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Mechanism of Passive Malic-Acid Efflux from Vacuoles of the CAM Plant *Kalanchoë daigremontiana*

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Summary. An analysis was carried out of the mechanism of malic-acid efflux from vacuoles of mesophyll cells of the crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana*. Following its accumulation in the vacuole as a result of nocturnal CO_2 fixation, the malic acid is passively transported back across the tonoplast in the subsequent light period and is decarboxylated in the cytoplasm. Malic-acid efflux was studied using leaf slices in solution or by following malic-acid utilization (deacidification) in leaves of intact plants. Samples of leaf-cell sap were taken at different times during the day-night rhythm to establish the relation between cell-sap pH and malate content. From the empirically determined pK values for malic acid in the cell sap, it was then possible to calculate the proportion of malate existing as the undissociated acid (H_2mal^0) and in the anionic forms ($Hmal^{1-}$ and mal^{2-}) for all times during the CAM rhythm. In leaf-slice experiments it has been found that the rate of malic-acid efflux increases exponentially with the malic-acid content of the tissue. This is shown to be related to the increasing amounts of H_2mal^0 present at high malic-acid contents. At low malic-acid contents (<65 mol m^{-3}), when H_2mal^0 is not present in significant amounts, efflux must be in the form of $Hmal^{-1}$ and/or mal^{2-}. At high malic-acid contents it is suggested that efflux occurs predominantly in the form of passive, noncatalyzed diffusion of H_2mal^0 across the tonoplast by a 'lipid-solution' mechanism. This is supported by the fact that the slope of the curve relating efflux to H_2mal^0 concentration, when corrected for the presumed contributions from $Hmal^{1-}$ and mal^{2-} transport and plotted on a log-log basis, approaches 1.0 at the highest malic-acid contents. Moreover, the permeability coefficient required to be consistent with such a mechanism ($P_{H_2mal^0}$ = 1.0 to 2.0 × 10^{-8} m sec^{-1}) is similar to that estimated from a Collander plot, using the partition coefficient of malic acid between ether and water. We suggest that $P_{H_2mal^0}$ may be important in determining the maximum amounts of malic acid that can be accumulated during the CAM rhythm.

Key Words crassulacean acid metabolism · *Kalanchoë* · malic acid · tonoplast · membrane permeability · lipid-solution mechanism

Introduction

Plants possessing crassulacean acid metabolism (CAM) show large day-night changes in the malic-acid content of their photosynthetic tissues. During the night CO_2 is assimilated into malic acid, which can accumulate to concentrations of more than 200 mmol kg^{-1} fresh weight of tissue (Lüttge, Smith & Marigo, 1982). The following day this malic acid is decarboxylated and the released CO_2 fixed *via* the C_3 photosynthetic carbon reduction cycle (Kluge & Ting, 1978; Osmond, 1978). As the entire cytoplasm occupies of the order of only 1% of the cell volume (Lüttge et al., 1982), the vacuole must constitute the main storage site for malic acid.

This day-night rhythm poses important questions concerning the mechanism of malic-acid transport into and out of the vacuole across the tonoplast. From the electrochemical potential difference for the relevant ionic species between cytoplasm and vacuole, it is clear that malic acid must be actively transported across the tonoplast during the dark period (Lüttge & Ball, 1979; Rona et al., 1980; Lüttge et al., 1982). The exact cytosolic concentrations of the transported species are not known, but one restriction is imposed by the requirement for control of cytosolic pH, which presumably remains around 7.5 to 8.0 (Lüttge et al., 1982; Marigo et al., 1982). Further, an upper limit to cytosolic malate concentrations will be set by the sensitivity of PEP-carboxylase, the enzyme responsible for dark fixation of CO_2, to feedback inhibition by malate (*see* Lüttge et al., 1982). The driving force for malic-acid accumulation in the vacuole is thought to be provided by a proton-translocating ATPase at the tonoplast, with the malate anions following the protons electrophoretically (Lüttge et al., 1981, 1982, 1984; Smith et al., 1984*a*,*b*).

In contrast to the nocturnal accumulation process, malic-acid efflux from the vacuole during the light period is passive (Lüttge & Ball, 1977). The malic acid can be transported down its electrochemical potential gradient from the vacuole into the cytoplasm (Lüttge & Ball, 1979), and experiments

with tissue slices from *Kalanchoë daigremontiana* and *K. tubiflora* have indicated that this efflux is not energy-requiring. For example, the efflux process has a Q_{10} below 2.0, and it is unaffected by a variety of metabolic inhibitors (Lüttge, Ball & Tromballa, 1975; Marigo, Lüttge & Smith, 1983).

As yet we do not have a clear understanding of the mechanism of malic-acid efflux across the tonoplast. At typical vacuolar pH values (which may be as low as 3.4 in *K. daigremontiana*) malate will exist as a mixture of the undissociated acid, H_2mal^0, and the anions, $Hmal^{1-}$ and mal^{2-}. But it is not known which of these species cross the tonoplast or at what rates. In this paper we have investigated the efflux process with the aim of deriving flux-concentration relationships for the various species of malic acid present in the vacuole. An evaluation is made of the transport mechanisms that could contribute to malic-acid efflux across the tonoplast during the light phase of the CAM rhythm.

