LIGHT-INDUCED VOLUME CHANGES
IN SPINACH CHLOROPLASTS

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ABSTRACT

A light-dependent mechanism that results in a slow, high-amplitude swelling of spinach chloroplasts *in vitro* has been discovered. The swelling is readily observed by optical and gravimetric methods, and by the use of an electronic particle counter; all show a 100 per cent increase of chloroplast volume in the light with an approximately 10-minute half-time. The existence of an osmotic mechanism for chloroplast swelling in the dark is confirmed. The volume of illuminated chloroplasts versus NaCl concentration represents the addition of osmotic and light effects. The action of light is enhanced by electron flow cofactors, such as phenazine methosulfate (PMS). However, neither conditions for ATP hydrolysis or synthesis nor NH_4Cl influence the time course and extent of swelling. Hence, high-amplitude chloroplast swelling is light- (or electron flow), but not energy-dependent. A remarkable inhibitory effect of inorganic phosphate on chloroplast swelling is observed in the light, but not in the dark. Another action of light on chloroplasts is known to result in a shrinkage of chloroplasts which is rapid, reversible, energy-dependent, and requires phosphate. Thus phosphate determines the action of light on chloroplast volume. Since shrinkage is reversible, but swelling is not, it may be that they reflect physiological and deteriorative processes, respectively. Chloroplasts and mitochondria appear to control their volume by similar mechanisms.

INTRODUCTION

Chloroplasts *in vitro* manifest active and passive mechanisms controlling their structure. There is an osmotic mechanism, which results in alterations in chloroplast volume in response to changes in tonicity of suspending media, and a light-dependent mechanism. Nishida (1) has described the passive osmotic swelling of spinach chloroplasts in sucrose solutions by optical, gravimetric, and volumetric techniques. The light-induced changes in chloroplast structure, which have been observed by light-scattering (2) and by the Coulter counter and electron microscopy (3), correspond to shrinkage both *in vitro* (2, 3) and *in vivo* (4). This action of light brings about low-amplitude volume changes in chloroplasts by energy-dependent mechanisms, *i.e.* a shrinkage occurs under conditions of electron transport, light-triggered ATPase, or both (5) which is characterized by being rapid and reversible (with half-times for its growth in light and decay in dark of about 20 seconds).

Chloroplasts and mitochondria (6, 7) resemble one another in that both systems manifest osmotic and low-amplitude volume changes. Moreover, mitochondria show a deteriorative type of swelling that is dependent upon electron transport (6). High-amplitude volume changes occur more slowly than low-amplitude, and are accompanied by extensive changes in mitochondrial structure

and function. This type of swelling change has not yet been described for chloroplasts. The close correspondence of the response of chloroplasts and mitochondria with regard to structural changes suggested that chloroplasts might manifest a similar high-amplitude swelling process.

In this paper, the discovery and characterization by various methods of a light-dependent high-amplitude swelling process in spinach chloroplasts will be described.[1] The factors which activate and inhibit this process have helped bring into focus the similarities and differences between the various mechanisms that control chloroplast volume *in vitro*.

METHODS

CHLOROPLAST ISOLATION: Spinach was purchased commercially and chloroplasts isolated at 4°C in one of the following two ways, designated Method I and Method II. In Method I, the spinach leaves were washed and the midribs removed. The leaves were then placed in a Waring blendor for 30 seconds in Tris-HCl (100 mM, pH 7.2) and NaCl (350 mM). The crude suspension was filtered through four layers of cheesecloth and then centrifuged at 200 g for 5 minutes. The supernatant was centrifuged for 15 minutes at 600 g and the chloroplast pellet was collected and resuspended in the isolation medium. Chloroplasts prepared by Method II were isolated in Tris-HCl (50 mM, pH 8.0) and NaCl (175 mM) with the above procedure except that the first centrifugation was for 1 minute at 200 g and the supernatant was then centrifuged at 200 g for 10 minutes. Although Method II appeared to yield a higher percentage of whole chloroplasts based upon a more defined peak in Coulter counter studies, both preparations gave comparable results. Chlorophyll was determined spectrophotometrically.

