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Electrochemical Investigation of Active Malic Acid Transport at the Tonoplast into the Vacuoles of the CAM Plant *Kalanchoë daigremontiana*

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Summary. The membrane potential of cells in leaf slices of the CAM plant *Kalanchoë daigremontiana* Hamet et Perrier in the light and in the dark is −200 mV on the average; it is reversibly depolarized by the metabolic inhibitors FCCP (5×10^{-6} M) and CN^- (5×10^{-3} M); it shows the light-dependent transient oscillations ubiquitously observed in green cells; it is independent of the amount of malic acid accumulated in the cells (in a tested range between 30 and 140 mM); and it is considerably hyperpolarized by the fungal toxin fusicoccin (30×10^{-6} M). Fusicoccin inhibits nocturnal malic acid accumulation in intact isolated phyllodia of the CAM plant *Kalanchoë tubiflora* (Harv.) Hamet but does not affect remobilization of malic acid during the day.

Electrochemical gradients for the various ions resulting from dissociation of malic acid, i.e., H^+, $Hmal^-$ and mal^{2-}, were calculated using the Nernst equation. With a very wide range of assumptions on cytoplasmic pH and malate concentration results of calculations suggest uphill transport of H^+ and $Hmal^-$ from the cytoplasm into the vacuole, while mal^{2-} might be passively distributed at the tonoplast. On the basis of the present data the most likely mechanism of active malic acid accumulation in the vacuoles of CAM plants appears to be an active H^+ transport at the tonoplast coupled with passive movement of mal^{2-} possibly mediated by a translocator ("catalyzed diffusion"), with subsequent formation of $Hmal^-$ ($2\,H^+ + mal^{2-} \rightarrow H^+ + Hmal^-$) at vacuolar pH's.

For various reasons, cells of higher plants may transport organic acids or organic acid anions across their tonoplasts and accumulate them in their vacuoles. This occurs, for instance, when uptake of inorganic cations from an outer medium surmounts anion uptake; organic acids are synthesized by CO_2 dark fixation; the protons are exuded, balancing the electrical charge of the cations taken up; the cations are accumulated in the vacuole together with the organic anions (Torii & Laties, 1966; Jacoby & Laties, 1971; Osmond, 1976). A similar mechanism operates when the anions of a salt taken up (e.g., NO_3^-, SO_4^{2-}) are metabolized; organic anions are needed to balance the electrical charge of the inorganic cations left behind, and the protons becoming

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available during organic acid synthesis by CO_2 dark fixation serve neutralization of OH^- ions formed in reduction of inorganic anions (Osmond, 1976; Smith & Raven, 1976). A third example is hormone (e.g., indole acetic acid) stimulated H^+/K^+ exchange, where organic acids provide protons for exchange with K^+, and K^+ is accumulated with the organic anion in the vacuole (Haschke & Lüttge, 1975*a–b*, 1977). In all these cases synthesis of organic acids (mainly of malic acid) serves a pH-stat mechanism providing protons during more or less extensive and continuous H^+ extrusion and electrical charge balance of inorganic cations accumulated (Osmond, 1976; Smith & Raven, 1976). In addition, organic acids accumulating in the vacuoles may serve as osmotic agents for the maintenance of turgor (Haschke & Lüttge, 1977; Wyn Jones, Brady & Speirs, 1979).

Malic acid accumulation in the leaf cells of plants having crassulacean acid metabolism (CAM) might be envisaged as just another example. In CAM, malic acid synthesized by CO_2 dark fixation during the night (dark phase) serves as storage of CO_2. By decarboxylation of malic acid during the day (light phase), CO_2 becomes available for photosynthesis via the Calvin cycle when stomata of the leaves remain closed to minimize transpirational loss of H_2O, i.e., when no CO_2 is available from the ambient atmosphere (Kluge, 1976). There is a fundamental difference, however, as compared with the examples mentioned above. In CAM the malic acid anions are always stoichiometrically accompanied by 2 H^+; coupling between malate and H^+ accumulation appears to be very tight; H^+ cannot be replaced by other cations (Lüttge, Ball & Tromballa, 1975*a*). Extracts of leaves of the CAM plant *Kalanchoë daigremontiana* Hamet et Perrier contain very little K^+, concentrations are on the order of 12 to 20 mM (*unpublished data;* Lüttge & Ball, 1974*b*; Lüttge *et al.*, 1975*a*). Watering *K. daigremontiana* plants regularly with 25 or 50 mM KCl leads to a rise of K^+ concentration in the leaf extracts up to about 100 mM, but this does not affect the 2 H^+ : 1 malate stoichiometry in CAM (Lüttge *et al.*, 1975*a*). Other inorganic ions like Na^+ and Cl^- are also at low levels in *K. daigremontiana* leaf extracts (Na^+, 5 to 16 mM; Cl^-, 8 to 21 mM; *unpublished results;* Lüttge & Ball, 1974*b*; Lüttge *et al.*, 1975*a*), but there appear to be high levels of free Ca^{2+} and Mg^{2+} (*unpublished results*).

The malic acid accumulated during the night in *K. daigremontiana* leaves normally reaches concentrations in the leaf extracts well above 150 mM; occasionally levels above 200 mM can be recorded (Lüttge & Ball, 1977). For purely geometric reasons this suggests that most of

Table 1. K_i-malate of PEP-C in leaves of CAM plants

Species	K_i [mM]	References
Kalanchoë tubiflora	3.6	Kluge & Osmond, 1972
Sedum praealtum	3	Kluge & Osmond, 1972
Kalanchoë fedtschenkoi	0.4	Bollig & Wilkins, 1979
Mesembryanthemum crystallinum	0.1–0.3	Winter, 1974; Greenway, Winter & Lüttge, 1978
Kalanchoë daigremontiana	0.4–0.8	I.C. Bollig-Buchanan (*personal communication*)

the malic acid accumulated, must be compartmented in the vacuoles. This is corroborated by attempts of compartmental analysis (Kluge & Heininger, 1973) and by work with isolated vacuoles (Buser & Matile, 1977). The best evidence for this comes from an evaluation of some properties of phosphoenol pyruvate carboxylase (PEP-C), i.e., the enzyme mediating CO_2 dark fixation which leads to malate synthesis in CAM. PEP-C is a cytoplasmic enzyme which is subject to feed-back inhibition by malate. The inhibition constant, K_i-malate, is on the order of 0.1–1 mM (larger values reported in early investigations may be overestimations, but there may also be seasonal variations; *see* Table 1). Therefore, malate concentrations in the cytoplasm above 1 mM seem to be very unlikely at any one time during the CAM rhythm; at larger cytoplasmic malate concentrations the synthesis of malate which is actually observed would appear impossible. Hence, there must be a steep concentration gradient for malic acid between the cytoplasm and the vacuole, and the transport into the vacuole must be thermodynamically uphill, i.e., active transport.

