

# Light-Induced Alkalinization of the Suspending Medium of Guard Cell Protoplasts from *Vicia faba* L.<sup>1</sup>

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## ABSTRACT

The light-dependent pH changes in the suspending medium of guard cell protoplasts (GCP) from *Vicia faba* were studied. Upon illumination, the medium was initially slightly alkalinized and then acidified. The extent of alkalinization was lower in CO<sub>2</sub>-free air than in normal air. This initial alkalinization was inhibited by DCMU. Acidification in CO<sub>2</sub>-free air became observable in shorter duration of light exposure than that in normal air. The rate of acidification was higher in CO<sub>2</sub>-free air than in normal air. The CO<sub>2</sub> level of the medium decreased in the light, and increased in the dark. <sup>14</sup>CO<sub>2</sub> uptake was enhanced 2- to 3-fold by light, but not in the presence of DCMU. These results indicate that photosynthetic CO<sub>2</sub> fixation does take place in GCP and that the initial alkalinization is due to this photosynthetic CO<sub>2</sub> uptake. Diethylstilbestrol, a nonmitochondrial membrane-bound ATPase inhibitor, inhibited the acidification, suggesting that the acidification resulted from H<sup>+</sup> extrusion by GCP. The acidification in light was also prevented by KCN, and partly by DCMU. Possible mechanisms of alkalinization and acidification are discussed in relation to guard cell metabolism.

We reported that guard cell protoplasts from *Vicia faba* responded to light by volume increase (8) accompanied by accumulation of K<sup>+</sup> and malate (10). A photosynthetic inhibitor, DCMU, prevented the light-induced swelling of GCP<sup>2</sup> (9). The results suggest that photosynthesis may play a role in accumulation of osmotica in guard cells.

Raschke and Humble (19) have observed that the floating solution of epidermal strips of *V. faba* is acidified during stomatal opening, and suggested a possible K<sup>+</sup>/H<sup>+</sup> exchange mechanism. Acidification of the bathing medium by illuminated *V. faba* epidermal tissue in CO<sub>2</sub>-free air was confirmed by Gepstein *et al.* (6). The acidification is probably caused by the guard cells, since neutral red staining indicated that most (about 90%) of the other epidermal cells had been killed during the preparation of epidermal tissue. Light-induced H<sup>+</sup> extrusion and K<sup>+</sup> accumulation were inhibited by vanadate which also blocked stomatal opening. The results suggest that the plasma membrane ATPase operates in light-driven H<sup>+</sup> extrusion by guard cells. Photosyn-

thesis is considered to be one of potential donors of ATP (12).

We studied the light-induced changes in pH of the suspending medium of GCP. GCP with pure and homogeneous populations are experimental materials suitable for the study of ion transport through the plasma membrane, because the lack of cell walls facilitate ion movements. We carried out the experiments in normal air as well as in CO<sub>2</sub>-free air. We now report initial alkalinization of the medium of *Vicia* GCP on illumination and light-enhanced CO<sub>2</sub> fixation by GCP, and discuss the effect of CO<sub>2</sub> on H<sup>+</sup> extrusion.

## MATERIALS AND METHODS

**Isolation of Guard Cell Protoplasts.** Guard cell protoplasts were isolated from the lower epidermis of *Vicia faba* leaves as reported previously (9). Purified protoplasts were kept in the dark on ice until use.

**Measurement of pH Changes in the Suspending Medium.** The pH of the suspending medium was continuously monitored using a small flat surface pH electrode (Fuji Kagaku Keisoku Co., Japan) at 25°C. The measurements were conducted in a closed vessel (22). A 1-ml sample of protoplast suspension was continuously stirred and illuminated with a white light from 300-W slide projector (Elmo Co., Japan) through a 50-mm water layer at 300 μE m<sup>-2</sup> s<sup>-1</sup>. The suspension medium contained 0.4 M mannitol, 10 mM KCl, and 1 mM CaCl<sub>2</sub>, and was weakly buffered by 0.1 mM Mes-Tris (pH 6.5).

