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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

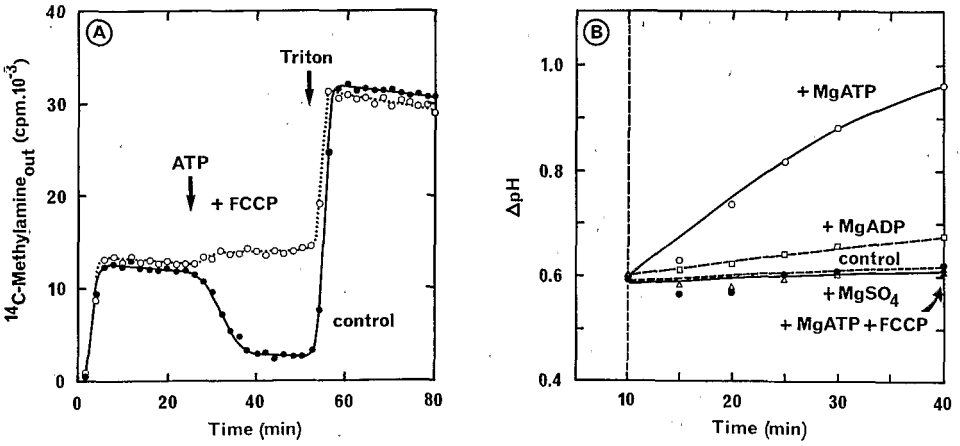

FIG. 1. — Time-course of change of ΔpH across the tonoplast (inside acid) and the effect of ATP and FCCP as measured by accumulation of methylamine.

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*).

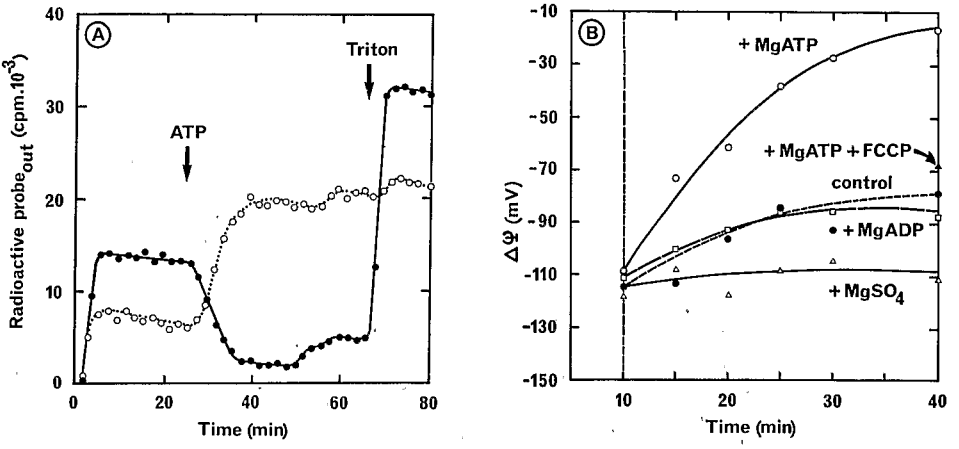

FIG. 2. — Time-course of change in $\Delta\psi$ across the tonoplast (inside negative) and the effect of ATP and some effectors as measured by accumulation of Rb^+ and $TPMP^+$.

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 $MgCl_2$),

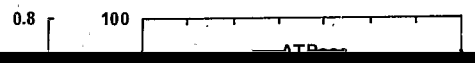

FIG. 3. — pH-dependence of ATP

results in a decrease of preexistent ΔpH (MARIN *et al.*, 1981 *a*). The addition of

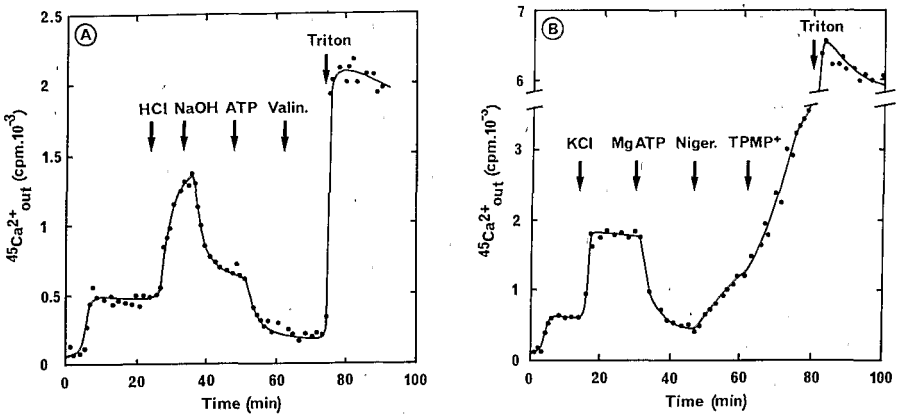

FIG. 7. — Effect of proton-motive force change on Ca^{2+} distribution on a vacuole suspension.

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

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.

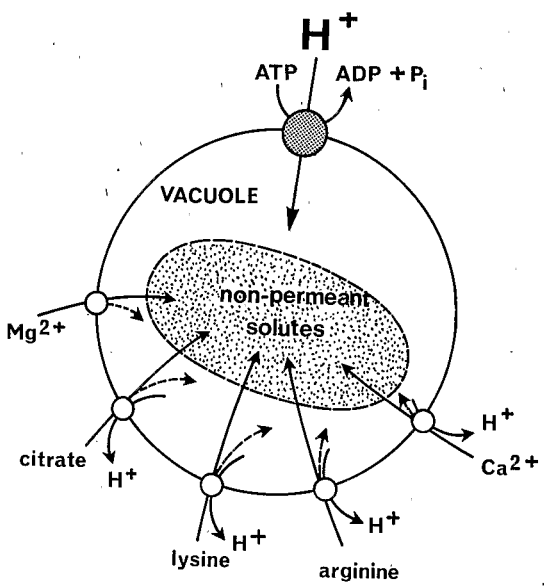

FIG. 9. — Possible mechanisms of incorporation and trapping of different solutes in the *Hevea* vacuole.

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).

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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