Earlier work has shown that in *K. daigremontiana* the efflux of malic acid from the vacuole is passive; it increases exponentially with increasing malate concentration in the tissue (Lüttge & Ball, 1977); it is insensitive to or increased by the uncoupler FCCP (carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone; Lüttge *et al.*, 1975*a*), independent of light (Lüttge & Ball, 1974*a*), and has a temperature coefficient (Q_{10}) below 2 (Lüttge *et al.*, 1975*a*). Uptake of malate by *K. daigremontiana* leaf cells from an external medium is also passive; it proceeds at a very low rate with a linear concentration isotherm (Lüttge & Ball, 1977).

Thus, malic acid transport into the vacuole seems to be the only active transport mechanism involved in malate distribution in the CAM leaf cells. It is not clear, though, in which form and by which mechanism malate is transported. The present electrophysiological characterization of *Kalanchoë* leaf cells aims to supply data for the evaluation of electro-

chemical driving forces for malic acid transport. Passive but presumably catalyzed movement of malate^{2-} coupled to active pumping of protons into the vacuoles seems to be the simplest mechanism consistent with data and calculations.

Materials and Methods

Plants of *Kalanchoë daigremontiana* Hamet et Perrier and *Kalanchoë tubiflora* (Harv.) Hamet were grown in the green house and transferred to a growth chamber with controlled light intensity and temperature several days before the experiments. *K. daigremontiana* plants used for most of the experiments were kept 12 hr in the light of HQL lamps at 40 W m^{-2} and 25 °C and 12 hr in the dark at 15 °C. In a few experiments (Fig. 7) the plants were kept in Xenon light at 160 W m^{-2} and 30 °C during the day to allow a more efficient utilization of the malic acid accumulated during the dark phase. The *K. tubiflora* plants used in the experiment of Fig. 8 during the 12 hr light period were kept the first 10.5 hr in the light of the Xenon lamp at 160 W m^{-2} and 30 °C and the last 1.5 hr in the light of HQL lamps at 25 W m^{-2} and 25 °C, the 12 hr dark phase was at 15 °C.

For the O_2 exchange and the efflux experiments (Figs. 3 and 7, respectively) 2 mm wide slices were cut from *K. daigremontiana* leaves. In the O_2 experiments slices were pretreated for 60 min with 0.1 mM $CaSO_4$ in 10 mM Na-phosphate buffer at pH 6.3, and O_2 exchange was then studied in the same solution with the addition of 2 mM $NaHCO_3$ in a Clark O_2-electrode assembly (Rank Brothers, Bottisham, Cambridgeshire, U.K.). Light was obtained from goose neck optics at 350 W m^{-2}. In the efflux experiments 1 g fresh weight (Fr. Wt.) of leaf slices were kept in 25 ml solution at 25 °C in a shaking water bath. Further experimental details are given in the legend of Fig. 7.

Experiments with phyllodia of *K. tubiflora* are described in detail in *Results*. The same light regime was used in these experiments as during pretreatment of the intact plants described above.

Electric membrane potential differences (PD) of *K. daigremontiana* leaf cells were studied with about 10 mm × 5 mm large pieces of leaves mounted in a perspex chamber of about 5 ml of volume so that micro-salt bridges (glass capillaries filled with 3 M KCl and having a tip diameter of <0.5 µm) could be inserted into intact leaf cells via a cut edge. Penetration of the thick cuticle of mature *K. daigremontiana* leaves with the glass capillaries proved impossible without breaking the tips. The reference salt bridge was made up of 3 M KCl in 2% agar in 1 mm wide perspex tubing. Salt bridges were connected via Ag/AgCl contacts to a Keithley differential electrometer Model 604 and a line recorder. These perspex chamber was flushed with experimental solution as indicated in the legends of figures at a rate of 10 ml min^{-1}. The tip potential of the reference electrode measured in experimental solution before and after the experiments was between 5 and 20 mV. Light was provided by goose neck optics and focussed on the leaf sample with a microscope condensor to give 380 W m^{-2}.

The cutting of pieces of leaves which was unavoidable in the PD measurements caused problems because the wounding considerably reduced the PD of the cells. Wound effects on membrane potentials of plant cells were reported earlier for other materials (Pitman *et al.*, 1970; Jones, Novacky & Dropkin, 1975; Higinbotham & Pierce, 1976; Mertz & Higinbotham, 1976; Sambeek & Pickard, 1976*a–b*; Sambeek, Pickard & Ulbright, 1976; Cheeseman & Pickard, 1977). For *K. daigremontiana* we confirmed this using the small adventitious plantlets growing between the dents of mature leaves. The leaf surface of these young plantlets can be penetrated by the micro-salt bridges without breaking the

tips. With intact leaves of plantlets we observed PD's of 126 ± 25 mV (SD, $n=25$), with leaves cut in two halves the PD was reduced to 84 ± 17 mV (SD, $n=6$). We concluded that this wounding effect explained the very low membrane potentials of -60 mV in cells of mature *K. daigremontiana* leaves observed earlier (Lüttge & Ball, 1977). Unfortunately, the leaflets of adventitious plantlets do not perform CAM (malate levels were 38 μmol g^{-1} Fr.Wt. and 25 μmol g^{-1} Fr.Wt. at the end of the dark phase and light phase, respectively) and thus were not suitable for the present study. When the pieces cut from mature leaves were washed in experimental solution for 24–48 hr prior to the experiments, we obtained higly negative PD values. This procedure was used for the experiments, where effects of inhibitors and of fusicoccin were measured. An alternative was deep penetration with the tip of the micro-salt bridge into the tissue, i.e., insertion into a cell far from the wound. This was done in the experiments of Fig. 5, because longer washing is impossible without altering the malate content of the tissue.

The solutions used are given in the figure legends. Fusicoccin (FC) was dissolved in a small amount of ethanol followed by dilution with water or experimental solution; similar concentrations of ethanol were added to the controls of experiments.

Malate concentrations in external solutions and in extracts of *K. daigremontiana* leaves and *K. tubiflora* phyllodia were determined enzymatically after Hohorst (1970). Titratable H^+ in external solutions and extracts was measured by adding 5 ml of solution or extract to 5 ml of 2 mM Na-phosphate buffer of pH 8 and titrating the lowered pH of the resulting solution back to pH 8 using 0.1 N NaOH (Lüttge *et al.*, 1975*a*).

