The control by $\Delta\bar{\mu}_{H^+}$ of the tonoplast-bound H^+ -translocating adenosine triphosphatase from rubber-tree (*Hevea brasiliensis*) latex

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The relationship between tonoplast-bound ATPase activity and the magnitude of the electrochemical proton gradient has been investigated on tightly sealed vesicles prepared from rubber-tree (*Hevea brasiliensis*) latex. A variety of methods have been used to modify, either alone or together, the two components of the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$). When the ΔpH component was decreased either by titration with $(NH_4)_2SO_4$ or by addition of protonophores or nigericin in the presence of K^+ , ATPase activity was stimulated. On the other hand, when the $\Delta\psi$ component was decreased either by addition of lipophilic cations or by addition of valinomycin in the presence of K^+ , ATPase activity decreased. It is concluded that activity of the tonoplast-bound ATPase is regulated by changes in the electrochemical proton gradient across the tonoplast, so that, once the maximum proton gradient is established across the tonoplast, any perturbation of the equilibrium state should result in the increased rate of ATP hydrolysis as the enzyme attempts to re-establish the initial gradient.

The vacuoles in higher plants constitute a highly specialized compartment that sequesters a great variety of molecules (Zirkle, 1937; Guillaiermond, 1941). Recently, when compartmentation studies between the different cell organelles became possible, it was shown that vacuoles accumulate, in addition to secondary plant products, amino acids,

glucides (sucrose, ascorbate), various organic acids and ions such as Na^+ , Mg^{2+} , Cl^- or K^+ (Wagner, 1982). The mechanism of this accumulation is largely unknown, except in few cases such as citrate in the latex of the rubber tree (*Hevea brasiliensis*) (Marin, 1982), arginine in *Saccharomyces cerevisiae* (Ohsumi & Anraku, 1981) and sucrose in *Saccharum* sp. (Thom & Komor, 1984).

Abbreviations used: $\Delta\bar{\mu}_{H^+}$, electrochemical potential difference of protons between the two bulk phases that the membrane separates; Δp , protonmotive force, given by the equation:

$$\Delta p = \Delta\bar{\mu}_{H^+}/F = \Delta\psi - 2.303RT \cdot \Delta pH/F$$

(R , T and F having their usual thermodynamic meanings); ΔpH , transmembrane pH gradient; $\Delta\psi$, electrical transmembrane potential; DCCD, NN' -dicyclohexylcarbodi-imide; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid; 2,4-DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide p -trifluoromethoxyphenylhydrazone; NEM, N -ethylmaleimide; S-13, 5-chloro-3- t -butyl-2'-chloro-4-nitrosalicylanilide; SF-6847, 3,5-di- t -butyl-4-hydroxybenzylidene malononitrile; TMT, trimethyltin chloride; TPMP $^+$, triphenylmethylphosphonium ion; TPP $^+$, tetraphenylphosphonium ion; Z has its usual thermodynamic meaning.

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It has been clearly established that the electrochemical proton gradient across the tonoplast membrane from *Hevea* latex is the driving force for citrate transport and its accumulation (Marin *et al.*, 1981b, 1982; Marin, 1982). This also seems to be the case for the basic amino acids in *Saccharomyces cerevisiae* (Ohsumi & Anraku, 1981). Protons enter the vacuole through a proton-translocating ATPase bound to the tonoplast in *Hevea* (Marin *et al.*, 1981a; Créten, 1982) or in *Saccharum* sp. (Thom & Komor, 1984). Consequently, an important role has been ascribed to this ATPase, which by driving an influx of protons is capable of generating and maintaining a ΔpH (acidic-inside) and a $\Delta\psi$ across the tonoplast membrane. Despite the mechanistic importance of this electrogenic H^+ pump, almost nothing is known of its basic properties, functioning and regulation. As described previously (Marin, 1982; D'Auzac *et al.*, 1982), tonoplast vesicles derived from *Hevea* latex

provide a convenient system for clarifying the properties of the tonoplast H^+ -translocating ATPase.

