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The Effect of Illuminating Spinach Chloroplasts on Their Membrane Permeability, Measured by a Dielectric Dispersion Technique

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Summary. A dielectric dispersion cell was modified to enable chloroplast suspensions placed between the electrodes to be illuminated directly. The dispersion (ε' *vs.* $1/\sqrt{\omega}$) for suspensions of intact spinach chloroplasts was linear between 0.2 and 5 MHz, the slope of the dispersion depending on the permeability of the internal membranes, the ionic concentration, and the chloroplast volume. The outer chloroplast envelope only contributed to the dispersion insofar as it retained ions within the stroma, thereby increasing the ionic concentration in the region of the internal membranes. When suspensions were illuminated, the slope of the dispersion decreased, due to chloroplast shrinkage. The permeability of the internal membranes remained constant following illumination, to within a 3% margin of error.

Terminology

Throughout the paper the following symbols are used:

ε' = dielectric constant of chloroplast suspension
ρ = volume concentration of chloroplasts
$\omega = 2\pi \times$ frequency of applied potential (s^{-1})
V = mean mobility of ions across the internal membranes ($m\,s^{-1}/V\,m^{-1}$)
c_0 = ionic concentration in the region of the membranes (m^{-3})
e = ionic charge (coulombs)
A = area of membranes per unit volume of suspension (m^{-1})
ε_0 = permittivity for free space ($F\,m^{-1}$)
k = Boltzmann's constant ($J\,°K^{-1}$)
T = temperature (°K)

The permeability of a membrane may depend on the rates of both active and passive transport of ions through the membrane. Passive transport through chloroplast internal membranes has been investigated by means of the dielectric dispersion technique described in a recent paper (Gordon, 1972), and is measured in terms of the mean ionic mobility V, that is,

the mean velocity of ions caused to move through the membranes by a unit electric field.

Although the concentration of ions near the membrane is c_0, it is possible that only a proportion of these ions actually move through the membranes, since transport may be limited to certain regions or sites in the membranes. V is the mean mobility for all c_0 ions, and thus it reflects the overall permeability of the membrane.

For a suspension of cane chloroplasts at 10 °C it was shown (Gordon, 1972) that between 0.5 and 50 MHz, ε' varied with frequency in a manner typical of electrodiffusion; that is, the movement of ions across a membrane under the opposing influences of an electric field and a charge concentration gradient (Cole, 1968). The slope $S_{\varepsilon'}$ of the dispersion ε' *vs.* $1/\sqrt{\omega}$ was shown to be

$$S_{\varepsilon'} \simeq \frac{3\rho^2}{(2+\rho)^2} \cdot \frac{c_0 e \sqrt{(Ve)}}{\varepsilon_0 A \sqrt{(2kT)}} \tag{1}$$

in the linear region. ρ was found to decrease as c_0 increased, due to the reported osmotic effect of added ions (Nishida & Koshii, 1964; Packer & Crofts, 1967). $A \simeq 160\ \mathrm{m^2/g}$ chloroplast protein (Mitchell, 1966), and since the protein/chlorophyll ratio is about 10 for spinach chloroplasts, then if the chlorophyll content of a suspension is known, A may be calculated. Measurement of $S_{\varepsilon'}$ therefore enables V to be estimated.

At lower frequencies, results are complicated by the high dielectric effect of the stroma proteins (Grant, Keefe & Takashima, 1968). At higher frequencies, instrumentation becomes difficult (Schwan, 1957), but as the dispersion characterized by Eq. (1) covers one and a half decades of frequency, accurate estimates of V may be made.

This method has the advantage that the membranes of intact chloroplasts may be investigated. In the present experiments, it was possible to examine the permeability of the chloroplast membranes at the same time that light-stimulated electron transfer was occurring.

Since membrane potentials are known to influence the permeability of some membranes (Cole, 1968), chloroplasts were investigated both under phosphorylating conditions, and phosphate-limited conditions. According to the chemiosmotic hypothesis (Mitchell, 1966), electron transfer in the chloroplast internal membranes leads to a charge separation or membrane potential across the membranes. Under phosphorylating conditions, the membrane potential would be partially dissipated by ATP formation, so one might expect a greater effect on the membrane permeability when phosphate-limited chloroplasts are illuminated.