Results

In an earlier communication we have mentioned preliminary measurements where membrane potentials (PD) of *K. daigremontiana* leaf cells

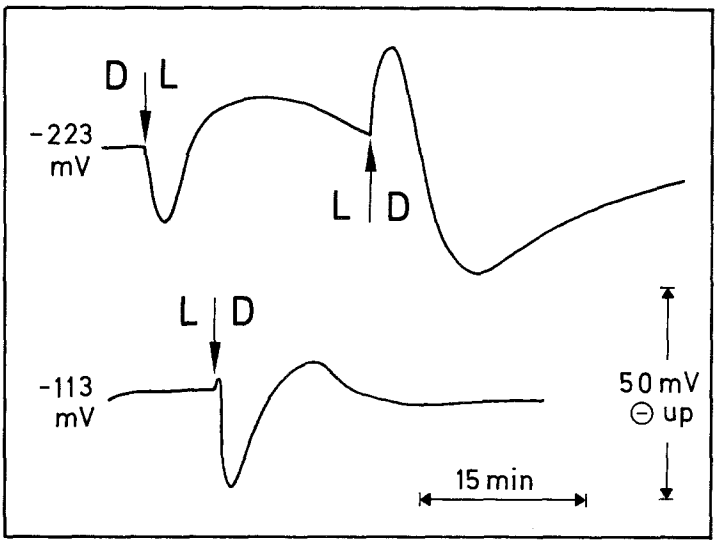

Fig. 1. Light-dark (*L/D*) and dark-light (*D/L*) triggered transients of membrane potential of cells of *K. daigremontiana* leaf slices. Experimental solution: 0.1 mM $CaSO_4$

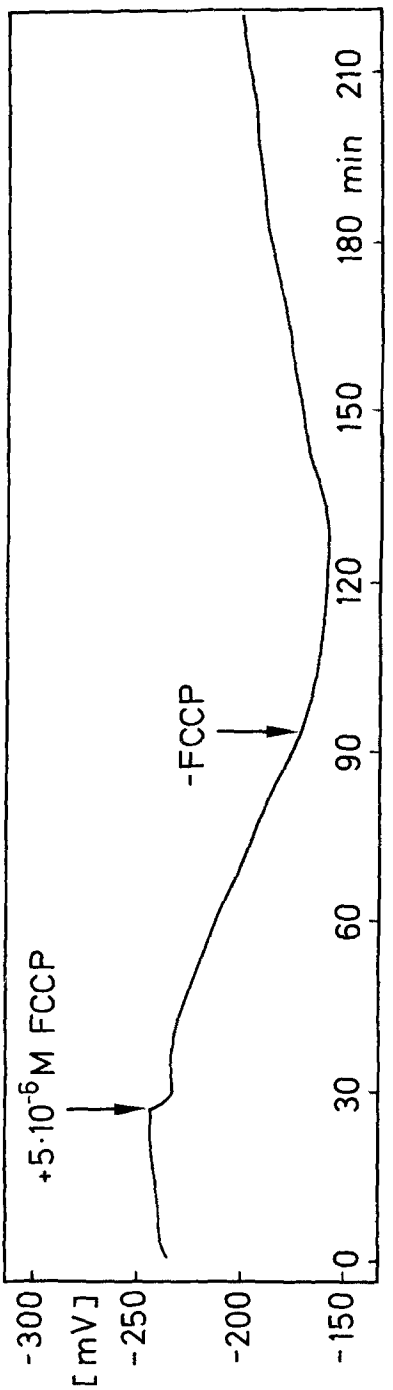

Fig. 2. Effect of FCCP on the membrane potential of a cell in a *K. daigremontiana* leaf slice in the light. (Similar responses are obtained in the dark.) Experimental solution: 0.1 mM $CaSO_4$ in 1 mM Na-phosphate buffer, pH 6

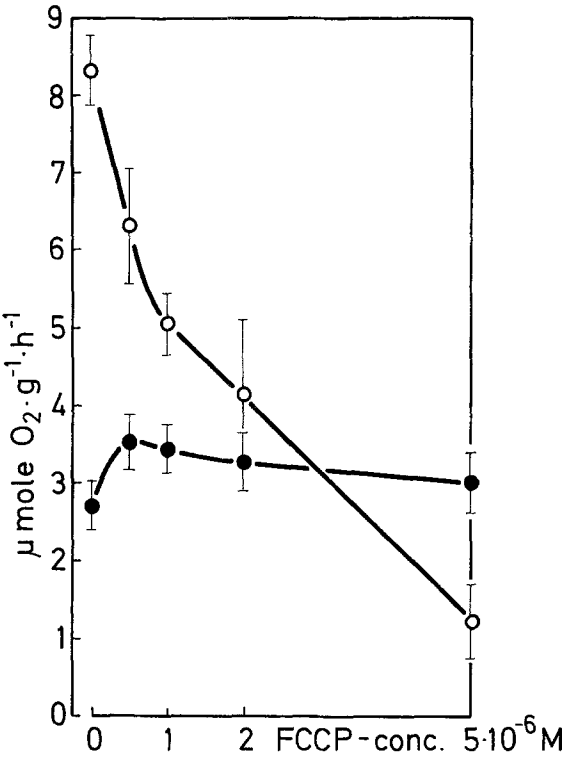

Fig. 3. Effect of FCCP on O_2 evolution in the light (open symbols) and O_2 uptake in the dark (closed symbols) of *K. daigremontiana* leaf slices. Experimental solution: 0.1 mM $CaSO_4$ + 2 mM $NaHCO_3$ in 10 mM Na-phosphate buffer, pH 6.3. Measurements were made between 7 a.m. and 3 p.m. Errors are SE

were not larger than about −60 mV (Lüttge & Ball, 1977). These were severe underestimations due to technical problems, which were overcome in the present work (*see Materials and Methods*). The PD of fully expanded leaves is roughly −200 mV on the average and can be well above this value in individual recordings (e.g., Figs. 2 and 5). In a series of measurements in May to July, 1977, the average PD was -199 ± 31 mV ($n=37$, error = SD, *see also* Fig. 5); a later series of experiments (December, 1977, to February, 1978) gave PD's of -206 ± 25 mV (SD, $n=46$) in the light and -206 ± 20 mV (SD, $n=10$) in the dark. Thus the resting potential is light independent. The PD of *K. daigremontiana*, however, shows the dark-light and light-dark transients (Fig. 1) ubiquitously observed in green cells (e.g., Pallaghy & Lüttge, 1970; Bentrup, 1974).