The present paper describes the regulation of the tonoplast-bound ATPase activity by the electrochemical proton gradient.

Experimental

Materials

All reagents were of analytical grade. Unless mentioned below, they were obtained from the following manufacturers: Labosi, Paris, France; Fluka Feinchemikalien GmbH, Ulm, Germany; Merck, Darmstadt, Germany; and Sigma Chemical Co., St. Louis, MO, U.S.A. ATP (as sodium salt) was from Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany.

[^{14}C]Dextran (1.30–5.18 TBq·mol $^{-1}$) was obtained from New England Nuclear Chemicals, Dreieich, Germany, ^3H_2O (3.33 GBq·mol $^{-1}$) from Amersham Buchler, Braunschweig, Germany, and [^{14}C]TPMP $^+$ (1.84 TBq·mol $^{-1}$) and [^{14}C]methylamine hydrochloride (1.52 TBq·mol $^{-1}$) both from Commissariat à l'Energie Atomique, Gif-sur-Yvette, France.

S-13 was a gift from Monsanto Co., Paris, France. Valinomycin was obtained from Eli Lilly and Co., Indianapolis, IN, U.S.A. Nigericin was generously given by Hoffman-LaRoche, Basel, Switzerland.

Plant material

Latex was obtained from rubber trees (*Hevea brasiliensis* Müll.-Arg.; clone Prang Besar 86) growing on the experimental plantation of the I.R.C.A. (Institut de Recherches sur le Caoutchouc en Afrique), Abidjan, Ivory Coast. The fluid cytoplasm was harvested in ice-cooled flasks as described previously (Marin & Trouslot, 1975).

Vacuole isolation and purification

Vacuoles were isolated, purified and freeze-dried as described elsewhere (Marin *et al.*, 1981b).

Preparation of tonoplast membranes

Tonoplast membranes were dispersed in 25 mM-Mes/25 mM-Hepes/5 mM- β -mercaptoethanol, adjusted to pH 6.0 with Tris base. As previously described (Marin *et al.*, 1981b; Marin, 1982), the procedure used allowed the preparation of a highly purified membrane fraction (D'Auzac *et al.*, 1982).

Standard incubation conditions

Experiments were conducted at 30°C in 50 mM-Pipes/5 mM- β -mercaptoethanol, adjusted to pH 6.8 with Tris base. Ammonium molybdate was added at a final concentration of 0.1 mM. ATP (as the Tris

salt) and Mg^{2+} (as $MgSO_4$) were added at a final concentration of 5 mM. Further details are given below where appropriate.

ATPase assay

ATPase activity was routinely determined in 1 ml of the reaction mixture. The reaction was initiated by the addition of a portion of tonoplast membranes containing about 100 μ g of protein and stopped by the addition of 250 μ l of ice-cooled 20% (w/v) trichloroacetic acid. P_i was assayed by the method of Heinonen & Lahti (1981) or by the procedure of Taussky & Shorr (1953). One unit of activity corresponded to 1 μ mol of P_i /min.

Protein assay

The amount of protein in the purified fraction was determined by the method of Lowry *et al.* (1951) as modified and described previously (Marin, 1983a). Under these conditions the components of the reaction medium do not interfere with the determination. Bovine serum albumin was used as standard.

Measurement of tracer uptake

Uptake of radioactively labelled compounds by tonoplast vesicles was used to determine the membrane potential, the transmembrane pH difference and the osmotic volume of the vesicles, as described previously (Marin *et al.*, 1981a,b; Marin, 1982). [^{14}C]TPMP $^+$ was used for the determination of membrane potential ($\Delta\psi$). [^{14}C]Methylamine distribution was used to measure Δ pH across the tonoplast. Intravesicular volumes were determined with ^3H_2O and [^{14}C]dextran.