Abbreviations

CAM = crassulacean acid metabolism
PAR = photosynthetically active radiation
PEP = phospho*enol*pyruvate

Symbols and Notation of Species Involved in Malic-Acid Dissociation Equilibria

H_2mal^0 = undissociated malic acid, subscript 0
$Hmal^{1-}$ = univalent malate anion, subscript $1^{\ominus}$
mal^{2-} = divalent malate anion, subscript $2^{\ominus}$
mal-Σ = total malate, i.e., $H_2mal^0 + Hmal^{1-} + mal^{2-}$, subscript Σ
c = concentration
J = tonoplast efflux
P = permeability coefficients with subscripts denoting the chemical species

Materials and Methods

Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie were grown in soil culture in a glasshouse as described previously (Smith et al., 1984*a*). Plants used for the experiments were 5 to 10 months old. About three weeks before experimentation they were transferred to a controlled-environment chamber. The conditions were 12-hr light (25 ± 1°C)/12-hr dark (16 ± 1°C), with a relative humidity of 60 to 75% in both phases. High-pressure mercury-vapor lamps provided photosynthetically active radiation, PAR (400 to 700 nm) at upper-leaf height of 400 μmol quanta m^{-2} sec^{-1} (as measured using an LI-190SB quantum sensor, Li-Cor, Inc., Lincoln, Nebraska).

Leaf-cell sap was usually obtained by freeze-thawing samples of leaf tissue and centrifuging the sap through two layers of filter paper. For the experiment described in Fig. 2, leaf tissue was homogenized in a Braun Melsungen Potter S homogenizer and then centrifuged. Cell-sap pH was measured in the clear supernatant with a glass combination electrode. L-malate was assayed enzymatically according to Hohorst (1970). In view of the very high proportion of the mesophyll-cell volume occupied by the vacuole in *K. daigremontiana* leaves (*ca.* 98%: *see* Lüttge et al., 1982), the sap extracts obtained by the above means can be taken to represent essentially the vacuolar contents.

The pK values for malic acid in the leaf-cell sap were determined by titrating samples (adjusted to pH 2.0 with HCl) with aliquots of 200×10^{-9} m^3 KOH (500 mol m^{-3}). Replicate samples were also titrated to which various amounts of pure L-malic acid had been added. Back-titration of the neutralized samples with HCl revealed no hysteresis in the pH response of the sap.

Results and Discussion

pK Values of Malic Acid in the Leaf-Cell Sap and Day-Night Changes in Vacuolar pH during CAM

The pK values for the two carboxyl groups in malic acid are usually quoted as $pK_1 = 3.5$ and $pK_2 = 5.1$ (at 25°C). However, these are estimates of the pK values at zero ionic strength (I). In view of the marked dependence of the pK values on I (Kortüm, Vogel & Andrussow, 1960; Sillén & Martell, 1964), these estimates do not give an accurate indication of the dissociation state of malic acid in the cell sap. For example, at $I = 4.0$ kmol m^{-3} the observed values at 25°C are $pK_1 = 2.62$ and $pK_2 = 4.00$ (Sillén & Martell, 1964).

To determine the pK values of malic acid relevant to the leaf-cell sap of *Kalanchoë daigremontiana*, sap samples were titrated with KOH over the range of observed sap pH values. For our sap extracts, I was calculated to be of the order of about 0.2 from the formula

$$I = 0.5\,\Sigma m_i z_i^2 \qquad (1)$$

where the summation is taken over all the different ions i in the sap, m = molality (taken as approximately equal to molarity in kmol m^{-3}) and z = electrical charge of each ion. (The value of I was calculated from unpublished data on the complete cation and anion content of the sap extracts obtained by Dr. M. Popp. A rough allowance was made for the proportion of the organic-acid anions likely to have been complexed with Ca^{2+} and Mg^{2+}.)

To facilitate the identification of the mid-points in the titration curves, sap samples were titrated

before and after the addition of various amounts of pure L-malic acid. The empirically determined pK values for malic acid at 20°C were as follows (mean ± SD, number of experiments):

$pK_1 = 3.18 \pm 0.097$ (5)
$pK_2 = 4.25 \pm 0.076$ (6).

Using these pK values, Fig. 1 shows the dissociation curves for malic acid (calculated according to the Henderson-Hasselbalch equation) in the pH range relevant for the discussion of CAM in *K. daigremontiana*. Although the pK values will again be slightly different under conditions in the cytoplasm, at the anticipated cytoplasmic pH of ≥7.5, mal^{2-} is the only species present in significant amounts. Considerable day-night changes must occur in the vacuoles, whose pH varies between about 6.0 at the end of the light period and 3.4 at the end of the dark period.

To assess the extent of these changes in malic-acid dissociation during the CAM rhythm, leaves of *K. daigremontiana* were harvested at different times of day. Malate levels in the sap gave mal-Σ, which is plotted *versus* the pH of the sap in Fig. 2*A*. The curve was fitted to the values (closed circles) by eye. Known amounts of L-malic acid were also added to the sap and the total malate concentration plotted *versus* the pH obtained after addition (open circles). A similar correlation between malate concentration and pH was observed.

