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# Citrate Uptake into Tonoplast Vesicles from Acid Lime (*Citrus aurantifolia*) Juice Cells

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**Abstract.** Citrate transport into the vacuoles of acid lime juice cells was investigated using isolated tonoplast vesicles. ATP stimulated citrate uptake in the presence or in the absence of a $\Delta\mu H^+$. Energization of the vesicles only by an artificial $K^+$ gradient (establishing an inside-positive $\Delta\psi$) also resulted in citrate uptake as was the case of a $\Delta pH$ dominated $\Delta\mu H^+$. Addition of inhibitors to endomembrane ATP*ases* showed no direct correlation between the inhibition to the tonoplast bound $H^+$/ATP*ase* and citrate uptake. The data indicated that, although some citrate uptake can be accounted for by $\Delta\psi$ and by a direct primary active transport mechanism involving ATP, under *in vivo* conditions of vacuolar pH of 2.0, citrate uptake is driven by $\Delta pH$.

**Key words:** Acid lime — ATP*ase* — Citrate uptake — Membrane potential — Tonoplast vesicles — Vacuolar pH

## Introduction

Uptake of citric acid into the vacuole of plant cells was first described for leutoids (latex containing vacuoles) of *Hevea brasilensis* where it accumulates to concentrations of over 50 mM (Marin et al., 1981). In these studies, citrate uptake was enhanced in the presence of ATP. The stimulatory effect of Mg/ATP on citrate uptake was interpreted as implicating the involvement of the tonoplast-bound $H^+$/ATP*ase*. Later, Marin and Christen (1985) proposed the existence of a citrate/$H^+$ antiport as the mechanism for citrate transport at the tonoplast of *Hevea* leutoids.

In isolated barley mesophyll vacuoles, accumulation of citrate was also ATP dependent and was substantially inhibited by the ionophore CCCP (Rentsch & Martinoia, 1991). The presence of valinomycin/$K^+$ also reduced the levels of citrate uptake to control levels. Inhibition by both CCCP and valinomycin/$K^+$ was taken as an indication that the membrane potential was the sole driving force for citrate uptake. In contrast to barley mesophyll vacuoles, ATP did not enhance the uptake of citrate into tonoplast vesicles from tomato pericarp (Oleski, Mahdavi & Bennett, 1987) where facilitated diffusion was suggested as the major transport mechanism.

In a more recent study, a different mechanism for citrate uptake into the plant vacuole was described for the juice cells of the acidless citrus cultivar ''pummelo 2240'' (Canel, Bailey-Serres & Roose, 1995). A primary active transport mechanism presumably involving a citrate$^{-3}$/ATP*ase* was proposed. The proposed mechanism was based on the stimulatory effect of ATP and the lack of inhibition by nigericin/$K^+$. In these studies, however, citrate uptake was also stimulated by ADP, $PO_4^-$, and the potential effect of $\Delta\psi$ was not taken into account.

Acid lime juice cells accumulate citric acid up to 350 mM and, therefore, should constitute a more efficient model system for the study of citrate uptake. In the present communication, we report on the results of our further investigation on the mechanisms of citrate uptake into the vacuoles of highly acidic citrus juice cells with emphasis on a primary active transport system as proposed for acidless citrus cultivars.

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**Table 1.** Comparison of $H^+$/ATP*ase* inhibition profile and $Cl^-$ dependence between acid lime and lemon low density membrane vesicles

| Inhibitors | % of ATPase activity | |
|---|---|---|
| | Lemon | Acid lime |
| $NO_3^-$ (50 mM) | 82.4[a] | 70.3 |
| Bafilomicin (6 μM) | 90.3[a] | 92.6 |
| Vanadate (100 μM) | 30.7[b] | 22.1 |
| $Cl^-$ dependance ($-Cl^-$) | 84.6[a] | 88.2 |

[a] Data from Müller et al., 1996
[b] Data from Müller et al., 1997
ATP*ase* activity was assayed in the presence of 3 mM ATP/$MgSO_4$.

