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Review

Energization of solute transport and accumulation at the tonoplast in *Hevea* latex

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Abstract. Some translocators of ions (Ca^{2+}, phosphate) and organic molecules (citric acid, lysine) are associated with the lutoidic membrane (tonoplast) of *Hevea brasiliensis* latex. They assure an uptake against a transmembrane concentration gradient. Their functioning is coupled with tonoplast ATPase activity, which ensures an active proton influx. Furthermore, vacuolar solutes appear to exist as two kinetically-distinct pools. The first pool corresponds to the permeant form of the solute. Its size is generally limited. The second one corresponds to the different molecules trapped inside the vacuome by different mechanisms. It contains the greater part of vacuolar solutes. The mechanism of tonoplast solute translocators has been studied. Hence, Ca^{2+} and citric acid appear to be uptaken according to an antiport mechanism which involves protons. Their relationships with the tonoplast proton pump are defined. No direct coupling exists between the carrier and the proton pump.

Key words: ATPase, lutoids, proton pump, tonoplast, solute transport, Hevea.

Résumé. *Un certain nombre de transporteurs d'ions (Ca^{2+}, phosphate, ...) et de molécules organiques (citrate, lysine, ...) sont associés à la membrane des lutoïdes (tonoplaste) du latex* d'Hevea brasiliensis. *Ils assurent l'absorption de ces solutés contre un gradient transmembranaire de concentration. Leur fonctionnement se trouve couplé à l'activité ATPase tonoplastique, qui assure un transport actif de protons. Par ailleurs, tout se passe comme s'il existait un pool de solutés de taille réduite, accessible à leur transporteur, et un pool de solutés piégés par différents mécanismes, ce dernier représentant l'essentiel du contenu vacuolaire. Des mécanismes de fonctionnement des différents transporteurs tonoplastiques étudiés sont proposés. Ainsi, le Ca^{2+} et le citrate seraient incorporés selon un mécanisme antiport assurant l'échange entre des molécules de solutés externes et des protons internes. Leurs relations avec la pompe à protons sont établies. En aucun cas, il ne peut s'agir de couplage direct.*

Mots clés : ATPase, lutoïdes, pompe à protons, tonoplaste, transport des solutés, *Hevea.*

Abbreviations. DCCD, *N*, *N'*-dicyclohexyl-carbodiimide; ΔpH, transmembrane pH gradient; ΔΨ, transmembrane electric potential difference; 2,4-DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl-cyanide *p*-trifluoromethoxyphenyl hydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino) ethanesulfonic acid; MOPS, 3-(*N*-morpholino) propanesulfonic acid; NEM, *N*-ethyl-maleimide; *p*CMB, *p*-chloromercuribenzoic acid; *p*-NPP, *p*-nitrophenyl-phosphate; S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4-nitro-salicylanilide; TEA, triethanol amine (or 2, 2', 2''-nitrilotriethanol); $TPMP^+$, triphenyl-methyl-phosphonium ion; TPP^+, tetraphenyl-phosphonium ion; Tris-base, *tris*-(hydroxymethyl)-aminomethane.

INTRODUCTION

The lutoids of *Hevea brasiliensis* latex form a dispersed vacuolar compartment whose properties are analogous to those of the central vacuome of higher plants (D'AUZAC *et al.*, 1982). *In vitro* these vacuoles take up such vari

phosphate, arginine, citrate or lysine against a transmembrane concentration gradient (RIBAILLIER, 1972; D'AUZAC and LIORET, 1974; D'AUZAC *et al.*, 1977 *a*; HANOWER *et al.*, 1977). This uptake occurs in an incubation medium, without the aid of energetic substrates, such as ATP. However, the presence of Mg-ATP with the vacuoles in the incubation medium or even in preincubation leads to an increase in the uptake level of the solutes (Ca^{2+}, citrate, arginine, lysine) which can be as much as twofold for citric acid, for example (D'AUZAC and LIORET, 1974). These uptakes correspond to accumulations and not to mere isotopic exchanges.

Yet, Mg-ATP tends to acidify the intravacuolar medium (LAMBERT, 1975). Protonophores, such as NH_4Cl or 2,4-DNP, which decrease the amplitude of the transmembrane pH gradient, cause a considerable reduction in the uptake of citrate and lysine (D'AUZAC and LIORET, 1974; D'AUZAC *et al.*, 1977 *a*; HANOWER *et al.*, 1977). These findings have obviously led to the hypothesis of an energetic coupling of the transports and an ATPase activity, this coupling being made possible by the intermediary of a pH gradient across the tonoplast.

Two materials, one derived from the other, were examined in order to test the validity of this hypothesis: on the one hand, the lutoids (vacuoles) of *Hevea brasiliensis* latex; and, on the other hand, the vesicles formed from the lutoidic (tonoplastic) membranes.

Characterization and properties of tonoplastic ATPase

The vacuoles of *Hevea* latex contain a high acid phosphatase activity capable of hydrolyzing ATP (PUJARNISCLE, 1971). The presence of this activity, typical of the vacuolar compartment of higher plants (NISHIMURA and BEEVERS, 1978; BOLLER and KENDE, 1979; BRISKIN and LEONARD, 1980), places a constraint on the demonstration of a specific ATPase activity associated with the tonoplast.

Inhibition of acid phosphatase activity by Mo

The addition of 0.1 mM ammonium molybdate (JACOB and SONTAG, 1974) to a reaction medium containing vacuolar membranes totally inhibits acid phosphatase activity, followed by the hydrolysis of *p*-NPP, but does not affect the hydrolyzing ATP activity, which may be imputed to a potential ATPase (D'AUZAC, 1975; D'AUZAC *et al.*, 1977 *b*).