Materials and Methods

Dispersion Cell

The cell previously described (Gordon, 1972) was modified to enable chloroplast suspensions placed between the electrodes to be illuminated directly (Fig. 1). White light from a tungsten filament spotlamp passed through heat filters before illuminating the sample at 50,000 lx. The platinum electrodes were connected to a Boonton-75C capacitance bridge for measurement of the dielectric constant of the sample between 0.2 and 0.5 MHz, and to a Hewlett-Packard-250B RX meter for measurement between 0.5 and 25 MHz. The accuracy of a single measurement of ε' was $\pm 1\%$. By means of the heat filters, the reflecting heat shield, and the water jacket maintained at 20 °C, the heating effect of illuminating the suspension was negligible. The cell constant was 4.9 mm and results were corrected for electrode polarization and inductive effects by replacing chloroplast suspensions by their supernatants (Schwan, 1957). At the high frequencies investigated, polarization effects were small.

Chloroplast Suspensions

The following media were used in preparing chloroplast suspensions:

M1: 0.33 M sorbitol solution containing 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM sodium iso-ascorbate and 10 mM $Na_4P_2O_7 \cdot 1\,H_2O$ adjusted to pH 6.5 at 4 °C with HCl.

M2: 0.33 M sucrose solution containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 50 mM tricine, adjusted to pH 7.6 at 20 °C with NaOH.

M3: 0.155 M sucrose solution containing 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM EDTA, 25 mM tricine, adjusted to pH 7.6 at 20 °C with NaOH.

Spinach *(Spinacia oleracea)* was field grown and sugar cane (*Saccharum officinarum*, var. unknown) was grown in a greenhouse maintained above 10 °C. Chloroplasts were isolated by a modification of the method of Cockburn, Walker and Baldry (1968). Thirty grams of spinach leaf tissue or 20 g of deribbed cane leaf tissue were cut into small pieces and ground in a domestic blender containing 200 ml of the semi-frozen medium M1. The macerate was squeezed through two layers and filtered through

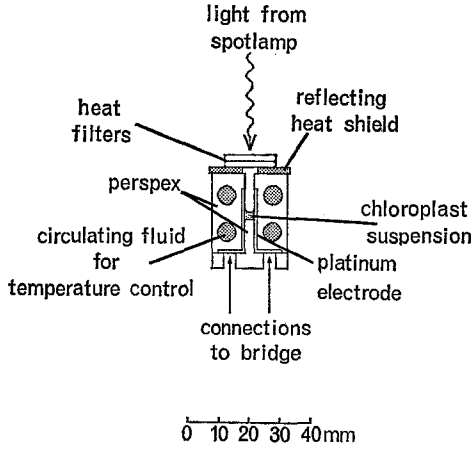

Fig. 1. Method of illuminating dispersion cell

eight layers of muslin, then centrifuged at 1,600 × *g* for 4 min at 4 °C. The supernatant was discarded and the pellet mixed and washed in 14 ml of M2. The suspension was then recentrifuged at 1,600 × *g* for 4 min. The supernatant was again discarded, and the pellet remixed into a small volume of M2 or M3 to which an appropriate molarity of KCl was added. Unless otherwise stated, the chlorophyll content of suspensions was adjusted to 100 µg/0.1 ml. Chlorophyll concentrations were measured in 80% acetone by the method of Bruinsma (1961).

Volume concentrations of chloroplast suspensions were measured using a Hawksley Micro-Haematocrit Centrifuge, operating at 12,000 × *g* for 6 min. The degree of chloroplast integrity was determined by viewing suspensions in the phasecontrast microscope (light ground illumination). Intact chloroplasts have a highly refractive appearance compared with outer-envelope-free chloroplasts (Walker, 1967).