From the curve in Fig. 2*A* and the relationships in Fig. 1, the concentrations of the various ionic species at different vacuolar pH values can be calculated (Fig. 2*B*). At the end of the light period (pH ~ 6.0) mal^{2-} is the dominant species; $Hmal^{1-}$ increases throughout the dark period (pH 6.0 to 3.4), and H_2mal^0 becomes increasingly important towards the end.

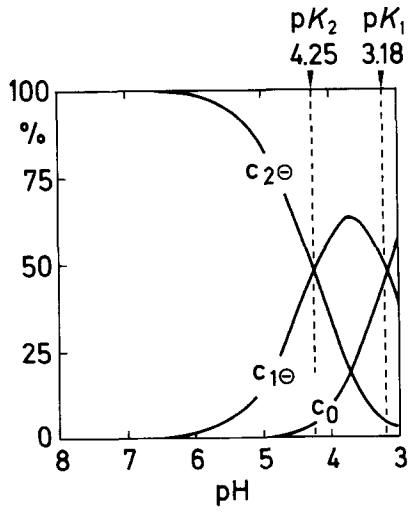

Fig. 1. Dissociation equilibria of malic acid based on the pK values determined in the leaf-cell sap of *K. daigremontiana*

Relations between Malic-Acid Efflux and Vacuolar Concentrations of mal^{2-}, $Hmal^{1-}$ and H_2mal^0

Efflux of malic acid from the leaf tissue of *K. daigremontiana* was studied previously using 2-mm-wide leaf slices submerged in 0.1 mol m^{-3} $CaSO_4$ solution (Lüttge & Ball, 1977). Since malic acid is concentrated in the vacuoles and cytosolic concentrations are negligible (*see* Introduction), the tissue efflux must correspond to efflux from the vacuoles. In the leaf-slice experiments, however, malic acid must cross two membranes on leaving the vacuole (tonoplast and plasmalemma) and must then diffuse through the "extracellular space" before reaching the bulk medium. Hence, the rate of malic-acid efflux from the leaf slices will be an underestimate of the actual flux across the cell membranes because of the diffusion barrier presented by the "extracellular space." The magnitude of this diffusion limitation will be determined by the dimensions of the leaf slices, a tortuosity factor given by the tissue geometry, the diffusion coefficient for malic acid in the cell walls, and the unstirred Nernst diffusion layers at the surface of the slices.

To assess the extent of the extracellular diffusion limitation on malic-acid efflux, experiments were conducted with leaf slices of various sizes. The approach was based on that of Kohn and Dainty (1966). We assumed that the cuticles of the upper and lower epidermis are impermeable to the malic acid and that efflux occurs solely *via* the cut edges of the slices. From the analysis of Philip (1958*a*,*b*) and Kohn and Dainty (1966) it can be predicted that, if extracellular solute diffusion is the rate-limiting process, there should be a direct dependence of the rate of malic-acid efflux on the square of the leaf-slice dimensions (here considered as length × width). The results presented in Fig. 3 show that such a relationship exists and allow a tentative extrapolation to the rates that would be observed in the absence of the extracellular diffusion limitation. This computed efflux is a factor of 1.8 ± 0.29 (mean ± SD, 5 experiments) higher than the rate observed with 2-mm-wide slices. With the corrections provided by this factor, efflux from the

