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Presence of Chloride Reduces Malate Production in Epidermis during Stomatal Opening[1]

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CAROL A. VAN KIRK AND KLAUS RASCHKE
Michigan State University/Energy Research and Development Administration Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

When stomata of isolated epidermis of *Vicia faba* are allowed to open in the presence of K^+ and iminodiacetate (an impermeant zwitterion), malate is formed in the epidermis; the increases in malate content follow a nearly linear relationship with stomatal aperture. Stomata of leaf sections of *V. faba* floated on water during opening also exhibit this relationship. When isolated epidermis is offered KCl, this relationship is not observed and less malate is detected at comparable stomatal apertures. The data indicate that Cl^-, if present at concentrations $\geq 10^{-5}$ eq liter^{-1}, can partially satisfy the anion requirement of guard cells of *V. faba* during stomatal opening. Discrepancies between earlier reports on the relative roles Cl^- and malate play as counterions for K^+ in guard cells of *V. faba* could now be explained as resulting from variations in the availability of Cl^- to guard cells.

Stomata open or close when guard cells gain or lose turgor because of changes in their osmotic contents (11, 19). From the results obtained with tracer studies (7, 8), and by direct measurement based on electron microprobe analysis (12, 22), K salts have been shown to be taken up by guard cells in amounts sufficient to cause these turgor changes. (In some halophytes, Na^+ can substitute for K^+ [6].) However, there has been less certainty as to the anion which accompanies K^+ into guard cell vacuoles. Some analyses have shown that Cl^- can move with K^+ into guard cells during stomatal opening (5, 15, 18, 20) but the extent of its participation has been observed to vary from about 5% in *Vicia faba* (12) to 100% in some individual stomates of *Zea mays* (20). In addition to this lack of evidence for the complete balance of K^+ by Cl^-, or some other inorganic anion, two observations indicate that organic anions might be involved. (*a*) When stomates open, the starch content is often seen to decrease (13). (*b*) When epidermal strips are floated on K salts of nonabsorbable acids, stomates open as widely as they open on KCl solutions (21). The second observation indicates that the uptake of external anions is not necessary for stomatal opening and implies that guard cells can produce organic anions to balance K^+.

In 1973 Allaway (1) reported that when stomata of *V. faba* opened, guard cells contained malate; the malate he detected could account for about half of the K^+ taken up during opening. Other work has supported Allaway's observations. Pallas and Wright (16) found that the organic acid content of *V. faba* epidermis increased upon stomatal opening. Pearson and Milthorpe (17) reported a nearly linear relationship between the level of epidermal malate and the stomatal aperture between 0 μm and 12 μm in *Commelina cyanea*. Recently, Outlaw and Lowry (14), using enzyme amplification techniques, have demonstrated that similar increases in organic acids can be observed if individual stomatal complexes are isolated and their acid contents determined.

Thus, evidence implicates chloride as well as malate as dominant counterions for K^+ during stomatal opening. We suspected that the availability of Cl^- to guard cells determined its use; we therefore measured malate production by isolated epidermis while stomata were opening in the absence or presence of Cl^-. When Cl^- was absent, iminodiacetate was used as a nonabsorbable counterion to K^+ in the solutions offered to the epidermal samples (21).

MATERIALS AND METHODS

Plant Material. Seeds of *V. faba* (improved Long Pod variety) were obtained from Lagomarsino Seeds Inc., Sacramento, Calif. The plants were grown either in a glasshouse (temperature: 23-29 C; humidity 70-80%; light supplemented with Sylvania Gro-Lux/WS fluorescent tubes to give 0.3 w m^{-2} to extend the natural light period to 20 hr) or in a growth chamber under 85 w m^{-2} for 16 hr, 85% relative humidity, and a temperature of 27 C during the light period and 23 C during the dark period. (Differences in growth conditions did not affect experimental results.) The soil consisted of 2 parts Bacto potting soil (Michigan Peat, Houston, Tex.) and 1 part Perlite (W. R. Grace & Co.). The plants were grown in 10-cm square plastic pots with a soil depth of about 12 cm. Once a week, the plants were watered with freshly prepared Hoagland solution (pH 6.5); on the other days deionized H_2O was used.

Preparation of Leaf Sections and Epidermal Strips. Second, third, and fourth fully expanded leaves from 3- to 5-week-old plants which had not yet flowered were cut in the morning and placed in distilled, deionized H_2O. Within 15 min, the leaves were rinsed, the upper and lower surfaces were wiped with moist tissue paper, and the leaves were cut into sections of 1 to 3 × 2 to 4 cm^2. The sections were rinsed at least three times with H_2O and floated abaxial side up on distilled, deionized H_2O.