Oxygen evolution or uptake by chloroplasts was assayed in the oxygen electrode at 20 °C by the method of Cockburn *et al.* (1968). A sample of 0.2 ml of the chloroplast suspension was mixed with another 2.8 ml of the same resuspending medium, before illumination in the electrode at 43,000 lx.

Results

Unilluminated spinach chloroplast suspensions at 20 °C showed linear dispersions between 0.2 and 5 MHz. For cane chloroplasts the linear region was between 0.5 and 25 MHz (Fig. 2).

If T, A and e remain constant, Eq. (1) shows that

$$\frac{(2+\rho)^2}{\rho^2} S_{\varepsilon'} \propto c_0 \sqrt{V} \tag{2}$$

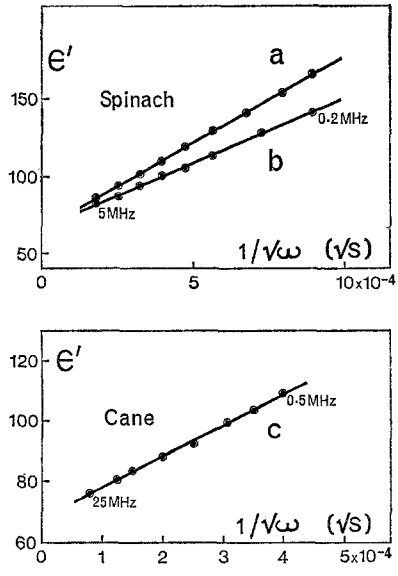

Fig. 2. Dispersion curves for chloroplast suspensions. Spinach chloroplasts were about 40% intact. (*a*) Resuspending medium M2+40 mM KCl; $\rho = 4.4\%$. (*b*) Resuspending medium M2+0 mM KCl; $\rho = 4.3\%$. (*c*) Resuspending medium M2+40 mM KCl; $\rho = 5.1\%$

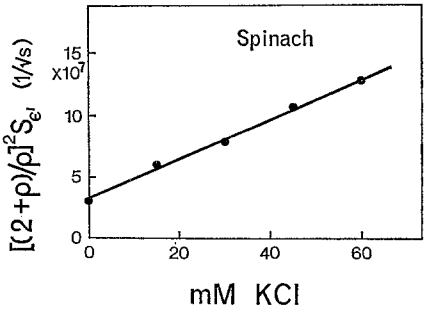

Fig. 3. Graph used to determine mobility of KCl ions. Suspension chlorophyll 94 μg/0.1 ml. Resuspending medium M2+quoted KCl. Chloroplasts outer-envelope-free

and therefore $[(2+\rho)/\rho]^2 S_{\varepsilon'}$ should be linear with increasing concentration of ions (e.g., KCl) in the resuspending medium, provided V is constant. This was tested for outer-envelope-free spinach chloroplasts (the reason for this will be given later) from 0 to 60 mM KCl, to cover the range of concentrations used in other investigations. A linear graph was obtained (Fig. 3) from which it was deduced that the mean mobility of ions of KCl through the internal membranes of this sample of spinach chloroplasts was 2.7×10^{-10} m $s^{-1}/V\ m^{-1}$. Other investigations in the same way gave a range of values of V for spinach chloroplasts from 1×10^{-10} to 4×10^{-10} m $s^{-1}/V\ m^{-1}$.

Effect of Outer Envelope

The dispersion for suspensions of outer-envelope-free chloroplasts is due to the movement of ions across the chloroplast internal membranes. Hope (1956) assumed that dispersions for intact *Chara* chloroplast suspensions were due to the capacitance of the membranes comprising the outer envelope, but the circular Cole-Cole plots necessary to support this view were not obtained for either cane or intact spinach chloroplast preparations, even when the frequency was reduced to 5 KHz. The unity loss tangent reported for the dispersions (Gordon, 1972) in fact yields linear Cole-Cole plots, indicating that electrodiffusion was the major factor in the dispersion even for intact chloroplasts, rather than a static capacitance.

