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Effects of Cations and Abscisic Acid on Chlorophyll *a* Fluorescence in Guard Cells of *Vicia faba*[1]

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ABSTRACT

The effects of cations and abscisic acid on chloroplast activity in guard cells of *Vicia faba* were investigated by analysis of the transient of chlorophyll *a* fluorescence. When epidermal strips containing guard cells as the only living cells were incubated in water and illuminated with strong light, chlorophyll *a* fluorescence rose rapidly to a high intensity and then declined slowly to a stationary level. The rate of this decline was enhanced by K^+ or Na^+, and the effect of these cations was greater when added with phosphate than with chloride as the anion. Ca^{2+} suppressed the enhancement by Na^+ and, to a lesser extent, that by K^+. Abscisic acid also suppressed the enhancement by K^+ and Na^+. Since the fluorescence decline reflects the increase of intrathylakoid H^+ concentration necessary for photophosphorylation, the acceleration of the decline by K^+ (or Na^+ in the absence of Ca^{2+}) implicates chloroplast activity in ion accumulation by guard cells in the light. The differential effects of phosphate and chloride suggest that chloroplast activity may be involved in malate formation in guard cells in the light.

Stomatal guard cells of most plant species contain chloroplasts. Action spectra (12, 20) and inhibitor data (7) suggest that these chloroplasts may play a role in stomatal opening (6), perhaps as a source of reducing or high energy phosphate equivalents for the import of K^+ and synthesis of organic counterions (25). The chloroplasts possess both photosystems (23) of photosynthesis and are capable of linear, light-driven electron transport (30). However, they do not contain certain enzymes of the Calvin-Benson cycle (22) and are therefore unlikely to fix CO_2 photosynthetically. The specific involvement of the chloroplasts in ion transport and counterion synthesis remains to be demonstrated, however.

The kinetics of Chl *a* fluorescence has been correlated with specific photosynthetic partial reactions and are sensitive indicators of photosynthetic activity (for reviews see 5, 16, 26). When dark-adapted mesophyll or algal cells are illuminated with strong light, the fluorescence transient begins from a low level (O or F_0) followed by three rapidly appearing phases, designated as I (intermediary hump), D (dip) and P (peak or F_∞) during the first second of illumination. From P, the fluorescence slowly declines to a quasi-stationary level, S, rises to a maximum level, M, and then declines to a terminal steady state level, T, within a few minutes of illumination. The fast changes, OIDP, are correlated with early photochemical events in the PSII reaction center and electron transport chain, whereas the later changes, PSMT, are correlated with alterations in energy state and membrane structure associated with photophosphorylation.

The present study was undertaken to evaluate the effects of cations and ABA on the fluorescence transients in guard cells. The observed dependence of the fluorescence kinetics on conditions known to affect stomatal aperture *in vivo* suggests that chloroplast activity in guard cells is involved in ion import and counterion production for stomatal opening.

MATERIALS AND METHODS

Preparation of Sonicated Epidermal Strips. Seeds of *Vicia faba* L. cv. Long Pod (Burpee Seed Co., Warminster, PA) were germinated in pots and grown in a growth chamber at 28°C day (14 h)/18°C night (10 h) at radiation intensities (daylight fluorescent) of 400 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 3 to 4 weeks. Plants remained in the dark for 2 h before each experiment.

Epidermal strips were peeled from the abaxial (lower) surface of young leaves and put into distilled H_2O cooled in an ice bath. The strips were taken only from the tips and margins of young leaves to avoid large veins and were gently rubbed under water to remove most adhering mesophyll cells. The strips were sonicated (20) at 0°C for 20 s (Sonic Dismembrator; Quigley-Rochester, Inc., Rochester, NY). Sonication was repeated three times with fresh cold water. Prior to sonication, epidermal strips contain intact epidermal cells and a few adhering mesophyll cells and chloroplasts (Fig. 1A). The epidermal cells were broken and the mesophyll cells removed by sonication, but the guard cells remained intact after this treatment, as shown by the uptake of a vital stain (Fig. 1B). Inspection of the peels by fluorescence microscopy showed little if any contamination by mesophyll chloroplasts. It has been shown that the guard cells prepared by this technique remain active in stomatal opening (20, 27), K^+ uptake (27), and malate formation (20) in the light. The sonicated strips were cut with a razor blade into small pieces (about 50 mm^2) prior to incubation in various media.