The PD is reversibly depolarized by the uncoupler FCCP (carbonyl-cyanide-*p*-trifluoromethoxy-phenylhydrazone; Fig. 2) which inhibits photosynthetic O_2 evolution in the light and at concentrations between 0.5

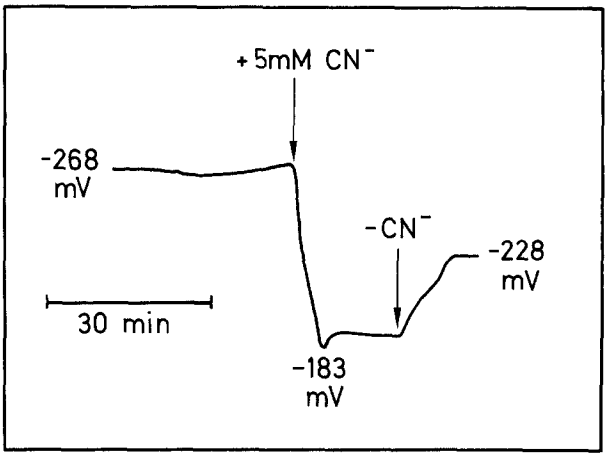

Fig. 4. Effect of cyanide on the membrane potential of cells in *K. daigremontiana* leaf slices in the light. The tissue was pretreated for 10–48 hr with 10 mM HEPES (=*n*-hydroxyethylpiperazine-N′-2-ethane sulfonic acid) buffer, pH 7, containing 0.1 mM $CaCl_2$ and 2.5 mM Na_2SO_4, so that upon addition of CN^- the CN^- replaced the SO_4^{2-} without any other change of the ionic composition of the medium

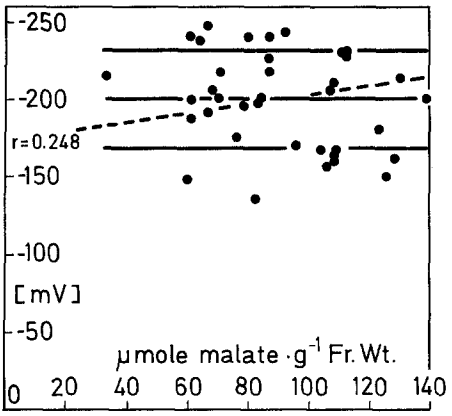

Fig. 5. Membrane potentials of cells in *K. daigremontiana* leaf slices with different malate levels obtained by harvesting the leaves at different times during the day. The three solid lines mark the mean of the points with the SE. The dotted line is the result of an attempt to calculate a linear regression line by the method of least squares; *r* is the correlation coefficient

and 2×10^{-6} M slightly increases respiratory O_2 uptake in the dark (Fig. 3). Cyanide also reduces the PD reversibly (Fig. 4). This suggests that the PD has a significant active component due to electrogenic mechanisms driven by energy metabolism of the cells. The slow response after addition and removal of FCCP may be due to problems of diffusion in the leaf slices.

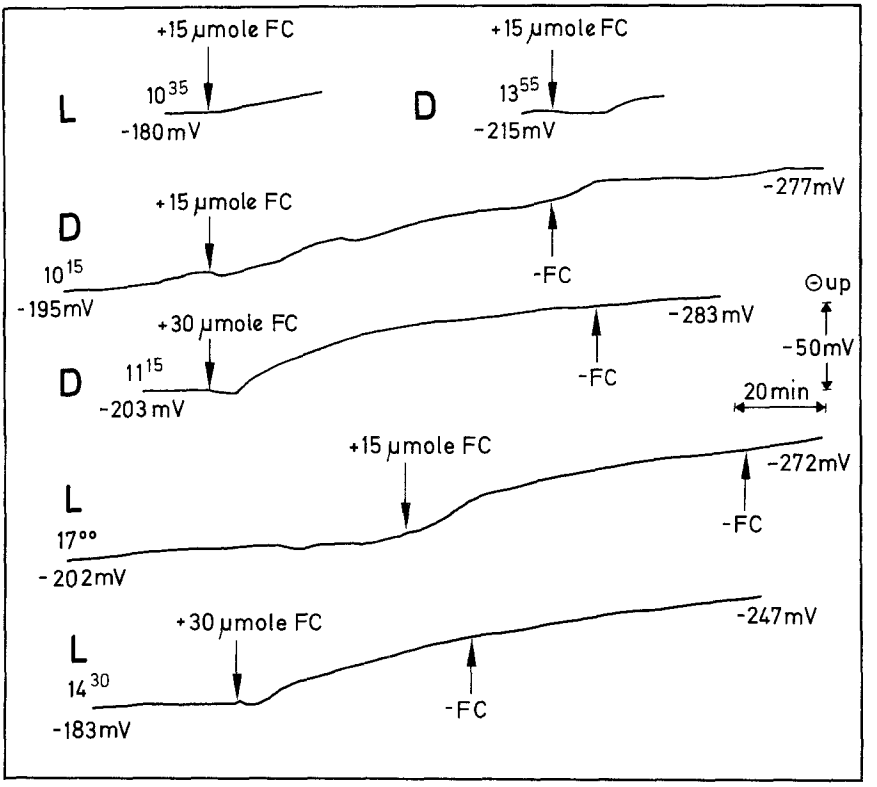

Fig. 6. Effect of fusicoccin (FC) on the membrane potential of cells in *K. daigremontiana* leaf slices in the light and in the dark at various times of the day as indicated at the beginning of the traces. Experimental solutions: upper four traces, 0.1 mM $CaSO_4$ in 1 mM Na-phosphate buffer, pH 6; lower two traces, 1 mM NaCl+0.1 mM KCl+0.05 mM $CaSO_4$ (=APW) in 1 mM K-phosphate buffer, pH 6

Figure 5 shows that the PD of *K. daigremontiana* leaf cells is independent of the amount of malate stored in the leaves, i.e., PD remains constant during the diurnal rhythm of CAM.