**Experiments in CO<sub>2</sub>-Free Air.** Water-saturated CO<sub>2</sub>-free air, obtained by passing commercial CO<sub>2</sub>-free air through water, was bubbled into the medium through a hypodermic needle until the pH rise in the medium stopped. Then GCP were transferred into the medium to a final volume of 1 ml, and incubated with continuous flow of CO<sub>2</sub>-free air over the surface of the medium in the vessel (capacity 8 ml) covered with Parafilm (American Can Co.). The gas flow rate was 300 ml min<sup>-1</sup>.

**CO<sub>2</sub> Concentrations.** The CO<sub>2</sub> concentration in the suspending medium was determined from the increase in pH that could be induced by bubbling N<sub>2</sub> through a supernatant (13). GCP suspension (1.2 ml) was transferred into centrifuge tube (capacity 1.5 ml) and centrifuged at 10,000g for 10 s (Microfuge B, Beckman). One ml of supernatant was transferred into a vessel and its initial pH was measured. Then the solution was purged by N<sub>2</sub> until the pH rise stopped. The medium was titrated back to initial pH by HCl, whose amount defined the neq CO<sub>2</sub> ml<sup>-1</sup>.

**CO<sub>2</sub> Uptake and Fixation Assays.** GCP were preincubated in 0.4 M mannitol, containing 10 mM KCl, 1 mM CaCl<sub>2</sub>, and 5 mM Mes-Tris (pH 6.5) at 25°C for 2 min in the dark. DCMU was added at the onset of preincubation. Then, 24 μl of <sup>14</sup>C-labeled NaHCO<sub>3</sub> (1.24 μCi) was injected into 2 ml of cell suspension at

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<sup>2</sup> Abbreviations: GCP, guard cell protoplast(s); DES, diethylstilbestrol; PEPC, phosphoenolpyruvate carboxylase; RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase.

a final concentration of 100  $\mu\text{M}$  in the light or in the dark. After incubated for 5 min, GCP were collected by silicone oil centrifugation as described previously (8), except that the bottom layer was replaced by 2.5 M NaOH. The bottom layer which contained the pelleted GCP was separated and mixed with 300  $\mu\text{l}$  of 0.1 M NaOH. The mixed solution was divided into two portions, one portion for the determination of the total uptake of  $^{14}\text{CO}_2$  and the other for the incorporation of  $^{14}\text{CO}_2$  into acid-stable products. Radioactivity in the sample was counted with a liquid scintillation spectrometer (model 3255, Packard) in Aquasol-2 scintillation fluid (New England Nuclear). The data for total uptake were corrected for the  $^{14}\text{CO}_2$  carried with the medium surrounding GCP into the bottom layer. The amount of the medium that was carried into the bottom layer was determined using [ $^{14}\text{C}$ ]mannitol. The correction ranged between 2 and 7%.

## RESULTS

GCP that were kept in the dark hardly changed the pH of the medium (Fig. 1). Upon illumination, the pH of the medium initially rose and then decreased (Fig. 1). The pH continued to decrease for 60 min. The profile of pH changes in  $\text{CO}_2$ -free air was different from that in normal air (Fig. 1). In normal air, acidification started about 12 min after the light was turned on. In the same GCP population, the duration of the initial alkalization in the light was shorter in  $\text{CO}_2$ -free air than in normal air, and the acidification started within 1 to 2 min of illumination (Fig. 1). These differences are listed in Table I. The extent of alkalization in the medium was lower in  $\text{CO}_2$ -free air than in normal air. The rate of acidification was six times higher in  $\text{CO}_2$ -free air than in normal air. DCMU completely inhibited the initial alkalization, and partly prevented the acidification (Table I). In the presence of DCMU, the pH of the medium in the normal air began to decrease about 9 min after the onset of light exposure.

