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Electrochemical Data on Compartmentation into Cell Wall, Cytoplasm, and Vacuole of Leaf Cells in the CAM Genus *Kalanchoë*

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Summary. Transcellular electrical profiles of *Kalanchoë* leaf cells were obtained by pushing a glass micro-saltbridge through cells with the tip consecutively in the cell wall, cytoplasm, and vacuole. The electrical resistance of the cell wall was too small to be detectable, that of the plasmalemma and tonoplast was about 0.18–0.21 and 0.16–0.18 Ωm^2, respectively. The electrical potential difference between the cytoplasm and the external medium, ψ_{co}, was ~ -180 mV, the potential difference between the vacuole and the medium, ψ_{vo}, was ~ -155 mV, and thus the mean potential difference at the tonoplast, ψ_{vc}, was about $+25$ mV. Potential difference, ψ_{vo}, was independent of proton concentration in the external medium between pH 9 and 5.5, and behaved like an H^+-electrode between pH 5 and 3. Depolarizations and hyperpolarizations of ψ_{vo} obtained by increasing and decreasing, respectively, the Na^+-concentrations in the medium were smaller than with changing K^+-concentrations, suggesting that permeabilities are $P_{Na^+}/P_{K^+} \simeq 0.23$. Assessment of K^+-compartmentation by flux analysis gave K^+-concentrations in the cytoplasm including chloroplasts (c_c) and vacuole (c_v) as c_c between 200 and 400 mmol kg^{-1} FrWt and $c_v \simeq 15$ mmol kg^{-1} FrWt. The Nernst criterion suggests that metabolically regulated K^+ transport out of the vacuoles concentrates K^+ in the cytoplasm. Fusicoccin (10^{-5} M) hyperpolarized ψ_{co} by about 100 mV and depolarized the positive ψ_{vc} by about 10 mV, the latter presumably being an insignificant effect. The evidence for the existence of proton pumps exchanging H^+ and K^+ at the plasmalemma and at the tonoplast is discussed.

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The most outstanding feature of crassulacean acid metabolism is the occurrence of large diurnal oscillations of malic acid levels in the leaf cells. Under experimental conditions the amplitude of these oscillations in *Kalanchoë* plants can amount to up to 180 mmol malic acid per kg FrWt for the 12-hr period between the end of the light phase (malic acid level: 20–40 mmol kg^{-1} FrWt) and the end of the dark phase (200–220 mmol kg^{-1}). Feedback inhibition of the malate producing system, whose major component is phosphoenol pyruvate carboxylase, by the product malate (*cf.* Kluge & Ting, 1978) and cytoplasmic pH-control (Lüttge, 1979) are important factors in regulation of these oscillations. Energy-dependent transport of malic acid from the cytoplasm into the vacuole clearly is involved. It has been suggested that a proton pump at the tonoplast directed towards the vacuole is driving this process (Lüttge & Ball, 1979).

Levels of inorganic ions, e.g., K^+, Na^+, Cl^-, in extracts of CAM leaves do not oscillate during the diurnal CAM rhythm (Lüttge & Ball, 1974). It is possible, however, that compartmentation of inorganic ions in CAM leaf cells participates in regulation of malic acid oscillations. In particular K^+ and Mg^{2+} are cations whose concentration in the cytoplasm and near the tonoplast could considerably influence enzymic reactions synthesizing and breaking down malate and a putative H^+-pumping ATPase at the tonoplast, respectively. The surprisingly low K^+ content of *Kalanchoë* leaves (below 20 mmol kg^{-1} FrWt) has long been a puzzling observation (Lüttge & Ball, 1974, 1979; Lüttge, Ball & Tromballa, 1975), and it has been suggested that a mechanism must be operative concentrating K^+ in the cytoplasm (Lüttge, 1979). Otherwise cytoplasmic reaction systems would be hampered by K^+ deficiency (WynJones, Brady & Spears, 1979).

Among other parameters, electrical characteristics

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of the tonoplast and plasmalemma (i.e., potential differences and resistances, responses to ion gradients such as those of H^+, K^+, Na^+), intracellular compartmentation of K^+, and K^+ fluxes at the tonoplast and plasmalemma need to be known to evaluate these possibilities. Such data are provided in the present report.

Materials and Methods

Plants

Plants of *Kalanchoë daigremontiana* Hamet et Perrier and *Kalanchoë tubiflora* (Harv.) Hamet were grown in soil culture in a glass house. Ion contents in the leaves were 10–20 mM K^+, 1–15 mM Na^+, and 10–20 mM Cl^- (Lüttge & Ball, 1979, and new analyses in the present study).

Electrical Measurements

For measurements of electrical membrane parameters several kinds of preparations were used. In experiments with material designated "intact leaves" in Table 1, whole leaves were cut from the plants and glass micro-electrodes were inserted into cells without further slicing. In the experiments of Tables 1 and 4 and Fig. 1, leaf slices were 2 × 5 mm large pieces of tissue with the cuticles removed on both surfaces. These small tissue blocks were used either shortly (i.e., 5–20 min) after preparation or after a washing period of 60–90 min at 25 °C in the light. Leaf slices of the experiments shown in Figs. 3–5 were 5 × 10 mm large with the cuticles left in place. Young plantlets in Table 1 were adventitious plantlets that had emerged from mature leaves. The second about 5 mm long leaves of 4 to 6 months old plantlets were used without slicing.

All preparations were kept in experimental solutions whose composition is indicated in the experiments of Figs. 3–5 in the legends. In the experiments of Fig. 1 and Table 1 the external medium contained 1 mM K^+, 1 mM Na^+, 0.25 mM Ca^{2+}, 0.25 mM Mg^{2+}, all as Cl^- salts. In Table 4 the solution was distilled water because fusicoccin was dissolved in distilled water, but similar results could be obtained with a salt solution as indicated above. Replacing this salt solution by distilled water caused a hyperpolarization of only −15 to −20 mV as compared to a hyperpolarization of −100 mV due to fusicoccin.

For measurements of electrical membrane potentials glass micro-saltbridges (micro-electrodes) filled with 3 M KCl and connected via Ag/AgCl contacts to an electrometer and recording devices (oscilloscope, line recorder) were applied in the usual way (Lüttge & Ball, 1979; Rona, 1973; Rona et al., 1980). The reference saltbridge was in the external solution excepting experiments with intact leaves. In the latter case the reference electrode was in contact with an agar bridge (8% agar-agar, plus 1 mM KCl, 1 mM NaCl, 0.25 mM $CaCl_2$ and 0.25 mM $MgCl_2$) touching the leaf surface. For the recordings of transcellular electrical profiles the micro-electrode was pushed slowly through the cell wall, cytoplasm, and vacuole and out of the cell through the cytoplasm and cell wall at the site opposite to electrode entry (Fig. 1) with the aid of an automatic micromanipulator (model MM6 of Oskar Uhl, Asslar-Wetzlar, W-Germany; Rona, Cornel & Heller, 1977). The electrode also could be kept for up to 5 min in the cytoplasm. In other cases the electrode was inserted directly into the vacuole. In this way potentials were obtained which often were stable for many hours and the effects of various parameters could be investigated (i.e., Figs. 3–5). Measurements of electrical resistance were made with the "one-electrode" technique (Brenneke & Lindemann, 1971; Anderson, Henderson & Higinbotham, 1974; Rona & Cornel, 1979; Rona, 1979) since it was extremely difficult to insert more than one electrode into the *Kalanchoë* leaf cells. A small constant current (4 nA, square signal of a frequency of 10 kHz) was injected into the cells via the same glass micro-electrode serving for measurement of membrane potentials. The difficulties of this method have been discussed by Etherton, Keifer and Spanswick (1977). Results obtained here are similar to those found in the literature for other plant materials (Walker, 1960; Greenham, 1966; Spanswick, 1970, 1972; Anderson et al., 1974; Rona & Cornel, 1979). Furthermore, for purposes of the present investigation, the relative resistances were more important than the correct absolute values. Resistance measurements were made under the same conditions as measurements of membrane potentials; often they were made simultaneously. The increase in resistance as the electrode passed from the cell wall into the cytoplasm and then into the vacuole and the decrease as the electrode was pushed out of the cell was an important control for identification of compart-