Measurement of Fluorescence Transient. The sonicated epidermal strips were incubated in water or in a medium containing ions ± ABA at room temperature for at least 30 min in the dark. After the incubation, the epidermal strips were layered on cheesecloth and placed in a stoppered Dewar flask, illuminated with strong blue light (1.5 mw cm^{-2}, Corning glass filters No. 4–76 and 3–73 placed in front of a 500-w tungsten lamp). A heat-absorbing water bath was placed between the filters and the lamp. Changes of Chl *a* fluorescence yield were measured at 685 nm according to the

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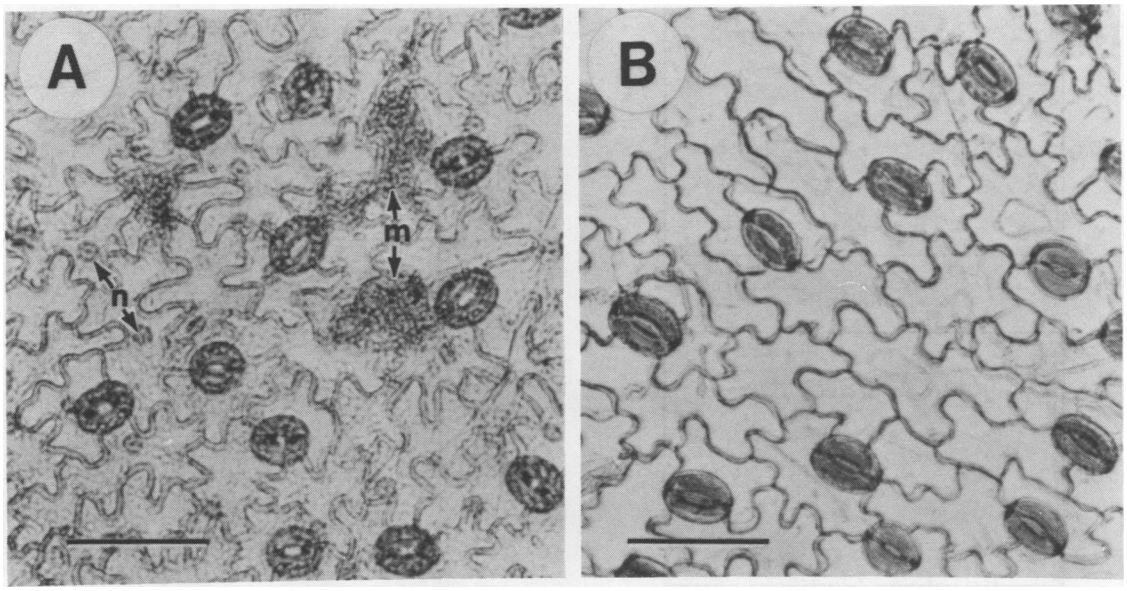

FIG. 1. Photomicrographs of epidermal strips of *Vicia faba* before (A) and after (B) sonication. The strips were stained with neutral red. An unsonicated strip (A) has many intact epidermal cells containing nuclei (n) and adhering mesophyll cells (m). A sonicated strip (B) contains no mesophyll or intact epidermal cells and shows stain confined to guard cells. The bars indicate 0.1 mm.

procedure of Mohanty *et al.* (17) and Munday and Govindjee (18) and were always repeatable after dark readaptation.