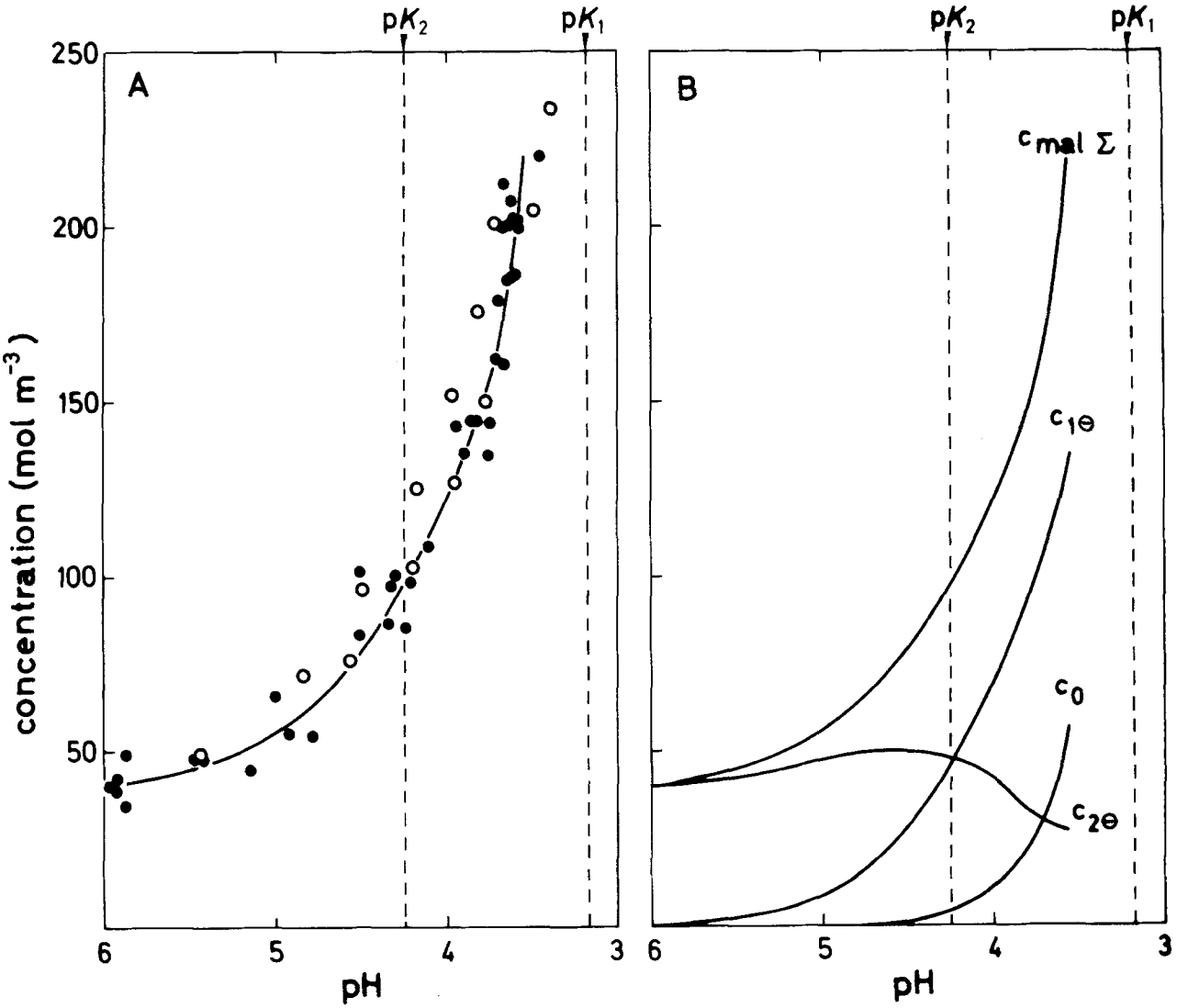

Fig. 2. *A*. Relationship between total malate concentration (mal-Σ) and pH of the leaf-cell sap without (closed circles) and with (open circles) added L-malic acid. *B*. Contributions of undissociated malic acid and malate anions to mal-Σ (curve replotted from *A*) at the observed pH values

slices is taken to represent vacuolar malic-acid efflux.

We can now consider quantitatively the relationship between malic-acid efflux from the leaf slices and the vacuolar content of the three species H_2mal^0, $Hmal^{1-}$ and mal^{2-}. Lüttge and Ball (1977) observed that the initial rates of malic-acid efflux from 2-mm-wide leaf slices increased exponentially with increasing malate concentrations (mal-Σ) in the vacuoles. This curve is shown as the heavy line in Fig. 4 (J_Σ), with the rates scaled up by the factor 1.8 to correct for the extracellular diffusion limitation. Such a concentration-dependence would not be expected for a passive transport process. However, these data can now be further analyzed using the results from Fig. 2*B* to reveal the relationship between malic-acid efflux and the vacuolar concentrations of the three species H_2mal^0, $Hmal^{1-}$ and mal^{2-}.

The three additional curves shown in Fig. 4 and denoted J_0, $J_{1\ominus}$ and $J_{2\ominus}$ represent the predicted dependence of efflux on the vacoular concentration of the individual species (c_0, $c_{1\ominus}$ and $c_{2\ominus}$) based on the assumption that malate can only cross the membrane in the one form (namely H_2mal^0, $Hmal^{1-}$ or mal^{2-}, respectively). It can now be seen that there is a linear relationship between malic-acid efflux and the concentration of H_2mal^0 in the tissue. This would be consistent with malic-acid efflux occurring (over the relevant range of mal-Σ concentra-

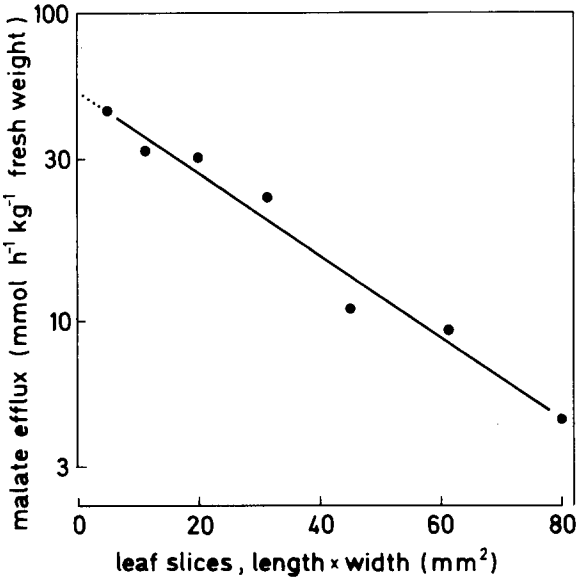

Fig. 3. Dependence of malic-acid efflux in 60 min from leaf slices (measured as malate appearing in the external medium) on the dimensions of the slices. About 1.0 g of leaf slices were incubated in 20×10^{-6} m^3 of 0.1 mol m^{-3} $CaSO_4$ solution (15°C) and were shaken gently during the experiment. The shape of the slices was kept constant at a length/width ratio of 5 : 1; the slices were approximately 2 mm thick. The results are from one experiment representative of five

