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The protonmotive potential difference across the vacuo-lysosomal membrane of *Hevea brasiliensis* (rubber tree) and its modification by a membrane-bound adenosine triphosphatase

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The vacuo-lysosomes of *Hevea brasiliensis* (rubber tree) constitute a suitable model system for the study of active transport and energization at the level of the membrane of plant vacuoles. The pH gradient (ΔpH) and the membrane potential ($\Delta\psi$) of vacuo-lysosomes were determined by means of the weak base methylamine and the lipophilic cation tetraphenylphosphonium. The values obtained depended strongly on the experimental conditions such as medium pH or K^+ concentration. Under experimental conditions, i.e., pH 7.5 outside and low K^+, the ΔpH amounts to about 0.9 unit, interior acid, and the $\Delta\psi$ to -120 mV, interior negative. The $\Delta\psi$ is presumably caused by the imposed K^+ gradient, and the internal acidification might be a consequence of the passive proton inflow along the electric field. This explanation is sustained by the ineffectiveness of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in destroying the ΔpH and $\Delta\psi$, whereas higher K^+ concentration decreased both. Under conditions existing *in vivo*, the membrane potential might be significantly lower. The presence of ATP increased the acidification of the intravesicular space by 0.5 pH unit to a ΔpH of up to 1.4 and shifts the membrane potential at least 60 mV to a more positive value. The change of the protonmotive potential did not occur with ADP; the pH-dependence of the change was identical with the pH-dependence of a vacuo-lysosomal membrane-bound ATPase, and the effect of ATPase was prevented by the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The change of protonmotive potential difference, brought about by the ATPase, was at least 90 mV. This is evidence that a vacuo-lysosomal ATPase in plants can function as an electrogenic proton pump that transfers protons into the vacuo-lysosomal space.

The milk sap (latex) of the rubber tree (*Hevea brasiliensis* Müll.-Arg. Kunth) consists of the fluid cytoplasmic content of the syncytial lactiferous system. This is made up of rubber particles, serum and organelles imbedded therein, such as Frey–Wyssling particles (a degenerated plastid compartment) and lysosomes (generally called lutoids) (Dickenson, 1964, 1969).

Abbreviations used: ΔpH, pH gradient; $\Delta\psi$, membrane potential; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazol-2-yl)benzene; TPP^+, tetraphenylphosphonium ion; Mes, 4-morpholine-ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

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The lysosomes can be separated from other latex constituents by differential centrifugation, and high yields of pure lysosomes can be readily obtained (Pujarniscle, 1968). The preparation of these lysosomes involves neither cell rupture treatments nor lytic attack by cell-wall-degrading enzymes. The chemical and physical properties of the membranes should correspond precisely to those *in vivo* (Moreau *et al.*, 1975; Dupont *et al.*, 1976).

The physiological and morphological features of the *Hevea* lysosomes reveal them as intermediate between 'true' lysosomes (Pujarniscle, 1968) and 'normal' plant vacuoles (Ribaillier *et al.*, 1971), and they can therefore be termed 'vacuo-lysosomes'. [Vacuoles generally seem to work as a lysosomal compartment (Matile, 1978) too.] Since the preparation of undamaged vacuoles from plant cells is difficult to perform (Matile, 1978; Wagner &

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Siegelman, 1975; Leigh & Branton, 1976), the *Hevea* vacuo-lysosomes might be regarded as a suitable model system in which to study the biochemistry and physiology of plant vacuoles, especially those processes which require a perfectly intact membrane, e.g., solute transport and energization of the vacuolar membrane.

The freshly-harvested vacuo-lysosomes of *Hevea* contain about 50 mM-citrate, which corresponds to an accumulation factor of about 10 when compared with the citrate concentration in the cytoplasm (5 mM; d'Auzac & Lioret, 1974). The properties of catalysed citrate transport and citrate accumulation are fully retained by the isolated purified vacuo-lysosomes (d'Auzac & Lioret, 1974; Montardy & Lambert, 1977) and by vesicular compartments generated from purified freeze-dried vacuo-lysosomal membranes. The mechanism of solute accumulation by vacuolar or lysosomal compartments is unknown. One possible model to explain solute accumulation would be the uptake of solutes driven by a chemiosmotic type of membrane energization, e.g., a protonmotive potential difference, similar to the mechanism of solute uptake in bacterial cells and through plant plasmalemma [for a review, see Harold (1977)].

