinach chloroplasts are known. Since these processes may be involved in stomatal control, the action of certain substances that cause their closure in plants was examined on spinach chloroplasts in vitro.

Phenylmercuric acetate and 3 alkenylsuccinic acids inhibit light-induced chloroplast swelling, which occurs by an energy-independent mechanism. Light-induced shrinkage of chloroplasts, an energy-dependent mechanism, is also inhibited by the alkenylsuccinic acids, but phenylmercuric acetate exerts a remarkable stimulation of the light effect. An examination of the action of these substances on electron flow (NADP reduction) and photophosphorylation revealed that these reactions are inhibited by all substances.

An explanation for the mechanism of stomatal control based upon the regulation of chloroplast volume by light- and energy-dependent processes is proposed.

Acknowledgment

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