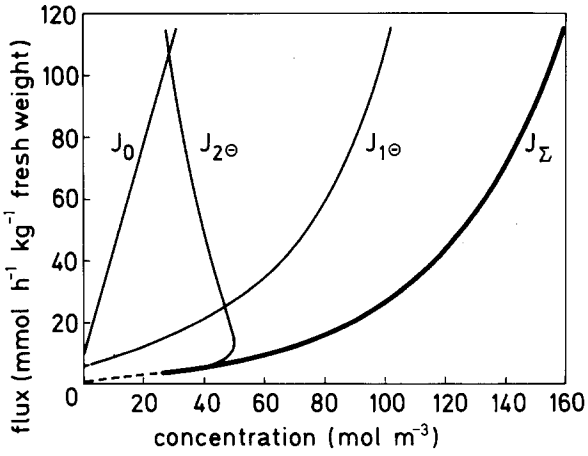

Fig. 4. Malate efflux from 2-mm-wide leaf slices of *K. daigremontiana*. J_Σ gives the experimental curve of the dependence of the initial rate of malate efflux to vacuolar malate concentration (c_Σ) of Lüttge and Ball (1977: Fig. 3) corrected for extracellular diffusion limitation. J_0, $J_{1\ominus}$ and $J_{2\ominus}$ give the rates of efflux plotted versus c_0, $c_{1\ominus}$ and $c_{2\ominus}$, respectively

tions) in the form of passive, noncatalyzed diffusion of H_2mal^0. The curves denoted $J_{1\ominus}$ and $J_{2\ominus}$ are not consistent with diffusion of $Hmal^{1-}$ and mal^{2-}, respectively, as the sole species. We note that if the normally quoted pK values for malic acid (at $I \rightarrow 0$) are used for the computation of c_0, $c_{1\ominus}$ and $c_{2\ominus}$ then the relationship between J_0 and c_0 is not linear!

Closer inspection of Fig. 4 reveals several important features. First, at mal-Σ concentrations below about 65 mol m^{-3}, H_2mal^0 is not present in significant amounts. The J_0 curve has an intercept at $c_0 = 0$, showing that there is still some efflux which cannot be due to H_2mal^0 and therefore must be in the form of $Hmal^{1-}$ and/or mal^{2-}. Similarly, when $c_\Sigma < 40$ mol m^{-3}, c_0 and $c_{1\ominus}$ are zero, so that the $J_{1\ominus}$ curve also has an intercept at $c_{1\ominus} = 0$. Hence, some efflux must in fact occur in the form of mal^{2-}, i.e. at $c_\Sigma < 40$ mol m^{-3}, c_0 and $c_{1\ominus} = 0$ and $J_\Sigma = J_{2\ominus}$.

Second, at the lower values of c_Σ the relationships between $J_{2\ominus}$ and $c_{2\ominus}$ and between $J_{1\ominus}$ and $c_{1\ominus}$ are approximately linear. The former can be described in the c_Σ range of 25 to 40 mol m^{-3} (where $c_\Sigma = c_{2\ominus}$) by the equation

$$J_{2\ominus} = 0.142 \cdot c_{2\ominus}. \quad (2)$$

Correcting for $J_{2\ominus}$, we can then describe $J_{1\ominus}$ in the c_Σ range of 40 to 65 mol m^{-3} by the equation

$$J_{1\ominus} = 0.333 \cdot c_{1\ominus}. \quad (3)$$

Equations (2) and (3) would be consistent with passive transport of mal^{2-} and $Hmal^{1-}$ at the lower values of c_Σ. In fact, Eqs. (2) and (3) may hold over the whole range of c_Σ values, with J_0 becoming the dominant flux at higher values of c_Σ. At $c_\Sigma = 160$ mol m^{-3}, for example, the ratio of the fluxes would then be $J_0 : J_{1\ominus} : J_{2\ominus} = 0.66 : 0.30 : 0.04$.

Third, if Eqs. (2) and (3) are valid for all values of c_Σ it is possible to compute the relationship between J_0 and c_0 by correcting J_Σ for the contributions from $J_{2\ominus}$ and $J_{1\ominus}$. The plot of log J_0 against log c_0 is slightly nonlinear with the slope increasing at the lowest values of log c_0 (*data not shown*). At the highest value of c_0 (at $c_\Sigma = 160$ mol m^{-3}), where J_0 is the dominant flux, the slope of the log/log plot is 1.16. Within experimental limits this is in good agreement with the slope of 1.00 expected for passive diffusion. It may be that the contributions of $J_{2\ominus}$ and $J_{1\ominus}$ to J_Σ are not accurately predicted by Eqs. (2) and (3) for all values of c_Σ.

Calculation of Permeability Coefficients from the Rates of Malic-Acid Mobilization *in vivo* and Efflux from Leaf Slices

On the basis of the arguments presented above, the mechanism proposed to account for malic-acid efflux out of the vacuole can be summarized as fol-