The method, introduced by D'AUZAC (1975), entails the inhibition of vacuolar acid phosphatase activity by ammonium molybdate and has enabled the confirmation of such an ATPase activity on the isolated *Beta vulgaris* tonoplast (LEIGH and WALKER, 1980; ADMON *et al.*, 1981). WAGNER (1981) has obtained the same result on the vacuole tonoplast isolated from *Hippeastrum* petals, by inhibiting acid phosphatase activity with potassium fluoride.

Intrinsic properties of tonoplastic ATPase activity

D'AUZAC (1977) proceeded with a biochemical analysis of the tonoplastic ATPase activity of *Hevea* latex. This activity hydrolyzes the ATP into ADP and Pi. It is remarkable that Pi does not inhibit the reaction. In contrast, ADP proves to be a noncompetitive inhibitor. The value of Ki is estimated at 0.4 mM. The optimum pH varies according to the nature of the buffer: it ranges from 6.6 (potassium phosphate medium, HEPES-Tris medium) to 7.75 (TEA-HCl, Tris-HCl, HEPES-NaOH media) (D'AUZAC, 1977; MARIN, unpublished results).

The ATPase activity is specific to ATP. Other nucleotides prove to be less efficient substrates. When the ATP/Mg^{2+} ratio is 0.5, the value of K_m for the ATP ranges from 0.4 to 0.8 mM. The total inhibition of ATPase activity, observed in the

presence of EDTA, indicates that the presence of a divalent cation is indispensable. Among the different cations tested, Mg^{2+} and Mn^{2+} seemed the most efficient. The optimum is obtained when the ATP/cation ratio is about 1.

In contrast, this activity is not affected by monovalent cations such as K^+ and Na^+. It does not seem justified, in light of the different results obtained with *Hevea* as well as with other plants, to compare this tonoplastic activity with the classic animal Na^+/K^+ ATPase activity.

An original aspect is the activation observed upon addition of mineral anions (Cl^-, HCO_3^-) or organic anions (malate, succinate, aspartate, fumarate, lysine). It can attain 150% of the initial activity. Activations by anions were first reported for a microsomal ATPase activity in turnip roots (RUNGIE and WISKICH, 1973).

The study of the action of diverse inhibitors shows that the tonoplastic ATPase is affected by the SH group reagents (*p*CMB, mersalyl, NEM). The inhibition observed never exceeds 50% of the initial activity, in the range of the concentrations used. Furthermore, the uncouplers are only efficient when they are used at relatively high concentrations (about 1 mM). In these conditions FCCP and DCCD inhibit by 50%. S-13 is just as effective (MARIN, 1981).

In no way does this involve an adsorbed or membranous activity from plasmalemma or mitochondrial contaminations, the inhibitors of these activities being without effect on this tonoplastic ATPase (MARIN and D'AUZAC, unpublished results).

Tonoplastic ATPase as an electrogenic proton pump

This tonoplastic ATPase activity has been characterized similarly on freshly isolated vacuoles (CRÉTIN, 1982), on ice-conserved vacuoles, and on tonoplastic vesicles obtained from lyophilized vacuolar membranes (MARIN, 1981).

The variation of the transtonoplastic pH gradient (ΔpH) and of the electrical potential difference ($\Delta\Psi$) across the tonoplast caused by ATP hydrolysis has been estimated using two different methodologies: flow dialysis (CRÉTIN, 1982) and differential centrifugation, with or without silicone layers (MARIN, 1981; MARIN *et al.*, 1981 *a* and *b*). The transmembrane ΔpH is calculated from the distribution, at equilibrium, of ^{14}C-methylamine; the $\Delta\Psi$ is obtained from that of a phosphonium ion (^3H-TPP^+ or ^{14}C-$TPMP^+$) or $^{86}Rb^+$ ion (the latter ion in the presence of valinomycin) (MARIN, 1981; MARIN *et al.*, 1981 *a* and *b*; CRÉTIN, 1982).

Methylamine accumulates in the vacuoles' internal medium or in tonoplastic vesicles indicating an acid internal pH. In the case of fresh vacuoles, this pH is about 5.5 to 6.0 when that of the medium is about 7.0 to 7.5 (MARIN *et al.*, 1981 *a*; CRÉTIN, 1982). In the case of the tonoplastic vesicles, this internal pH is a function of the medium pH initially used in their preparation (MARIN, 1981). Nevertheless, any addition of Mg-ATP to the reaction medium containing the vacuoles leads to a rapid increase in the quantity of methylamine initially accumulated (*fig.* 1 A and B) indicating an increase of transtonoplastic ΔpH (CRÉTIN *et al.*, 1982; MARIN, 1981; MARIN *et al.*, 1981 *a* and *b*; CRÉTIN, 1982). The addition of Mg-ATP is thus immediately followed by a net influx of protons which increases the internal pH. This acidification of the internal medium is specific to Mg-ATP. It is not observed in the presence of Mg-ADP or $MgSO_4$.