## Materials and Methods

### Plant Material

Acid limes (*Citrus aurantifolia*) were collected from the lime orchard located at the Citrus Research and Education Center in Lake Alfred, FL. Selection of the fruit was carefully observed to ensure fruit at phases of rapid citrate accumulation.

### Tonoplast Isolation

For the isolation of tonoplast vesicles from acid lime juice cells, we followed the procedure of Müller et al. (1996) with few modifications. Approximately 150 mL of juice cell extract was squeezed directly into 100 mL of homogenization buffer (Müller et al., 1996). The concentration of the buffer was increased to 2 M MOPS/KOH (pH 8.0) to neutralize the increase in acidity. The juice was added to the stirring solution to keep exposure of the membranes to the low pH as short as possible. The homogenate was filtered through 200 μm pore nylon mesh and centrifuged at 7,000 × *g* for 10 min. The supernatant was further centrifuged for 2 hr at 100,000 × *g*. The resulting pellet was resuspended in resuspension buffer (Müller et al., 1996), layered on a 8, 17, 26% sucrose gradient and centrifuged for 90 min at 100,000 × *g*. Membrane vesicles at the 8/17 and 17/26 sucrose interfaces were collected, pelleted at 100,000 × *g* for 30 min and resuspended in 1 mL of storage buffer containing 50 mM MES/BTP (pH 7.5), 1 mM DTT, 1 mM EDTA, 20 mM KCl. Vesicles were stored in aliquots at −80°C until needed. All isolation procedures were conducted at 4°C.

### Citrate Uptake into Tonoplast Vesicles

Uptake studies with tonoplast vesicles were performed using a vacuum separation method at 625 mm Hg constant vacuum. Stock solutions of [$^{14}$C]citric acid were prepared at a radiological concentration of 25 μCi/μmol. Tonoplast vesicles (15 μg membrane protein) were incubated at 30°C in a solution containing 50 mM HEPES/BTP/KOH (pH 7.5), 25 mM KCl, 1 mM DTT, 0.01 mg/mL BSA. ATP, $MgSO_4$ and gramicidin were added as required. Uptake was started by the addition of the radiolabeled substrate to a final concentration of 100 μM and stopped at indicated times by pipetting 100 μL aliquots onto pre-rinsed cellulose nitrate filters (pore size 0.22 μm, 25 mm in diameter; Whatman Int. LTD., Maidstone, UK). After vacuum was applied, the vesicles were washed with 5 mL storage buffer at pH 7.5.

Radioactivity retained in the vesicles was determined by scintillation spectroscopy after immersing the filter discs in 5 mL of Scintiverse BD SX 18–4 (Fisher Scientific, Pittsburgh, PA).

### Simultaneous Measurements of ATPase Activity and pH Gradient Formation

Simultaneous measurements of ATP*ase* activity and formation of a pH gradient between the interior of the vesicles and the media were performed as described by Palmgren (1990). Measurement of ATP*ase* activity was coupled to pyruvate kinase/lactic dehydrogenase and followed as the oxidation of NADH at 340. Formation of a pH gradient was measured at 490 nm following the quenching of acridine orange. Reactions were started by the addition of 0.5 mM $MgSO_4$ to the reaction mixture containing 50 μg tonoplast vesicle protein. All reactions were carried out at 30°C in a Shimadzu UV-160 (Kyoto, Japan).

### Membrane Potential Determinations

Membrane potentials were measured in protoplasts obtained from maturing acid lime juice cells. Protoplasts were prepared by incubating 5–6 juice sacs (cut in halves) in 10 mL of a solution containing 800 mM MES buffer (pH 5.7), 500 mM mannitol, 1 mM $CaCl_2$, 0.1% (w/v) PVP-40, 5 mM spermidine, 1% Cellulysin (Calbiochem), 30 units/mL pectinase (Sigma P-5146) and 0.02% aprotinin. A mild suction was applied and the tissue incubated for 90–120 min at 30°C. After incubation, the vesicle halves were transferred to a similar solution without the hydrolytic enzymes and $CaCl_2$ and gently squeezed to release the protoplasts.