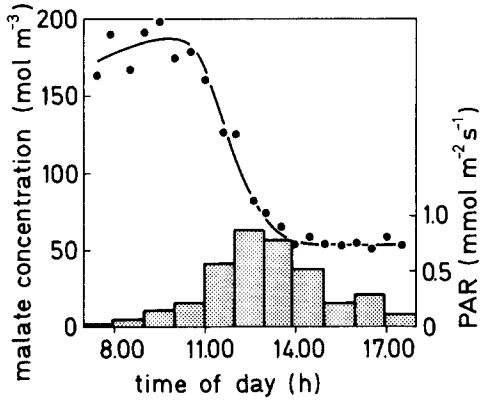

Fig. 5. Deacidification of leaves of intact *K. daigremontiana* plants observed during a typical light period (circles) under glasshouse conditions. The points are averages for five plants (one leaf sampled per plant). PAR is indicated by the histogram averaged over intervals of 60 min; the total daily PAR was 14.8 mol m^{-2}, which was similar to the values for the days immediately preceding the experiment

lows. At the lower values of c_Σ (<65 mol m^{-3}) J_Σ is accounted for by $J_{2\ominus}$ and $J_{1\ominus}$; at the higher values of c_Σ (>65 mol m^{-3}) J_0 becomes the dominant flux. From the concentration-flux relationships derived above, it is possible to calculate the membrane permeability coefficients (P) that are required of each species to be consistent with the observed efflux rates.

Permeability coefficients for electrically neutral solutes are given by

$$P = J/\Delta c, \tag{4}$$

where Δc is the concentration difference across the membrane. Permeability coefficients for electrically charged solutes can be obtained from

$$P = \frac{J}{c} \cdot \frac{RT}{zF\Delta\psi} \cdot \frac{1 - e^{zF\Delta\psi/RT}}{e^{zF\Delta\psi/RT}}, \tag{5}$$

where R is the gas constant, T the Kelvin temperature, F the Faraday, z the electrical charge and $\Delta\psi$ the electrical potential across the membrane. Flux rates and concentrations can be obtained from efflux experiments with leaf slices or from observations on the rate of malic-acid mobilization during the light period *in vivo* (*see below*). For conversion of the units mmol hr^{-1} kg^{-1} fresh weight of tissue to a membrane flux in mol m^{-2} sec^{-1}, the total mesophyll-cell surface area was taken as 100 m^2 kg^{-1} fresh weight (Schnellbächer, 1982), i.e., 10^5 m^2 m^{-3} or 10^5 m^{-1}. As discussed in the Introduction, cytoplasmic mal-Σ concentrations will be very small, and mal^{2-} is the only species occurring at cytoplasmic pH values. Thus cytoplasmic concentrations will be taken as zero. The transtonoplast electrical potential in *K. daigremontiana* leaves was measured to be +25 mV, vacuolar phase positive (Rona et al., 1980).

Because the rates of malic-acid efflux observed from leaf slices in solution are measured under unnatural conditions, we must consider whether they are representative of the fluxes across the tonoplast in intact leaves. Figure 5 shows the time-course of the decrease in malic-acid levels in leaves of *K. daigremontiana* during a typical light period. Rates of malic-acid efflux from the vacuoles can be obtained by differentiation of this curve assuming that the curve depicts the instantaneous flux of malic acid out of the vacuole into the cytoplasm. This neglects unstirred-layer effects at the tonoplast which should be small, however, in view of the cytoplasmic membrane dynamics and the very short diffusion pathways. Re-uptake must also be small as malate is decarboxylated in the cytoplasm in the light. However, in intact leaves efflux also depends on factors other than the thermodynamic parameters mentioned above, e.g. on PAR (Fig. 5). Thus only the maximum rate of J_Σ = 56 mmol hr^{-1} kg^{-1} fresh weight, observed at c_Σ = 110 mol m^{-3}, will be used for calculations; here the limitations imposed by other metabolic processes, e.g. photosynthesis, should be minimal. This value of J_Σ is of the same order as that given in Fig. 4 for the leaf-slice experiments for c_Σ = 110 mol m^{-3}, *viz.* 34 mmol hr^{-1} kg^{-1} fresh weight. With the leaf slices, though, the malic acid must cross two membranes (tonoplast and plasmalemma) to reach the external medium. Interpretation of the efflux data in terms of transport at the tonoplast is thus more difficult. In any case, the observed fluxes clearly represent the minimum values for the efflux at the tonoplast.

At c_Σ = 110 mol m^{-3} the contribution of $J_{2\ominus}$ to J_Σ is likely to be small (*see above* and Fig. 4), so for malic-acid efflux from the vacuoles *in vivo* values of P were only calculated for H_2mal^0 and $Hmal^{1-}$. These are presented in Table 1 calculated according to the assumption that either $J_\Sigma = J_0$ or $J_\Sigma = J_{1\ominus}$. Table 1 also presents values of P calculated from the leaf-slice data of Fig. 4. Although the malic acid must cross two membranes in series, the assumption was made that the efflux rates are determined solely by diffusion at the tonoplast. The values used in the computations were corrected for $J_{1\ominus}$ and $J_{2\ominus}$ at $c_0 = 0$ and for $J_{2\ominus}$ at $c_{1\ominus} = 0$. For $Hmal^{1-}$, P would be apparently concentration-dependent because $J_{1\theta}$ in Fig. 4 is nonlinear. There is reasonable agreement

(to within an order of magnitude) between the P values calculated from the two different experiments. The values derived from the leaf-slice experiment are presumably lower because of the assumption that the tonoplast alone determines the rate of efflux.