Phosphonium ions ($TPMP^+$, TPP^+) and ^{86}Rb (in the presence of valinomycin) accumulate in the vacuoles and in the tonoplastic vesicles which suggests a transmembrane electric potential difference, negative inside. In the case of fresh vacuoles, the estimated $\Delta\Psi$ in a medium containing 10 mM KCl is about -65 to

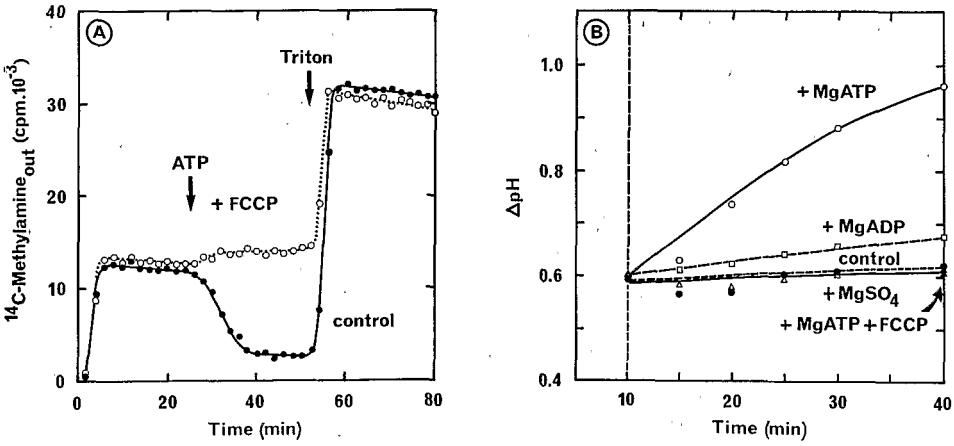

FIG. 1. — Time-course of change of ΔpH across the tonoplast (inside acid) and the effect of ATP and FCCP as measured by accumulation of methylamine.

A, fresh vacuoles were preloaded 20 min with ^{14}C-methylamine (6 μCi.ml^{-1}) in the presence (○– – –○) or absence (●———●) of FCCP (3.3 μM) at medium pH 7.0 (KCl absent). Then, the suspension was transferred to the upper chamber of the flow dialysis cell, as described elsewhere (CRÉTIN, 1982). As indicated by arrows, MgATP (5 mM final, pH 7.0) was added in both assays. The experiments were ended by addition of 100 μl Triton X-100 (1%; w/w). The radioactivity present in the exit flux was monitored during the experiment. It represents the changes in the external pH medium. B, tonoplast vesicles were incubated for 30 min in the usual medium, at pH 7.5, as described elsewhere (MARIN, 1981), in presence of ^3H_2O (0.5 μCi.ml^{-1}) and either ^{14}C-methylamine (0.1 μCi.ml^{-1}) or ^{14}C-dextran (0.1 μCi.ml^{-1}). The samples were preincubated for 10 min, after which the following were added (all at 5 mM, pH 7.5): ○———○, MgATP; □– – –□, MgADP; △———△, $MgSO_4$; ▲, MgATP+FCCP (1 mM); ●---●, control (no additions). The ΔpH was calculated from the accumulation factor of methylamine (MARIN, 1981; MARIN *et al.*, 1981 *a* and *b*).

−80 mV according to the nature of the probe used (CRÉTIN, 1982). In the case of tonoplastic vesicles, without KCl in the medium, it is about −110 to −120 mV (MARIN, 1981; MARIN *et al.*, 1981 *b*). In both materials, the addition of Mg-ATP is immediately followed by a rapid change in the transmembrane distribution of the potential probes (*fig.* 2 A and B), which corresponds to a membrane depolarization of 50 to 80 mV.

Moreover, the addition of such molecules as 2,4-DNP, FCCP or S-13 to a tonoplastic vesicle suspension is immediately followed by simultaneous variations of ΔpH and ΔΨ (MARIN, 1981). This is also observed on fresh vacuoles (CRÉTIN, 1982). In addition, the variation of external pH affects in a consistent manner the ATPase activity and ΔpH or ΔΨ (*fig.* 3) (MARIN, 1981).

The tonoplastic ATPase of *Hevea* latex is thus capable when working *in situ*, of changing the ΔpH and the ΔΨ. The hypothesis retained is that the tonoplastic ATPase activates an electrogenic proton pump (MARIN, 1981; MARIN *et al.*, 1981 *a* and *b*; CRÉTIN, 1982).

Relationships between the accumulation of solutes and the proton-motive force

Influence of transtonoplastic ΔpH on solute uptake

Several methods have been employed to change the amplitude of the transtonoplastic pH gradient of fresh vacuoles or of vacuolar vesicles. The addition

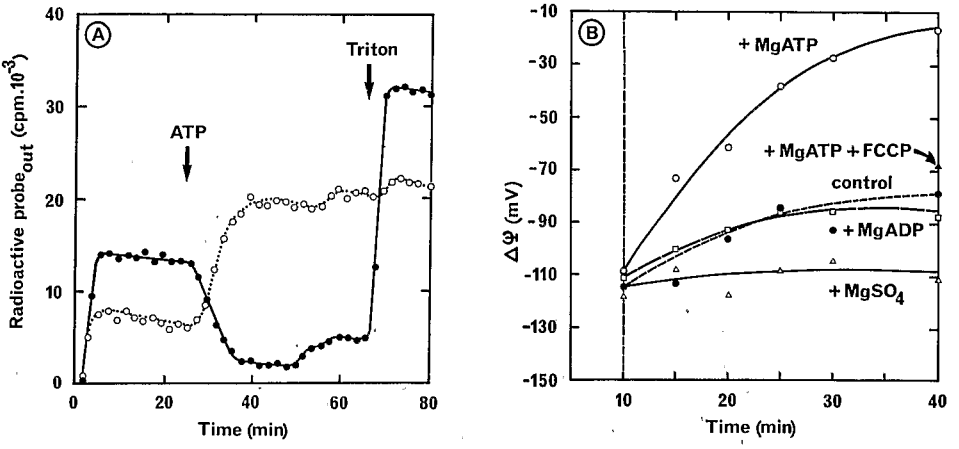

FIG. 2. — Time-course of change in $\Delta\psi$ across the tonoplast (inside negative) and the effect of ATP and some effectors as measured by accumulation of Rb^+ and $TPMP^+$.