The experiment was started, after the preincubation period (10 min), by the addition of 5 mM-ATP, in the presence of 5 mM- $MgSO_4$. Assays were performed so that each series of vials contained ^3H_2O and a ^{14}C -labelled compound with a $^{14}C/^3H$ ratio of 0.2. At the time indicated, the vesicles were sedimented by centrifugation in an Eppendorf model 3200 desk microcentrifuge for 3 min at 10000g.

Further experimental details were described previously (Marin, 1983a).

The distribution of the labelled compounds was determined from their relative activities in the pellet and supernatant (Marin *et al.*, 1981a,b; Marin, 1982). The distribution of the probe between the tonoplast vesicles and the medium was corrected for adherent water as follows:

$$\frac{C_{in}}{C_{out}} = R - (R - 1) \cdot \frac{K}{1 - K}$$

where C_{in} is the internal concentration of the probe and C_{out} its concentration in the medium, $R = ^{14}C$ -

labelled compound/³H₂O space, and $K = [^{14}\text{C}]\text{dextran}/^3\text{H}_2\text{O space}$.

The internal pH of tonoplast vesicles was calculated from the accumulation of the lipid-soluble base methylamine by using the following equation (see Marin *et al.*, 1981a,b; Marin 1982):

$$\text{pH}_{\text{in}} = \text{pH}_{\text{out}} - \log \frac{C_{\text{in}}}{C_{\text{out}}}$$

For the calculation of Δψ, as TPMP⁺ was demonstrated to be distributed according to the Nernst equation across the tonoplast (see Marin *et al.*, 1981a,b; Marin, 1982), it follows that:

$$\Delta\psi = \frac{RT}{ZF} \ln \frac{C_{\text{in}}}{C_{\text{out}}}$$

The values reported for each experiment are from one representative preparation.

Results

Electrochemical proton gradient and the effect of MgATP

When tonoplast vesicles were incubated in the used buffered medium, in the absence of any permeant anions, at pH 7.0, an intravesicular pH of 6.4 could be measured from the distribution of [¹⁴C]methylamine. When MgATP was added, the ΔpH reached 0.9 pH unit after 30 min, as described previously (Marin, 1983a). The membrane potential (Δψ), as measured by [¹⁴C]TPMP⁺ uptake, was estimated to be -140 mV, negative-inside. After 30 min, in the presence of MgATP, there was a depolarization that amounted to 120 mV, as described previously (Marin, 1983a). Under these conditions, the ATPase activity was considered as constant, at about 1.1 units/mg of protein, during the first 10 min, but decreased considerably afterwards. Such a situation was not observed with the fragmented tonoplast vesicles, where the ATPase activity was constant, at about 1.6 units/mg of protein, over at least 30 min.

As shown in Table 1, when the tonoplast ATPase was inhibited by DCCD, NEM, DIDS or

TMT (Marin, 1983b), no significant changes of ΔpH and Δψ were observed, thus indicating a relationship between the activity of the tonoplast ATPase and the magnitude of the electrochemical proton gradient (Δμ_H⁺) across the tonoplast membrane.

As this tonoplast-bound ATPase is pumping protons inwardly, electrogenically leading to the establishment of Δμ_H⁺, as for the other proton pumps [see Amzel & Pedersen (1983) and Maloney (1982)], it would be expected that the rate of the ATPase activity would be linked to the magnitude of Δμ_H⁺. Consequently, the relationship between tonoplast-bound ATPase activity and Δμ_H⁺ was investigated under a variety of conditions where the two components of the Δμ_H⁺ were modified either alone or together.

Effect of the collapse of ΔpH on the tonoplast ATPase activity

The results shown in Table 2 indicate that NH₄⁺ [as NH₄Cl or (NH₄)₂SO₄], methylamine and ethylamine were able to decrease the acidity of the tonoplast vesicles. When added at 50 mM, the original transtonoplast ΔpH was almost completely dissipated and, in parallel, the ATPase activity was stimulated by 76–109% (Table 2). This stimulation of the tonoplast ATPase could be shown to be related to the dissipation of the original ΔpH. Thus, as the concentration of (NH₄)₂SO₄ in the incubation medium was increased, the ΔpH decreased and the tonoplast ATPase increased (Fig. 1). The relationship between the magnitude of transtonoplast ΔpH and the extent of tonoplast ATPase activity is shown in Fig. 1. In addition, the ATPase activity of the fragmented tonoplast membranes and the ATPase activity solubilized from these membranes (Marin & Gidrol, 1985) were relatively insensitive to NH₄⁺ and to SO₄²⁻. Consequently, as this increase of tonoplast ATPase activity was not associated with some salt effect, and was not observed in the presence of a detergent concentration sufficient to disrupt the tightly sealed vesicles (Triton X-100 at