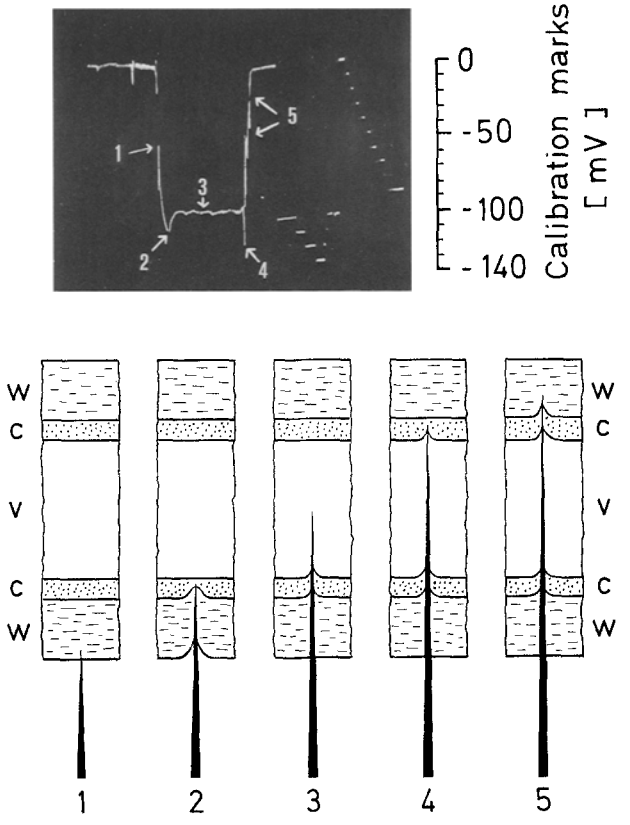


Fig. 1. Example of a transcellular electrical profile. This profile was obtained by the electrode penetrating a cell in a freshly cut leaf slice (i.e., 5 min after preparation) of *K. daigremontiana*, where absolute values of electrical potentials are comparatively low (*see* Table 1): ψ_{wo} (*1,5*) is −40 to −48 mV; ψ_{co} (*2,4*) is −110 to −122 mV; ψ_{vo} (*3*) is −90 to −100 mV. The first ψ_{co}-value (*2*) is smaller than the second one (*4*) and is an underestimation because the electrode entering the cell has to be pushed against the mechanic resistance of the cell wall and thus passes too rapidly through the cytoplasm into the vacuole. This problem does not occur upon exit of the electrode from the vacuole into the cytoplasm so that the second value (*4*) is more reliable (Rona et al.; 1977)

ments during measurements of transcellular electrical profiles in addition to microscopic observation of the localization of the tip of the electrode (Rona et al., 1980).

Potassium Flux Analysis

Analysis of samples of 2-mm wide leaf slices suspended in 5 mM KCl plus 0.1 mM $CaSO_4$ showed that there was very little change in K^+ content of the tissue over a period of about 36 hr. The rate of net K^+ uptake was small (*see* Fig. 2) and for the purpose of calculation of fluxes was taken to be zero. In this case the fluxes at the plasmalemma (ϕ_p) and tonoplast (ϕ_t) can be determined as follows (Walker & Pitman, 1976):

a) Tissue was put in labeled solution for 16 hr and then transferred to unlabeled solution of otherwise identical composition to measure the rate of tracer loss from the tissue. The labeling solution was 5 mM KCl + 0.1 mM $CaSO_4$, and ^{86}Rb was used as a tracer for K^+ at a specific activity of approximately 0.5 mCi/mmol K^+. The substitution of ^{86}Rb for K was checked by comparison of ^{42}K and ^{86}Rb uptakes from the same solution (Fig. 8).

b) When uptake and elution are at the same temperature, fluxes can be calculated from the analysis of the efflux curve (as in Fig. 6) into exponential components. The basis for these calculations is given in Walker and Pitman (1976). During uptake over a period t' the amount of tracer in the tissue will rise to

$$Q'_T = A(1-e^{-k_1 t'}) + B(1-e^{-k_2 t'}).$$

During elution to unlabeled solution Q'_T will fall as

$$Q'_T = A(1-e^{-k_1 t'})e^{-k_1 t} + B(1-e^{-k_2 t'})e^{-k_2 t}.$$

Analysis of the rate of loss of tracer from the tissue and the decrease in Q'_T can be used to estimate the rate constants k_1, k_2; the amounts $Q'_c = A(1-e^{-k_1 t'})$ and $Q'_v = B(1-e^{-k_2 t'})$; the rate of tracer loss at $t=0$ (Φ_{co}) and the rate of tracer loss in the slow component extrapolated to $t=0$ (Φ_{vo}). In this case, A and B can be calculated from Q'_c and Q'_v as t' is known, and ϕ_p and Q_T calculated since

$$A = (\phi_p - k_2 Q_T)/(k_1 - k_2)$$

$$B = (k_1 Q_T - \phi_p)/(k_1 - k_2).$$

This calculated value of Q_T can be compared with Q_T from chemical analysis to give a check on the method. The cytoplasmic content Q_c can be estimated from the relationship that $(k_1 + k_2) = \phi_p/Q_c + Q_T k_1 k_2/\phi_p$. The flux ϕ_t can be calculated since

$$\phi_p/\phi_t = \frac{\Phi_{co} - \Phi_{vo}}{\Phi_{vo}} \cdot \underline{S}_v \text{ (see below)}$$

and

$\underline{S}_v$ is $Q'_v/Q_v = Q'_v/(Q_T - Q_c)$.

c) When uptake is at a different temperature from efflux this simple (and exact) calculation is not valid. However, during the efflux we can write

$$\Phi_{co} = \phi_p \cdot \underline{S}_c = \frac{\phi_p(\phi_p + \phi_t \cdot \underline{S}_v)}{\phi_p + \phi_t}$$

where $\underline{S}_c$ is the specific activity in the cytoplasm at the end of the uptake and

$$\Phi_{vo} = \phi_p \cdot \underline{S}_{ce} = \phi_p \frac{(\phi_t \cdot \underline{S}_v)}{\phi_p + \phi_t}$$

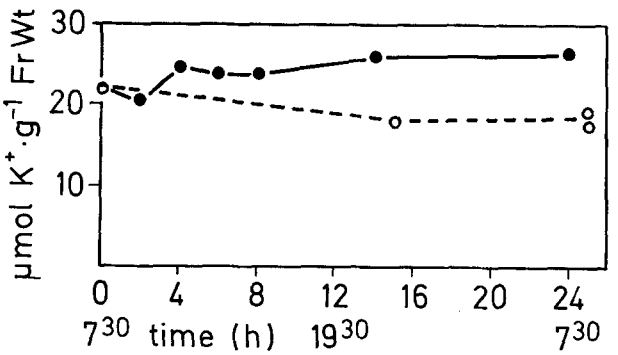


Fig. 2. K^+ levels in *K. daigremontiana* leaf slices which were pretreated in 5 mM KCl plus 0.1 mM $CaSO_4$ for 15 hr then transferred to fresh solution at time zero (7^{30}) at 21 °C (closed circles) and 6 °C (open circles). Samples were taken, blotted dry, and analyzed for K^+ as indicated

where $\underline{S}_v$ is the vacuolar specific activity at the end of the uptake (start of the elution) and $\underline{S}_{ce}$ is the specific activity during elution extrapolated to $t=0$.