A, the experiment was conducted on fresh vacuoles, as described in figure 1-A, with some modifications, in presence of KCl (10 mM), ^{14}C-methylamine (6.6 μCi.ml^{-1}), ^{86}Rb (5 μCi.ml^{-1}) and valinomycine (10 μg.ml^{-1}). Additions were as follows: Mg-ATP (5 mM final, pH 7.0), then KCl (120 mM, pH 7.0) and finally 100 µl Triton X-100 (1%; w/w). ●———●, ^{14}C-methylamine evolution in exit flux (external medium); ○- - -○, ^{86}Rb evolution in exit flux (from CRÉTIN, 1982). B, experimental conditions used with tonoplast vesicles were identical to those in figure 1 B except that ^{14}C-methylamine was replaced by ^{14}C-TPMP$^+$ (0.1 μCi.ml^{-1}). Additions were as follows (all at 5 mM, pH 7.5): ○———○, MgATP; □———□, MgADP, △———△; $MgSO_4$; ▲, MgATP+FCCP (1 mM); ●- - -●, control (no additions) (from MARIN, 1981 and MARIN *et al.*, 1981 *b*).

of NaOH or HCl in the reaction medium has been shown to be sufficient to respectively increase or decrease the preexistent ΔpH (MARIN, 1981; CRÉTIN, 1982). If tonoplastic vesicles are loaded with KCl during their preparation, any addition of nigericin still cause an acidification of the internal medium by an exchange H^+/K^+ (*fig.* 4 A). The incubation of fresh vacuoles or of tonoplastic vesicles with the ionophore A-23187, whether or not in the presence of $CaCl_2$ (or of $MgCl_2$),

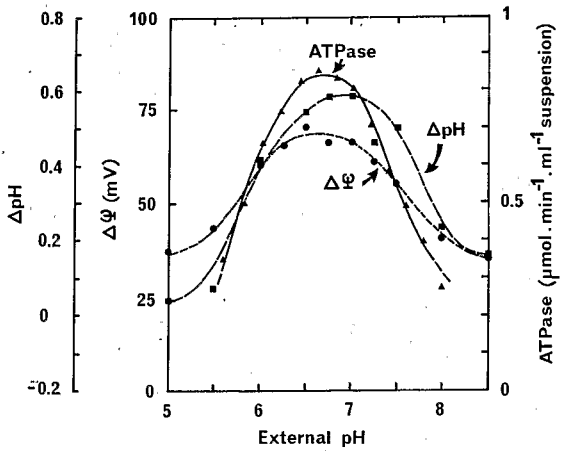

FIG. 3. — pH-dependence of ATP hydrolysis, ATP-induced $\Delta\psi$ change and ATP-induced ΔpH change by tonoplast vesicles.

Tonoplast vesicles were incubated in the usual medium (as in figure 1 B). The pH was adjusted to the desired value by addition of Tris-base. MgATP was added at a final concentration of 5 mM after 10 mn incubation. ^{14}C-TPMP$^+$ or ^{14}C-methylamine were added to give 0.1 μCi.ml^{-1}. The transtonoplastic distribution of lipophilic ions or Pi release were followed for 30 min: ▲———▲, ATP hydrolysis; ●---●, ATP-induced $\Delta\psi$ change; ■———■, ATP-induced ΔpH change (from MARIN, 1981).

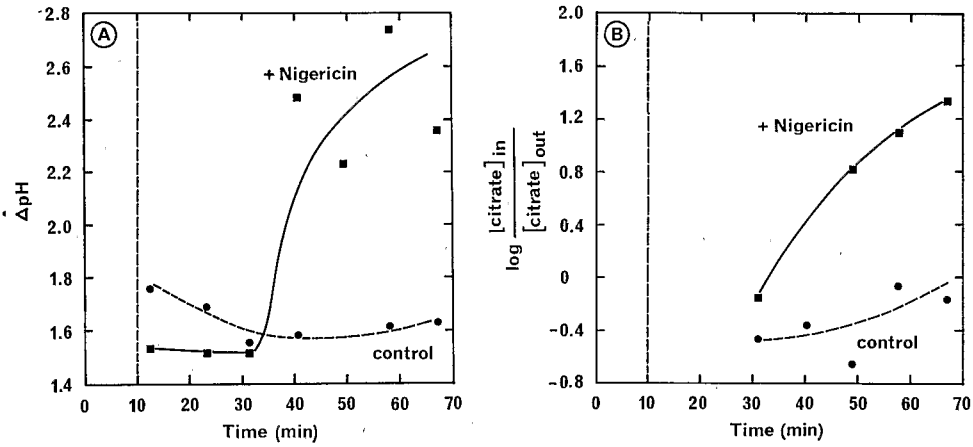

FIG. 4. — Effect of nigericin on ΔpH and citrate accumulation by tonoplast vesicles.