CHLOROPLAST VOLUME DETERMINATIONS: The chloroplast suspensions were diluted to 0.2 mg chlorophyll/ml (unless otherwise indicated) in the following media: for chloroplasts prepared according to Method I, Tris-HCl (20 mM, pH 8.0) and NaCl (35 mM); for Method II, Tris-HCl (50 mM, pH 8.0) and NaCl (175 mM). Two-ml aliquots of chloroplast suspensions with additions as indicated in individual experiments were transferred to 3-ml graduated protein (or "chlorocrit") tubes (Bauer-Schenck Kimax Protein tubes) which can be read to 0.001 ml. The tubes (dark ones covered with aluminum foil) were preincubated in a constant temperature bath at 25°C. A 150-watt tungsten reflector flood

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[1] A preliminary report of this research has been published (*Biochem. and Biophys. Research Comm.*, 1965, 18, 474.)

lamp was used as a light source (25,000 lux) for both the incubation and centrifugation at 1,000 g (International Clinical Centrifuge, Model CL). Specially constructed transparent lucite tube holders which fit into the trunnions of the clinical centrifuge were employed to permit illumination of the light samples. Packed volumes were determined by reading the pellet volume on the graduated tubes. The variability between identical samples has been found to be approximately 5 per cent. For fresh-weight determinations, 5-ml aliquots of chloroplast suspensions (200 μg chlorophyll/ml) were used. After incubation, a chloroplast pellet was formed by centrifuging at 27,000 g for 10 minutes; the supernatant was next removed, and the pellet weighed.

Light-induced absorbancy decreases of chloroplasts (isolated by Method I) were followed at 540 mμ in a Beckman DB spectrophotometer (1). The reaction mixture, containing Tris-HCl (20 mM, pH 8), NaCl (350 mM), PMS (phenazine metholsulfate, 20 μM), and chloroplasts (9 to 17 μg chlorophyll/ml), was illuminated in test tubes under continuous shaking conditions in a water-bath at 25°C. The absorbancy (OD × 1,000) of light and dark samples were measured at 0 and 30 minutes by reading against a reagent blank. Zero time readings were taken several minutes after mixing chloroplasts with the reaction medium to allow completion of osmotic volume changes. The results are given as the difference between these two readings (decrease of absorbancy) resulting from swelling $(-\Delta E_{540m\mu})$.

A Coulter counter, model B, with a Particle Size Distribution Plotter was also used to measure chloroplast volume. Calibration of the 100-μ orifice was made with pollen grains (3,884 μ^3) and checked with human erythrocytes (about 80 μ^3). Chloroplasts were isolated by Method II and the reaction mixture contained Tris-HCl (50 mM, pH 8), NaCl (175 mM), chloroplasts (15 μg chlorophyll/ml), and, where indicated, PMS (20 μM). Just before measurement, the chlorophyll concentration was diluted to 5 mμg/ml which corresponded to approximately 7,500 chloroplasts/ml. Chloroplast volume is defined as the total volume of all particles greater than 12 μ^3, divided by the number of such particles.