However, if the dispersion technique is to be used to investigate the permeability of the internal membranes of viable spinach chloroplasts – which must be intact to evolve oxygen – it is important to determine what effect the outer envelope does have on the dispersion.

Table 1. Spinach chloroplasts

Resuspending medium	$[(2+\rho)/\rho]^2 S_{\varepsilon'}(1/\sqrt{s})$	
	Outer-envelope-free	50% intact
M2 + 0 mM KCl	2.5×10^7	4.4×10^7
M2 + 50 mM KCl	10.3×10^7	18.3×10^7

Table 2. Cane chloroplasts

Resuspending medium	$[(2+\rho)/\rho]^2 S_{\varepsilon'}(1/\sqrt{s})$	
	Osmotically shocked	Normal preparation
M2 + 0 mM KCl	2.8×10^7	2.8×10^7
M2 + 50 mM KCl	11.8×10^7	11.0×10^7

The outer envelope was removed by osmotically shocking chloroplasts in deionized distilled water instead of M2 during the isolation process. When comparison was made between intact and outer-envelope-free chloroplasts resuspended in identical media, $[(2+\rho)/\rho]^2 S_{\varepsilon'}$ was found to be greater for intact chloroplasts (Table 1). This was not caused by the osmotic shock given to the outer-envelope-free chloroplasts (which might, for example, have affected V) since cane chloroplasts treated in the same way as the spinach showed no differences in $[(2+\rho)/\rho]^2 S_{\varepsilon'}$ (Table 2).

Since cane chloroplasts were outer-envelope-free whether they were osmotically shocked or not, as shown by electron microscopy (J. Coombs, *personal communication*) it appeared that the increased value of $[(2+\rho)/\rho]^2 S_{\varepsilon'}$ for intact spinach chloroplasts was in some way related to the presence of the outer envelope. However, it was apparently not due to the capacitance of the outer envelope, since during the course of an experiment in which a suspension remained 40% intact, $[(2+\rho)/\rho]^2 S_{\varepsilon'}$ decreased with time towards the value for outer-envelope-free chloroplasts (Fig. 4).

From Eq. (2) these results imply that, although intact and outer-envelope-free chloroplasts were suspended in media whose ionic concentrations were the same, c_0 (the ionic concentration in the region of the internal membranes) was dependent on whether the outer envelope was present or not. Thus, it seems that when the outer membrane was present, certain

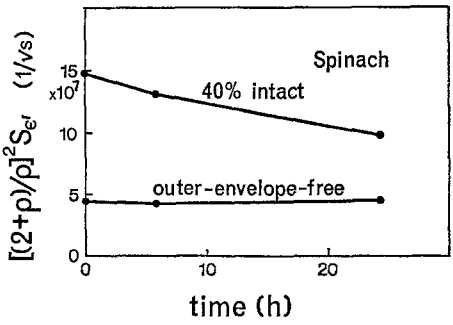

Fig. 4. Variation in $[(2+\rho)/\rho]^2 S_{\varepsilon'}$ with time for intact and outer-envelope-free chloroplasts. Resuspending medium M2+0 mM KCl

ions were retained within the stroma surrounding the internal membranes, thereby increasing c_0. This could be caused by a buffering action delaying the loss of ions, or to the retention of proteins in the stroma with fixed charges holding back an equivalent amount of mobile counterions, or to some ion-selective property of the outer membrane itself (Saltman, Forte & Forte, 1963; Heldt & Sauer, 1971). The osmotic shrinkage caused by increased KCl in the medium would further increase c_0, and hence $[(2+\rho)/\rho]^2 S_{\varepsilon'}$ (Table 1). The results of Fig. 4 are consistent with ions slowly leaking from the stroma.

Thus, when calculating the permeability of internal membranes (Fig. 3), a sample of outer-envelope-free chloroplasts was used, since c_0 was given by the concentration of KCl in the resuspending medium, and therefore was accurately known.

Illuminated Chloroplasts

An intact chloroplast preparation should be capable of stoichiometric oxygen evolution linked to CO_2 fixation through electron transfer in the internal membranes (Walker & Hill, 1967). ATP should be coupled to electron transfer, which will be rate-limited if insufficient ADP and phosphate are present in the preparation.