Intracellular microelectrodes were made from pyrex glass drawn with a Narishige glass puller and filled with 3M KCl. The electrode tips were less than 0.4 μm in diameter with electrical resistances of 40–50 mΩ. The external reference electrode was placed into the protoplast bathing solution. The intracellular potentials were measured as the difference between the microelectrode placed within the cell (with a micromanipulator) and the reference electrode.

### Protein Determination

Protein was determined by the Coomassie blue protein assay (Bradford, 1976) using bovine plasma gamma globulin as a standard.

## Results and Discussion

### Tonoplast Vesicles

Yield and purity of tonoplast vesicles from acid lime juice cells was very similar to those reported by Müller et al. (1996) for lemon fruit. Acid lime membrane vesicles collected from the lowest density interface (8/17% sucrose) exhibited similar inhibitor profiles and $Cl^-$ dependency when compared to corresponding lemon vesicles (Table 1). In addition, proton permeability (retention of established ΔpH after addition of EDTA) was equivalent for both fruits (Fig. 1 in this manuscript and Fig. 1 Müller et al., 1996). Lemon vesicles retained approximately 85% of the established ΔpH whereas lime vesicles retained 88% after EDTA was added to interrupt ATP*ase* activity. The acid lime tonoplast fraction was

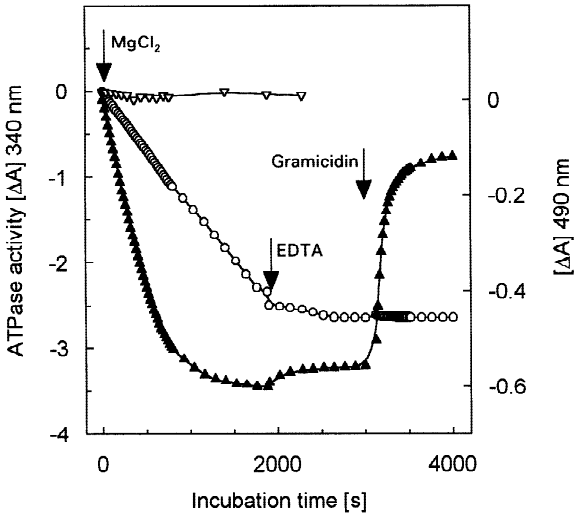


**Fig. 1.** Simultaneous measurements of $H^+$/ATP*ase* activity (○, left hand scale) and $H^+$ gradient formation (▲, right hand scale) in tonoplast vesicles from acid lime juice cells in the presence of Mg/ATP. Reactions were started with the addition of $Mg^{+2}$ to a final concentration of 3 mM. When appropriate, EDTA and gramicidin were added at 4 mM and at 10 μM, respectively. (▽ denotes pH gradient formation in samples with gramicidin added at time 0). Similar results were obtained in all five replicates.

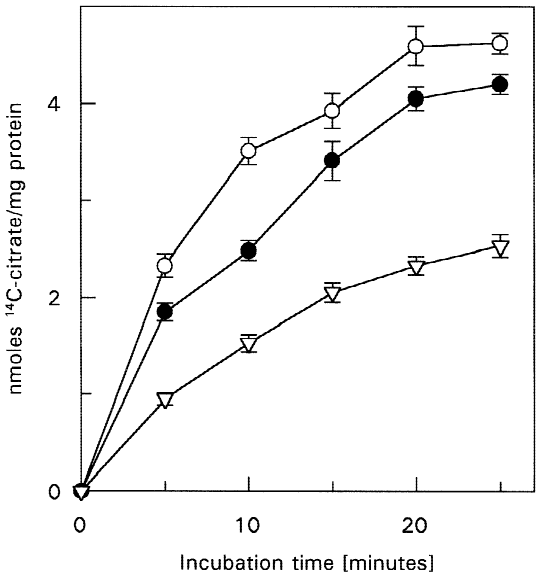


**Fig. 2.** Accumulation of [$^{14}$C]citrate by tonoplast vesicles from acid lime juice cells incubated at pH 7.5 in the presence of 10 μM gramicidin (▽), 3 mM Mg/ATP (○), 3 mM Mg/ATP plus 10 μM gramicidin (●). The results are the average of 6 experiments ± SD.