Epidermal strips were made by peeling an area of approximately 5 × 10 to 15 mm^2 from the lower epidermis of the leaf sections with dissecting forceps; the strips were peeled at an obtuse angle which caused 60 to 80% of the epidermal cells to rupture. The strips were rinsed five to 10 times in distilled H_2O and any adhering mesophyll cells were removed by rubbing a dissecting needle along the underside of the strip.

Experimental Procedures: Stomates Opened *in Situ*. Leaf sections were floated on dionized H_2O, abaxial side up, under mercury vapor lamps suspended above a 5-cm water filter (irradiance: 85 w m^{-2}) and in a stream of humidified air (flow rate: about 50 l hr^{-1}) containing either zero or ambient CO_2 (approximately 300 μl liter^{-1}). After about 4 to 5 hr, epidermal strips were made

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and were mounted in H_2O on microscope slides. The area of the strips was measured with a ruler and stomatal apertures were measured under the microscope with an eyepiece micrometer. The tissue was then plunged into boiling 80% ethanol, extracted, and assayed for malate as described below.

Experiments on Isolated Epidermis. Leaf sections were floated on double distilled H_2O in a large Petri dish that was covered with aluminum foil to keep the sections under dark conditions and thus inhibit stomatal opening. Epidermal strips were made as described above and floated on double distilled H_2O in the dark until all strips for 1 day's experiment were made. The strips were then transferred to 2 ml of treatment solution that was contained in a disposable 5-ml plastic beaker. The solutions contained 10 mM MES buffer (pH 5.6), 90 to 110 meq liter^{-1} K^+, and Cl^- at concentrations varying from zero to 100 meq liter^{-1}; IDA[2] balanced K^+ not balanced by Cl^-. No Cl^- could be detected in the double distilled H_2O (the detection limit was 0.03 meq liter^{-1}) or in H_2O in contact with crushed plastic beakers for 24 hr. Impurities in the chemicals used resulted in an estimated maximal concentration of 0.001 meq Cl^- liter^{-1}.

The relative proportions of K^+, Cl^-, and IDA in the incubation media were attained as follows. For solutions containing between 0 and 10 meq Cl^- liter^{-1}, 100 mM KIDA was prepared by titrating 0.1 N KOH and 10 mM MES with IDA, allowing sufficient time for the IDA to dissolve. Then KCl was added to supply the Cl^- in varying amounts as required. When 100 meq Cl^- liter^{-1} were required, 100 mM KCl was added to a solution of 10 mM MES; the pH was adjusted with KOH. The beakers were placed in plastic Petri dishes which were then clamped shut and placed in the same light conditions used for experiments on leaf sections. The Petri dishes had one air inlet, and several air outlets; humidified air containing either ambient or zero CO_2 was forced through each of these dishes at a flow rate of 12 liters hr^{-1} each. The Petri dishes were kept under these conditions for 4 hr. Areas and apertures were then measured and the tissue was transferred to boiling ethanol for extraction.

Extraction and Malate Assay. Malate was extracted from epidermal tissue in three changes of boiling 80% alkaline ethanol (alkalinity achieved by adding 5 drops of 2 N NaOH to 1 liter of 80% ethanol; it was observed that 80% ethanol containing a few drops of either HCl or NaOH resulted in higher malate, about 8 and 12%, respectively, per unit area of epidermal tissue than pure 80% ethanol). The volume of each change was about 5 ml and each was boiled for 5 to 10 min. The extracts were evaporated to dryness in a water bath at 50 C. Filtered air was bubbled through the extracts to speed evaporation. The evaporated extracts were kept in a freezer until they were resuspended in either double distilled H_2O or 0.2 M hydrazine buffer (pH 9) and assayed for malate. This procedure was checked by "extracting" a known amount of malate, evaporating to dryness, resuspending, and assaying; recoveries varied between 96 and 102%.