RESULTS

Light-induced, high-amplitude chloroplast swelling was first discovered when the sedimentation of chloroplasts was being studied under relatively simple conditions by means of packed volume (Fig. 1). The effect of light was observed by performing an experiment in which one tube was incubated and centrifuged in the light, and its control in the dark. The pellet volume becomes smaller in the dark, and larger in the light as centrifuga-
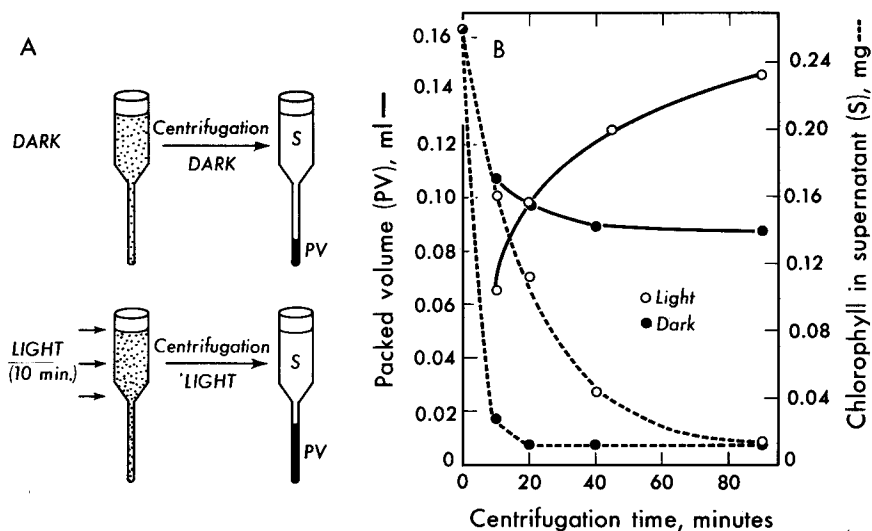
FIGURE 1 Influence of light on the sedimentation and packed volume of spinach chloroplasts. Chloroplasts (isolated by Method I) were incubated for 10 minutes under conditions as described in Methods. *A*, Use of "chlorocrit tubes." *B*, Light-induced pellet formation and time course for the removal of chlorophyll-containing material from the supernatant fraction.

tion proceeds. The packed volume in the light is approximately 70 per cent larger after 90 minutes, when essentially all of the chlorophyll-containing material in the supernatant has been removed. The results show that in the dark nearly all the particles are brought down in 20 minutes, whereas in the light it takes 60 to 90 minutes to achieve the same state. Although light obviously causes an unusual change in the pattern of sedimentation of chloroplasts,[2] it is clear that after all the chlorophyll-containing material in the supernatant fraction is removed, the pellet is much larger in the light.

Since volume changes of chloroplasts may be brought about by an osmotic mechanism (1), a test for the existence of this type of swelling was performed by determining the volume of the chloroplasts. Fig. 2 shows that packed volume continuously decreases as the NaCl concentration is increased in accordance with the expected osmotic effect. However, other factors are involved, since packed volume is always greater in the light and the curve does not have the same shape as in the dark. In spite of this, a measurement of the packed volume as a function of tonicity reveals an increase

of volume (about twofold) over a very wide range of NaCl concentration. It is concluded that the light-induced increase of packed volume occurs independently of the osmotic mechanism.

To confirm if packed volume measurements are a reliable indicator of chloroplast volume, a comparison was made between several methods for assessing swelling. Fig. 3 shows that a light-dependent decrease in absorbancy of the chloroplast suspension occurs. The absorbancy change is relatively independent of the NaCl concentration over the same range as in the packed volume studies, although absorbancy experiments are made at low chlorophyll concentrations compared to packed volume. Packed volume results have also been validated by performing absorbancy experiments at 200 μg chlorophyll/ml, by taking readings immediately after diluting the samples ten- to twentyfold.

Nishida has shown (1), and we verify here, that decreases in absorbance correspond to swelling. Further proof for the correctness of this interpretation is given in Table I, in which it is shown that absorbancy decreases correspond with increases in packed volume, wet weight, and volume as determined by the Coulter counter. All methods show a large light-induced swelling with a much smaller swelling in the dark, even when the chlorophyll concentration ranges from 5 mμg/ml