The role of  $\text{CO}_2$  flux through the plasma membrane of GCP in alkalization of the medium was assessed by changes in  $\text{CO}_2$  levels of the medium and  $^{14}\text{CO}_2$  uptake by GCP. The  $\text{CO}_2$  concentration was estimated from the increase in pH produced by purging the solution with  $\text{N}_2$ . The increase of pH from 6.51 to 6.74 by  $\text{N}_2$  purge in the cell-free medium was estimated to be the  $\text{CO}_2$  concentration of 11 neq  $\text{ml}^{-1}$  by HCl titration. Decreases in the  $\text{CO}_2$  level were observed in the suspension that was exposed to light (Table II). The  $\text{CO}_2$  level increased in the dark (Table II). GCP showed  $^{14}\text{CO}_2$  uptake and fixation in the dark as well as in the light (Table III). Incorporation of  $^{14}\text{CO}_2$  into acid-stable products was enhanced 3-fold by light (Table III). DCMU in-

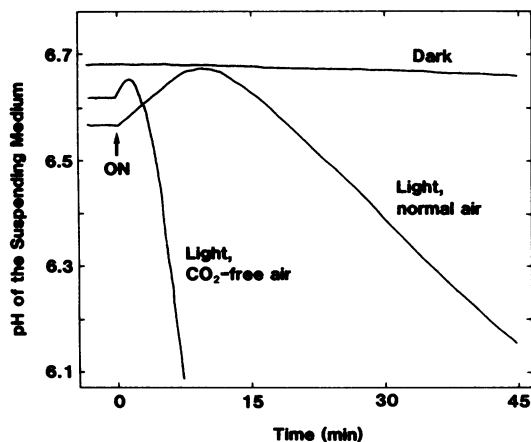


FIG. 1. Changes in pH of the suspending medium of GCP. One and one-half ml of suspension containing  $1.5 \times 10^6$  GCP was continuously stirred in the dark or in whitelight.

Table I. Effect of Light,  $\text{CO}_2$ , and DCMU on Alkalinization and Acidification of the Suspending Medium of Guard Cell Protoplasts from *V. faba* L.

GCP ( $8.2 \times 10^5 \text{ ml}^{-1}$ ) were preincubated at  $25^\circ\text{C}$  for 5 min in the dark and followed by incubation in the light or in the dark. DCMU ( $5 \mu\text{M}$ ) was added at the onset of preincubation. The extent of alkalization was estimated from the differences in pH between initial and plateau. The rates of acidification were determined from the changes in acidity of the medium from 15 to 30 min after the start of the assay. The acidity was measured by titration with NaOH.

Treatment	DCMU	Extent of Alkalinization $\text{neq H}^+ \text{ ml}^{-1}$	Rate of Acidification $\mu\text{eq H}^+ \text{ mg}^{-1} \text{ Chl min}^{-1}$
Light, normal air	-	13	1.3
Light, normal air	+	0	0.8
Light, $\text{CO}_2$ -free air	-	3	7.6
Dark, normal air	-	0	0.1

Table II.  $\text{CO}_2$  Levels of the Medium

$\text{CO}_2$  was purged with  $\text{N}_2$  from medium decanted from suspensions of  $1.5 \times 10^6$  GCP  $\text{ml}^{-1}$ . The increase in pH after removal of  $\text{CO}_2$  was titrated back to initial pH by HCl, whose amount defined the neq  $\text{ml}^{-1}$ .

Treatment	Initial pH	pH after $\text{CO}_2$ Depletion	$\text{CO}_2$ Concentration $\text{neq ml}^{-1}$
Initial	6.61	6.87	27
Light, 30 min	6.37	6.43	9
Dark, 30 min	6.55	7.23	61
Cell-free medium	6.51	6.74	11
+100 neq $\text{CO}_2 \text{ ml}^{-1}$	7.08	7.86	96

Table III.  $\text{CO}_2$  Uptake and Fixation by Guard Cell Protoplasts

GCP were preincubated in the absence or presence of  $5 \mu\text{M}$  DCMU at  $25^\circ\text{C}$  for 2 min in the dark, followed by incubation with  $\text{NaH}^{14}\text{CO}_3$  in the light or in the dark for 5 min. Incubation was stopped by spinning down GCP into 2.5 M NaOH by silicone oil centrifugation. Mean values from triplicate experiments are presented with  $\pm\text{SD}$ .

Treatment	$\text{CO}_2$ Uptake	$\text{CO}_2$ Fixation
	$\mu\text{mol mg}^{-1} \text{ Chl}$	
Light	$2.67 \pm 0.20$	$2.35 \pm 0.03$
Light + DCMU	$1.13 \pm 0.06$	$0.80 \pm 0.01$
Dark	$1.19 \pm 0.22$	$0.77 \pm 0.01$

Table IV. Effect of DES, KCN, and DCMU on Decrease in the pH of the Medium

The rate of acidification in control was determined from the change in acidity of the medium from 15 to 30 min after light-on. After 30 min of light-on, inhibitor was added to the suspending medium. The rates of acidification were monitored for the following 15 min and expressed as the percentage of control.