Malate was assayed by enzymic analysis according to Goldberg and Passonneau (9). In this method, malate is oxidized to oxaloacetate with concomitant reduction of NAD by the action of malate dehydrogenase. The NADH formed was measured fluorimetrically using an Aminco Bowman spectrophotofluorometer (model XLS 1AM2). The reaction was run in a total volume of 2.005 ml. The medium consisted of 0.2 M hydrazine buffer (pH 9), 0.12 mM NAD, 0.2 mM EDTA. Samples of evaporated epidermal extract resuspended in 0.2 M hydrazine buffer (pH 9) were included; the volume of the aliquot was varied according to the estimated concentration of the sample but was usually 0.2 ml. To start the reaction, 5 μl of malate dehydrogenase, which provided 0.05 units of enzyme/assay, were added. Fluorescence was read before the start of the reaction and after 1-hr incubation at room temperature following the addition of the enzyme. Each sample was assayed in duplicate. The assay gave about 60% conversion of malate; ng quantities of malate could be measured.

Epidermal "contaminants," most probably phenolics, were extracted along with malate. These compounds fluoresced, as indicated by background fluorescence in the assay reaction mixture, and absorbed both the exciting and emitting wavelengths of NADH. Because the relative concentration of contaminants varied from extract to extract, it was necessary to calibrate each malate determination individually. This was done by adding a known quantity of L-malate to the assay mixture. The NAD conversion in this reaction mixture was compared with that observed in an identical reaction mixture not containing the malate "spike." The amount of malate originally present in the aliquot of extract was determined according to the following equation:

$$c = \frac{F_2 - F_1}{F_3 - F_2} \times c'$$

where F_1 = fluorescence of assay mixture before addition of enzyme; F_2 = fluorescence of assay mixture containing resuspended extract 1 hr after adding malate dehydrogenase; F_3 = fluorescence of an assay mixture containing both resuspended extract and a malate spike 1 hr after adding malate dehydrogenase; c' = concentration in mol liter^{-1} of the spike; and c = concentration in mol liter^{-1} of the resuspended extract.

This equation holds as long as the range of a linear relation between fluorescence and concentration is not exceeded.

Sources of Reagents. EDTA: Fisher Chemical Co.; hydrazine hydrate: J. T. Baker Chemical Co. or Mallinckrodt; IDA: J. T. Baker Chemical Co.; malate dehydrogenase: Sigma Chemical Co., grade III; L-malic acid: Eastman Organic Chemical Co.; NAD: Sigma Chemical Co., grade V.

RESULTS

Malate Production in Isolated Epidermis. Epidermal strips of *V. faba* were floated on solutions of about 100 meq liter^{-1} K^+, and varying amounts of Cl^-. The K^+ concentration was kept high and nearly constant in order to eliminate effects on stomatal aperture resulting from K^+ availability. In order to recognize relationships between malate content and stomatal opening, epidermal samples whose stomata were open to various degrees had to be analyzed. The endogenous variability of the tissue was used for this purpose and the range of opening extended by exposing strips to either CO_2-free air or air containing CO_2 at the ambient concentration; some of the strips were analyzed immediately after the leaf sections were peeled.

Figure 1A shows that as stomata attained their various apertures in the absence of Cl^-, the malate level increased in a nearly linear fashion. When Cl^- was present at 10^{-5} to 10^{-1} eq liter^{-1}, this relationship between malate and aperture broke down (Fig. 1B). Thus, the ready availability of Cl^- seemed to make the formation of organic counterions for K^+ unnecessary. Data of the initial conditions merged with those obtained after 4-hr exposure to light in CO_2-free or CO_2-containing air. At no point were the stomates completely closed; neither did the epidermal malate content fall to zero.

In comparing Cl^- offered to the epidermal strips and the final aperture attained by their stomates (Fig. 2), much scatter is evident. If the mean of the final apertures measured is plotted in relation to the logarithm of Cl^- concentration, then between 10^{-5} and 10^{-3} eq Cl^- liter^{-1} an increase in aperture with increasing Cl^- concentration is seen; this increase appears to saturate at 10^{-3} eq Cl^- liter^{-1}. However, the large scatter in these data makes this conclusion tenuous at best.

Malate Content of Epidermis Sampled from Leaves. Leaf sections of *V. faba* were floated on distilled H_2O in light and CO_2-free air for 4 hr. The lower epidermis was then isolated; adhering mesophyll cells were removed. Stomatal aperture and tissue area

[2] Abbreviation: IDA: iminodiacetic acid, iminodiacetate.