[2] Perhaps the slower sedimentation in the presence of light reflects density decreases or changes in the surface characteristics of chloroplasts that accompany the swelling.
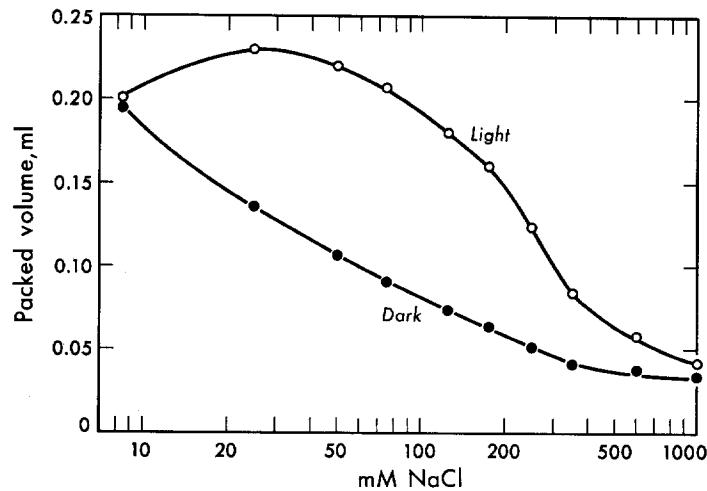
FIGURE 2   Influence of sodium chloride concentration on the packed volume of chloroplasts. The reaction mixture contained 20 mM Tris-HCl (pH 8), NaCl, and chloroplasts isolated by Method II. Tubes were incubated for 10 minutes at the indicated salt concentration and then centrifuged for 2 hours before reading pellet volume.
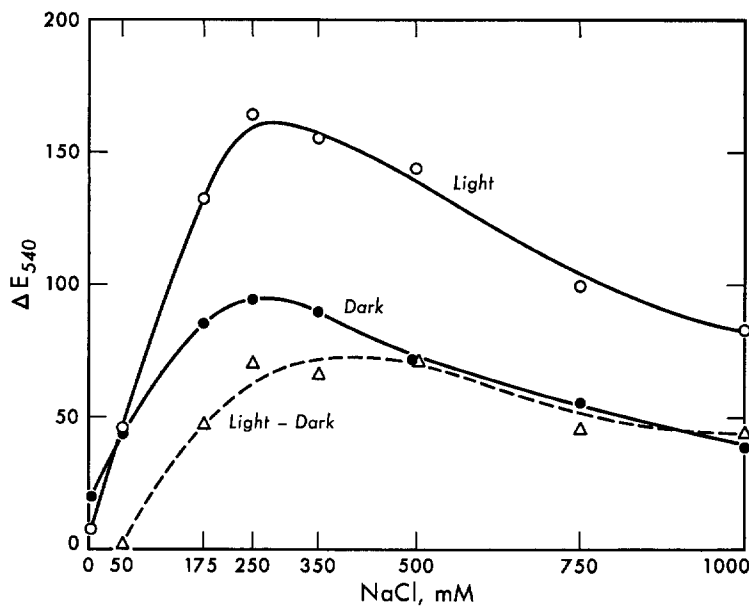

FIGURE 3   Influence of sodium chloride concentration on absorbancy decreases of chloroplast suspensions. Conditions are as in Methods; chlorophyll was 9 μg/ml.

(Coulter counter) to 200 μg/ml (packed volume and fresh weight). It has been found that PMS is an activator of the light-induced swelling process, and hence it was used in the experiments performed to compare the light-induced volume changes by various methods.

The influence of PMS on the time course for the swelling of illuminated chloroplasts is shown in Fig. 4, in which the Coulter counter was employed to monitor the volume changes. It is found that in the presence of PMS, the extent and rate of the swelling of the illuminated chloroplasts is greater. Following illumination, extensive swelling occurs in the first 20 minutes. After this time, swelling

TABLE I

Comparison of Methods for Measuring Light-Dependent High-Amplitude Chloroplast Swelling

The reaction mixture contained Tris-HCl (20 mM, pH 8), NaCl (350 mM), PMS (20 μM), and chloroplasts isolated by Method I, except for Coulter counter measurements. Other conditions are described in Methods except that for absorbancy determination, samples were diluted 20-fold (just before reading) from an initial concentration of 200 μg chlorophyll/ml.

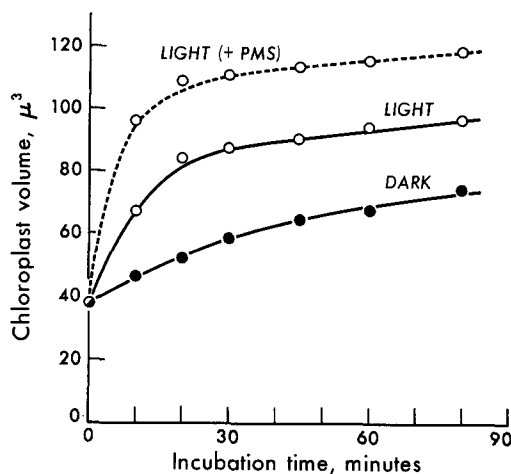| Time	Packed volume		Fresh weight		Absorbancy		Coulter counter	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
min.	*ml*	*ml*	*mg*	*mg*	$E_{540\ m\mu}$	$E_{540\ m\mu}$	μ^3	μ^3
0	0.36	0.34	47.0	47.0	385	395	58	58
30	0.72	0.46	75.0	53.0	240	348	118	71