Table 1. Effect of MgATP on the electrochemical proton gradient in *Hevea* tonoplast vesicles
Tonoplast vesicles were incubated for 30 min at 30°C, as described in the Materials and methods section, with the additions indicated.

Addition(s)	ΔpH	Δψ (mV)	ATPase activity (units/mg of protein)
None (control)	0.60	-140	-
+ 5 mM-MgATP	1.51	-20	1.10
+ 5 mM-MgATP + 0.8 mM-DCCD	0.71	-135	0.11
+ 5 mM-MgATP + 0.4 mM-NEM	0.65	-130	0.05
+ 5 mM-MgATP + 0.1 mM-DIDS	0.91	-130	0.17
+ 5 mM-MgATP + 0.1 mM-TMT	0.70	-130	0.15

Table 2. *Effect of ΔpH collapse on tonoplast ATPase activity*

Tonoplast vesicles were preincubated for 30 min at 30°C, as described in the Materials and methods section, with the additions indicated, and the ΔpH and $\Delta\psi$ were measured. The ATPase activity was then initiated by the addition of 5 mM-ATP and 5 mM-MgSO₄ at an external pH of 7.0. Abbreviations used: n.d., not determined; HCl, hydrochloride.

Addition(s)	ΔpH	$\Delta\psi$ (mV)	ATPase activity (units/mg of protein)
None (control)	0.7	-140	1.20
50 mM-(NH ₄) ₂ SO ₄	0.025	n.d.	2.51
50 mM-NH ₄ Cl	n.d.	n.d.	2.68
50 mM-Methylamine HCl	0.1	n.d.	2.32
50 mM-Ethylamine HCl	0.1	n.d.	2.11
2 μM -SF-6847	n.d.	n.d.	1.91
0.05 μM -Nigericin	0.8	-136	1.14
0.05 μM -Nigericin + 10 mM-K ₂ SO ₄	0.1	-140	2.30

Fig. 1. *Dependence of tonoplast ATPase activity on ΔpH*

(a) Reduction of transtonoplast pH gradient in the presence of (NH₄)₂SO₄ and (b) effect of this decreased ΔpH on the activity of the tonoplast ATPase. The experimental conditions were identical with those of Table 2, except that 255 mg of intact tonoplast vesicles were present. In addition, small titrating amounts of (NH₄)₂SO₄ were also added in a final volume of 2.0 ml. ΔpH was measured just before the initiation of the reaction by the addition of 5 mM-ATP and 5 mM-MgSO₄.

0.05%), it is likely that this stimulation is related to the dissipation of the initial transtonoplast ΔpH .

Another way to dissipate the transtonoplast ΔpH is to increase the relatively low H⁺-permeability of the tonoplast by the use of protonophores such as S-13, SF-6847, FCCP or 2,4-DNP. These molecules permit H⁺ to equilibrate according to its electrochemical potential. As shown in Table 2, their presence in the incubation medium resulted in a stimulation of the tonoplast ATPase activity between 53 and 85%. This effect was dose-dependent (Fig. 2). In each case, at low concentra-

tions the protonophore stimulated the tonoplast ATPase activity but, at higher concentration, it inhibited the ATPase. The extent of this stimulation varied with the preparation used, but the same dependency on the concentration of protonophore was always observed. It was actually difficult to understand how these different protonophores affect the ATPase when used at high concentrations.