In the present experiments, spinach chloroplast suspensions contained up to 60% intact chloroplasts demonstrating light-dependent oxygen evolution (Fig. 6*B*). The outer-envelope-free chloroplasts within the suspension were capable of electron transfer linked to light-dependent oxygen uptake (Mehler, 1951).

Spinach Chloroplasts – 100% Outer-Envelope-Free. On illuminating chloroplasts which had been osmotically shocked before being resuspended

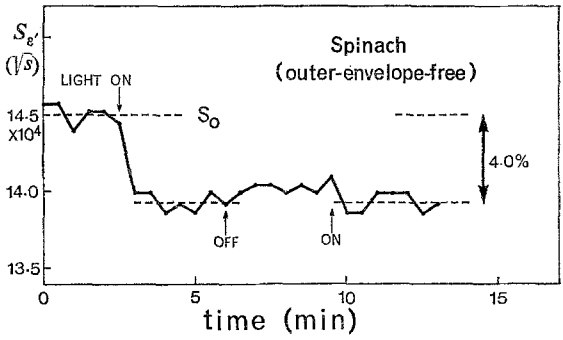

Fig. 5. Decrease in $S_{\varepsilon'}$ on illuminating outer-envelope-free chloroplasts. Resuspending medium M2 + 60 mM KCl + 4 mM CO_2 (in the form of $NaHCO_2$)

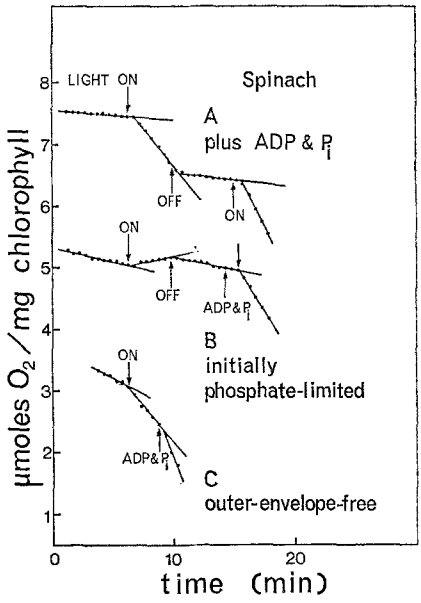

Fig. 6. Oxygen uptake by spinach chloroplasts. Samples A and B 60% intact, and resuspended in medium M3 + 40 mM KCl. Sample C resuspended in medium M2 + 60 mM KCl + 4 mM CO_2. Where indicated, 7.5 mM ADP and 7.5 mM di-sodium hydrogen orthophosphate were added

in medium M2, $S_{\varepsilon'}$ decreased by 4% below the initial value of S_0 (Fig. 5). When the light was subsequently switched off, $S_{\varepsilon'}$ slowly increased again. In the oxygen electrode the chloroplasts demonstrated phosphate-limited electron transfer (Fig. 6*C*). The dielectric constant of the supernatant did not vary following illumination.

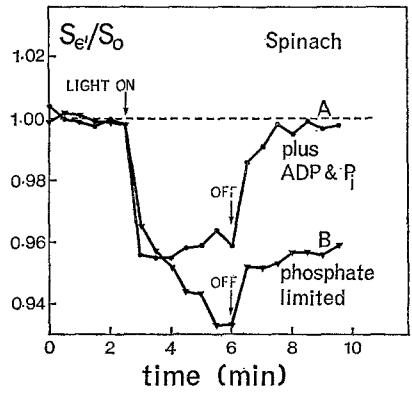

Fig. 7. Decrease in $S_{\varepsilon'}$ on illuminating 60% intact spinach chloroplasts. (*A*) Resuspending medium M3 + 40 mM KCl + 7.5 mM ADP + 7.5 mM di-sodium hydrogen orthophosphate. (*B*) Resuspending medium M3 + 40 mM KCl

Spinach Chloroplasts – 60% Intact. Chloroplasts from a single preparation were either resuspended in medium M3 (sample B) or M3 to which ADP and phosphate were added (sample A). Both samples were coupled, but electron transfer was rate-limited in the case of sample B (Fig. 6*A* and *B*). By expressing variations in $S_{\varepsilon'}$ in terms of $S_{\varepsilon'}/S_0$ the percentage decrease in $S_{\varepsilon'}$ was immediately apparent (Fig. 7).