FIGURE 4 Time course of chloroplast swelling measured with the Coulter counter. Chloroplasts were suspended in Tris (50 mM, pH 8), NaCl (175 mM), with or without PMS (20 μM).

continues, but at a reduced rate which is comparable to the rate in the dark.

The influence of other cofactors and inhibitors of high-amplitude swelling are presented in Table II. Cofactors for electron flow such as flavine mononucleotide, PMS (as already described), and ferricyanide stimulate swelling in the light, but not in the dark; NADP plus ferredoxin causes an inhibition of the light-stimulated swelling. The extent of swelling with ferricyanide is less than for PMS. A test was also made for the effect of photophosphorylation and ATPase conditions, and it was found that neither affected the high-amplitude swelling. Addition of ATP plus MgCl₂ after chloroplast swelling has occurred does not induce shrinkage. However, inorganic phosphate

TABLE II

Test for Light- and Energy-Dependence of High-Amplitude Swelling of Chloroplasts

Reaction conditions are as described in Methods, except that PMS was omitted and additions were made as indicated.

Conditions	Light	Dark
	% Decrease E_{540}	% Decrease E_{540}
Electron flow		
None	40	12
PMS (20 μM)	65	13
Flavine mononucleotide (20 μM)	48	11
Fe(CN)₆⁻³ (3 mM)	53	11
NADP (5 mM) + ferredoxin	24	10
Photophosphorylation		
PMS (20 μM), MgCl₂ (5 mM), Pᵢ (2 mM), ADP (2 mM)	65	12
ATPase		
PMS (20 μM), ATP (2 mM), MgCl₂ (5 mM)	63	12
PMS (20 μM), Pᵢ (5 mM)	25	11

has been found to be a potent inhibitor of the light-dependent swelling.

Since the action of phosphate on high-amplitude swelling could be important as a control mechanism, a more detailed study of its inhibitory effect was undertaken, as shown in Fig. 5. A remarkable action of phosphate was observed on the swelling of illuminated chloroplasts. A progressive inhibition of swelling is found as the phosphate concentration is raised to 20 mM. The inhibition by phosphate is
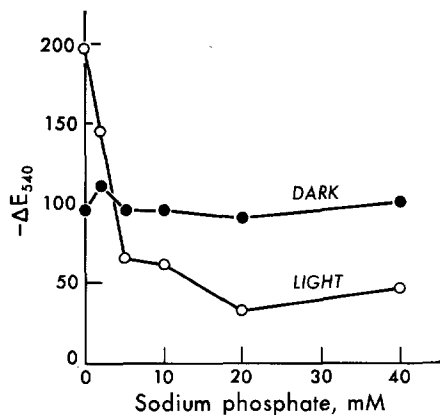
FIGURE 5 Influence of inorganic phosphate on light-dependent volume changes of chloroplasts. Assay conditions are as described in Methods, except that absorbancy readings were made after 20 minutes' incubation and the chlorophyll concentration was 17 μg/ml. The absorbancy was 665 at zero time.

so potent that it leads to shrinkage when compared to the dark control, which is unaffected by phosphate up to 40 mM. Potassium phosphate, sodium phosphate, and sodium arsenate show the same degree of inhibition[3]. Control experiments with sodium chloride showed that the effect of phosphate was not due to osmotic changes. Although phosphate is clearly a potent inhibitor of swelling, it was important to test if it were a contraction factor. A series of experiments was performed in which phosphate was added at various times. As long as swelling was incomplete, its further progress was inhibited by phosphate, but phosphate was not observed to cause contraction. The concentration of phosphate giving a 50 per cent inhibition of high-amplitude swelling is between 5 and 10 mM. The action of phosphate on swelling is probably not energy-dependent, since NH$_4$Cl does not abolish its inhibitory effect and because sodium arsenate acts like sodium phosphate.