Additive	% Control
None	100
DES, $5 \mu\text{M}$	19
KCN, 2 mM	25
DCMU, $5 \mu\text{M}$	88

hibited light-enhanced  $\text{CO}_2$  fixation, suggesting that  $\text{CO}_2$  fixation was photosynthetic. This photosynthetic  $\text{CO}_2$  fixation was reproducible among three different batches of GCP preparation: the mean value of  $^{14}\text{CO}_2$  fixation with SD were  $1.16 \pm 0.35$  for 5 min in the dark and  $2.93 \pm 0.52 \mu\text{mol mg}^{-1} \text{ Chl}$  for 5 min in the

light. This result suggests that the initial alkalinization of the medium is due to CO<sub>2</sub> uptake caused by photosynthetic CO<sub>2</sub> fixation in GCP.

Effects of inhibitors on acidification were examined after 30 min of light illumination (Table IV). DES, a nonmitochondrial membrane-bound ATPase inhibitor, prevented 81% of control. KCN inhibited 75% of control. The inhibitory effect of DCMU on acidification was only 12%. The inhibitory effect of DCMU was less when it was added after light-on. The difference may depend on CO<sub>2</sub> level in the medium.

## DISCUSSION

Accumulation of K<sup>+</sup> in guard cells, which is responsible for the turgor change during stomatal opening (12), has been interpreted on the basis of chemiosmotic mechanism (29). The hypothesis predicts that guard cells excrete H<sup>+</sup> during stomatal opening. If it is the case, extracellular pH will decrease. Upon illumination, the acidification of the medium followed a brief alkalinization (Fig. 1). The light-induced acidification was consistent with earlier reports using epidermal strips (6, 19). The acidification can be caused either by OH<sup>-</sup> influx or by H<sup>+</sup> efflux. In accordance with convention, the flux is referred to H<sup>+</sup> efflux.

Light-induced H<sup>+</sup> extrusion was inhibited by 5 μM DES (Table IV). The effect of this inhibitor on H<sup>+</sup> transport depends on its capability of inhibiting a membrane-bound ATPase (3). Another inhibitor, vanadate, blocked the acidification of the medium by epidermal tissue (6). Light-induced hyperpolarization of guard cell membrane potential was observed (14, 30). These results support the operation of light-driven H<sup>+</sup> pump in guard cells (29).

It is generally believed that plasma membrane ATPase derives its supply of ATP from both respiration and photosynthesis during stomatal opening (12). KCN inhibited the pH decrease as much as DES did. The previous study showed that KCN decreased the ATP level in GCP (23). The resultant shortage of an energy supply probably caused the inhibition of the pH decrease. The inhibitory effect of DCMU was less than that of KCN. The results suggest that ATP produced by respiration is contributing much more to the operation of a H<sup>+</sup> pump than photosynthesis.

This is the first report on the alkalinization of the suspending medium of GCP upon illumination. Light-induced alkalinization can result from H<sup>+</sup> influx (11, 18) as well as from CO<sub>2</sub> uptake (15). Both alkalinization and the decrease of CO<sub>2</sub> in the medium only occur when the suspension is exposed to light (Tables I, II). Alkalinization was limited by CO<sub>2</sub> concentration of the medium (Table I) and appeared to reflect its change. <sup>14</sup>CO<sub>2</sub> uptake by GCP was stimulated by light exposure (Table III). Both alkalinization and stimulation of <sup>14</sup>CO<sub>2</sub> uptake were inhibited by DCMU (Table III). Therefore, we concluded that alkalinization of the medium was due to photosynthetic CO<sub>2</sub> uptake.