Solutions. All reagents were obtained from Sigma. ABA solutions were prepared daily. ABA was first dissolved in ethanol, and 0.1 ml of this solution was added to 10 ml of a medium containing ions. The same amount of ethanol was added as a control.

RESULTS

Fluorescence of Chl *a* in Guard Cells and Mesophyll Cells. Typical kinetics of Chl *a* fluorescence were observed when isolated guard cells were illuminated in epidermal strips in water, and when mesophyll cells were illuminated on the abaxial surface of intact, excised leaves in air (Fig. 2). However, the time courses of these transients differed. The guard cells showed a faster rise from D to P and a higher O to I transient than did mesophyll cells (*cf.* traces a and b, Fig. 2A). These differences have also been observed by Zeiger *et al.* (30) between mesophyll chloroplasts and guard cells from albino sections of leaves of *Chlorophytum comosum*. The rise from D to P level is due to photoreduction of an electron acceptor and Chl *a* fluorescence quencher, Q, by electrons from PSII (3). Thus, the rate of the D to P rise indicates the rate of accumulation of Q^-, which depends on the rate of electron flow from PSII to Q, the number of Q type acceptors, and the rate of reoxidation of Q^- by the intersystem electron transport chain.

Figure 2B shows slow fluorescence transients of the same samples used for Figure 2A. Mesophyll cells exhibited typical fluorescence kinetics (trace d) from P to T levels through the intermediary levels of S and M. In contrast, guard cells exhibited a very slow decline from P to S level with little evidence of the SMT phase (trace c). It has been reported that the P to S decline reflects the presence of the high energy state in chloroplasts involving a decrease of intrathylakoid pH necessary for photophosphorylation (2, 4, 10, 11, 15, 16, 19).

Enhancement of the P to S decline by K^+ and Na^+. In guard cells, the P to S decline of the fluorescence transient of Chl *a* was greatly accelerated by K^+ when added with PO_4^{3-} as counterion (Fig. 3A). A similar enhancement was observed with sodium phosphate. Calcium phosphate caused only weak enhancement (Fig. 3B), suggesting that the effect was due largely to the monovalent cations. To describe the effect quantitatively, the rate of the decline, $-dF/dt$ (Fig. 3A), was calculated and expressed as a percentage of the maximum value. The rate of the P to S decline increased about 8-fold as K-phosphate concentration increased, and the effect saturated at a concentration of about 1 mM (Fig. 4).

The enhancement of the P to S decline by monovalent cations was completely suppressed by CCCP[2] (Fig. 3C), an uncoupler of photophosphorylation. A similar suppression of the P to S decline by uncouplers has been observed with intact mesophyll chloroplasts (4, 11) and algal cells (16), and has been interpreted as a dissipation of the high energy state in chloroplasts.

The P to S decline in guard cells incubated with 1 mM KCl was intermediate between that in the presence and absence of 1 mM K-phosphate (Fig. 3D). The $-dF/dt$ value in the presence of KCl was about one fifth that obtained with 1 mM K-phosphate (*cf.* Fig. 3, A and D). Similarly, sodium phosphate was more effective than NaCl (1st column, Table I).

Interaction of Ca^{2+} and Monovalent Cations. $CaCl_2$ suppressed the enhancement by K-phosphate of the P to S decline (*cf.* Figs. 3, A and E). The suppression was saturated at about 5 mM Ca^{2+} (Fig. 5). The $-dF/dt$ value obtained at saturation was about 40% of the value obtained in the absence of $CaCl_2$.

Table I summarizes the rates of the P to S decline in guard cells incubated with K^+ or Na^+ added with PO_4^{3-} or Cl^- (column 1), and also shows the effects of Ca^{2+} on these rates (column 2). The enhancement by sodium phosphate or NaCl was strongly suppressed by Ca^{2+} while suppression of the enhancement by potassium phosphate or KCl was much weaker. Both K^+ and Na^+ ions are transported into guard cells in the absence of Ca^{2+} but in the presence of Ca^{2+} there is greater selectivity in favor of potassium uptake (24).