As shown in Table 2, other ionophores have also been used to control the ΔpH . Nigericin is a carboxylic carrier-type ionophore that allows an

Fig. 2. Effect of protonophores upon ATPase activity of tonoplast vesicles

The experimental conditions were identical with those of Fig. 1, except that 20 mM-Tris/Pipes, pH 7.0, was present, together with the concentrations of S-13 (a), FCCP (b) or 2,4-DNP (c) indicated in the Figure.

exchange between K^+ and H^+ (Pressmann, 1976; Reed, 1979). At low concentration (≤ 50 nM), nigericin affected the ΔpH without modifying the membrane potential, when the tonoplast vesicles were incubated in the presence of K_2SO_4 (Fig. 3). With these low concentrations of nigericin, and in presence of 10 mM- K_2SO_4 , tonoplast ATPase was stimulated by 92% (Table 2).

In addition, when the tonoplast vesicles were exposed to Triton X-100 (0.05%), a procedure that destroys the integrity of the tonoplast membrane, or when the control experiments were carried out with solubilized ATPase (Marin & Komor, 1984), no significant effects by the different molecules tested, as listed in Table 2, were observed. Consequently, this stimulation of tonoplast ATPase involves the integrity of tonoplast vesicles.

All these data indicate clearly that the tonoplast ATPase responds to changes of ΔpH through the tonoplast membrane.

Effect of the collapse of $\Delta\psi$ on the tonoplast ATPase activity

Tonoplast vesicles incubated in the absence of any permeant anions were found to possess a resting membrane potential of -140 ± 20 mV, as measured by the distribution of [^{14}C]TPMP $^+$ (Marin, 1983a). This membrane potential could be decreased by a variety of methods (Table 3). Thus the addition of 5 mM-TPP $^+$ or 5 mM-TPMP $^+$ to the incubation medium was followed by an almost complete dissipation of the membrane potential that was associated with an inhibition of about 15% of the ATPase activity. These cations did not affect the activity of solubilized ATPase (results not shown).

In the absence of any K^+ in the incubation medium, valinomycin addition did not change the

two components of $\Delta\bar{\mu}_H^+$ (Table 3), and there was no effect on the ATPase activity. But, in the presence of K_2SO_4 , valinomycin addition decreased $\Delta\psi$ (Table 3). When ATPase activity was measured under similar conditions, there was a slight stimulation at low concentrations of valinomycin (0–2 μM) and a greater inhibition at higher concentrations (generally above 3 μM) (Fig. 4). As this ionophore reacts non-competitively with $MgATP^{2-}$ over this range of concentrations, it would be expected that valinomycin would interact with some sites essential for the ATPase activity.

The membrane potential could be decreased by incubating the tonoplast vesicles in the presence of different concentrations of K_2SO_4 and low concentration of valinomycin (0.5 μM) (Fig. 5). Under these conditions it was observed that ATPase activity was decreased markedly as the tonoplast membrane potential became more positive (Fig. 6).

In addition, the ATPase activity of the fragmented tonoplast membranes and the ATPase activity solubilized from these membranes (Marin & Komor, 1984) were insensitive to the different treatments used to collapse $\Delta\psi$ (described in Table 3).

Thus it can be concluded that dissipation of the tonoplast negative potential inhibits the tonoplast ATPase activity.

Effect of the collapse of the two components of the proton electrochemical gradient on the tonoplast ATPase activity

When the magnitude of the proton electrochemical gradient ($\Delta\bar{\mu}_H^+$) after the various treatments specified in Tables 2 and 3 is plotted against the ATPase activity, the relationship shown in Fig. 7 is obtained.