**Table 2.** Effect of $Mg^{+2}$ on $H^+$/ATPase activity and [$^{14}$C]citrate transport into tonoplast vesicles from acid lime juice cells

| $Mg^{+2}$ Concentration (mM) | Transport activity (nmoles [$^{14}$C]citrate · mg Protein$^{-1}$ (after 25 Minutes of incubation) | $H^+$/ATPase activity (nmoles P*i* · mg prot. · min$^{-1}$) |
|---|---|---|
| 0.00 (0%) | 5.44 ± 0.22 | 0.00 |
| 0.05 (0.8%) | 6.17 ± 0.55 | 3.89 |
| 0.10 (1%) | 7.29 ± 0.43 | 4.87 |
| 0.20 (4%) | 7.58 ± 0.38 | 19.48 |
| 0.50 (20%) | 8.65 ± 0.39 | 97.40 |
| 1.00 (73%) | 9.11 ± 0.84 | 355.50 |
| 3.00 (100%) | 5.89 ± 0.41 | 487.00 |

ATP and [$^{14}$C]citrate were maintained at constant concentrations of 3 mM and 100 μM, respectively.

further characterized kinetically and by immunorecognition to V and P-ATP*ases*, and the results confirmed the vacuolar nature of the fraction (object of separate manuscript). These results indicate that the tonoplast of both lemon and acid lime juice cells are very similar in that both contain a vanadate-sensitive, nitrate and bafilomycin insensitive ATP*ase*. Therefore, although vacuolar in nature, acid lime tonoplast purity cannot be determined entirely by conventional patterns of inhibition to endomembrane ATP*ase* inhibitors (Müller et al., 1996, 1997).

Figure 1 demonstrates the capacity of the tonoplast vesicles to form a pH gradient in the presence of Mg/ATP. Inclusion of 10 μM gramicidin at the initial reaction time prevented the formation of the pH gradient as would be expected by the action of the protoionophore. In samples where a ΔpH was allowed to form, addition of gramicidin prompted a total collapse of the $H^+$ gradient. However, inclusion of EDTA to terminate the activity of the ATP*ase* (Fig. 1) did not result in a significant reduction in the ΔpH as reported for sweet limes (Echeverria, Gonzalez & Brune, 1997) demonstrating the impermeability of the membrane and membrane components to protons. The above data demonstrate the usefulness of the vesicles to evaluate citrate uptake.

## Uptake of [$^{14}$C]Citrate by Tonoplast Vesicles

### *Effect of Mg/ATP*

Figure 2 shows an apparent stimulation of citrate uptake by Mg/ATP when compared to controls (–Mg/ATP). The stimulation of citrate uptake occurred even in the absence of a $\Delta\mu H^+$ (10 μM gramicidin) and despite the fact that free citrate was reduced in the presence of Mg/ATP due to the chelating effect of $Mg^{+2}$ on citrate. The maximum vesicular citrate concentration attained in the presence of Mg/ATP was of approximately 210 μM, which is about twofold over the external concentration. This is in sharp contrast to the results of Oleski et al. (1987) where the presence $Mg^{+2}$ reduced citrate uptake. Our results showed that the increases in citrate uptake in the presence of ATP were not due to the increase in free citrate as a consequence of $Mg^{+2}$ chelation by ATP and

**Table 3.** Effect of $H^+$/ATP*ase* inhibitors (DCCD, NBD, Vanadate, thapsigargin), ionophores (gramicidin) and anion channel blockers (DIDS) on uptake of [$^{14}$C]citrate and tonoplast bound $H^+$/ATP*ase* activity