DISCUSSION

It has been established that a 100 per cent increase of chloroplast volume can occur upon illumination. The features which characterize this chloroplast swelling are: (1) requirement for light; (2) enhancement of the effect of light on swelling by cofactors for cyclic flow; (3) absence of the involve-

[3] Other anions, particularly organic acids, have also recently been found to inhibit swelling.

ment of the energy-transfer pathway, based upon the inability of NH$_4$Cl, photophosphorylation, and ATPase conditions to modify the action of light on swelling; (4) marked inhibition of swelling by phosphate in illuminated chloroplasts; (5) slow time course, since more than 1 hour is required for completion; and (6) irreversibility.

Since two other mechanisms have been described for chloroplast volume changes, it is important to bring the features which control these processes into focus with one another. The passive osmotic mechanism for changing chloroplast volume may not require much comment. It has already been studied in some detail by Nishida (1) and is clearly shown in the dark curve of Fig. 2. In addition, the existence in isolated chloroplasts of osmotic sensitivity indicates some degree of structural integrity. The action of light seems to be superimposed on the osmotic effect, as light causes volume changes over a wide range of osmolarity.

Light-induced chloroplast shrinkage (2, 3), however, is an important mechanism for the metabolic control of chloroplast volume. Since light can also cause swelling under the circumstances described above, it is important to clarify the characteristics of these two light-dependent processes (2, 3, 5). Shrinkage of chloroplasts requires: (1) light; (2) cofactors for electron flow; (3) the presence of the energy-transfer pathway, based upon the involvement of photophosphorylation, ATPase, and inhibition by NH$_4$Cl; (4) and phosphate. Also, it is a relatively rapid process (10 to 100 seconds) and is reversible in darkness. It is apparent from the requirements for the two processes that phosphate availability could play a central role in determining what action light will have on chloroplast volume.

It is of some interest that corresponding processes for the control of volume have also been recognized and described in mitochondria (6, 7). Whether chloroplasts swell or shrink in response to light is an important question for plant cell physiology because (a) chloroplasts frequently occupy a substantial volume in the plant cell, (b) the type of volume change would influence ionic concentrations in chloroplasts and, therefore, in other cellular compartments, (c) physiological processes, dependent on osmoregulatory mechanisms in the cell such as stomatal control (8), may be affected, and (d) this action of light may trigger processes such as chloroplast deterioration.

REFERENCES

1. NISHIDA, K., Osmotic swelling of isolated chloroplasts, *Plant and Cell Physiol.*, 1963, **4**, 247.
2. PACKER, L., Structural changes correlated with photochemical phosphorylation in chloroplast membranes, *Biochim. et Biophysica Acta*, 1963, **75**, 12.
3. ITOH, M., IZAWA, S., and SHIBATA, K., Shrinkage of whole chloroplasts upon illumination, *Biochim. et Biophysica Acta*, 1963, **66**, 319.
4. KUSHIDA, H., ITOH, M., IZAWA, S., and SHIBATA, K., Deformation of chloroplasts on illumination in intact spinach leaves, *Biochim. et Biophysica Acta*, 1964, **79**, 201.
5. PACKER, L., and MARCHANT, R. H., Action of adenosine triphosphate on chloroplast structure, *J. Biol. Chem.*, 1964, **239**, 2061.
6. LEHNINGER, A. L., Water uptake and extrusion by mitochondria in relation to oxidative phosphorylation, *Physiol. Revs.*, 1962, **42**, 467.
7. PACKER, L., Size and shape transformations correlated with oxidative phosphorylation in mitochondria. I. Swelling-shrinkage mechanisms in intact mitochondria, *J. Cell Biol.*, 1963, **18**, 487.
8. PACKER, L., and SIEGENTHALER, P. A., Action of stomatal inhibitors on light-dependent changes of chloroplast volume, *Fed. Proc.*, 1965, **24**, 608.