Hence

$$\phi_p/\phi_t = \frac{\Phi_{co} - \Phi_{vo}}{\Phi_{vo}} \cdot \underline{S}_v, \quad \text{and} \quad \phi_p/(\phi_p + \phi_t)$$

can be determined and substituted with $\underline{S}_v$ in Φ_{vo} to give ϕ_t. The content of the cytoplasm, Q_c, can be calculated from $k_1 = (\phi_p + \phi_t)/Q_c$ (which is true when $Q_c \ll Q_v$ and $\phi_t \ll Q_v$). The value of $\underline{S}_v$ needed in these calculations can be that calculated for the end of the uptake period at 25° using the method *b* above. The calculated value of Q_T at 6° then uses the estimate of Q_c at 6° plus Q_v at the end of the uptake period.

Elutions were made at 6 and at 25 °C. Separate measurements of tracer uptake were performed by adding tissue to labeled solution and extracting samples at intervals. Each sample was rinsed for 2 min in ice cold, unlabeled solution, blotted and weighed, and then counted.

Stock solutions of ^{86}Rb (RbCl in aqueous solution, 1–12 mCi/mg Rb) and of ^{42}K (target material KCl) were obtained from Amersham Buchler, Braunschweig. Counting was performed with a methane gas flow counter on planchets.

Potassium levels in tissue samples were determined by flame photometry of aqueous extracts.

Sampling of Leaf Material for Experiments in Relation to the Diurnal Rhythm of CAM.

It was observed earlier that the electrical potential difference between the vacuole of cells in *K. daigremontiana* leaf slices and the medium, ψ_{vo}, is independent of the phase of the diurnal rhythm of CAM at which the leaves were harvested (Lüttge & Ball, 1979). Total K^+ levels in the tissue do not change during the rhythm (Lüttge et al., 1975). K^+ and Cl^- uptake by *K. daigremontiana* leaf slices also is independent of the phase of the rhythm (Lüttge & Ball, 1974). Thus in the present attempt to investigate compartmentation of K^+ and electrical properties of plasmalemma and tonoplast, it seemed justified to pay no attention to the phase of the rhythm at which the plant material was sampled. In the experiments of flux analysis requiring a loading period of 15 hr plus a washout period of 24 hr or more this would have been useless anyhow. Sampling usually was during the light phase; in the experiments of Fig. 1 and Tables 1 and 4 it was between 2 and

6 p.m. This is a first approach only and more rapid methods are needed to establish ion levels in the various compartments in relation to the CAM rhythm, i.e., to test the possibility of diurnal oscillations in the ion compartmentation.

Symbols and Abbreviations:

c = concentration
CAM = crassulacean acid metabolism
FC = fusicoccin
FrWt = fresh weight
FS = free space
HEPES = *n*-hydroxy-ethyl-piperazine-N'-2-ethanesulfonic acid
k = rate constant
MES = 2-(*n*-morpholino)-ethanesulfonic acid
N = Nernst
P = permeability coefficient
Q = quantity, content
Q' = amount of tracer
R = electrical resistance
r = specific electrical membrane resistance
S = specific activity
t = time

Used as subscripts only (two symbols in series indicate a direction):
c = cytoplasm
i = inside the cells
o = external medium
p = plasmalemma
t = tonoplast
T = total of c and v
v = vacuole
w = cell wall

Greek symbols:
ψ = electrical potential difference at a membrane
ϕ = flux
Φ = experimental measure of tracer exchange

Results and Discussion

Transcellular Electrical Profiles

Figure 1 depicts the gradual penetration of a *Kalanchoë* cell with a glass micro-electrode and gives an example for a corresponding transcellular electrical profile. Table 1 summarizes data obtained from numerous such experiments.

The potential obtained by penetrating the cell wall is rather independent of the material and conditions (Table 1). Similar results were obtained with *Lupinus luteus* roots *(unpublished)* and isolated cells of *Acer pseudoplatanus* L. (Rona et al., 1980). The electrical resistance of the cell wall is too small to be detected with the methods used; this is expected in view of the high permeability of the cell wall which is

Table 1. Electrical potential differences and resistances obtained from transcellular electrical profiles of *K. daigremontiana* and *K. tubiflora* leaf cells under various conditions[a]

Materials and conditions	$w \rightarrow o$	$c \rightarrow o$			$v \rightarrow o$			$v \rightarrow c$		
	ψ [mV]	ψ [mV]	R [MΩ]	r [Ωm²]	ψ [mV]	R [MΩ]	r [Ωm²]	ψ [mV]	R [MΩ]	r [Ωm²]
Kalanchoë daigremontiana:										
Intact leaves	−45 ± 6(9) −30 to −60 (80)	−183 ± 11(13) −170 to −221 (80)	7 ± 2(13) 3.5 to 10 (80)	0.18	−159 ± 7(15) −130 to −198 (80)	13 ± 2(12) 11 to 19 (80)	0.34	+24	6	0.16
Leaf slices 5 to 15 min after preparation	−36 ± 11(8)	−90 ± 8(13)			−64 ± 7(11)			+26		
Leaf slices 60 to 90 min after preparation	−38 ± 10(9)	−174 ± 7(12)			−153 ± 9(17)			+21		
Young plantlets	−31 ± 12(8)	−120 ± 12(13)			−95 ± 14(10)			+25		
Kalanchoë tubiflora:										
Intact leaves	−33 ± 7(6) −20 to −40 (6)	−167 ± 9(15)	8 ± 2(12) 4 to 12 (12)	0.21	−141 ± 5(12)	15 ± 2(19) 13 to 20 (19)	0.39	+26	7	0.18

[a] Averages with SD and numbers of replicates in brackets and in some cases ranges of individual measurements are given. Cell wall resistances (R_{wo}) were below detectable values. Specific resistances, r, were calculated on the basis of an average diameter of 0.091 mm (±0.014 SD) of the approximately spherical cells of *K. daigremontiana* leaves measured on sections of 266 cells (by courtesy of D. Kramer and J.A.C. Smith). This may underestimate the specific resistance in cells of a complex leaf tissue, because due to the electrical coupling between individual cells (Spanswick, 1972; Drake, 1979) a large surface may be in effect.

readily accessible to ions and thus is usually considered as free space (Grignon & Lamant, 1973; Rona, 1979).

Resistance increases markedly upon penetration into the cytoplasm. Although the role of the cell wall should not be generally neglected (Heller, Grignon & Rona, 1974; Demarty, Ripoll & Thellier, 1980), the electrical potential difference between the cytoplasm and the medium (ψ_{co}) can be considered to be largely a plasmalemma potential. ψ_{co} is slightly but not significantly larger in cells of intact leaves of *K. daigremontiana* than of *K. tubiflora*. Cells of young 4–6 months old plantlets obtained from vegetative propagation on leaves of older plants have lower ψ_{co} than those of adult leaves. This agrees with earlier observations where ψ_{vo} of cells in adult leaves was about −200 mV on average but cells in the leaflets of the small adventitious plantlets growing between the dents of mature leaves only had ψ_{vo} of −126 mV (Lüttge & Ball, 1979). Slicing much reduces ψ_{co} but recovery is already observed after about 1 hr of washing. Membrane potential changes due to slicing and recovery due to "adaptive ageing" (Van Steveninck, 1976) are well known from a variety of plant materials including leaves (*see* Lüttge & Ball, 1979, and references therein).