| Treatment of vesicles | Citrate uptake (nmoles) [$^{14}$C]citrate · mg prot. · 25 min$^{-1}$) | $H^+$/ATP*ase* activity (%) |
|---|---|---|
| Vesicles + ATP/$Mg^{+2}$ | 7.87 ± 0.39 | 100 |
| Vesicles − ATP/$Mg^{+2}$ + Gramicidin (10 μM) | 4.25 ± 0.26 | 0 |
| Vesicles + ATP/$Mg^{+2}$ + Gramicidin (10 μM) | 7.08 ± 0.68 | 100 |
| Vesicles + ATP/$Mg^{+2}$ + Vanadate (100 μM) | 7.08 ± 0.45 | 25 |
| Vesicles + ATP/$Mg^{+2}$ + DCCD (100 μM) | 7.87 ± 0.72 | 70 |
| Vesicles + ATP/$Mg^{+2}$ + NBD-Cl (12 μM) | 4.97 ± 0.17 | 92 |
| Vesicles + ATP/$Mg^{+2}$ + DIDS (10 μM) | 4.25 ± 0.23 | 72 |
| Vesicles + ATP/$Mg^{+2}$ + Thapsigargin (100 nM) | 7.87 ± 0.67 | 100 |
| Vesicles + ATP/$Mg^{+2}$ + Thapsigargin (2 μM) | 7.49 ± 0.48 | 100 |

ATP and $Mg^{+2}$ were maintained at a concentration of 3 mM and 0.5 mM, respectively.

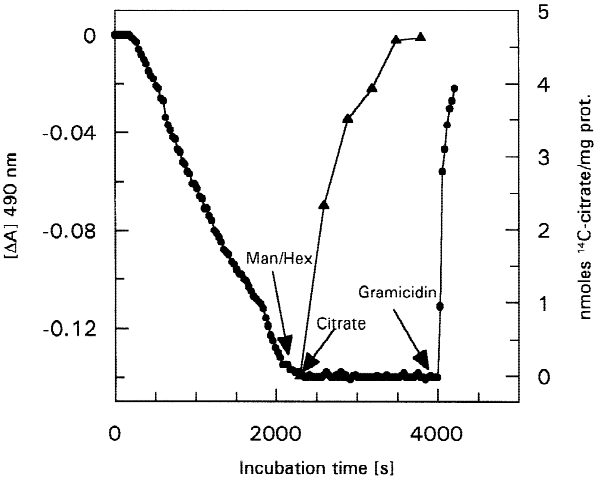


**Fig. 3.** Changes in $H^+$ gradient (●, left hand scale) by tonoplast vesicles from acid lime juice cells in the presence of Mg/ATP. Mannose/hexokinase (ATP trap) was added to deplete remaining ATP and stop the $H^+$/ATPase activity. Citrate was added after pH gradient ceased to increase (depletion of ATP) and uptake followed for 30 min (▲, right hand scale). Gramicidin was added to a final concentration of 10 μM. Similar results were obtained in the three replicates.

supports the concept of a primary citrate active transport system in citrus fruits (Canel et al., 1995).

To further characterize citrate uptake despite of the chelating effect of $Mg^{+2}$, citrate accumulation was measured in the absence of $Mg^{+2}$ and in the presence of increasing concentrations of $Mg^{+2}$ (Table 2). Addition of $MgSO_4$ up to 1 mM resulted in an increase in citrate uptake above control levels despite the fact that the concentration of free citrate must have declined. At higher $Mg^{+2}$ concentrations, citrate uptake declined as expected due to chelation.

There were negligible levels of $H^+$/ATP*ase* activity at $Mg^{+2}$ concentrations below 0.5 mM. However, under the same conditions, citrate uptake increased sharply (Table 2) suggesting that citrate (at least in part) could be taken up independently from the action of the tonoplast bound $H^+$/ATP*ase*.

Having observed that the tonoplast $H^+$/ATP*ase* appears not to be entirely coupled with the increases in citrate uptake in the presence of ATP, several ATP*ase* inhibitors were tested (Table 3). Strong inhibition by vanadate of the vanadate sensitive lime tonoplast-bound ATP*ase* did not affect citrate uptake. However, the anion channel blocker DIDS and the V-ATP*ase* inhibitor NBD-Cl which had a mild inhibitory effect on the tonoplast-bound ATP*ase*, completely suppressed citrate uptake. Similar effect on citrate uptake by DIDS was observed in tomato and barley tonoplast vesicles (Oleski et al., 1987; Rentsch & Martinoia, 1991). The effect of other inhibitors, albeit at a lesser inhibitory degree, also showed no direct correlation between the activity of the $H^+$/ATP*ase* and citrate uptake under the present experimental conditions.