We also showed that CO<sub>2</sub> fixation by guard cells was stimulated by light and that the stimulation was completely inhibited by DCMU (Table III). This is the first report that photosynthetic CO<sub>2</sub> fixation occurs in guard cells. There are a few reports on CO<sub>2</sub> fixation by GCP from *Vicia* (2, 20) or from *Commelina* (1). The CO<sub>2</sub> uptake rate of *Vicia* GCP was similar in both light and the dark (20). In the present study, however, CO<sub>2</sub> uptake was stimulated by light, and the CO<sub>2</sub> taken up was incorporated into acid-stable products. The results indicate that photosynthesis play a role in the production of osmotica by CO<sub>2</sub> fixation during light-induced swelling of GCP. The differences in amount between CO<sub>2</sub> taken up and CO<sub>2</sub> fixed were small and similar in the light and in the dark (Table III). It is likely that CO<sub>2</sub> uptake is induced passively by the decrease in free CO<sub>2</sub> due to CO<sub>2</sub> fixation in guard cells. The inhibitory effect of DCMU on CO<sub>2</sub> fixation thus seemed to be an indirect effect on CO<sub>2</sub> uptake.

In mesophyll cells of *Vicia*, photosynthetic CO<sub>2</sub> fixation is catalyzed by RuBisCO. However, the carboxylase activity of RuBisCO in *Vicia* guard cells has yet to be detected by histochemical techniques or in extracts of GCP (17). Recently RuBisCO was found in both *Vicia* and *Pisum sativum* guard cells by immunocytochemical technique (7), although neither its activity *in vitro* nor the operation of the enzyme *in vivo* has been detected. A higher level of PEPC than that of RuBisCO was found in leaf epidermal tissues of *Commelina* (4, 26, 28) and *Tulipa gesneriana* (28) and *Vicia* GCP (21). In these literatures, PEPC is concluded to have a predominant role in CO<sub>2</sub> fixation in guard cells. Accumulation of radioactivity was observed in malate by <sup>14</sup>CO<sub>2</sub> labeling in stomatal systems (16, 27). Carboxylation of PEP was found to be a major source of accumulating malate in isolated epidermal tissue of *C. cyanea* (25). These results raise the possibility that CO<sub>2</sub> fixation by PEPC is stimulated by light in addition to the possibility of CO<sub>2</sub> reduction by the photosynthetic carbon reduction pathway.

Changes in pH of the medium may reflect both CO<sub>2</sub> and H<sup>+</sup> flux. The pH in the suspending medium of GCP that were kept in the dark remained constant despite the increase in CO<sub>2</sub> concentration, presumably compensated by H<sup>+</sup> influx in the dark (16). The present study shows that not only H<sup>+</sup> extrusion but also CO<sub>2</sub> uptake by guard cells are stimulated by light. Higher rates of acidification were observed in CO<sub>2</sub>-free air than in normal air. It is conceivable that the difference is due to CO<sub>2</sub> availability. There are two possibilities for the slower rate of acidification in normal air. Depletion of CO<sub>2</sub> from the weakly buffered incubation medium causes its alkalinization. Therefore, if CO<sub>2</sub> uptake simultaneously occurs during H<sup>+</sup> extrusion, the acidification of the medium becomes observable only when H<sup>+</sup> extrusion exceeds CO<sub>2</sub> uptake. If so, the observed rate of acidification should be slower than the actual rate of H<sup>+</sup> extrusion. Alternatively, CO<sub>2</sub>-induced cytoplasm acidification might dissipate the proton motive force thus leading to prevention of H<sup>+</sup> extrusion (29). In this case, the H<sup>+</sup> extrusion is directly depressed, so K<sup>+</sup> uptake should also be decreased if stoichiometric H<sup>+</sup>/K<sup>+</sup> exchange occurs. Our previous study showed that ambient CO<sub>2</sub> indeed had some effect on the contribution of K<sup>+</sup> to volume increase (8), suggesting that K<sup>+</sup> uptake was influenced by CO<sub>2</sub>. It was reported that CO<sub>2</sub> inhibits the H<sup>+</sup> pump and consequently Cl<sup>-</sup> influx without effects on the ATP level in *Niella* (24). Enhanced levels of CO<sub>2</sub> depolarized the membrane potential of all the cells of the stomatal complex of *Tradescantia virginiana* (5). Additional studies are needed for elucidation of the role of CO<sub>2</sub> in stomatal movement.

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