Both samples showed a decrease in $S_{\varepsilon'}$ following illumination, although the percentage decrease was greater for the phosphate-limited chloroplasts, which also showed a slower increase in $S_{\varepsilon'}$ when the light was switched off. Thus the slow increase in $S_{\varepsilon'}$ in the dark phase for outer-envelope-free chloroplasts (Fig. 5) was probably also related to their lack of ADP and phosphate.

On ceasing illumination of intact chloroplasts capable of phosphorylation, $S_{\varepsilon'}$ increased to its original value within about 2 min (Figs. 7*A* and 8). $S_{\varepsilon'}$ decreased again when pre-illuminated chloroplasts received a further period of illumination (Fig. 8), showing that the light had not caused irreversible damage to the chloroplast membranes.

Reason for Decreased $S_{\varepsilon'}$. From Eq. (2)

$$S_{\varepsilon'} \propto \frac{\rho^2}{(2+\rho)^2} c_0 \sqrt{V} \tag{3}$$

and therefore a decrease in $S_{\varepsilon'}$ of about 5% on illumination could either be caused by a 10% reduction in the ionic mobility or a 2.5% chloroplast shrinkage (Good, Izawa & Hind, 1966), if c_0 remains effectively constant (*see* Discussion).

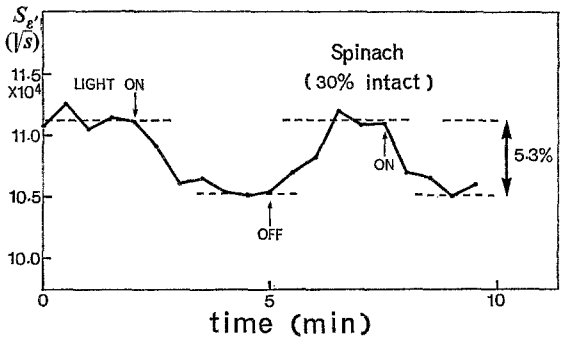

Fig. 8. Decrease in $S_{\varepsilon'}$ on illuminating a suspension of 30% intact chloroplasts. Resuspending medium M2+30 mM KCl+3 mM CO_2+15 mM ADP and 15 mM di-sodium hydrogen orthophosphate

A separate measurement was therefore made of the packed chloroplast volume by the micro-haematocrit method. Although this method is direct, it takes about 6 min to perform. It will be seen from Fig. 7 that any changes in sample A would have been almost completely reversed in this time, and therefore phosphate-limited chloroplasts (prepared as sample B) were investigated. In a typical experiment, $S_{\varepsilon'}$ decreased by $5.4 \pm 1.6\%$ while ρ decreased by $2.2 \pm 0.6\%$ which would give rise to a $4.0 \pm 1.1\%$ decrease in $[\rho/(2+\rho)]^2$. The slight discrepancy between the decrease in $S_{\varepsilon'}$ and $[\rho/(2+\rho)]^2$ is most probably caused by a partial reversal of shrinkage for these chloroplasts, so that the decrease in $S_{\varepsilon'}$ on illumination can be explained in terms of chloroplast shrinkage alone.