### *Effect of Citrate on the Steady-State pH Gradient*

The steady-state pH gradient generated by the acid lime tonoplast bound $H^+$/ATP*ase* was unaffected by the addition of up to 6 mM citrate. The lack of absorbance recovery was noted after the pumping activity of ATP*ase* was terminated by the establishment of an ''ATP trap'' (depletion of ATP by added mannose and hexokinase). This was necessary since pumping of protons by the $H^+$/ATP*ase* would have masked any extrusion of protons during a possible citrate/$H^+$ exchange (Fig. 3). Addition of gramicidin resulted in the total collapse of the remaining pH gradient. Therefore, lack of absorbance recovery during citrate accumulation argues against a $H^+$/citrate exchange as suggested for *Hevea* leutoids (Marin & Christen, 1985).

**Table 4.** Citrate transport into tonopast vesicles at different imposed conditions of $\Delta\mu H^+$.

| Treatment of vesicles | Transport Activity (nmoles [$^{14}$C]citrate · mg prot. · 25min$^{-1}$) | | | |
|---|---|---|---|---|
| | pH 7.5 | | pH 5.5 | |
| | −ATP/$Mg^{+2}$ | +ATP/$Mg^{+2}$ | −ATP/$Mg^{+2}$ | +ATP/$Mg^{+2}$ |
| Vesicles (Control) | 5.44 ± 0.41 | 7.87 ± 0.34 | 8.85 ± 0.35 | 9.59 ± 0.33 |
| Vesicles + CCCP 10 μM | 4.87 ± 0.38 | 5.69 ± 0.36 | 4.33 ± 0.59 | 4.75 ± 0.82 |
| Vesicles + Nigericin 15 μM | 5.44 ± 0.57 | 6.38 ± 0.75 | 5.85 ± 0.40 | 5.90 ± 0.62 |
| Vesicles + Valinomycin 15 μM ($K^+$, 0) | 4.86 ± 0.37 | 6.26 ± 0.29 | 5.34 ± 0.17 | 6.28 ± 0.17 |
| Vesicles + Valinomycin 15 μM ($K^+$, 25 mM) | 5.61 ± 0.34 | 6.37 ± 0.63 | 6.07 ± 0.25 | 6.68 ± 0.51 |
| Vesicles + Valinomycin 15 μM ($K^+$, 150 mM) | 6.35 ± 0.77 | | | |

Different $\Delta\psi$ were imposed by variations to the external $K^+$ concentration.
$Mg^{+2}$ and ATP, when present, were added at a concentrations of 0.5 mM and 3 mM respectively.

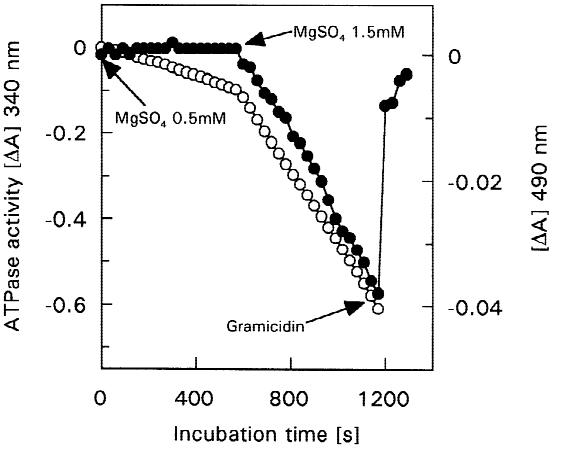


**Fig. 4.** Tonoplast bound ATP*ase* activity (○, ΔA at 340 nm) and $H^+$ gradient formation (●, ΔA at 490 nm) by acid lime juice cell tonoplast vesicles in the presence of 0.5 mM and 1.5 mM $MgSO_4$. Gramicidin was added to a final concentration of 10 μM. Similar results were obtained in the three replicates.