Suppression of the P to S Decline by ABA. The enhancement of the P to S decline by K-phosphate was suppressed by ABA (Fig. 3F). The suppression was 50% at an ABA concentration of 1 μM and was saturated above 10 μM (Fig. 6). The enhancement by sodium phosphate, NaCl and KCl were also suppressed by ABA (Table I). In all cases, the suppressive effects of ABA were increased in the presence of Ca^{2+} and, except for sodium phosphate, the suppressive effects of Ca^{2+} and ABA were synergistic. ABA is a potent inhibitor of stomatal opening that blocks cation transport into guard cells (13, 28).

[2] Abbreviation: CCCP, carbonyl cyanide 3-chlorophenylhydrazone.

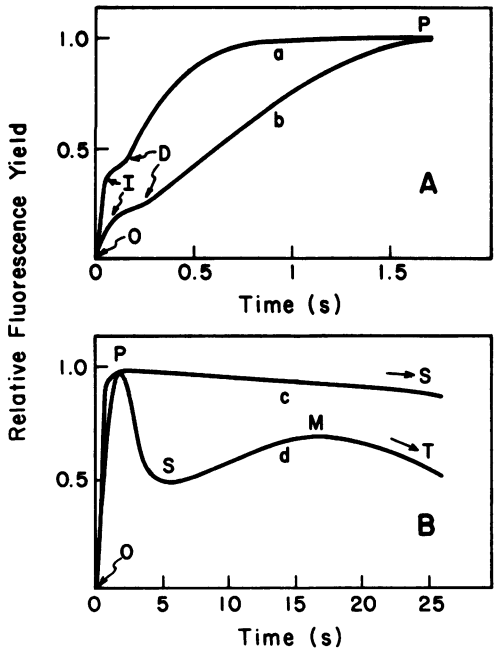

FIG. 2. Fast (A) and slow (B) changes of Chl *a* fluorescence of guard cells (traces a and c) observed with sonicated epidermal strips incubated in distilled H_2O and of mesophyll cells (traces b and d) observed by irradiating the abaxial surface of an intact, excised leaf in air. The fluorescence yield was normalized at the P level.

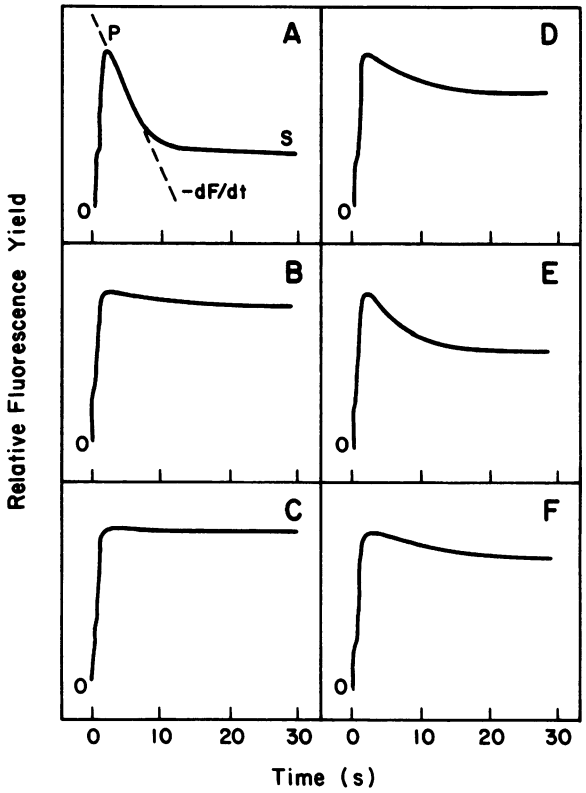

FIG. 3. Slow changes of Chl *a* fluorescence yield of guard cells (sonicated epidermal strips) normalized at the P level. The strips were incubated in the dark at least for 30 min at room temperature in a medium containing 1 mM K-phosphate (A), 5 mM calcium phosphate (B), 1 mM K-phosphate and 40 μM CCCP (C), 1 mM KCl (D), 1 mM K-phosphate, and 5 mM $CaCl_2$ (E) and 1 mM K-phosphate, 5 mM $CaCl_2$, and 100 μM ABA (F). The pH of the media was adjusted to 7.0.