The fungal toxin fusicoccin (FC), which is known to stimulate active H^+ extrusion at the plasmalemma in a large variety of plant species (Marrè, 1977), higly hyperpolarizes the PD of *K. daigremontiana* leaf cells in the presence and in the absence of K^+ in the experimental solution (Fig. 6). This hyperpolarization is not reversible by removal of FC, but this was also found with other plant material (Schaefer, 1978; E. Marrè, *personal communication*). There is no apparent effect of FC on efflux of malate and H^+ from leaf slices of *K. daigremontiana* irrespective of whether or not K^+ is present in the external medium (Fig. 7*a–b*). It is possible that the large passive malic acid efflux from

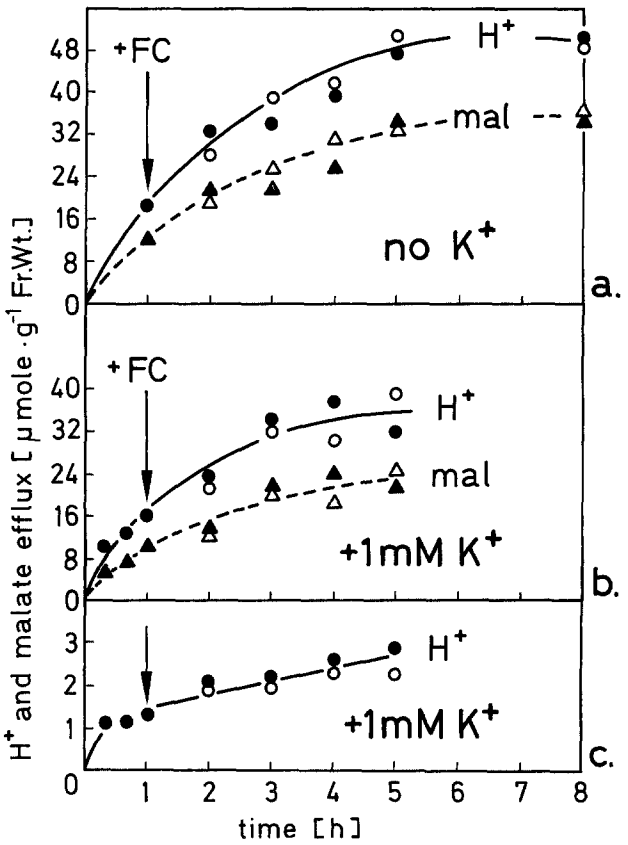

Fig. 7. H^+ efflux (circles) and malate efflux (triangles) in the dark in the presence (open symbols) and absence (closed symbols) of 30 µM fusicoccin (FC), which was added after 1 hr (arrows), in a 0.1 mM $CaSO_4$ solution (*a*) and in solution containing additionally 1 mM KCl (*b* and *c*). Initial malate levels of the tissue: (*a*) 118 µmol g^{-1} Fr.Wt.; (*b*) 100 µmol g^{-1} Fr.Wt.; (*c*) 42 µmol g^{-1} Fr.Wt.

slices of *K. daigremontiana* leaves having accumulated malic acid during the night masks an effect of FC on a proton extrusion pump at the plasmalemma. To test this possibility, the effect of FC on H^+ efflux was also tested with leaf slices of a low initial malate level obtained from leaves harvested towards the end of the light phase. Malic acid efflux from this material is much lower, but again FC had no stimulating effect on H^+ extrusion (Fig. 7*c*). Similarly in the presence of mannitol lowering the turgor of the cells and thus inhibiting passive malic acid efflux (Lüttge & Ball, 1974*a*; Lüttge, Kluge & Ball, 1975*b*; Lüttge, Ball & Greenway, 1977), FC did not enhance apparent H^+ efflux (results not shown).

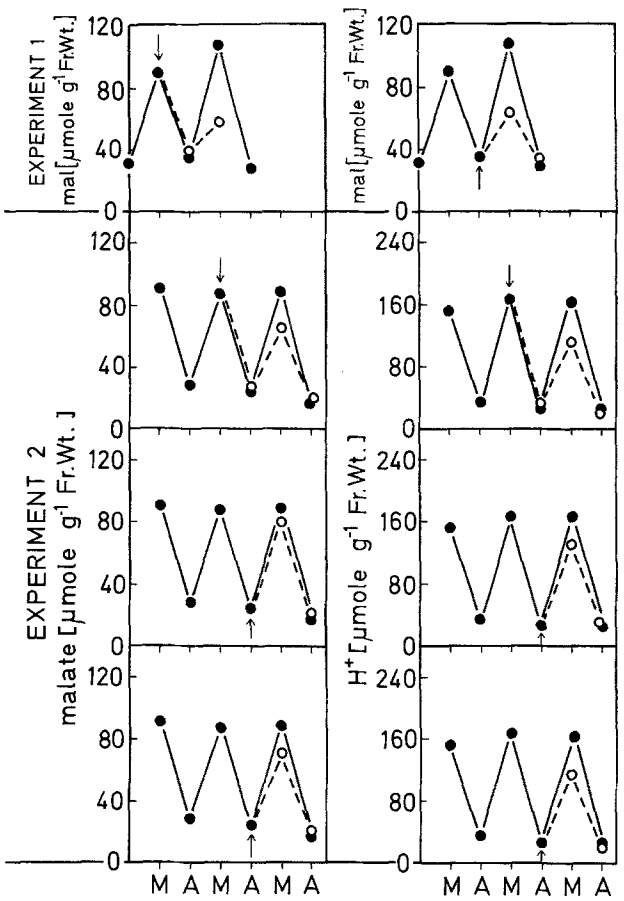

Fig. 8. Effect of fusicoccin (FC) on the accumulation and remobilization of malic acid (malate in experiment 1; malate *and* titratable protons in experiment 2) of intact isolated phyllodia of *Kalanchoë tubiflora*. Time of FC addition is indicated by arrows. The control (solid lines and circles) is always the same in the different graphs of a given experiment (1 or 2). Samples analyzed after FC addition are represented by open circles and dotted lines. FC concentration was 30 µM except for the bottom graphs of experiment 2, where 60 µM was used. *M*=morning (end of the dark phase), *A*=afternoon (towards the end of the light phase)

Isolated phyllodia of *K. tubiflora* were used to test the effect of FC on the diurnal malic acid rhythm. The diffusion pathways in the narrower phyllodia seem less tortuous than in the large leaves of *K. daigremontiana*; and many biochemical investigations of Kluge and coworkers using these phyllodia indicate that solutes are readily distributed (Kluge, 1969). Groups of 5–7 isolated phyllodia were placed with their lower ends in 15 ml of artificial pond water (APW = 1 mM NaCl + 0.1 mM KCl + 0.05 mM $CaSO_4$) with or without FC in small beakers. Incisions

had been made in the lower parts of the phyllodia dipped in the solution to facilitate uptake of water and solutes driven into the phyllodia by transpiration. There is a small but consistent inhibition of nocturnal malic acid accumulation by FC, but malic acid mobilization in the light is not affected (Fig. 8).