Resistance increases further as the tip of the electrode passes into the vacuole. The electrical potential between the vacuole and the medium (ψ_{vo}) is the sum of gradients across the tonoplast (ψ_t) and plasmalemma plus cell wall (ψ_{co}). The differences between the two *Kalanchoë* species and the effects of slicing, "adaptive ageing" and leaf age discussed above for ψ_{co} are also reflected in ψ_{vo}.

The electrical potential difference at the tonoplast can be estimated as

$$\psi_t = \psi_{vo} - \psi_{co}.$$

Table 1 shows that for all conditions and materials ψ_t is very similar. This is an important result, suggesting that regulation of the overall electrical potential difference, ψ_{vo}, resides at the plasmalemma, changed conditions such as age, slicing, and "adaptive ageing" affecting ψ_{co} but not ψ_t. This agrees with experiments on cells of the moss *Mnium cuspidatum*, where depolarization by cyanide was exclusively due to plasmalemma depolarization (Fischer, Lüttge & Higinbotham, 1976). Electrogenicity resides largely at the plasmalemma. This reflects itself also in the finding that potentials at the tonoplast are only very small and positive.

The electrical resistance of the plasmalemma R_p is equal to R_{co} because R_{wo} is not detectable, and

$$R_t = R_{vo} - R_{co}.$$

The specific resistances of the plasmalemma and tonoplast are not significantly different (Table 1).

Responses of ψ_{vo} to Gradients of H^+, K^+ and Na^+

It was demonstrated earlier that ψ_{vo} of leaf cells of *K. daigremontiana* is independent of the amount of malic acid stored in the vacuoles and hence of the pH gradients between the vacuoles and the cytoplasm or the vacuoles and the outer medium (Lüttge & Ball, 1979). Figure 3 shows that ψ_{vo} is also independent of the external pH between pH 9 and about 5.5. Below pH 5, ψ_{vo} behaves like an H^+-electrode, for which at

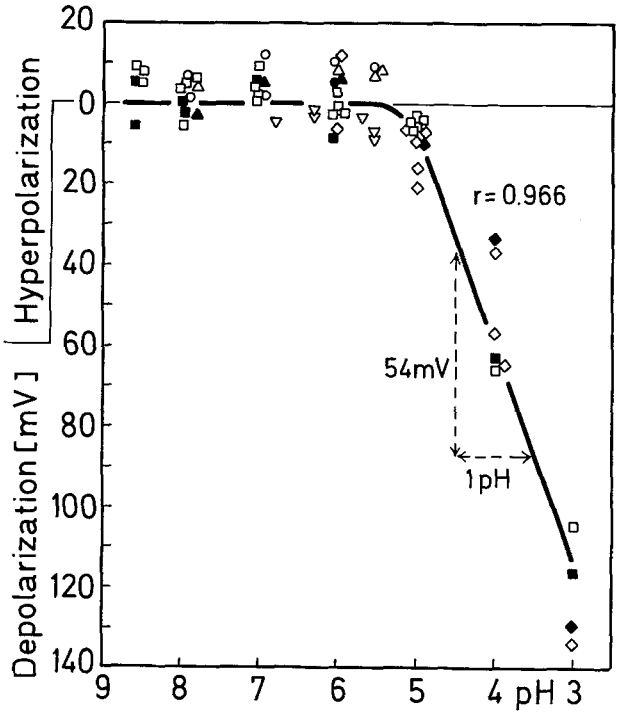
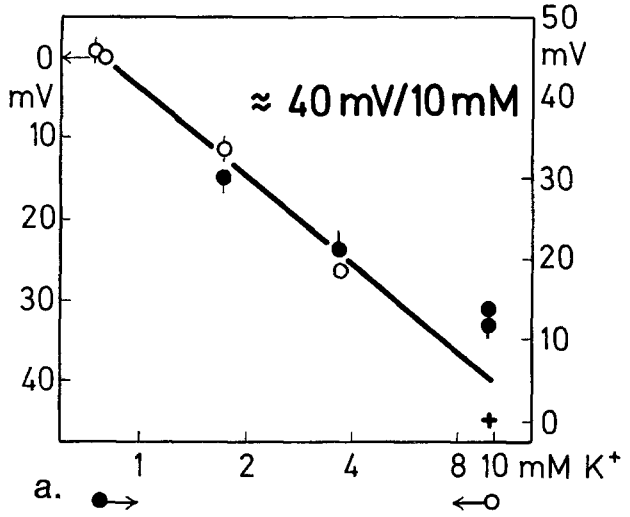
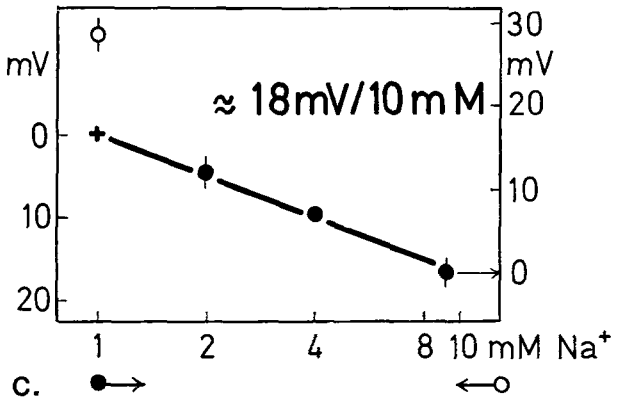


Fig. 3. Dependence of ψ_{vo} of cells in *K. daigremontiana* leaf slices on pH in the external medium. Leaf slices were incubated overnight at pH 6 or 7 in the same MES and HEPES buffers, respectively, which were subsequently used during the experiments. After impalement of electrodes establishment of a constant resting potential at pH 7 was attended, then the pH was changed, and the de- and hyperpolarizations obtained stepping pH up and down and back to the initial pH 7 are plotted. Different symbols denote different buffer solutions, open and closed symbols refer to experiments in the light and in the dark, respectively. All solutions contained 0.1 mM $CaCl_2$; K^+-concentrations (K_2SO_4) were 0.75 to 0.85 mM excepting the squares, where the medium contained no K^+. Na^+ concentrations varied since the pH of the solutions was adjusted with NaOH. By additions of Na_2SO_4, Na^+ concentration was kept constant, however, within a given buffer series as follows: circles — 10 mM buffer, 6.8 mM Na^+; upright triangles — 1 mM buffer, 0.7 mM Na^+; inverted triangles — 0.1 mM buffer, 0.13 mM Na^+; squares and diamonds — 1 mM buffer, 1 mM Na^+. The tissue contained 20 ± 3 mmol $K^+ \cdot kg^{-1}$ FrWt and 2.7 ± 0.8 mmol $Na^+ \cdot kg^{-1}$ FrWt (SD)