The vesicles were generated in a medium in which the usual osmoticum mannitol (300 mM) was replaced by KCl (185 mM) and in which the buffers were at 5 mM instead of 25 mM. The pH was 7.5. The incubation in presence of ^{14}C-methylamine (0.1 μCi . ml^{-1}) and $^{3}H_2O$ (0.5 μCi . ml^{-1}) was conducted as described in figure 1 B. After 31 min, nigericin (10 μg . ml^{-1}) was added to the medium. The ^{14}C-citrate assay was carried out as described elsewhere (MARIN, 1981). The samples were incubated with ^{14}C-citric acid (0.5 μCi . ml^{-1}) and $^{3}H_2O$ (2.5 μCi . ml^{-1}). ΔpH was calculated from the accumulation factor of methylamine (MARIN, 1981). Citrate uptake was expressed as log of the accumulation ratio, where C_{in} = internal concentration and C_{out} = external concentration. ●---●, control; ■———■, nigericin (from MARIN *et al.*, 1981 *b*).

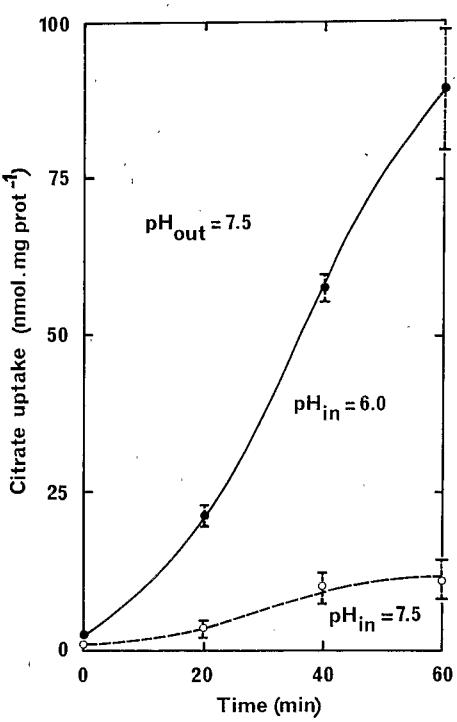

FIG. 5. — Effect of pH of vesiculation medium on citrate uptake by tonoplast vesicles.

Tonoplast vesicles were generated either in phosphate-mannitol medium (pH 6.0), or in G.E.M. medium (pH 9.5). These media differed from the usual medium by the nature of the buffer: 50 mM potassium phosphate for the first medium; 20 mM glycine + 10 mM EDTA for the second one. Vesicles were both incubated in 1.8 ml of phosphate-mannitol medium (pH 7.5) in presence of 90 μl of ^{14}C-citrate (0.1 μCi . ml^{-1}). Aliquots (100 μl) were subsampled and treated as described elsewhere (MARIN, 1981). ●———●, pH = 6.0; ○---○, pH = 7.5.

results in a decrease of preexistent ΔpH (MARIN *et al.*, 1981 *a*). The addition of FCCP, of S-13 or of the ionophore 1799 to a vesicular suspension equally leads to a marked decrease in the initial ΔpH (MARIN, 1981; CRÉTIN, 1982; MARIN and BLASCO, 1982). It is also possible to form tonoplastic vesicles in a given pH medium; this method makes it possible to obtain transtonoplastic ΔpH of varied amplitudes (MARIN, 1981). Finally, the use of high NH_4Cl concentration in the incubation medium leads often to a considerable decrease of the initial ΔpH, corresponding to a relative alkalinization of the internal medium (MARIN, 1981; CRÉTIN, 1982).

In each case, any increase of the transtonoplastic ΔpH, regardless of the manner in which it is obtained, is immediately followed by an increase in the uptake of ^{14}C-citric acid (*fig.* 4 B and 5), of ^{14}C-lysine (*fig.* 6) and of $^{45}Ca^{2+}$ (*fig.* 7 A and B) (HANOWER *et al.*, 1977; MARIN, 1981; MARIN *et al.*, 1981 *b*; MARIN *et al.*, 1982). In contrast, a ΔpH decrease causes the opposite effect (*fig.* 5, 6 and 7).

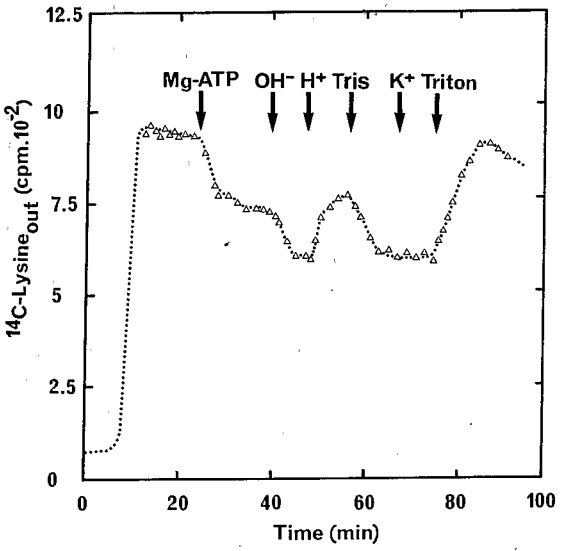

FIG. 6. — Effect of proton-motive force change on lysine distribution on a vacuolar suspension.

Fresh vacuoles were incubated in the usual medium (as in figure 1 A), in the presence of 0.8 mM cold lysine and ^{14}C-lysine (6 μCi . ml^{-1}), at pH 7.5. After washing, they were resuspended in the same medium without radioactivity. Additions were as follows in the upper chamber of flow dialysis cell: MgATP (2.5 mM, pH 7.5), NaOH (until pH 8.2), HCl (until pH 6.2), Tris (until pH 7.8), KCl (10 mM) and Triton X-100 (0.1 %; w/w). Radioactivity in the exit flux (external medium) was monitored during the experiment.