Discussion

1) Electrochemical Gradients for H^+ and Malate at the Tonoplast

a) Problems and assumptions in calculations. The electrochemical gradients for H^+ and the two charged species of the dicarboxylic acid anion malate, i.e., $Hmal^-$ and mal^{2-}, can be evaluated using the Nernst equation:

$$E_{\text{Nernst}} = -\frac{RT}{zF} \ln \frac{c_i}{c_o} \tag{1}$$

where E_{Nernst} is the Nernst potential in mV, R is the universal gas constant, T the temperature in °K, F the Faraday and z the electrical charge of the ion transported; c is the concentration in an inner (i) and outer compartment (o) separated by a membrane. At 25 °C

$$E_{\text{Nernst}} = -\frac{59}{z} \log \frac{c_i}{c_o}. \tag{2}$$

If the membrane potential (PD) is close to the Nernst potential the respective ion could be distributed passively solely due to physical driving forces, provided that the membrane has an adequate permeability to the ion in question. Discrepancies between E_{Nernst} and PD suggest that active transport is involved (Nernst criterion).

A number of problems, however, are encountered using Eqs. (1) or (2) for a discussion of malic acid transport at the tonoplast of CAM leaf cells:

i) In the PD measurements the tip of the electrode is in the vacuole and membrane potentials (Ψ) of the tonoplast and the plasmalemma in series are recorded:

$$\text{PD} = \Psi_{\text{plasmalemma}} + \Psi_{\text{tonoplast}}. \tag{3}$$

In some higher plant cells where separate measurements have been possible, $\Psi_{\text{tonoplast}}$ was found to be zero or slightly positive (Etherton & Higinbotham, 1960; Denny & Weeks, 1968; Zurzycki, 1968; Etherton,

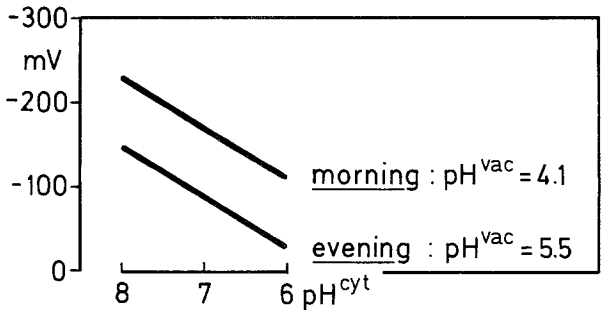

Fig. 9. Possible ranges of H^+ Nernst potentials (vacuole negative, cytoplasm zero) at the tonoplast of *K. daigremontiana* cells for a cytoplasmic pH range of 6–8 and vacuolar pH's of 4.1 and 5.5 at the end of the dark phase (morning) and light phase (evening), respectively

1970; Ginsburg & Ginzburg, 1974; Lüttge & Zirke, 1974; Dunlop, 1976; Smith & Raven, 1976), and we may assume that this is similar in *K. daigremontiana;* only in exceptional cases of a few marine algae were larger positive tonoplast potentials observed (MacRobbie, 1970).

ii) The cytoplasmic malate concentration (c^{cyt}_{mal}) is unknown. From the K_i-malate of PEP-C (Table 1) it follows though, that c^{cyt}_{mal} must be within the range of 0.01 to 1 mM (*see* introduction).

iii) The vacuolar malate concentration (c^{vac}_{mal}) is less difficult to assess; it must be very close to the malate concentration in the brei obtained by homogenization of leaf tissue, because the volume of the vacuoles is very much larger than that of the cytoplasm. Based on many measurements of diurnal variations of malic acid levels, concentrations of 200 mM at the end of the dark phase and 20 mM at the end of the light phase were chosen for the calculations below.

iv) The pH of the cytoplasm (pH^{cyt}) is unknown. Where indirect measurements of cytoplasmic proton concentrations were possible, pH^{cyt} was 7 or somewhat above (*Acetabularia*, Dodd & Bidwell, 1971; *Neurospora*, Slayman & Slayman, 1968; *Chara*, Walker & Smith, 1975; higher plants, Drawert, 1968, p. 516). Thus, it seems safe to assume the rather wide range of pH 6 to 8 for the calculations made below.

v) For similar reasons as discussed for vacuolar malate concentration, the pH in the vacuole (pH^{vac}) must be close to the pH of leaf tissue brei. We recorded values of pH 4.1 and pH 5.5 at the end of the dark phase and light phase, respectively.

b) Possible range of H^+ Nernst potentials at the tonoplast during the diurnal malic acid rhythm. Figure 9 shows the possible range of H^+ Nernst potentials ($E^{H^+}_{Nernst}$) obtained with the assumptions made above

Table 2. Dissociation equilibria of malic acid given as percentages of the species H_2mal (=undissociated acid), $Hmal^-$ and mal^{2-} at pH values relevant for CAM

	pH	H_2mal	$Hmal^-$ [%]	mal^{2-}
Likely vacuolar pH's:				
leaf brei at the end of the dark phase	4.1	19.9	72.8	7.3
leaf brei at the end of the light phase	5.5	0.3	29.3	70.4
Possible range of cytoplasmic pH's:	6.0	0.1	11.6	88.3
	7.0	0.0	1.3	98.7
	8.0	0.0	0.1	99.9

(*a/iv* and *a/v*). The lowest $E^{H^+}_{Nernst}$ (i.e., ~ -30 mV) is found in the evening with $pH^{cyt}=6$, i.e., at the unlikely lower end of the pH^{cyt} range assumed above. With a $\Psi_{tonoplast}$ of zero or slightly positive, H^+ would have to be actively transported into the vacuole at any time of the CAM rhythm and within the wide range of conditions assumed to be possible.

c) Dissociation equilibria of malic acid. Dissociation equilibria of the dicarboxylate malic acid are determined by the pK values:

$$pK_1 = 3.537,$$
$$pK_2 = 5.119.$$

The percentage of the various possible species, i.e., H_2mal, $Hmal^-$ and mal^{2-}, can be obtained in good approximation by the following relationships:

$$pH - pK_1 = \log \frac{c_{Hmal^-}}{c_{H_2mal}} \tag{4}$$

and

$$pH - pK_2 = \log \frac{c_{mal^{2-}}}{c_{Hmal^-}}. \tag{5}$$

Table 2 lists some values for pH's relevant in the present study. With these values the concentrations of $Hmal^-$ and mal^{2-} in the cytoplasm and vacuole for the discussions in sections *d* and *e* were obtained from the assumptions made in sections *a/ii* and *a/iii*.