Inherent in the calculation of the values for Table 1 is the assumption that efflux of each species considered is by simple, noncatalyzed diffusion through the lipid phase of the tonoplast. Treating each species in turn as the only solute to which the tonoplast is permeable is moreover an extreme condition. We will now consider whether P values of the order given in Table 1 are likely for the tonoplast. In particular, is $P_{H_2mal^0}$ of the tonoplast likely to be high enough to allow for simple diffusion of H_2mal^0 as the main component of J_Σ at high values of c_Σ?

Estimated Values for the Passive Permeability of the Tonoplast to mal^{2-}, $Hmal^{1-}$ and H_2mal^0

The values of P considered in this section refer to simple diffusion of the solutes across membranes by a 'lipid-solution' mechanism, i.e., in the absence of carriers that might catalyze the transmembrane flux. Relevant data are available from studies on lipid bilayers and a number of plant cell membranes.

Investigations of the passive permeability of lipid bilayers to the four-carbon maleic acid have indicated a value of P for the monovalent maleate anion of 4×10^{-11} m sec^{-1} (Prestegard, Cramer & Viscio, 1979). These authors point out that this value may be higher than the P of lipid bilayers to other anions (*see also* Stein, 1967). For the plasmalemma of *Spinacia oleracea* mesophyll-cell protoplasts, $P_{NO_3^-}$ has been determined as 5×10^{-10} m sec^{-1}, though this is said to represent an upper limit (Kaiser & Heber, 1983, and *see* Day, Jenkins & Hatch, 1981). The exact value for a particular species will depend upon factors such as the degree of charge delocalization on the anion. Nevertheless, comparison with the values in Table 1 suggests that the likely values for $P_{mal^{2-}}$ and $P_{Hmal^{1-}}$ would be at least one, and possibly two or more, orders of magnitude too small to account for malic-acid transport across the tonoplast by a lipid-solution mechanism for mal^{2-} and $Hmal^{1-}$. In fact, there is evidence from work with isolated vacuoles of *K. daigremontiana* for the existence of a carrier catalyzing the passive exchange of malate anions across the tonoplast (Buser-Suter, Wiemken & Matile, 1982). We assume therefore that "facilitated" diffusion is the principal mechanism by which mal^{2-} and $Hmal^{1-}$ cross the tonoplast.

Table 1. Permeability coefficients (P) calculated assuming passive and noncatalyzed diffusion of H_2mal^0, $Hmal^{1-}$ or mal^{2-} [a]

Transported species	Concentration [mol m^{-3}]	P [$\times 10^8$ m sec^{-1}]
Deacidification *in vivo* (*ex.* Fig. 5)		
H_2mal^0	7	2.22
$Hmal^{1-}$	59	0.45
Efflux experiments (*ex.* Fig. 4)		
H_2mal^0	up to 30	0.94
$Hmal^{1-}$	2.5 to 102	0.13–0.51
mal^{2-}	25 to 40	0.12

[a] The values were calculated assuming that the tonoplast was permeable only to the species considered.

A much more detailed literature exists on the permeability of lipid bilayers and plant cell membranes to organic acids. Collander has studied the passive permeation of a wide range of solutes into the giant cells of the algae *Chara ceratophylla* (Collander & Bärlund, 1933) and *Nitella mucronata* (Collander, 1954). For both species there is a good correlation between $P \cdot M^{1.5}$ (where M = molecular weight) and the partition coefficient K for the particular solute between organic solvents and water (*see also* Stein, 1967). (This general relation also holds for the permeation of neutral solutes across rabbit gallbladder epithelium and is discussed further by Diamond and Wright (1969).) From these correlations it is in principle possible to estimate $P_{H_2mal^0}$ from the relevant value of K. From the diethyl ether-water partition coefficient for L-malic acid of 0.014 (taken as the mean of the values given by Dermer and Dermer (1943) and Collander (1950)), averaging the estimates derived from the *C. ceratophylla* and *N. mucronata* data gives $P_{H_2mal^0} \sim 4 \times 10^{-8}$ m sec^{-1}.

The permeability required for a lipid-solution mechanism of H_2mal^0 transport at rates compatible with the time-course of deacidification is $P_{H_2mal^0} = 2.2 \times 10^{-8}$ m sec^{-1} (Table 1). Due to the possible underestimation of vacuolar efflux this is a minimum value and thus compares well with the value of $P_{H_2mal^0} \simeq 4 \times 10^{-8}$ m sec^{-1} obtained from the Collander plots. But some indication of the probable accuracy of the latter is needed. Although such studies have not been made with malic acid, investigations have been carried out on the permeability of lipid bilayers to two other four-carbon organic acids, namely *n*-butyric acid (Orbach & Finkelstein, 1980; Walter, Hastings & Gutknecht, 1982) and maleic acid (Prestegard et al., 1979). A comparison of

Table 2. Estimated and measured values of the membrane permeability coefficients (P) of *n*-butyric acid, maleic acid and L-malic acid

	n-butyric acid $CH_3(CH_2)_2COOH$	Maleic acid $COOH(CH)_2COOH$	L-malic acid $COOH \cdot CH_2 \cdot CHOH \cdot COOH$
P (m sec^{-1}), from:			
1. Collander plots[a]	7×10^{-4} [b]	4×10^{-7} [c]	4×10^{-8} [c]
2. Lipid-bilayer studies	$6.4–9.5 \times 10^{-4}$ [d]	4×10^{-7} [e]	—