DISCUSSION

Guard cells represent a small fraction of the cells in the leaf. Therefore, methods for studying them must be highly specific. The sonicated epidermal strips used in this work represented an efficient means of isolating guard cells free of contamination by other living cells or mesophyll chloroplasts while retaining full capacity for stomatal function (20). The fluorescence transients provided a probe of fundamental chloroplast behavior while conditions known to affect stomatal opening were varied. Although the detailed interpretation of fluorescence transients in intact cells is not completely worked out, it is clear that this fluorescence is specific to the chloroplast, whose function in stomatal opening has remained enigmatic (6). Thus, fluorescence transients of sonicated epidermal strips allow the chloroplast activity of guard cells to be studied in relation to stomatal opening.

The initial portion (OIDP) of the transient of Chl *a* fluorescence indicates that guard cell chloroplasts contain functional PSII which mediates the flow of electrons to intersystem electron carriers, as has been shown by Zeiger *et al.* (30). Inasmuch as the kinetics of the fast transient are influenced by the number of acceptors, Q, and the rates of electron flow to and from Q, the faster D to P rise in guard cells than in mesophyll cells suggests that there are differences in these parameters between the chloroplasts of these two cell types. Guard cell chloroplasts do not contain ribulose-1,5-bisphosphate carboxylase nor glyceraldehyde phosphate dehydrogenase of the Calvin-Benson cycle (22) and therefore do not function in photosynthetic carbon reduction. Since the fast fluorescence transient was measured in the absence of external cations, the guard cells also were unable to function in ion transport. It is therefore possible that the differences between the fast transients of guard cells and mesophyll cells were associated with a relative lack of electron acceptors in guard cells due to lack of CO_2 reduction and ion accumulation compared with mesophyll cells, which remained in contact with CO_2 containing air. Alternatively, the differences may have been associated with chloroplast differences not related to utilization of the products of

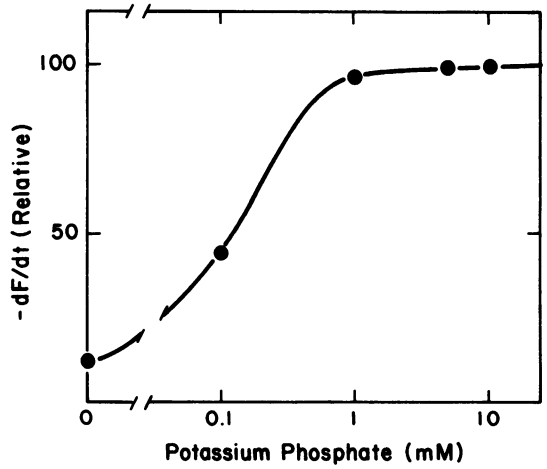

FIG. 4. Effect of different concentrations of K-phosphate on the rate of the P to S decline (−dF/dt) of the fluorescence transients in guard cells. The −dF/dt value obtained with 10 mM K-phosphate was taken as 100. The pH of the media was adjusted to 7.0.

electron transport.

For slower phases of the fluorescence transient, Briantais *et al.* (2) have shown a linear relationship between the P to S decline and the intrathylakoid proton concentration in mesophyll chloroplasts. In intact leaves, the P to S decline is associated with an increase in *A* at 535 nm because of shrinkage of the chloroplasts (10). Since this shrinkage apparently accompanies light-dependent

Table I. *Rates of the P to S Decline (−dF/dt) in Guard Cells Incubated with K^+ or Na^+ Salts of Phosphate or Chloride in the Presence and Absence of $CaCl_2$ and/or ABA*

The −dF/dt value obtained with 1 mM potassium phosphate was taken as 100. The concentrations of $CaCl_2$ and ABA were 5 mM and 100 μM, respectively.