Fig. 3. Effect on nigericin on the magnitude of the proton electrochemical gradient across the tonoplast membrane. Tonoplast vesicles were incubated for 30 min at 30°C with 5 mM-ATP and 5 mM-MgSO₄ at an external pH of 7.0. The incubation medium was supplemented with nigericin at the concentration indicated in presence of 10 mM-K₂SO₄. Δψ (○) and ΔpH (Δ) were measured in the same preparations of tonoplast vesicles. The magnitude of the proton electrochemical gradient (●) was calculated from the values of Δψ (interior-negative) and ΔpH (interior-acid) by using the following equation:

$$\Delta\bar{\mu}_{H^+} = \Delta\psi - 58.8\Delta pH$$

At pH 7.0, the optimum pH₀ value for the tonoplast ATPase activity, the Δμ_H⁺ varied between -83 mV (Table 3) and -93 mV (Table 2) for the results reported in the present paper. As ΔpH and Δψ contributed to Δμ_H⁺, the tonoplast ATPase does not sense and respond to ΔpH independently of the membrane potential. Consequently, this would not be significantly different from the situation found with the mitochondrial and thylakoid enzymes; even the structure of the tonoplast enzyme seems to be different (Marin, 1984; Marin & Komor, 1984).

Fig. 4. Effect of valinomycin concentration on ATPase activity of tonoplast vesicles

The experimental conditions were identical with those of Fig. 1, except that 20 mM-Tris/Pipes, pH 7.0, was present, together with the amount of valinomycin indicated in the Figure and in the presence of 10 mM-K₂SO₄.

Inhibitors

When ATP-dependent changes in ΔpH and in Δψ were measured in tonoplast vesicles in the presence of various concentrations of the ATPase inhibitors DCCD or TMT, inhibition of proton translocation paralleled inhibition of ATPase activity (Fig. 8). If one of the two components of Δμ_H⁺ was dissipated before the addition of these inhibitors, no subsequent stimulation of tonoplast ATPase was observed (results not shown). Consequently, the ATPase being inhibited by the inhibitor can no longer be stimulated by an appropriate adjustment of Δμ_H⁺. This regulation by Δμ_H⁺ could involve a component (or a site) sensitive to TMT and to DCCD similar to that described for the chloroplast ATPase activity (Gould, 1976).

Discussion

The present results are interpreted to show that the activity of the *Hevea* tonoplast ATPase is effectively modulated by changes in the transmembrane H⁺ gradient across the tonoplast. A similar suggestion has been made for the electrogenic H⁺ translocase on vacuoles from *Saccharomyces cerevisiae* (Okorokov & Lichko, 1983). A relationship between ATPase activity and Δμ_H⁺ helps to explain otherwise-inconclusive information found in the literature concerning the activity of tono-

Table 3. *Effect of Δψ collapse on tonoplast ATPase activity*
The experimental conditions were as described for Table 2.

Addition(s)	ΔpH	Δψ (mV)	ATPase activity (units/mg of protein)
None (control)	0.8	-130	1.05
5mM-TPP ⁺	0.7	-10	0.91
5mM-TPMP ⁺	0.7	-12	0.88
0.5 μM-Valinomycin	0.8	-130	1.10
0.5 μM-Valinomycin + 10mM-K ₂ SO ₄	0.8	-68	0.85
5 μM-Valinomycin	0.8	-125	1.02
5 μM-Valinomycin + 10mM-K ₂ SO ₄	0.8	-10	0.88
1 mM-NH ₄ Cl	0.4	-71	0.96

Fig. 5. *Effect of valinomycin on the magnitude of the proton electrochemical gradient across the tonoplast membrane*
The experimental conditions were identical with those of Fig. 4, except that the incubation medium was supplemented with valinomycin at the concentration indicated in presence of 10mM-K₂SO₄. O, Δψ; Δ, -ΔpH; ●, Δμ_H⁺.

plast-bound ATPase from fungi and higher plants (reviewed by Marin, 1985).
Tonoplast vesicles prepared from *Hevea* latex can be regarded as tightly sealed (Marin, 1982). They constitute a homogeneous population of closed

Fig. 6. *Dependence of tonoplast ATPase activity on Δψ*
The experimental conditions were identical with those of Fig. 1, except that 180mg of intact tonoplast vesicles were present. In addition, small titrating amounts of K₂SO₄ in the presence of 5 μM-valinomycin were also added in a final volume of 2.0ml. Δψ was measured just before the initiation of the reaction by the addition of 5mM-ATP and 5mM-MgSO₄.