Without added KCl, the ions in the medium M2 (predominantly Mg^{++}, Mn^{++} and Cl^-) and the stroma ions contributed significantly to the dispersion (Figs. 2 and 3). When the concentration of KCl was reduced (Fig. 9)

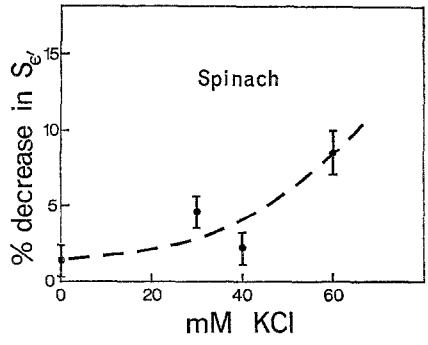

Fig. 9. Effect of KCl concentration on the per cent of decrease in $S_{\varepsilon'}$ on illumination. 50% intact chloroplasts resuspended in medium M2+4 mM CO_2

the decrease in $S_{\varepsilon'}$ on illumination was reduced, so that in the absence of any KCl the percentage decrease was about $1.4 \pm 1.0\%$, and chloroplast volume changes were too small to be detected. Thus, if there was any variation in the permeability of the internal membranes to the ions in the stroma or the surrounding medium, it was less than $2.8 \pm 2.0\%$.

At increased concentrations of KCl, the decrease in $S_{\varepsilon'}$ on illumination was shown to be consistent with shrinkage, and thus the permeability of the membranes to KCl was not affected to within 3%.

Discussion

The observed shrinkage was probably caused by the transport of osmotically active ions from the chloroplast in response to proton uptake (Packer & Crofts, 1967). Both shrinkage and swelling of chloroplasts on illumination have been observed by other workers (Good *et al.*, 1966), the magnitude of volume changes depending on the medium in which the chloroplasts were resuspended. Similarly, the decrease in $S_{\varepsilon'}$ was found to depend on the resuspending medium (Figs. 5, 7 and 9). The results of Fig. 7 suggest a partial suppression of shrinkage when ADP and phosphate were added to the resuspending medium, a result also reported by Dilley and Vernon (1964). The incomplete reversal of shrinkage for phosphate-limited chloroplasts (Figs. 5 and 7*B*) may be compared with the incomplete reversal of light-induced scattering increases for spinach chloroplasts under conditions of ATP hydrolysis, reported by Packer and Crofts (1967).

Since variations in $S_{\varepsilon'}$ correspond to chloroplast volume changes reported by other workers, and since chloroplast shrinkage was detected in the present experiments using the micro-haematocrit method, it is unlikely that the membrane permeability is affected by illumination, either for phosphate-limited chloroplasts, or for chloroplasts capable of phosphorylation and light-dependent oxygen evolution.

From Eq. (3), if the decrease in $S_{\varepsilon'}$ can be accounted for in terms of chloroplast shrinkage, this means that either variations in c_0 are small following illumination, or that they are reflected by variations in ρ. The latter is almost certainly true since, as already mentioned, chloroplast shrinkage is apparently caused by an increase in the number of osmotically active ions outside the chloroplast. It is also reasonable to suppose that variations in c_0 would be small following illumination since (1) when

protons are taken up by the chloroplasts (thereby increasing the ionic concentration within the chloroplasts), other ions are excreted in exchange, so the net effect on c_0 could be small and (2) the majority of the ions comprising c_0 were externally added, thereby providing a "sink" for local fluctuations in ionic concentration.

However, the concentration of the stroma ions would increase while ρ decreased, so that the percentage decrease in $S_{\varepsilon'}$ would be proportionately less. At most, the percentage decrease in ρ would be equal to the percentage increase in c_0 (this would be in the absence of any externally added ions) so that the percentage decrease in $S_{\varepsilon'}$ would equal the percentage decrease in $\rho/(2+\rho)^2$ rather than $[\rho/(2+\rho)]^2$. Therefore, a decrease in $S_{\varepsilon'}$ would still indicate chloroplast shrinkage.

Thus, the dispersion method could also prove a useful tool for investigating chloroplast volume changes. This method has the advantage that the dispersion depends primarily on chloroplast volumes, rather than their axial ratios (Fricke, 1924), while light-scattering methods are significantly affected by conformational as well as volume changes (Itoh, Izawa & Shibata, 1963).

I would like to thank Dr. C. Bucke for helpful discussion during this work.

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