In the case of citric acid, it is known that the increase of tracer uptake corresponds to a true increase of the accumulation ratio (MARIN, 1981). Furthermore, the citrate influx seems to be associated with a H^+ efflux since, in the absence of ATP, the addition of citrate to the medium results in an alkalinization of the internal medium and in an acidification of the external medium (MARIN, 1981).

Influence of transtonoplastic ΔΨ on solute uptake

The amplitude of the transmembrane electric potential difference can be changed by using the specific properties of ionophores, such as valinomycin in the presence of KCl (MARIN, 1981; CRÉTIN, 1982). Tonoplast depolarization can also be obtained by the addition of increasing KCl quantities to a vacuolar or vesicular suspension (MARIN, 1981; MARIN *et al.*, 1981 *a;* CRÉTIN, 1982; MARIN *et al.*, 1982). Here again, regardless of the manner in which the ΔΨ variation is obtained, any depolarization of tonoplastic vesicles involves an increase of citric acid uptake (*fig.* 8) (MARIN, 1981; MARIN *et al.*, 1981 *b*).

On the other hand, the addition of citric acid to the medium causes an hyperpolarization of tonoplast (MARIN, 1981). This strongly suggests translocation

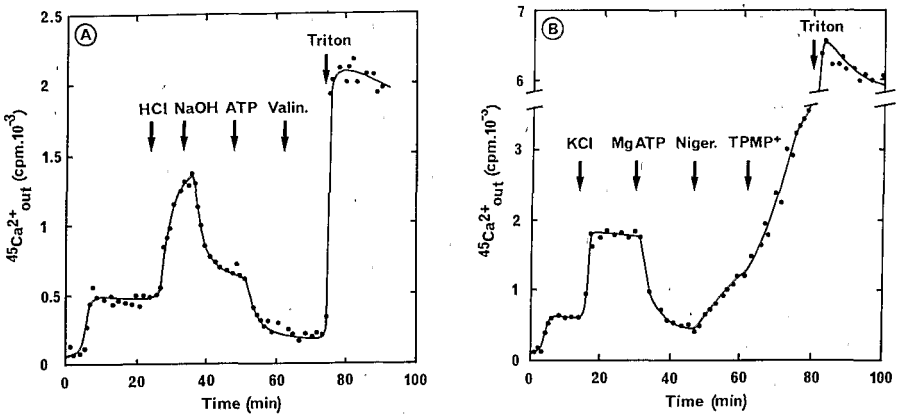

FIG. 7. — Effect of proton-motive force change on Ca^{2+} distribution on a vacuole suspension.

A, fresh vacuoles were incubated in the usual medium (as in figure 1 A) in the presence of 0.5 mM $CaCl_2$ and $^{45}Ca^{2+}$ (0.8 µCi.ml^{-1}) at pH 7.5. Additions were as follows in the upper chamber of flow dialysis cell: in A, HCl (until pH 6.0), NaOH (until pH 7.0), CaATP (2.5 mM at pH 7.2), valinomycin (0.8 µg.ml^{-1}) and Triton X-100 (0,1%; w/w). B, experiment was conducted as described in A, with some modifications, in presence of 5 µCi $^{45}Ca^{2+}$. Additions were as follows: KCl (130 mM), MgATP (5 mM), nigericin (16 µg.ml^{-1}), $TPMP^+$ (7.5 mM) and Triton X-100 (0.1%; w/w).

in tonoplastic vesicles and in vacuoles to result in a negative net charge transfer. This is in good accordance with the effect of tonoplast depolarization on citrate uptake to suggest that the electrical force is involved in this transport.

As for Ca^{2+}, which has only been studied on fresh vacuoles, the membrane depolarization, obtained by the addition of large quantities of KCl or $TPMP^+$, leads to a considerable Ca^{2+} efflux (*fig.* 7 B) (MARIN *et al.*, 1982). It is suggested that this Ca^{2+} efflux originates in an internal pool of sequestrated ions (*see* below).

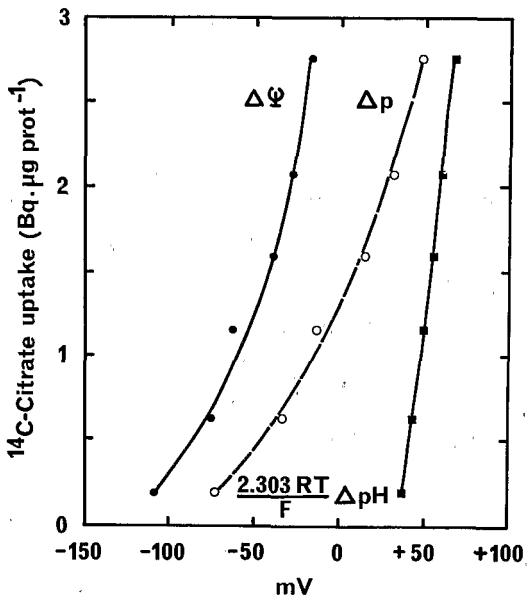

FIG. 8. — Relation between the components of the proton motive force (Δp) and citrate incorporation by tonoplast vesicles.

The data are collated from results described elsewhere (MARIN *et al.*, 1981 *b*). ●——●, Δψ; ■——■, 2.303 RT/F ΔpH; ○---○, Δp calculated as the sum of the two components. Uptake is measured after 30 min incubation at 30°C.

Reversibility of the solute uptake

Although solute uptake follows a linear kinetics during 60 min for citric acid as example (D'AUZAC and LIORET, 1974; MARIN, 1981), the data presented above do not measure the influx of the solutes but the rate of net uptake of the tracer. In certain cases, when the ΔpH is increased or when the ΔΨ is relatively more positive, it has been shown that the accumulation ratio exceeds 1 (MARIN, 1981; MARIN *et al.*, 1981 *b*).