structures where the tonoplast ATPase is exposed to the external medium. They are considered as active and capable of generating a Δμ_H⁺ (Marin *et al.*, 1981b; Marin, 1982, 1983a). The present results indicate that the *Hevea* tonoplast ATPase is responsive to changes in both components of the electrochemical proton gradient. Thus collapse of the ΔpH from 1.0 to 0.1pH unit with different treatments resulted in increased rates of ATP hydrolysis. Whereas altering the initial negative membrane potential to less negative values result-

Fig. 7. Dependence of tonoplast ATPase activity on the magnitude of the proton electrochemical gradient across the tonoplast membrane

This is a compilation of the data shown in Tables 2 and 3. The magnitude of the proton electrochemical gradient was calculated by using the following equation:

$$\Delta\mu_{H^+} = \Delta\psi - 58.8 \Delta\text{pH}$$

ed in decreased rates of ATP hydrolysis, it was also observed that, when the initial membrane potential was very negative (from -150 to -160 mV), the rate of ATP hydrolysis could become even greater than usual (Fig. 6 and Table 3).

Consequently, at tonoplast level, any process which consumes $\Delta\mu_{H^+}$ is able to re-activate the tonoplast ATPase. This is the case for the citrate translocator in *Hevea* latex (Marin, 1982). Thus citrate uptake caused a transient hyperpolarization of tonoplast membrane. In parallel, the tonoplast ATPase activity was stimulated. The data reported here help to explain how citrate stimulates the tonoplast ATPase of *Hevea* latex. The uphill transport of citrate across the *Hevea* tonoplast is supported by the $\Delta\mu_{H^+}$ energy formed at the expense of ATP hydrolysis catalysed by the tonoplast H^+ -translocating ATPase. This transport operates in the $\Delta\mu_{H^+}$ -consuming direction and consequently, as the hydrolytic activity of tonoplast ATPase is controlled by $\Delta\mu_{H^+}$, any change of this $\Delta\mu_{H^+}$ re-activates it immediately. This is true of all the known energy-dependent transport in vacuoles (Marin *et al.*, 1982; Marin, 1985).

The present data clearly indicate control of the tonoplast ATPase by $\Delta\mu_{H^+}$. However, it is too early to attempt a complete description of this control from all the data described for this enzyme (Marin, 1985). At present it is thought that, as suggested for the chromaffin-granule H^+ -transporting ATPase

Fig. 8. Effect of trimethyltin and DCCD on the tonoplast ATPase activity and on the proton-pump activity of tonoplast vesicles. Tonoplast vesicles ($100 \text{ mg of protein} \cdot \text{ml}^{-1}$) were incubated in the presence of different radioactive probes used to monitor ΔpH (b) and $\Delta\psi$ (c), and TMT (●) or DCCD (○) at the indicated concentrations. After 30 min of incubation at 30°C , samples were taken off, centrifuged and processed as described in the Materials and methods section. ΔpH was estimated to 0.8 and $\Delta\psi$ was calculated as -125 mV . The ATPase reaction (a) was initiated by the addition of 5 mM-ATP and 5 mM-MgSO_4 . Control ATPase activity was $0.35 \text{ unit/mg of protein}$ under these experimental conditions.

(Johnson *et al.*, 1982), once the maximum proton gradient is established through the tonoplast, the magnitude being based on the thermodynamic properties of the proton pump and the proton leak or backflow through the membrane, any perturbation of the equilibrium state should result in an increased rate of ATP hydrolysis as the enzyme attempts to re-establish the initial gradient. Such a property is characteristic of any system involved in chemiosmotic proton circuits in biological membranes (Skulachev, 1981). The tonoplast ATPase may be considered as a new $\Delta\bar{\mu}_{H^+}$ generator. Nevertheless, this enzyme belongs to a new class of proton-translocating ATPases, an intermediate type between the F_1F_0 -type enzyme (such as the reversible proton-translocating ATPase of mitochondria, chloroplasts and bacteria) and the other proton (or ion)-translocating enzymes, as classified and defined by Maloney (1982). Recent data described for the vacuolar membrane ATPase of *Neurospora crassa* confirm this point of view (Bowman, 1983). Consequently, it becomes important to develop further studies on the modalities of the regulation of tonoplast ATPase by $\Delta\bar{\mu}_{H^+}$, according to the model procedure described for the bacteriorhodopsin proton pump (Hellingwerf, 1979).