The integrity of *Hevea* vacuo-lysosomes should offer an opportunity to prove or disprove involvement of a protonmotive force in vacuolar transport processes.

Since an ATP-splitting enzyme has been shown to be associated with the membrane of the vacuo-lysosomes (d'Auzac, 1975, 1977), the assumption of a chemiosmotic driving force seemed a promising working hypothesis (d'Auzac *et al.*, 1977*a*,*b*).

In the present paper, data are presented that demonstrate the direction and magnitude of the pH gradient (ΔpH) and electric membrane protential ($\Delta\psi$) across the membrane of isolated vacuo-lysosomes. Furthermore, the influence of the membrane-bound ATPase on these parameters has been measured. The results are compatible with the existence of a proton-translocating ATPase that pumps protons into the vacuo-lysosomes and generates a protonmotive potential difference sufficient to explain the observed citrate-accumulation ratio.

Materials and methods

Material

Hevea brasiliensis latex was harvested in an ice-cooled flask as previously described (d'Auzac, 1975, 1977) from rubber-tree buddings of the clones GT_1 or TJ_1 (I.R.C.A. experimental plantation, Lange-dedou, Ivory Coast), which were selected for high production and latex homogeneity (d'Auzac & Lioret, 1974).

Preparation of vacuo-lysosomal fraction

All experiments were performed on the vacuo-lysosomal fraction prepared as described by d'Auzac (1975, 1977).

Latex diluted with 1–3 vol. of triethanolamine/mannitol buffer (300 mM-mannitol/50 mM-triethanolamine/2 mM-mercaptoethanol), adjusted to pH 7.5 with HCl, was centrifuged at 39000 *g* for 60 min at 4°C. The pellet, resuspended in 20–30 ml of the same buffer, was the crude vacuo-lysosomal fraction. The agglomerated polyisoprenoid particles that collect above the supernatant and constitute the rubber fraction were discarded. After two washings with triethanolamine/mannitol buffer, the crude vacuo-lysosomes were free from contaminating latex structures such as Frey–Wyssling particles and rubber bodies (Pujarniscle, 1968). The isolation of the vacuo-lysosomal fraction was performed in Africa (Laboratoire de Physiologie Végétale, I.R.C.A., Station Bimbresso, Abidjan, Ivory Coast) and the purified pellet, stored in ice, was shipped by airmail to Montpellier (Laboratoire de Physiologie Végétale Appliquée, U.S.T.L., Montpellier, France) to be processed.

The vacuo-lysosomes were usually resuspended in buffer (300 mM-mannitol/25 mM-Mes/25 mM-Mops/25 mM-Hepes, together with sufficient triethanolamine base to adjust the pH to 7.5) and twice washed with this medium. The experiments were conducted 36–48 h after latex collection.

Experimental standard incubation conditions

Experiments were performed at pH 7.5 and at a temperature of 30°C, unless otherwise indicated. The standard reaction mixture was the buffer mixture 300 mM-mannitol/25 mM-Mes/25 mM-Mops/25 mM-Hepes, adjusted to pH 7.5 with triethanolamine base. The medium contained neither Mg^{2+} nor citrate. Further details are given in the legends to the Figures.

Measurement of the distribution of radioactive methylamine, TPP^+, water and dextran across the vacuo-lysosomal membrane

Tracer uptake was measured after incubation of radioactive molecules with intact lysosomal vesicles at room temperature in various media as described in the Figure legends, for instance in the presence of ^3H_2O (0.5 μCi/ml) and either 10 μM-[^{14}C]methylamine (0.1 μCi/ml), or [^{14}C]dextran (0.1 mg/ml; 0.1 μCi/ml). When [^{3}H]TPP^+ (100 μM; 0.5 μCi/ml) was used, ^3H_2O was replaced by [^{14}C]dextran. [^{14}C]Dextran of average mol.wt. 70000 was purchased from New England Nuclear Corp., Dreieich, Germany, [^{14}C]methylamine and ^3H_2O were from C.E.A., Saclay, France, and [^{3}H]TPP^+ was generously given by Dr. P. Geck, G. Embden Institut, Frankfurt, Germany.