### *Effect of Different Components of $\Delta\mu H^+$ on Citrate Uptake*

To examine the means by which citrate uptake is coupled to $\Delta\mu H^+$, citrate uptake was assayed under conditions in which the overall $\Delta\mu H^+$ was dominated by either a $H^+$ concentration gradient (ΔpH) or membrane electrical potential ($\Delta\psi$). Acid lime tonoplast contains only trace levels of PP*iase* activity, therefore, creation of a $\Delta\mu H^+$ independent from ATP for the following studies was achieved by a pH jump (Bush, 1989). Vesicles with an internal pH of 5.5 would have a higher $H^+$ concentration and would generate a larger ΔpH when incubated in a medium at pH 7.5 (during citrate uptake) than vesicles with an internal pH of 7.5. Citrate uptake into control vesicle samples (-ATP) with internal pH of 5.5 increased significantly (65%) over vesicles at pH 7.5 (Table 4). Addition of the protoionophore CCCP (collapsing the $\Delta\mu H^+$) to control vesicles (-ATP) at different internal pH reduced citrate uptake to basal values suggesting some involvement of the $\Delta\mu H^+$ in citrate uptake (Table 4). The noted slight reduction in samples at pH 7.5 (-ATP) after addition of CCCP was probably due to the retention of some electrochemical potential during isolation of the vesicles.

Disruption of ΔpH by the electroneutral $H^+/K^+$ exchange across the tonoplast of vesicles at pH 5.5 by nigericin resulted in a significant reduction in citrate uptake (Table 4). Similar reductions in citrate uptake were observed as the result of an equilibration of $\Delta\psi$ by valinomycin (25 mM $K^+$).

Energization of vesicles (internal pH of 7.5) solely by $\Delta\psi$ established by artificial $K^+$ gradient (using valinomycin and 150 mM K-gluconate) stimulated citrate uptake over basal rates (Table 4). The generated $\Delta\psi$ was estimated at +50 mV (positive inside). In all cases, inclusion of ATP resulted in significant increases in citrate uptake even in the total absence or absence of one of the components of the electrochemical gradient. The increase in citrate uptake in the presence of ATP was not related to the $H^+$/ATP*ase* since the $Mg^{+2}$ concentration in the reaction mixture (0.5 mM) was purposely maintained low enough to suppress the $H^+$/ATP*ase* and not allow the formation or magnification of $\Delta\mu H^+$ (Fig. 4, Table 4). The increases in $\Delta\mu H^+$-independent citrate uptake driven by ATP appear to be substrate specific since other high energy donors such as UTP, GTP or PP*i* did not reproduce the effect of ATP (*data not shown*).

The data presented in this communication indicate that more than one mechanism are likely to participate in the overall process of citrate accumulation into isolated tonoplast vesicles from acid lime juice cells. Our data support the existence of a primary active transport mechanism for citrate uptake as suggested by Canel et al.

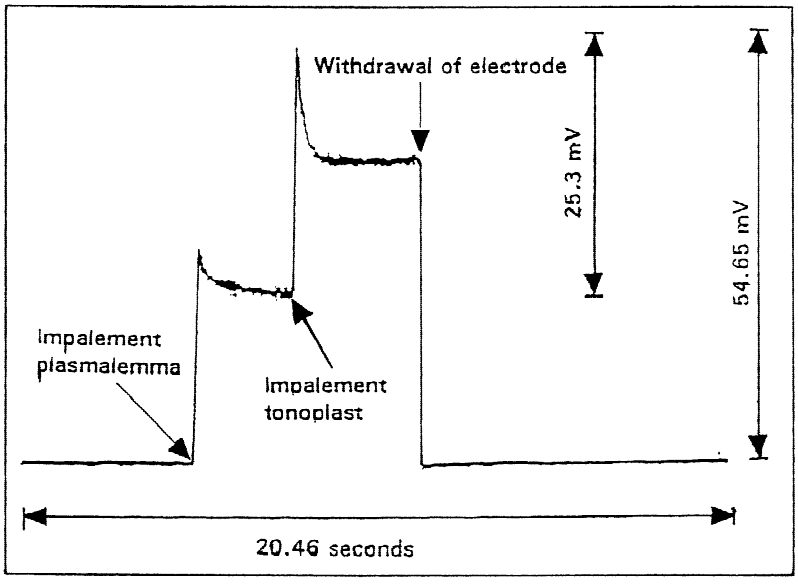


**Fig. 5.** Electrical profiles of protoplasts from acid lime as the electrode penetrates the plasmalemma, tonoplast and after withdrawal of the electrode. The experiment was conducted 6 times. The figure presented is a representative sample. Results of vacuolar $\Delta\psi$ had a variation of 4.3 mV.