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References

- Amzel, L. M. & Pedersen, P. L. (1983) *Annu. Rev. Biochem.* **52**, 801–824
- Bowman, E. J. (1983) *J. Biol. Chem.* **258**, 15238–15244
- Crétin, H. (1982) *J. Membr. Biol.* **65**, 175–184
- D'Auzac, J., Crétin, H., Marin, B. & Lioret, C. (1982) *Physiol. Vég.* **20**, 311–331
- Gould, J. M. (1976) *Eur. J. Biochem.* **62**, 567–575
- Guilliermond, A. (1941) *The Cytoplasm of Plant Cell*, pp. 1–247, Chronica Botanica Co., Waltham, MA
- Heinonen, J. K. & Lahti, R. J. (1981) *Anal. Biochem.* **113**, 313–317
- Hellingwerf, K. (1979) Ph.D. Dissertation, University of Amsterdam
- Johnson, R. G., Beers, M. F. & Scarpa, A. (1982) *J. Biol. Chem.* **257**, 10701–10707
- Lowry, O. H., Rosebrough, R. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maloney, P. C. (1982) *J. Membr. Biol.* **67**, 1–12
- Marin, B. (1982) *Trav. Doc. ORSTOM* **144**, 1–409
- Marin, B. (1983a) *Planta* **157**, 324–330
- Marin, B. (1983b) *Plant Physiol.* **73**, 973–977
- Marin, B. (1984) *Abstr. Congr. Eur. Soc. Plant Physiol.* 4th, Strasbourg, 29 July–3 August 1984 458
- Marin, B. (1985) *Biochemistry and Function of Vacuolar Adenosine Triphosphatase in Fungi and Plants*, Springer-Verlag, Heidelberg, p. 259
- Marin, B. & Gidrol, X. (1985) *Biochem. J.* **226**, 85–94
- Marin, B. & Komor, E. (1984) *EBEC Reports*, vol. 3A (*Eur. Bioenerg. Conf.* 3rd, Hannover, 2–7 September 1984) (Schäfer, G., ed.), pp. 389, Congress-Edition, Hannover
- Marin, B. & Trouslot, P. (1975) *Planta* **124**, 31–41
- Marin, B., Marin-Lanza, M. & Komor, E. (1981a) *Biochem. J.* **198**, 365–372
- Marin, B., Smith, J. A. C. & Lüttge, U. (1981b) *Planta* **153**, 486–493
- Marin, B., Crétin, H. & D'Auzac, J. (1982) *Physiol. Vég.* **20**, 333–346
- Ohsumi, Y. & Anraku, Y. (1981) *J. Biol. Chem.* **256**, 2079–2082
- Okorokov, L. A. & Lichko, L. P. (1983) *FEBS Lett.* **155**, 102–106
- Pressmann, B. C. (1976) *Annu. Rev. Biochem.* **45**, 501–530
- Reed, P. W. (1979) *Methods Enzymol.* **55**, 435–454
- Skulachev, V. P. (1981) in *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P. & Hinckle, P. C., eds.), pp. 3–46, Addison-Wesley Publishing Co., London
- Taussky, H. H. & Shorr, E. (1953) *J. Biol. Chem.* **202**, 675–685
- Thom, M. & Komor, E. (1984) *Eur. J. Biochem.* **138**, 93–99
- Wagner, G. J. (1982) *Recent Adv. Phytochem.* **16**, 1–45
- Zirkle, C. (1937) *Bot. Rev.* **3**, 1–30