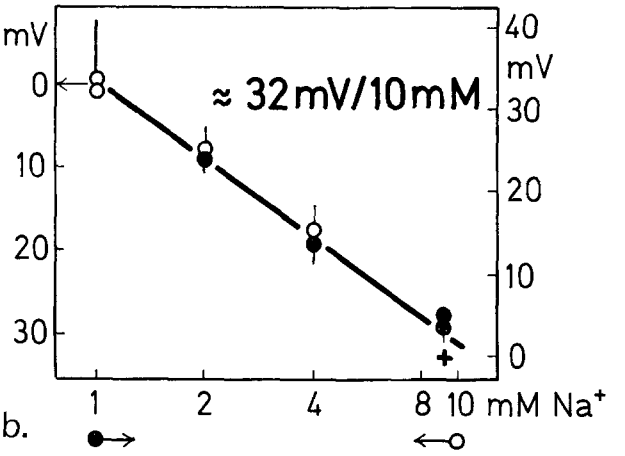


Fig. 4. Dependence of ψ_{vo} of cells in *K. daigremontiana* leaf slices on K^+ and Na^+ concentration in the external medium. Depolarization (•); Hyperpolarization (○). Leaf slices were preincubated overnight in solutions as indicated below. After establishment of a constant resting potential following electrode impalement in the same solution as during pretreatment (= crosses in the graphs serving as points of reference), K^+ or Na^+ concentrations were stepped up or down and back to the original concentration and the resulting de- and hyperpolarizations were plotted. (*a*): Preincubation with 0.1 mM $CaSO_4$ and 9.6 mM K^+ (as sulphate); K^+ and Na^+ levels in the tissue after preincubation were 23 ± 0.6 and 2.7 ± 0.2 mmol kg^{-1} FrWt; K^+ varied in experiment. (*b*): Preincubation with 0.1 mM $CaSO_4$ and 9.2 mM Na^+ (as sulphate); K^+ and Na^+ levels in the tissue 16 ± 1.4 and 7 ± 0.9 mmol kg^{-1} FrWt; Na^+ varied. (*c*): Preincubation with 0.1 mM $CaSO_4$ and 1 mM Na^+ (as sulphate); K^+ and Na^+ levels in the tissue 17 ± 3.3 and 3 ± 0.1 mmol kg^{-1} FrWt; Na^+ varied. Errors are SD

room temperature a depolarization of 58 mV per pH unit would be observed. For higher plants a similar pH dependence was obtained with *Impatiens balsamina* root cells (Jones, Novacky & Dropkin, 1975). In the ranges tested pH-response of *K. daigremontiana* was independent of the kind (MES or HEPES), strength, and K^+ and Na^+ content of the buffer solutions. By contrast to observations with *Nitellopsis obtusa* (Gyenes et al., 1978) the response was not affected by light.

Figures 4 and 5 show the dependence of ψ_{vo} on K^+ and Na^+ concentrations and ratios, respectively, in the outer medium. Unfortunately, these experiments could not be performed with cells whose electrogenic mechanisms were inhibited and which had only a diffusion potential. Due to equilibration problems of *K. daigremontiana* leaf slices ψ_{vo} responds rather sluggishly to inhibitors (Lüttge & Ball, 1979). Electrode impalements are usually lost before a stable difussion potential is attained after addition of an inhibitor to the medium. Thus it is difficult to estimate the permeability coefficients from the data of Figs. 4 and 5 without further assumptions. P_{Na^+}/P_{K^+} can be calculated from the Goldman equation

$$\Delta E = -\frac{RT}{F} \ln \frac{c_i^{K^+} P_{K^+} + c_i^{Na^+} P_{Na^+}}{c_o^{K^+} P_{K^+} + c_o^{Na^+} P_{Na^+}},$$

where R, T and F have the usual thermodynamic meanings. The assumption must be made that the condition of constant field holds for a plant tissue, for which justification may be limited. If it is further assumed that the hyperpolarization of about -38 mV obtained in Fig. 5 by replacing 8 mM K^+ by 8 mM Na^+ is a diffusion and not an electrogenic effect and that the internal concentrations $c_i^{Na^+}$ and $c_i^{K^+}$ do not change during the experiment, P_{Na^+}/P_{K^+} can be calculated as about 0.23. A similar relationship, i.e., $P_{Na^+} < P_{K^+}$, is obtained with algal cells (*see* Table 6.5 in Raven, 1976), with many plant roots (*see* Pitman, 1976), and with isolated cells of *Acer pseudoplatanus* (Pennarun et al., 1978), but in maize root epidermis it was found that the situation can also be inverse

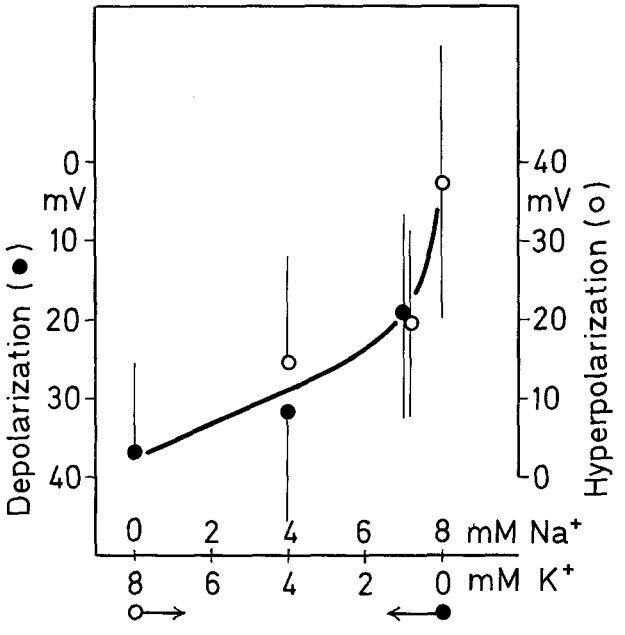


Fig. 5. Dependence of ψ_{vo} in *K. daigremontiana* leaf slices on the Na^+/K^+ ratio in the external medium. Hyperpolarizations obtained by stepwise replacing 8 mM K^+ by Na^+ (open symbols) and depolarizations due to stepwise replacement of Na^+ by K^+ (closed symbols) are plotted

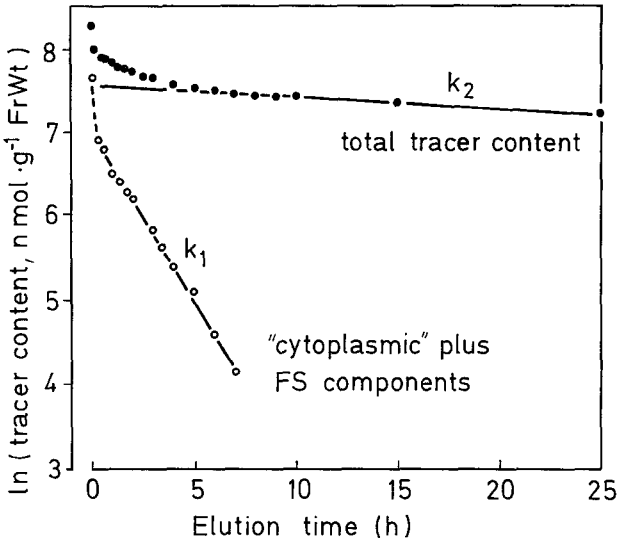


Fig. 6. Time course of tracer exchange at 6 °C after loading for 16 hr in ^{86}Rb labeled 5 mM KCl solution. The "cytoplasmic" plus FS components (open points) are calculated as the differences between the straight line and total tracer content (solid points). In this example $k_1 = 1.055 \times 10^{-4}\,sec^{-1}$ and $k_2 = 2.95 \times 10^{-6}\,sec^{-1}$

depending on the Na^+ and K^+ concentrations in the medium (Michalov, 1977*a*, *b*).

Analysis of K^+-Fluxes and Intracellular Compartmentation of K^+

Figure 6 gives an example of the tracer exchange plotted as $\log_e$ (tracer in tissue) *vs.* time of elution. There was a rapidly exchanging component of about 1.1 μmol g^{-1} FrWt with a rate constant of $k_{FS} = 4.5 \cdot 10^{-3}\,sec^{-1}$ (i.e., $t_{1/2}$ about 150 sec) due to elution of the apoplastic free space.

Results for elution of the more slowly exchanging components at 6° and 25° for 2 experiments are given in Table 2. Data at 25° were analyzed using uptake and efflux information as outlined above (p. 27). For comparison with the calculated value of Q_T, the equivalent estimates from analysis of the tissue in the two experiments were 16 and 17.4 μmol g^{-1}_{FrWt} (Table 2). Data at 6° were analyzed using the efflux data only together with the value of S_v from the 25° experiment. The fluxes at the plasmalemma were not altered by the temperature difference, but the fluxes into the vacuole were higher at 25° than at 6°. Estimates of the cytoplasmic content were about 2–3 μmol g^{-1}_{FrWt} with about 12–15 μmol g^{-1}_{FrWt} in the vacuoles.