When vacuoles are incubated in the presence of radioactive tracers for 30-60 min and then placed in a non radioactive medium, the incorporated molecules show no size efflux. Exsorption, measured after 45 min, represents only about 10% of the organic acid content (MONTARDY and LAMBERT, 1977). These authors note a 87% retention for citrate, 94% for malate and 88% for succinate. Moreover, this value is 98% for lysine, after an hour of exsorption (HANOWER *et al.*, 1977). These results have been confirmed by flow dialysis (CRÉTIN, unpublished results).

In the case of Ca^{2+}, the addition of divalent cations to the reaction medium, even at high concentrations (10 mM for Ca^{2+} and 25 mM for Mg^{2+}), does not suffice to free the incorporated $^{45}Ca^{2+}$; this implies not only the absence of a transmembrane isotopic exchange but also the lack of adsorption at the tonoplast's external face. The same holds for the other solutes (citrate, lysine) (MARIN *et al.*, 1982). Consequently, the tracer uptakes reported above cannot be explained by a mere isotopic exchange between the extra- and intravacuolar medium. Furthermore, the uptakes take place against a concentration transmembrane gradient as evidenced for citric acid (D'AUZAC et LIORET, 1974; D'AUZAC *et al.*, 1977 *a;* MARIN, 1981; MARIN *et al.*, 1982).

The results obtained with tonoplastic vesicles, at least for citrate, are not the same (MARIN, 1981). In effect, after a one-hour loading, one observes a considerable efflux, up to 80% of the incorporated citrate concentration in one hour. This efflux is temperature-dependent and increases with the external citrate concentration. Yet, upon uptake of ^{14}C-citrate by the vesicles, the isotopic enrichment of the internal compartment does not exceed 8% of the external specific activity. As a result, isotopic equilibrium is never reached.

These experimental findings on vacuoles and on tonoplastic vesicles suggest the existence of an internal compartmentation of the vacuolar citrate in two clearly distinct pools, only one of them being directly accessible to isotopic exchange (MARIN, 1981; MARIN *et al.*, 1982). This can only be accounted for by a kinetic pool since no morphological compartmentation is visible. No direct indication of this phenomenon has been obtained. Nevertheless, the presence of Mg^{2+} in *Hevea brasiliensis* latex in stoichiometric concentrations with citrate (COUPÉ, 1977; JACOB, unpublished results) leads to the supposition that one part of the internal citrate is able to chelate this cation (MARIN, 1981). Acid-base equilibrium calculations and associations with Mg^{2+} predict an accumulation of the complexe $(citrate)^{3-} - Mg^{2+}$ as also the formation of a pool of $(citrate)^{2-}$. This later seems to be the permeant form as judged from the shape of the curves of citrate uptake *vs* pH (MARIN, 1981). Kinetic simulations based on this proposed model of two citrate pools are quite reconcilable with the experimental results (MARIN, 1981). No information is available for Ca^{2+} and the amino-acids.

A model of the transport and the accumulation of vacuolar solutes

The data on the citrate transport and its accumulation may be summarized as follows: (*a*) from the reversibility of the incorporation of ^{14}C-citrate and from the

absence of isotopic dilution in the vesicles, it is inferred that two kinetic citrate pools exist in this material; (*b*) from the comparison of the pH on the linear initial rate of the ^{14}C-citrate uptake and the calculated dissociation curves, it seems that $(citrate)^{2-}$ is the transported form; (*c*) from the 1 : 1 stoichiometry of the accumulation of Mg^{2+} and citrate in fresh vacuoles and from the calculated curves for acid-base equilibrium and Mg complexes formation, it is supposed that the unpermeant accumulated form is predominantly the complexe $(citrate)^{3-} - Mg^{2+}$; (*d*) the acidification of the medium and the alkalinization of the vesicles which follow citrate addition to the medium in absence of ATP, suggest that there is a $n\,H^+ - m$ citrate antiporter; the acceleration of the citrate influx by decreasing the transmembrane pH gradient (making the external pH more acid) confirms this hypothesis; (*e*) the electrogenic character of citrate translocation observed in absence of ATP infers that a net negative charge is transported. But, theoretical considerations of the relationships observed between the transmembrane $\Delta\Psi$ and the citrate flux indicate that the transported net charge must not exceed 1. The artificial or natural (by ATP) variations of $\Delta\Psi$ result in variations of citrate influx, in accordance with this hypothesis.

The tentative model which account for the whole set of data is as follows: $(citrate)^{2-}$ is transported in exchange for $1\,H^+$. One Mg^{2+} is simultaneously transferred (*fig.* 9). These processes account for the electric character ($z_{net} = -1$), the effect of the pH of the medium, the effect of the ΔpH and the 1 : 1 Mg^{2+}-citrate stoichiometry. In the conditions of pH and Mg concentration which prevail in the vacuoles of *Hevea* latex, the unpermeant complex $(citrate)^{3-} - Mg^{2+}$ is the major form. The formation of this complexe from $(citrate)^{2-}$ and Mg^{2+} which enter the vacuoles, displaces the equilibrium accumulation of the total vacuolar citrate. The demonstration of the electrogenic pumping of H^+ into the vacuoles by the tonoplast ATPase explains how the citrate transport is energized. From the preceeds, it is clear that both the ΔpH and $\Delta\Psi$ components of the proton-motive force are used by the citrate system. No sufficient information is available for Ca^{2+} and the amino-acids.