d) Possible range of $Hmal^-$ Nernst potentials at the tonoplast during the diurnal malic acid rhythm. With the assumptions and data discussed above in *a/ii*, *a/iii*, and *c*, the ranges of $Hmal^-$ Nernst potentials at the tonoplast shown in Fig. 10 are obtained. Under all possible circum-

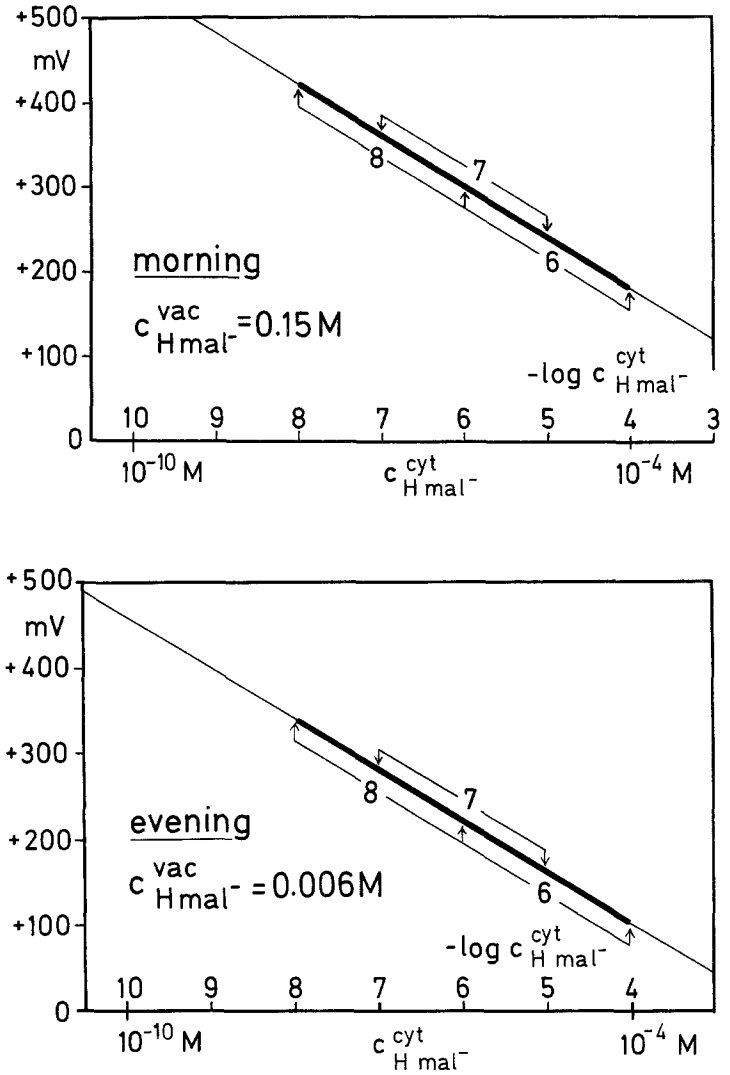

Fig. 10. Possible ranges of $Hmal^-$ Nernst potentials at the tonoplast of *K. daigremontiana* cells for a range of cytoplasmic $Hmal^-$ concentrations ($c^{cyt}_{Hmal^-}$, as given on the abscissa) which appears possible between pH 6 and 8 in the cytoplasm as indicated by the numbers on the lines referring to pH^{cyt}. The range of $c^{cyt}_{Hmal^-}$ covered by the bold parts of the lines is 10^{-8}–10^{-4} M

stances tested with a wide range of assumptions $E^{Hmal^-}_{Nernst}$ is highly positive. The lowest values of $\sim +100$ mV are obtained at the end of the light phase for the unlikely low pH^{cyt} of 6. Thus, during malate accumulation in the vacuole with a $\Psi_{tonoplast}$ of zero or slightly positve $Hmal^-$ must be actively transported into the vacuole at any time.

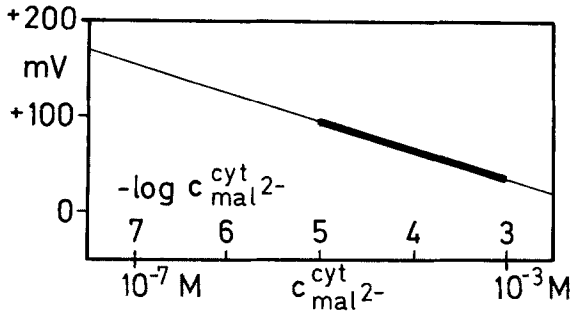

Fig. 11. Possible ranges of mal^{2-} Nernst potentials at the tonoplast of *K. daigremontiana* cells for a range of 10^{-5}–10^{-3} M cytoplasmic mal^{2-} concentrations (bold part of the line)

e) Possible range of mal^{2-} Nernst potentials at the tonoplast during the diurnal malic acid rhythm. With the same assumptions and data the range of possible mal^{2-} Nernst potentials is obtained as depicted in Fig. 11. The range obtained is rather independent of cytoplasmic pH between 6 and 8 since $pK_2 = 5.119$. Interestingly, the absolute concentration of mal^{2-} in the vacuole is not subject to much change during the diurnal malic acid rhythm. This is due to changes in vacuolar pH balancing changes in total malate concentration ($c_{mal\text{-}total}$) as shown by the following calculations:

$$\textit{End of dark phase: } c^{vac}_{mal\text{-}total} = 0.20\,M;$$

$$pH^{vac} = 4.1;\ c^{vac}_{mal^{2-}} = 7.3\,\% = 0.015\,M.$$

$$\textit{End of light phase: } c^{vac}_{mal\text{-}total} = 0.02\,M;$$

$$pH^{vac} = 5.5;\ c^{vac}_{mal^{2-}} = 70.4\,\% = 0.014\,M.$$

Nernst potentials are only slightly positive unless extremely low values of c^{cyt}_{mal} are assumed. It seems well possible that, by contrast to H^+ and $Hmal^-$, mal^{2-} is distributed passively at the tonoplast between the cytoplasm and vacuole.