An alternative approach to estimating Q_c and Q_v is to measure the time course of tracer uptake, which is given by the expression for Q'_T given on p. 27. In Fig. 7 a comparison of uptake at 2 temperatures was fitted to uptake expressions of this type, but including a term for free space content (*FS*). This analysis yielded the following values for fluxes (pmol $g^{-1}\,sec^{-1}$) and Q_c (μmol g^{-1})

	ϕ_p	ϕ_t	Q_c
6°	305	40	1.0
25°	430	100	2.0

The fluxes are higher than those in Table 2, due to differences in the tissue, but the results show the same general values and effect of temperature on ϕ_t. Note that there was also some effect on ϕ_p and a lower value of Q_c at 6° than at 25°. Other values for fluxes are from data of Fig. 8 (also at 25°) i.e.: $\phi_p = 235$, $\phi_t = 40$ pmol $g^{-1}\,sec^{-1}$ and $Q_c = 1.2$ μmol g^{-1}.

Figure 8 shows the validity of using ^{86}Rb as a tracer for K^+, by comparing ^{42}K and ^{86}Rb uptakes to the same tissue.

There is thus support both from uptake and efflux measurements for a cytoplasmic K^+ content of about 2 μmol g^{-1} FrWt and vacuolar K^+ of about 15–17 μmol g^{-1} FrWt.

Table 2. K^+ fluxes and intracellular compartmentation in adult leaves of *Kalanchoë daigremontiana* based on efflux analysis

Temp. (°C)	Data							Calculated values				
	Q'_c	Q'_v	Q_T	k_1	k_2	Φ_{co}	Φ_{vo}	ϕ_p	ϕ_t	Q_c	Q_v	Q_T
	(μmol g^{-1} FrWt)			($\sec^{-1} \times 10^{-6}$)		(pmol g^{-1} FrWt sec^{-1})		(pmol g^{-1} FrWt sec^{-1})		(μmol g^{-1} FrWt)		
6	1.12	2.35	16.0	100	1.8	115	4.2	140	32	1.65	11.1	12.8
25	1.04	1.82	16.0	105	2.9	115	5.3	145	42	1.8	11.1	12.9
6	1.37	2.87	17.4	87	2.5	125	7.2	150	41	2.4	12.8	15.2
25	1.21	2.87	17.4	78	3.8	105	10.8	150	77	3.0	12.8	15.8

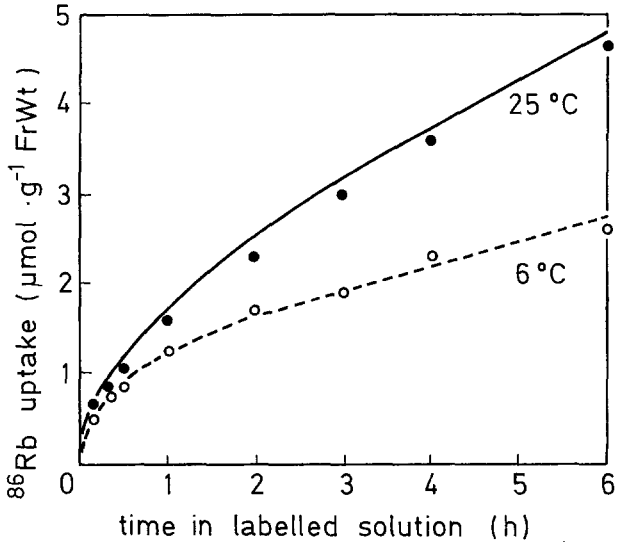


Fig. 7. Uptake of ^{86}Rb from 5 mM KCl + 0.1 mM $CaSO_4$ at 25 °C (closed circles) and 6 °C (open circles). The lines are calculated using the relation of uptake $= FS + A(1-e^{-k_1 t}) + B(1-e^{-k_2 t})$ (*see* text). Values of A and B were 1.0×10^{-6} mol g^{-1} FrWt and 17.0×10^{-6} mol g^{-1} FrWt at 25 °C and 0.7×10^{-6} mol g^{-1} FrWt and 17.3×10^{-6} mol g^{-1} FrWt at 6 °C, and k_1, k_2 were 263×10^{-6} sec^{-1} and 9.8×10^{-6} sec^{-1} at 25 °C and 333×10^{-6} sec^{-1} and 4.2×10^{-6} sec^{-1} at 6 °C

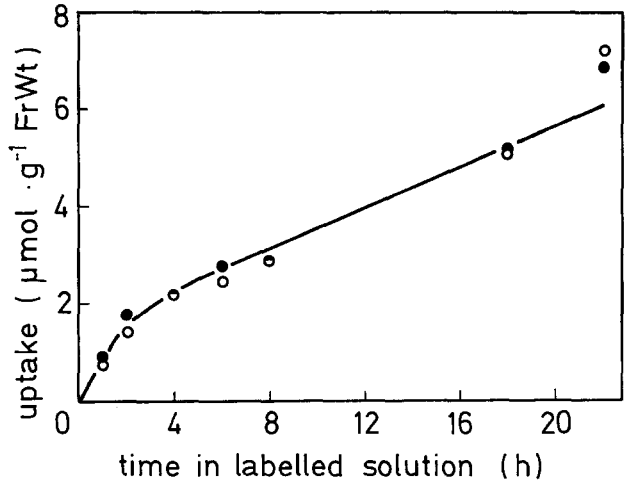


Fig. 8. Uptake of ^{86}Rb (open circles) and ^{42}K (closed circles) at 25 °C from 5 mM KCl and 0.1 mM $CaSO_4$. The line is calculated (as in Fig. 7) using $A = 0.7 \times 10^{-6}$ mol g^{-1}; $B = 17.3 \times 10^{-6}$ mol g^{-1}; $k_1 = 235 \times 10^{-6}$ sec^{-1} and $k_2 = 3.97 \times 10^{-6}$ sec^{-1}

K^+ Electrochemical Gradients at the Plasmalemma and Tonoplast

Results obtained by electrical measurements (Table 1) and compartmental analysis (Table 2) can be used to evaluate K^+-electrochemical gradients at the plasmalemma and tonoplast, of adult *K. daigremontiana* leaves, e.g., by the Nernst criterion

$$E^{K^+}_{N,p} = -\frac{RT}{F} \ln \frac{c_c^{K^+}}{c_o^{K^+}}; \quad E^{K^+}_{N,t} = -\frac{RT}{F} \ln \frac{c_v^{K^+}}{c_c^{K^+}}.$$

The relative volume of the cytoplasm is needed for calculation of c_c and c_v from Q_c and Q_v, respectively. (Note that Q_c and Q_v are *contents* in the compartments c and v on a total tissue FrWt basis, whereas c_c and c_v are *concentrations*.) On electronmicrographs of *K. daigremontiana* leaves D. Kramer *(unpublished)* has estimated the volume of the cytoplasm including chloroplasts as 0.5–1 % of the total cell volume. Table 3 compares K^+ Nernst potentials obtained on this basis with the measured electrical potential differences. Unfortunately the electrical and the flux measurements were made in two different laboratories at external K^+-concentrations of 1 and 5 mM, respectively. Calculations for both alternatives are given in Table 3. The calculations suggest that metabolic energy is needed to transport K^+ out of the vacuole into the cytoplasm and out of the cytoplasm to the medium. It should be noted that for the intact (aerial) leaves the plasmalemma fluxes are not so relevant, a large volume of external solution being absent. Experiments with *K. daigremontiana* leaf slices incubated for 15 hr in various KCl concentrations suggest that the tissue may be in equilibrium with a 10 mM K^+ solution because the net change of K^+ in the leaf slices was zero in this solution (Fig. 9). (This was also the reason for choosing for the flux analyses an external K^+ concentration of 5 mM, which is close enough to the equilibrium concentration – *see* Fig. 9 – and still low enough to allow sufficient specific radioactivity of the labeling solutions.)