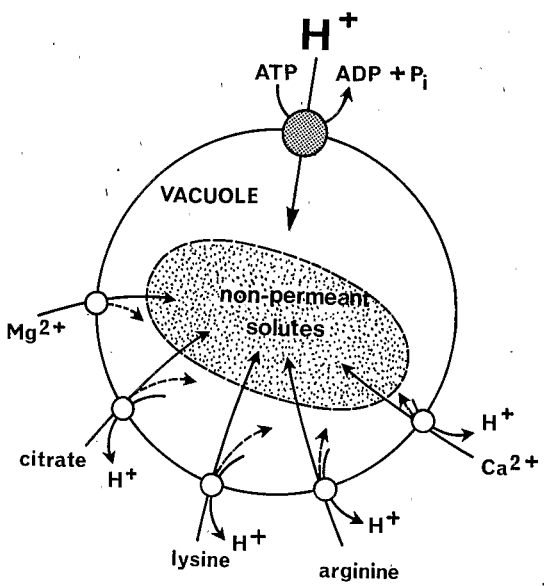

FIG. 9. — Possible mechanisms of incorporation and trapping of different solutes in the *Hevea* vacuole.

This figure shows the present status of the knowledge of solute transport in the *Hevea* vacuole. The tonoplast ATPase functions as a proton pump. The mechanism of citrate translocation is a citrate-proton antiporter associated with a Mg^{2+} influx. A mechanism is tentatively proposed for the other solutes (lysine, arginine, Ca^{2+}), as also the occurrence of two kinetic pools of solutes inside the vacuole.

CONCLUSION

The work carried out on the lutoids (vacuolar compartment) of *Hevea brasiliensis* latex has enabled a better understanding of the energetic coupling of solute uptakes and of their accumulations associated with tonoplastic ATPase function.

It is necessary to recall the principal characteristics which make this such an interesting biological material. *Hevea brasiliensis* latex enables the simple isolation of large quantities of intact vacuoles and the preparation of vesicles from their tonoplast. In most of the cases, two differential centrifugations suffice. Such simplicity in the preparation contrasts with the often sophisticated methods used to obtain these organelles from higher plants (MATILE, 1978; BOUDET *et al.*, 1981). In addition, the lyophilization of purified aliquots of vacuoles, so obtained and free from contamination, does not change their native properties. They conserve most of the activities observed *in vitro* in fresh vacuoles. It has been possible to reconstitute functional tonoplastic vesicles from the lyophilized vacuolar membranes for transport studies.

In this review, we have indicated that the uptake of most of the solutes studied (citrate, lysine, arginine, calcium) is accomplished against a visible transmembrane gradient of concentration, in the absence of any source of metabolic energy. This uptake often follows linear kinetics during the first 30 min.

The rate is a function of the external solute concentration; it is dependent on temperature and pH. Certain aspects of the uptake kinetics suggest the existence of two kinetic pools of the solutes present in this vacuome: the smaller pool represented the permeant form; the larger (containing the major fraction of the vacuolar solutes) corresponding to the non-permeant form (which can be identified with the complexes formed between the different intravacuolar molecules and the incorporated solutes). This hypothesis arises from generalization of the situation for citrate and its interaction with magnesium, which the simulations have reinforced. In other words, the concentration gradient is only apparent since it does not concern the permeant form but the sum of the immobilized and permeant forms. Furthermore, these solute uptakes decrease in the presence of either protonophores or ATPase activity inhibitors. There is, therefore, a relationship between solute uptake and tonoplastic ATPase functioning.

The transmembrane ΔpH constitutes the driving force of all the solute uptakes cited. Moreover, uptake of citric acid, and also of Ca^{2+}, are changed by the addition of any lipophilic molecule (such as TPP^{+} at high concentration) or of any ionophore (such as valinomycin, in the presence of KCl) known to change the preexistent $\Delta\Psi$. Thus, these uptakes prove also to be dependent on $\Delta\Psi$. Consequently, the proton-motive force is involved in the uptake process, at least with citric acid. Consequently, we believe that the proton-motive force would be involved in the functioning of tonoplastic translocators in the same manner as at the plasmic and bacterial membrane and that it would thus be a force in most of the solute transport systems involving cotransport and uniport (SLAYMAN and SLAYMAN, 1975; HAROLD, 1977 *a* and *b*; BAKER, 1978; EDDY, 1978).

Tonoplastic ATPase catalyzes an electrogenic efflux of protons from the cytoplasm in the same way as plasmalemmic ATPase. It is suggested that it might also be involved in the regulation of cytoplasmic pH (MARIN and BLASCO, 1982; D'AUZAC *et al.*, 1982). Although the active transport of protons by tonoplastic ATPase favors solute transport against a concentration gradient, ATPase and solute transport are not directly linked. Fresh vacuoles can absorb solutes in the absence

of ATP by utilizing the preexistent ΔpH. However, it is the tonoplastic ATPase function which will enable the maintenance of the phenomenon. Consequently, the coupling is indirect.

Granted, research on the plant vacuome is recent, contrary to that done with other plant cellular compartments. Nevertheless, the *Hevea brasiliensis* latex vacuoles represent, to our knowledge, one of very rare plant materials of which the vacuolar and cytoplasmic compartment composition is readily determined. In addition, it is the only plant material in which it has been clearly demonstrated that the tonoplastic ATPase functions as an electrogenic proton pump and that it provides the energy needed for the functioning of the different tonoplastic translocators. It is obvious that the precise mechanism of the coupling remains, for the large part, to be elucidated.

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