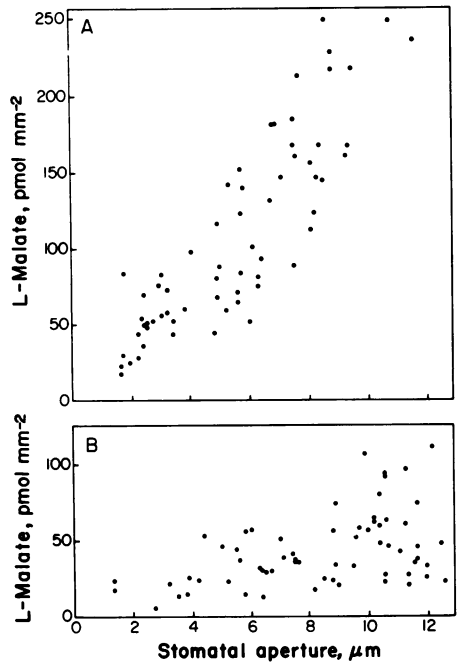

FIG. 1. Malate content of isolated epidermis of *V. faba* related to stomatal aperture. Strips peeled from leaf sections kept in the dark were either analyzed immediately (= initial conditions with narrow apertures) or floated on buffer in the light for 4 hr. The range of endogenous variation in stomatal aperture was extended by incubating strips in the absence or presence of CO_2 (300 μl liter^{-1}) in the air passing over the strips. A: strips exposed to 10 mM MES buffer (pH about 5.6) and 100 mM KIDA in the absence of Cl^-; epidermal malate increases linearly with increases in stromatal aperture; aperture (μm) = 1.74 + 0.0357 malate (pmol mm^{-2}); r = 0.866, N = 65. B: strips exposed to 10 mM MES buffer (pH about 5.6), 90 to 100 meq K^+ liter^{-1}, and Cl^- between 10^{-2} and 10^2 meq liter^{-1}; in the presence of Cl^-, the linear relation seen in A broke down.

were measured, and malate was extracted and assayed. As shown by open circles in Figure 3 (compare with closed circles which represent the data of Fig. 1A), epidermal malate was almost always greater in these tissues than in corresponding epidermis whose stomates had been allowed to open after stripping. Also, stomata were wider and more uniformly open if they were allowed to open on the leaf than on solutions of salts of K^+. A direct comparison of apertures is possible because restraints reducing stomatal opening in the complete leaf were released during peeling.

DISCUSSION

Since almost all epidermal cells except guard cells ruptured during preparation of the epidermal strips or while the strips floated on solutions, almost all of the solutes found in the strips at the end of each experiment must have been contents of guard cells.

In isolated epidermis of *V. faba* exposed to solutions free of Cl^-, stomatal aperture and malate content were linearly related to each other (Fig. 1A). This observation correlates well with the linear relationship between K^+ content of guard cells and stomatal aperture reported earlier (7, 22, 23). The stomatal frequency of our material was about 60 mm^{-2}; therefore the malate content of one pair of guard cells was about 4.2 pmol at an aperture of 10 μm. This quantity is about twice as large as the amount of a divalent anion needed to balance the amount of K^+ required to produce the same increase in opening, as was determined earlier on the same species (12). The discrepancy disappears if we recognize that in the earlier study guard cell volumes were about half as large as estimated on other occasions (Table 4.6 of ref. 11). Therefore it appears possible that import of K^+ and production of malic acid fully account for the change in stomatal aperture which occurred in the absence of external Cl^- in epidermal strips of *V. faba*.

The observed correlation between epidermal malate content and stomatal aperture is evidence against Bowling's (3) malate switch hypothesis when applied to stomata of *V. faba*. Bowling proposed that stomatal movement results from a shuttle of malate between guard cells and their neighboring cells. He suggested that changes in the pH in guard cells switch malate between its mono- and divalent forms. The divalent form is locked in the cells but the monovalent form can move from cell to cell, following its concentration gradient. According to Bowling's hypothesis, the

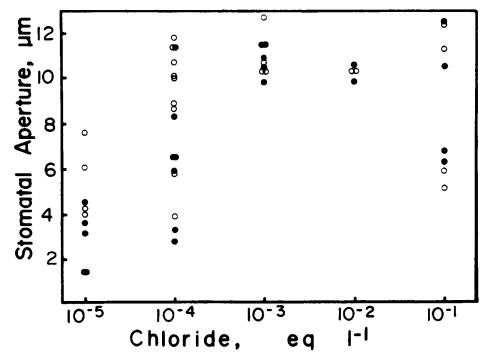

FIG. 2. Final aperture in epidermal strips of *V. faba* in relation to external Cl^- concentration. Same data as in Figure 1B. (○): strips exposed to CO_2-free air; (●): strips exposed to CO_2-containing air.