(1995). This conclusion was based primarily on the increased citrate uptake by Mg/ATP in the presence or absence of a $\Delta\mu H^+$. The system appears to be ATP specific, inhibited by the anion channel blocker DIDS but not affected by thapsigargin, a strong inhibitor of ABC transport systems (Hussain et al., 1995). Under our experimental conditions, however, such $\Delta\mu H^+$-independent ATP-stimulated citrate uptake accounted for only a two-fold accumulation of citrate from the medium at all external concentrations tested. The maximum vesicular concentration attained was of 1 ≈ mM when the media citrate was increased to 500 μM (and free $Mg^{+2}$ kept at a constant level).

As suggested by Rentsch & Martinoia (1991), some citrate uptake from the media into the vesicle lumen could be accounted for solely by the $\Delta\psi$. In our experiments, establishment of a $\Delta\psi$ similar to that found in vivo (Fig. 5; Sze, 1985) of +25 mV (positive inside) resulted in a 23% increase accumulation of citrate compared to the incubation media. This value may be an underestimate since transport of $citrate^{-3}$ into the experimental vesicles would dissipate the established $\Delta\psi$, whereas in vivo, the membrane potential is maintained. However, a constant $\Delta\psi$ of +25 mV would only generate approximately a 10-fold uptake of citrate. In acid lime juice cells, with a vacuolar citrate concentration of 350 mM, a cytosolic citrate concentration of 35 mM would be required, a highly unlikely figure.

Accumulation of citrate in the tonoplast vesicles was also obtained by the establishment of an artificial pH gradient alone (Table 4). This ΔpH was established by incubating vesicles with internal pH of 5.5 in an incubation media at pH 7.5 in the presence of valinomycin/$K^+$. Uptake of citrate increased 40% above the basal levels of vesicles incubated with CCCP. This value is also an underestimate from the expected 6-fold accumulation if the pH were maintained. Protonation of $citrate^{-3}$ as it enters the vesicle lumen increases the pH and consequently slows down the pH driven citrate accumulation.

In vivo, the vacuolar pH of acid lime juice cells declines from an estimated 5.5 at an early stage of development (Echeverria, 1990) to 2.0 at maturity (Echeverria et al., 1992). The acidification of the vacuole is accompanied by the accumulation of citrate (free acid) to concentrations of up to 350 mM. The uptake of $citrate^{-3}$ into the vacuole would bind protons and raise the pH. Thus, the drastic decline in pH during development is brought about by the active proton pumping rather than by citrate uptake (Müller et al., 1996).

The present data do not allow precise quantification of each component to the overall process of citrate accumulation under in vivo conditions. However, the measured transtonoplast $H^+$ gradient (Echeverria et al., 1992) indicates that the vacuolar pH of 2.0 in the juice cells is the dominant driving force. Citrate is transported into the vacuole as $citrate^{-3}$ and immediately protonated to the neutral form (citric acid). Removal of $citrate^{-3}$ by protonation inside the vacuole would prevent a build up of $citrate^{-3}$ and will allow for its continued diffusion down an electrical and concentration gradient. In addition, a portion of the citrate uptake may result from the ATP-driven $citrate^{-3}$ pump which will operate until $citrate^{-3}$ concentration builds up inside.

Maintenance of a $citrate^{-3}$ equilibrium across the tonoplast would allow for an accumulation of citrate (neutral state) in the vacuole of over 10,000 times. An in

vivo vacuolar citrate concentration of 350 mM can, therefore, be achieved at a very low cytosolic citrate ($\leqslant$35 $\mu$M).

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