Ions	−dF/dt			
	−ABA		+ABA	
	$-CaCl_2$	$+CaCl_2$	$-CaCl_2$	$+CaCl_2$
None	4	2		
K-phosphate, 1 mM	100	43	47	15
KCl, 1 mM	19	16	11	3
Sodium phosphate, 1 mM	83	19	50	15
NaCl, 1 mM	17	6	8	2

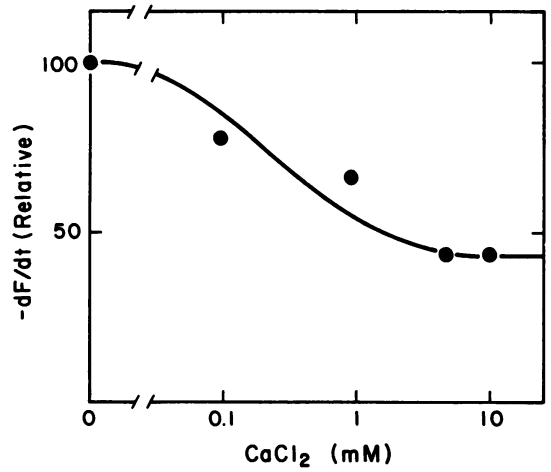

FIG. 5. Effect of different concentrations of $CaCl_2$ on the rate of the P to S decline (−dF/dt) of the fluorescence transients in guard cells incubated with 1 mM K-phosphate (pH 7.0). The −dF/dt value obtained in the absence of $CaCl_2$ was taken as 100.

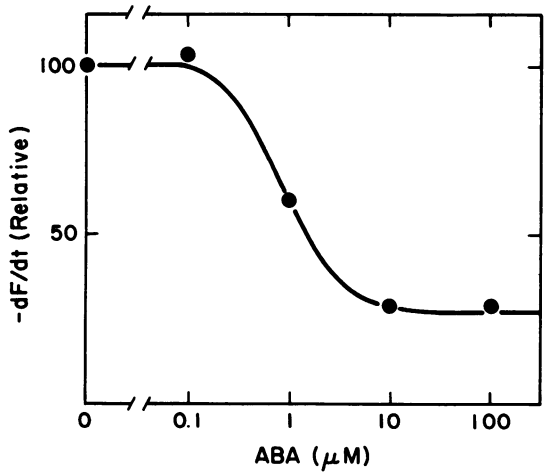

FIG. 6. Effect of different concentrations of ABA on the rate of the P to S decline (−dF/dt) of the fluorescence transients in guard cells incubated with 1 mM K-phosphate and 5 mM $CaCl_2$ (pH 7.0). The −dF/dt value obtained in the absence of ABA was taken as 100.

uptake of protons into the intrathylakoid space and redistribution of cations, the P to S decline does not simply reflect the removal or depletion of Q^-, as earlier suggested (3), but also reflects the formation and dissipation of the high energy state in chloroplasts (2, 4, 10, 11, 15, 16, 19). It has been shown previously (11, 26) in algal and mesophyll cells that the P to S decline is suppressed by uncouplers which dissipate the proton gradients of the high energy state. We observed a similar suppression in guard cells in the presence of the uncoupler CCCP.

In leaves, it was shown (10) that the P to S decline exhibits a three-way behavior. The first occurs when electron and/or ATP consuming reactions are minimal, in which case the high energy state is not observed by fluorescence. The second takes place as these reactions increase, and the P to S decline then becomes large. The third is observed at very high reaction rates, which decrease the P to S decline because of a rapid dissipation of the high energy state.