Thus it appears that metabolically regulated K^+ transport out of the vacuoles concentrates K^+ in the

Table 3. K^+-electrochemical gradients at the plasmalemma and tonoplast of adult leaves of *Kalanchoë daigremontiana*

					Plasmalemma		Tonoplast	
Q_c	Q_v	c_o	c_c	c_v	$E^{K^+}_{N,p}$	ψ_p	$E^{K^+}_{N,t}$	ψ_t
[mmol kg^{-1} FrWt]		[mM]			[mV]		[mV]	
2	15	5	400[a]	15	−112	~−180	+84	+25
2	15	1	400[a]	15	−154	~−180	+84	+25
2	15	5	200[b]	15	−95	~−180	+66	+25
2	15	1	200[b]	15	−136	~−180	+66	+25

[a] Cytoplasm = 0.5% of cell volume [b] 1% cytoplasm.

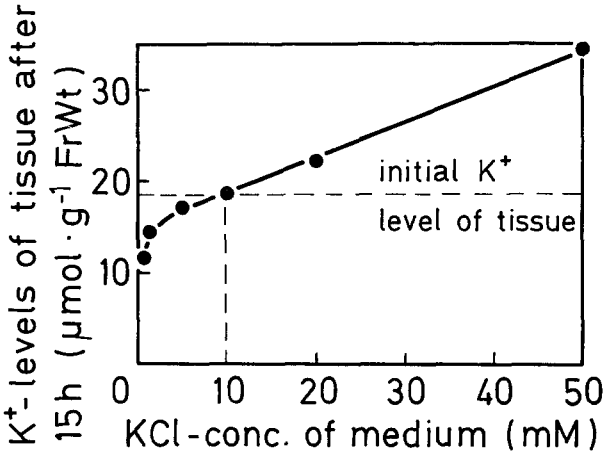


Fig. 9. Changes of K^+ levels of *K. daigremontiana* leaf slices after 15 hr incubation in KCl solutions of varied concentration (+0.1 mM $CaSO_4$) at 21 °C in the dark

Table 4. Effect of 10^{-5} M fusicoccin (FC) on electrical potential differences in leaf slices of *Kalanchoë daigremontiana* 60–90 min after preparation[a]

Before addition of FC:	$-182 < \psi_{co} < -165$	$-160 < \psi_{vo} < -140$
After addition of FC:	$-280 < \psi_{co} < -266$	$-270 < \psi_{vo} < -250$
Difference caused by FC:	$-101 < \Delta\psi_{FC} < -98$	$\Delta\psi_{FC} \simeq -110$

[a] Ranges of 18 individual measurements are given in mV

cytoplasm, so that sufficiently large levels are attained in spite of the very low overall K^+ content of *Kalanchoë* leaf cells.

The Effects of Fusicoccin (FC) on the Plasmalemma and the Tonoplast

Table 4 shows that FC hyperpolarizes ψ_{co} and ψ_{vo}. This confirms earlier results showing an FC-elicited hyperpolarization of ψ_{vo} of *K. daigremontiana* leaf cells (Lüttge & Ball, 1979). The hyperpolarization of ψ_{co} of about 100 mV is consistent with the literature, suggesting that all higher plant cells have an eletrogenic proton extrusion pump at the plasmalemma which is specifically stimulated by FC (Marrè, 1977, 1979). In the transcellular electrical profiles obtained with FC it was very difficult to distinguish between cytoplasm and vacuole, and thus it may be preferable to consider the FC effect somewhat less specifically as an "internal" hyperpolarization. Nevertheless, it seems to be clear enough that the large hyperpolarizing action of FC is effective exclusively at the plasmalemma. Taking the data of Table 4 at face value, FC would depolarize the positive potential at the tonoplast by about 10 mV. Although this is almost 50% of the tonoplast potential, in absolute terms it is only a small and not very significant change.

K^+-Transport and Putative H^+ Pumps at the Plasmalemma and Tonoplast of CAM Leaf Cells

It has been postulated that *Kalanchoë* leaf cells have proton pumps at the plasmalemma and tonoplast extruding protons out of the cytoplasm to the external medium and into the vacuole, respectively (Lüttge & Ball, 1979).

Transcellular electrical profiles and earlier uncoupler experiments (Lüttge & Ball, 1979) clearly show that there is a metabolism-dependent electrogenic transport mechanism at the plasmalemma. Evidence that this is an H^+ extrusion pump largely comes from the fusicoccin experiments. In the same way as it has been demonstrated for other higher plant cells, K^+ uptake in exchange for H^+ could be associated with this H^+ extrusion pump (Marrè, 1977, 1979; Pitman, Schaefer & Wildes, 1975 *a, b*).

Evidence for H^+ pumping across the tonoplast into the vacuole mainly rests on an evaluation of intracellular H^+ electrochemical gradients (Lüttge & Ball, 1979). An H^+ pump at the tonoplast could be

responsible for accumulation of malic acid in the vacuoles of the CAM leaves. Exchanging K^+ for H^+, it could also mediate metabolism-dependent K^+ transport from the vacuole into the cytoplasm. It appears that this mechanism is not electrogenic. It also differs in other respects from the ion pump at the plasmalemma, e.g., it is relatively unaffected by fusicoccin. It is now well established that ATPases, which perhaps pump protons, exist at the tonoplast and other vacuolar membranes (e.g., those of vacuo-lysosomal membranes of *Hevea* latex) (Matile, 1978; Doll, Rodier & Willenbrink, 1979; Guy, Reinhold & Michaeli, 1979; Marin, Marin-Lanza & Komor, 1980).

In vitro studies with isolated tonoplasts of CAM leaf cells are urgent to test the existence of a membrane-bound ATPase and its biochemical properties; e.g., its dependence on pH and K^+ and Mg^{2+} ions. Experiments with intact leaves are required to investigate the possibility of diurnal variations of the intracellular compartmentation of K^+ and Mg^{2+}, and perhaps other inorganic ions during the CAM rhythm. Taken together, such approaches would allow one to evaluate the idea suggested by the present results, that inorganic ions possibly might be involved in CAM regulation.

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References

Anderson, W.P., Hendrix, D.L., Higinbotham, N. 1974. Higher plant cell membrane resistance by a single intracellular electrode method. *Plant Physiol.* **53**:122

Brenneke, R., Lindemann, B. 1971. A chopped current clamp for current injection and recording of membrane polarization with single electrodes of changing resistance. *T.I.T.J. Life Sci.* **1**:53

Demarty, M., Ripoll, C., Thellier, M. 1980. Ion exchange in plant cell walls. *In:* Plant Membrane Transport: Current Conceptual Issues. R.M. Spanswick, W.J. Lucas, and J. Dainty, editors. pp. 33–44. Elsevier/North-Holland, Amsterdam-New York-Oxford

Doll, S., Rodier, F., Willenbrink, J. 1979. Accumulation of sucrose in vacuoles isolated from red beet tissue. *Planta* **144**:407

Drake, G. 1979. Electrical coupling, potentials and resistances in oat coleoptiles: Effects of azide and cyanide. *J. Exp. Bot.* **30**:719

Etherton, B., Keifer, D.W., Spanswick, R.M. 1977. Comparison of three methods for measuring electrical resistances of plant cell membranes. *Plant Physiol.* **60**:684