[a] Estimates are averages of the values derived from the relationship for *Chara ceratophylla* (Collander & Bärlund, 1933) and *Nitella mucronata* (Collander, 1954).
[b] Based on K_{ether} (Collander, 1950).
[c] Based on K_{ether} averaged from Dermer and Dermer (1943) and Collander (1950).
[d] Lower value: Orbach and Finkelstein (1980); higher value: Walter, Hastings and Gutknecht (1982).
[e] Prestegard et al. (1979).

these values with those (estimated as above for malic acid) from the Collander plots is presented in Table 2. For both *n*-butyric acid and maleic acid there is good agreement with the measured values for lipid bilayers. (The estimated value for *n*-butyric acid is based on a nonlinear extrapolation of the relationship between $P \cdot M^{1.5}$ and K_{ether} (Fig. 2 of Collander, 1954). (The nonlinearity for values of $K_{ether} > 1$ is presumably the result of unstirred-layer effects (Orbach & Finkelstein, 1980; Walter et al., 1982).) This agreement suggests that the estimated value of $P_{H_2mal^0}$ can be viewed with some confidence, and we suggest it is accurate to within a factor of five (*cf.* Stein, 1967). Stein (1981) emphasizes that interpretation for different solutes on the basis of their K values rests on the assumption that the solvent is a good model of the permeability barrier of the membrane (*see also* Leo, Hansch & Elkins, 1971). This seems to be the case for ether (Orbach & Finkelstein, 1980; Stein, 1981).

The estimated value of $P_{H_2mal^0}$ given in Table 2 is thus quantitatively consistent with passive diffusion of H_2mal^0 by a lipid-solution mechanism as the principal component of malic-acid efflux out of the vacuole at high values of c_Σ. This would result in the transfer of H^+ out of the vacuole by a "protonophore"-like mechanism (*cf.* McLaughlin & Dilger, 1980) in amounts equivalent to those proposed to be pumped by the ATPase in the accumulation phase (Lüttge et al., 1982; Smith et al., 1984*a*). For completeness, we must also consider the possibility of efflux of free H^+ out of the vacuole. Molecular "slippage" of the H^+-ATPase would amount to, at the very most, about 15% of the maximum catalytic activity of the pump (Rottenberg, 1979; Raven & Beardall, 1982). However, in view of the large pH differences across the tonoplast, the passive, noncatalyzed diffusion of free H^+ might be of importance. For the maximum rate of deacidification observed at $c_\Sigma = 110$ mol m^{-3} (Fig. 5), the rate of H^+ transport across the tonoplast must be 312 nmol m^{-2} sec^{-1}. Taking vacuolar pH to be 4.1 (from Fig. 2) and cytoplasmic pH as between 7.5 and 8.0 (Lüttge et al., 1982), the required value of P_{H^+} would be 4×10^{-6} m sec^{-1}. Although the measured values for the apparent P_{H^+} of lipid-bilayer membranes range over about five orders of magnitude (*see* Deamer & Nichols, 1983), the highest P_{H^+} observed is $\sim 10^{-6}$ m sec^{-1}, and the true P_{H^+} may be $\ll 10^{-6}$ m sec^{-1} (Gutknecht & Walter, 1981; Nozaki & Tanford, 1981; Deamer & Nichols, 1983; Elamrani & Blume, 1983). We thus reject the concept of passive, noncatalyzed diffusion of H^+ as a quantitatively important contributor to J_Σ.

Conclusions

The main conclusion from the above analysis is that, at high tissue malic-acid contents, efflux of malic acid from the vacuole occurs predominantly in the form of the undissociated acid, H_2mal^0. The efflux rates appear to be quantitatively consistent with passive, noncatalyzed diffusion of H_2mal^0 across the tonoplast by a 'lipid-solution' mechanism. At low malic-acid contents ($c_\Sigma < 65$ mol m^{-3}), when H_2mal^0 is not present in the vacuole in significant amounts, efflux is in the form of $Hmal^{1-}$ and/or mal^{2-}. These anionic species also cross the tonoplast passively, but their transport is presumed to be carrier-mediated (*cf.* Buser-Suter et al., 1982).

In the light period of the CAM rhythm, the rate of malic-acid decarboxylation will only be partly determined by the effective permeability of the tonoplast to the various species of malic acid. The process will ultimately be rate-limited by the strength of the metabolic 'sink' in the cytoplasm, which in turn depends on the rate of C_3 photosynthesis (*cf.* Fig. 5). Although the exact value of $P_{H_2mal^0}$ is not known, it is clear that the back-flux of H_2mal^0 into

the cytoplasm constitutes a 'leak' against which the proton pump at the tonoplast must work during the dark period to bring about malic-acid accumulation. We do not know how quantitatively significant this back-flux is at the lower values of c_Σ. However, the flux-concentration relationship for H_2mal^0 at the higher values of c_Σ suggests that the back-flux may become so important that it limits the extent of malic-acid accumulation in the dark period. Thus, in addition to thermodynamic restrictions on the activity of the proton pump (Lüttge et al., 1981; Smith, Marigo & Lüttge, 1982), $P_{H_2mal^0}$ may also determine the maximum attainable values of c_Σ. Clarification of the characteristics of the efflux processes at the tonoplast will be an important aim of future studies with isolated vacuoles from CAM plants.

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