2) Possible Mechanism of Malic Acid Transport at the Tonoplast during Active Malate Accumulation

As pointed out in the introduction, in CAM there is strict stoichiometry of $2H^+$ moving together with 1 mal^{2-} and tight coupling must occur during transport. It is unlikely, that the electrically neutral species H_2mal

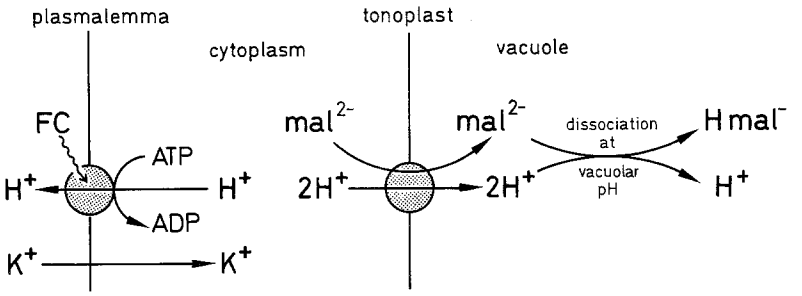

Fig. 12. Possible mechanism of malic acid transport at the tonoplast during active malate accumulation. FC=fusicoccin. H^+ pumps are indicated by hatched circles

is transported, because at any of the likely cytoplasmic and vacuolar pH values very little undissociated acid will be present (*see* Table 2). An uphill active movement of both H^+ and $Hmal^-$ would be energetically expensive. With the present state of our knowledge, the mechanism drawn in Fig. 12 seems to be preferable. In this scheme the driving force for malate accumulation is an H^+ pump across the tonoplast; mal^{2-} follows passively and is changed to $Hmal^-$ due to the dissociation equilibrium in the vacuole. This, would be in agreement with the possible electrochemical gradients as discussed in section 1 above.

H^+-cotransport or OH^--countertransport mechanisms such as those assumed to drive solute uptake at the plasmalemma of fungal and plant cells (Slayman & Gradmann, 1975; Tanner *et al.*, 1977; Novacky, Ullrich-Eberius & Lüttge, 1978*b*; Novacky *et al.*, 1978*a*) are difficult to envisage as being operative in malate transport into the vacuole. In these mechanisms H^+ is actively pumped in one direction across the membrane creating an electrochemical driving force which then takes H^+ + transported solute back in the opposite direction. Thus, for an H^+/malate cotransport in CAM, the proton pump at the tonoplast directed inwards to the vacuole would operate in the wrong direction. Conversely, an H^+/malate countertransport, for which the proton pump at the tonoplast in *K. daigremontiana* would operate in the right direction, does not explain the concomitant stoichiometric accumulation of malate *and* protons in the vacuole.

Naturally, the thermodynamic considerations based on the evaluation of likely concentration gradients of H^+, $Hmal^-$ and mal^{2-} at the tonoplast do not allow any conclusions about the molecular nature of the transport processes involved. The conclusion obtained by the thermodynamic evaluations even bears two problems in this respect. Firstly, if mal^{2-} were transported across the tonoplast by genuine diffusion under

physical forces (electrical gradient established by the H^+-pump) and without chemical interaction with other species and with membrane components, the tight coupling of transport of 1 mal^{2-} together with 2 H^+ would be difficult to explain. Secondly, it is hard to imagine a membrane passively permeable to mal^{2-} but not to $Hmal^-$. In barley roots effects of external pH clearly show that malate enters the cells as $Hmal^-$ not as mal^{2-} (Cram, 1974). In his malate-switch hypothesis trying to explain the action of stomata, Bowling (1976) postulates that mal^{2-} is strictly impermeant. (As in CAM in Bowling's hypothesis, malate movements and changing pH values of compartments play a large role. That Bowling's model fully explains functioning of stomata is unlikely, however, because Cl^- has been shown to be able to replace malate partially (Kirk & Raschke, 1978; Raschke & Schnabl, 1978) or entirely (Schnabl & Ziegler, 1977) during opening and closing movements.) Naturally it would be desirable to know the relative permeabilities of $Hmal^-$ and mal^{2-} at the tonoplast of CAM leaf cells, but this reaches far beyond our present technical possibilities.

The best way to avoid these difficulties in the hypothesis of malic acid transport in CAM is to postulate a translocator which could mediate mal^{2-} transport by catalyzed diffusion and which is linked energetically to the proton pump. Experiments with isolated vacuoles may offer the opportunity to study the molecular mechanisms. Obviously isolated vacuoles of tulip petals have a vacuolar anion ATPase activity correlated with H^+ transport (Lin, Wagner & Hind, 1977). Vacuoles isolated from *Hevea* latex ("lutoïds") have a citrate transport system which seems to be based on the operation of two independent carriers, a citrate translocator and an ATPase acting as a proton pump and providing the energy for citrate transport (d'Auzac, 1975, 1977; Matile, 1978).

3. Explanation of Fusicoccin Effects

Unfortunately, at present the experiments with fusicoccin do not contribute to further clarification. The only primary action of FC known in plant cells seems to be an enhancement of electrogenic H^+ extrusion at the plasmalemma with an uptake of K^+ for exchange with H^+, all other effects observed being secondary consequences thereof (Marrè, 1977). Such a mechanism would readily explain the hyperpolarization of PD by FC shown in Fig. 6, however, it cannot immediately explain the inhibition of malic acid accumulation in the experiments with phyllo-

dia of *K. tubiflora* (Fig. 8). An FC-dependent H^+ extrusion would tend to increase the cytoplasmic pH which would lead to increased malate synthesis as observed in other systems (Marrè, 1977) because PEP-C has an alkaline pH-optimum. FC causes stomata to open (Marrè, 1977); but again this would work in the wrong direction to explain the inhibition of malate accumulation since it would facilitate rather than inhibit CO_2 uptake required for malate synthesis by CO_2 dark fixation. An action of FC on active H^+ transport across the tonoplast at the moment seems unlikely. At present there is no reason to assume that FC acts on cellular membranes other than the plasmalemma. Since it stimulates the proton pump of the plasmalemma it is difficult to envisage that it should inhibit proton pumping at the tonoplast. Thus, the simplest explanation of the FC inhibition of malic acid accumulation in CAM leaves at the moment is a competition for protons between the FC enhanced pump at the plasmalemma and the malate accumulating pump at the tonoplast as shown in Fig. 12. This also would explain why the FC inhibition of acid accumulation, though consistently observed, is sometimes rather small (Fig. 8). The apparent lack of an effect of FC on H^+ efflux from *K. daigremontiana* leaf slices (Fig. 7, especially Fig. 7*c*) may be due to a large buffering capacity of the cell walls. It is often difficult in higher plants to demonstrate pH changes of the medium during the operation of H^+ transport mechanisms at the plasmalemma (Novacky *et al.*, 1978*a–b*).

Conclusions

The proton pump at the tonoplast of *Kalanchoë* leaf cells and the malate transport coupled with it seem to have rather unusual properties. But surprise may be expected since little is known in general about transport mechanisms at the tonoplast of higher plants. Leaf cells of CAM plants, which shuttle malic acid between their cytoplasm and vacuoles, seem to provide a unique system to study a transport process at the tonoplast without interference of the plasmalemma.

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