Fischer, E., Lüttge, U., Higinbotham, N. 1976. Effect of cyanide on the plasmalemma potential of *Mnium*. *Plant Physiol.* **58**:240

Greenham, C. G. 1966. The relative electrical resistances of the plasmalemma and tonoplast in higher plants. *Planta* **69**:150

Grignon, C., Lamant, A. 1973. Distribution de quatre cations dans les cellules libres d'*Acer pseudoplatanus* L. *C.R. Acad. Sci.* **276**:1685

Guy, M., Reinhold, L., Michaeli, D. 1979. Direct evidence for a sugar transport mechanism in isolated vacuoles. *Plant Physiol.* **64**:61

Gyenes, M., Andrianov, V.K., Bulychev, A.A., Kurella, G.A. 1978. Light-induced H^+ accumulation in the vacuole of *Nitellopsis obtusa*. *J. Exp. Bot.* **29**:1185

Heller, R., Grignon, C., Rona, J.P. 1974. Importance of the cell wall in the thermodynamic equilibrium of ions in free cells of *Acer pseudoplatanus* L. *In:* Membrane Transport in Plants. U. Zimmermann and J. Dainty, editors. pp. 239–243. Springer-Verlag, Berlin-Heidelberg-New York

Jones, M.G.K., Novacky, A., Dropkin, U.H. 1975. Transmembrane potentials of parenchyma cells and nematode-induced transfer cells. *Protoplasma* **85**:15

Kluge, M., Ting, I.P. 1978. Crassulacean Acid Metabolism. Analysis of an Ecological Adaptation. 209 pp. Springer-Verlag, Berlin-Heidelberg-New York

Lüttge, U. 1979. Malic acid transport across the tonoplast of *Kalanchoë* leaf cells: Tonoplast biophysics and biochemistry in relation to crassulacean acid metabolism (CAM). *In:* Plant Membrane Transport: Current Conceptual Issues. R.M. Spanswick, W.J. Lucas, and J. Dainty, editors. pp. 49–60. Elsevier/North Holland, Amsterdam-New York-Oxford

Lüttge, U., Ball, E. 1974. Mineral ion fluxes in slices of acidified and de-acidified leaves of the CAM plant *Bryophyllum daigremontianum*. *Z. Pflanzenphysiol.* **73**:339

Lüttge, U., Ball, E. 1979. Electrochemical investigation of active malic acid transport at the tonoplast into the vacuoles of the CAM plant *Kalanchoë daigremontiana*. *J. Membrane Biol.* **47**:401

Lüttge, U., Ball, E., Tromballa, H.-W. 1975. Potassium independence of osmoregulated oscillations of malate^{2-} levels in the cells of CAM-leaves. *Biochem. Physiol. Pflanzen* **167**:267

Marin, B., Marin-Lanza, M., Komor, E. 1980. The protonmotive potential difference across the vacuo-lysosomal membrane of *Hevea brasiliensis* and its change by a membrane-bound adenosine-triphosphatase. *Eur. J. Biochem. (in press)*

Marrè, E. 1977. Effects of fusicoccin and hormones on plant cell membrane activities: Observations and hypothesis. *In:* Regulation of Cell Membrane Activities in Plants. E. Marrè and O. Cifferi, editors. pp. 185–202. Elsevier/North-Holland, Amsterdam

Marrè, E. 1979. Fusicoccin: A tool in plant physiology. *Annu. Rev. Plant Physiol.* **30**:273

Matile, Ph. 1978. Biochemistry and function of vacuoles. *Annu. Rev. Plant Physiol.* **29**:193

Michalov, J. 1977*a*. Sodium permeability and conductance of maize primary root epidermis. *Z. Pflanzenphysiol.* **84**:1

Michalov, J. 1977*b*. Potassium ion permeability and conductance properties of maize primary root epidermis. *Z. Pflanzenphysiol.* **84**:377

Pennarun, A.M., Van de Sype, G., Grignon, C., Heller, R. 1978. Electrochemical state of potassium and sodium in free cells of *Acer pseudoplatanus* L. *Physiol. Plant.* **42**:331

Pitman, M.G. 1976. Ion uptake by plant roots. *In:* Transport in

Plants II. Part B, Tissues and Organs. Encyclopedia of Plant Physiology New Series, U. Lüttge and M.G. Pitman, editors. Vol. 2. pp. 95–128. Springer-Verlag, Berlin-Heidelberg-New York

Pitman, M.G., Schaefer, N., Wildes, R.A. 1975*a*. Stimulation of H^+ efflux and cation uptake by fusicoccin in barley roots. *Plant Sci. Lett.* **4**:323

Pitman, M.G., Schaefer, N., Wildes, R.A. 1975*b*. Relation between permeability to potassium and sodium ions and fusicoccin-stimulated hydrogen-ion efflux in barley roots. *Planta* **126**:61

Raven, J.A. 1976. Transport in algal cells. *In:* Transport in Plants II. Part A, Cells. Encyclopedia of Plant Physiology New Series, U. Lüttge and M.G. Pitman, editors. Vol. 2, pp. 129–188. Springer-Verlag, Berlin-Heidelberg-New York

Rona, J.P. 1973. Premières mesures du potentiel électrique sur des protoplastes et des vacuoles isolées d'*Acer pseudoplatanus*. *C.R. Acad. Sci. Paris* **277**:185

Rona, J.P. 1979. Evolution of electric resistance and potential during plasmolysis and the formation of protoplasts from free cells of *Acer pseudoplatanus* L. Vth International Symposium Bioelectrochemistry and Bioenergetics, Weimar

Rona, J.P., Cornel, D. 1979. Résistances électriques chez les cellules libres, les protoplastes et les vacuoles isolées d'*Acer pseudoplatanus* L. *Physiol. Vég.* **17**:1

Rona, J.P., Cornel, D., Heller, R. 1977. Direct measurement of the potential difference between the cytoplasm of free cells of *Acer pseudoplatanus* L. and the external medium. *Bioelectrochem. Bioenerg.* **4**:185

Rona, J.P., van de Sype, G., Cornel, D., Grignon, C., Heller, R. 1980. Plasmolysis effect on electrical characteristics of free cells and protoplasts of *Acer pseudoplatanus* L. *Bioelectrochem. Bioenerg.* **7**:2

Spanswick, R.M. 1970. Electrophysiological techniques and the magnitude of the membrane potentials and resistances of *Nitella translucens*. *J. Exp. Bot.* **21**:617

Spanswick, R.M. 1972. Electrical coupling between cells of higher plants: A direct demonstration of intracellular communication. *Planta* **102**:215

Van Steveninck, R.F.M. 1976. Cellular differentiation, aging and ion transport. *In:* Transport in Plants II. Part B, Tissues and Organs. Encyclopedia of Plant Physiology New Series. U. Lüttge and M.G. Pitman, editors. Vol. 2, pp. 343–371. Springer-Verlag, Berlin-Heidelberg-New York

Walker, N.A. 1960. The electric resistance of the cell membranes in a *Chara* and *Nitella* species. *Aust. J. Biol. Sci.* **13**:468

Walker, N.A., Pitman, M.G. 1976. Measurements of fluxes across membranes. *In:* Transport in Plants II. Part A, Cells. Encyclopedia of Plant Physiology New Series. U. Lüttge and M.G. Pitman, editors. Vol. 2, pp. 93–128. Springer-Verlag, Berlin-Heidelberg-New York

WynJones, R.-G., Brady, C.J., Speirs, J. 1979. Ionic and osmotic relations in plant cells. *In:* Recent Advances in the Biochemistry of Cereals. D.L. Laidman, and R.-G. WynJones, editors. pp. 63–103. Academic Press, London-New York

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