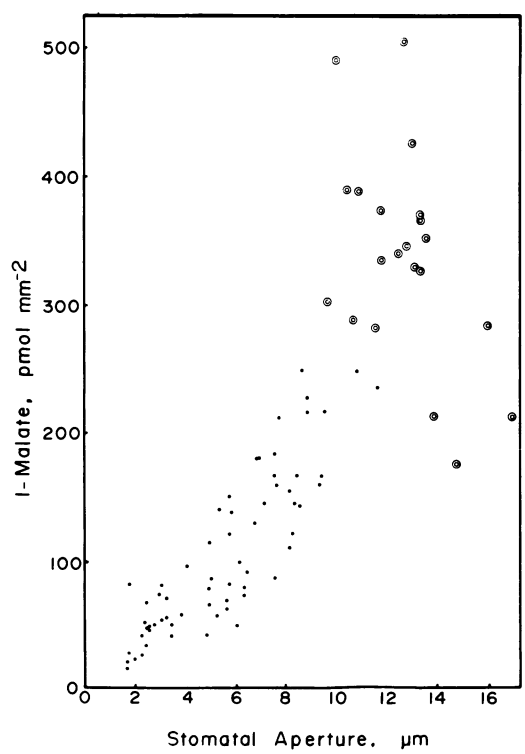

FIG. 3. Epidermal malate in *V. faba* after stomates were allowed to open in leaf sections (○) or in isolated epidermis (●). Data for isolated epidermis are the same as in Figure 1A. Leaf sections were floated on distilled H_2O in the light for 4 hr; CO_2-free air was passed over the sections. Epidermal strips were then made and extracted. On leaf sections, stomates attain a wider aperture and the concentration of malate in the epidermis is on the average correspondingly higher than in isolated epidermis, except at apertures >14 μm.

total malate content of the epidermis stays constant while the distribution of malate changes between guard cells and the other epidermal cells. In our experiments, the epidermal cells were ruptured. Malate accumulated in the epidermis although no exogenous malate was offered. Guard cells of *V. faba* must have been able to produce this acid.

When Cl^- was present in the solution supporting epidermal strips the linear relationship between malate content and stomatal aperture was no longer observed (Fig. 1B). At comparable apertures, malate contents were much lower than in epidermes without a supply of Cl^-. Presumably, Cl^- can be used as a counterion for K^+ by guard cells and guard cells produce malate when the supply of Cl^- is insufficient, using the following mechanism to balance charges and maintain pH. When stomata open, guard cells export H^+ in exchange for K^+; OH^- is left behind. Hydroxyl ions can then be exchanged for Cl^- if Cl^- is available. If not, the cytoplasmic pH will rise, which stimulates the synthesis of oxaloacetic acid and, as a consequence, that of malic acid. Stimulation occurs because bicarbonate is a substrate for P-enolpyruvate carboxylase (2), and the elevated pH increases the ratio of HCO_3^- to CO_2. Secondly, the activity of P-enolpyruvate carboxylase might also increase; this has been observed *in vitro* in the pH range of 6 to 8.5 (24). Experiments conducted on roots lend support to this idea: absorbable inorganic anions can replace organic anions (10). An inhibition of an uptake of malate and bicarbonate by Cl^- has also been observed in barley root cells (4). An alternative to the mutual replaceability of Cl^- and malate in guard cells would be an effect of Cl^- on the organic acid metabolism in guard cells in the direction of a change from malic to some other organic acid. Studies with radioactive chloride favor the first view (15). Direct measurements of Cl^- in guard cells are required to state unequivocally which of the two explanations of our results is correct.

The proposal that availability of Cl^- determines its use by guard cells can be invoked to explain the reported variability in the relative importance of Cl^- and malate as counterions to K^+ in guard cells of *V. faba*. Humble and Raschke (12) fertilized their plants with Hoagland solution which contains Cl^- only as a trace element. Correspondingly, only 5% of the K^+ was balanced by Cl^-. The plants used in our investigation were grown the same way; the malate content of epidermis taken from leaves was therefore high (except at apertures > 14 μm) and matched that of isolated epidermis floated on solutions containing no Cl^- (Fig. 3). Allaway (1) reported that the malate content of epidermis with open stomata accounted for about one-half of the positive charges of K^+. The nutrient supply to his plants was not given, but his leaf samples were supplied with a 0.1 mM $CaCl_2$ solution while stomata opened. Pallaghy and Fischer (15) exposed isolated epidermis to 10 mM $^{42}K^{35}Cl$ and found that the ratio of Cl to K varied between 0.07 and 0.48; on the average, about 30% of the K^+ was balanced by Cl^-.

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