In guard cells, the slow P to S decline in the absence of external ions is consistent with minimum consumption of electrons and/or ATP because CO_2 reduction could not occur and external ions were not present for accumulation by the cells. When K^+ was added, the enhancement of the P to S decline suggests that electrons and/or ATP were consumed which allowed the high energy state to be observed. The K^+ effect showed the strict concentration dependence that would be expected if this consumption were for ion uptake. This electron consumption might also serve to reoxidize Q^-, further enhancing the P to S decline. Although K^+ concentrations were as high as 10 mM in our experiments, the P to S decline remained large at high concentrations, suggesting that the rate of dissipation of the high energy state did not exceed the rate of formation during ion uptake.

ABA, which inhibits K^+ transport into guard cells (13, 28), suppressed the P to S enhancement by K^+. Since previous work showed no direct effect of ABA on photophosphorylation of isolated chloroplasts (1) or on photosynthetic rates of mesophyll cells (14), the effect of ABA is unlikely to be directly on the photosynthetic apparatus. Ca^{2+}, which inhibits Na^+ transport into guard cells more than K^+ transport (24), suppressed the P to S enhancement by Na^+ more than that by K^+. Thus, enhancement of the P to S decline by K^+ and suppression by Ca^{2+} and ABA suggest a role for guard cell chloroplasts in ion transport during stomatal opening.

It is unlikely that the cations acted directly on the chloroplasts, because the K^+ concentration inside the guard cells is substantial when prepared by sonication. Shimada *et al.* (27) reported a K^+ concentration of 7 to 10 nmol/mg dry weight in sonicated epidermis rinsed in 0.1 mM Ca^{2+}. Considering guard cell volumes (8) and guard cell density of about 140 mm^{-2} (Grantz, unpublished), estimated K^+ concentrations in the guard cells of sonicated epidermis were approximately 100 mM, which compares favorably with 77 mM found in guard cells of closed stomata by electron microprobe analysis (8). This internal K^+ concentration is above that required for photosynthesis in isolated chloroplasts (50–100 mM) (9). Thus, direct ion effects on the guard cell chloroplasts should have been saturated for our experiments and the observed changes in fluorescence should have reflected chloroplast involvement in ion accumulation by the cells.

The degree of enhancement by K^+ of the P to S decline in guard cells was related to the specific counterion presented. Since *Vicia* guard cells do not contain key enzymes of the Calvin-Benson cycle (22), NADPH produced by chloroplast activity must be consumed by some reaction other than reductive CO_2 fixation. This reaction could be reduction to malate of oxaloacetate derived from starch in guard cell chloroplasts. The negative charges of malate are known to balance the positive charges of imported K^+ in guard cells of *Vicia faba* (21) in the absence of sufficient external Cl^- to be taken up with K^+ (29). The effect of monovalent cations was larger in these experiments when they were accompanied by phosphate than by chloride (Table I). Thus, in guard cells incubated with K-phosphate, NADPH may be rapidly oxi-

dized by oxaloacetate to form malate and regenerate the final electron acceptor, NADP, of the electron transport chain. The high rate of NADPH depletion would then allow a high rate of noncyclic photosynthetic electron flow and lead to a large enhancement of the P to S decline. Oxidation of NADPH may be slower in guard cells incubated with KCl because of the uptake of external Cl^- and the concomitant decrease in internal malate production.

These results indicate that guard cell chloroplasts may play important roles in the accumulation of solute for stomatal opening. The differences induced by chloride and phosphate when presented as counterions for K^+ suggest that one role is to supply reducing equivalents for the formation of organic counterions when appropriate inorganic counterions are not available externally. Another role, to supply energy for ion uptake via photophosphorylation, is suggested by the formation of the high energy state in guard cell chloroplasts in the presence of external ions. The results with CCCP, ABA, and Ca^{2+} support these dual roles. It therefore seems that guard cell chloroplasts differ fundamentally from those of mesophyll cells, in that the products of the photosynthetic light reactions are directed toward the accumulation of cellular osmotica rather than toward the fixation of CO_2.

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