After incubation, the vacuo-lysosomes were pelleted by centrifugation in an Eppendorf model 3200 Desk Microfuge with a 3 min run at full speed (about 10000 *g*).

From each tube, a 100 μl sample was withdrawn from the supernatant. A slice was made across the bottom of the tube so that approximately half of the pellet was cut. This procedure avoided, in part, contamination with supernatant. Both samples were added to 0.2–0.3 ml of 0.1% Triton X-100 and left overnight. A portion (200 μl) of each homogeneous suspension was added to 2.5 ml of dioxan scintillation mixture (80 g of naphthalene, 4 g of PPO and 0.4 g of POPOP blended with 1 litre of dioxan). They were counted for ^{14}C and ^{3}H radioactivity. Data were corrected for spill-over and quenching.

$^{3}H_2O$, [^{3}H]TPP^{+}, [^{14}C]methylamine and [^{14}C]-dextran were determined by their relative specific radioactivities in the pellet and the supernatant as described by Johnson & Scarpa (1976) for the determination of transmembrane ΔpH and by Komor & Tanner (1976) for the quantitative analysis of $\Delta\psi$. The intravesicular volume was calculated as the water-accessible but dextran-inaccessible space; it comprised approx. 1 μl/mg of protein. The experiments were usually performed in triplicate and representative examples are described in the present paper; the variation of results from the same batch of vacuo-lysosomes was not more than $\pm$10%. Greater variability (about 30%) is observed between different batches of harvested latex. Therefore the experimental results from the same harvest of latex are given in the present paper.

Protein determination

Protein concentrations were measured by using the Ciocalteu reagent with dried crystalline bovine plasma albumin as the standard. The samples were processed as described by Marin *et al.* (1974).

Results

Permeation of methylamine into vacuo-lysosomes

The distribution of lipid-permeable weak acids (e.g. dimethyloxazolidinedione) or bases (e.g. methylamine) has been successfully used for the determination of intracellular or intravesicular pH values (Waddell & Bates, 1969; Rottenberg *et al.*, 1972). The pH determinations are based on the assumption that the uncharged compounds can easily pass across the membrane, whereas the charged forms are impermeant. The ratio of charged to uncharged molecules can be calculated from the entire amount of the compound in the cell, with the assumption that the uncharged form is equally distributed on both sides of the membrane. Since the ratio of charged to uncharged compound is governed by the pH value, the latter can be deduced. For most thoroughly studied situations, these assumptions have proven to be correct (reviewed by Smith & Raven, 1979).

The time-course of uptake of methylamine (Fig. 1) indicates a very rapid penetration of this probe into the intravesicular space, the internal amount staying constant for at least 30 min. Methylamine seems to be freely dissolved in the intravesicular space and not bound to macromolecules or to the membranes, since an osmotic rupture of the vesicles or short heat treatment liberates 90% of the internal radioactivity. Variation of methylamine concentration or vacuo-lysosome concentration by a factor of 2 had no influence on the time course and plateau value for methylamine distribution. At an external pH of 7.5, which is near the pH value of the *Hevea* latex, the methylamine distribution indicated an internal pH of 6.5; if the small amount of absorbed label is taken into account, a value of 6.6 is obtained. (A different value was obtained if the distribution of dimethyloxazolidinedione was measured, but since a very large correction for adsorbed material had to be made in this case, the data were regarded as unreliable.) An internal acidity of the vacuo-lysosomes had been also concluded from measurement of external pH before and after lysis of the vacuo-lysosomes (Lambert, 1975).

Dependence of intravesicular pH value on pH value of the external medium and on medium composition

The pH value of the cytoplasm of cells stays at

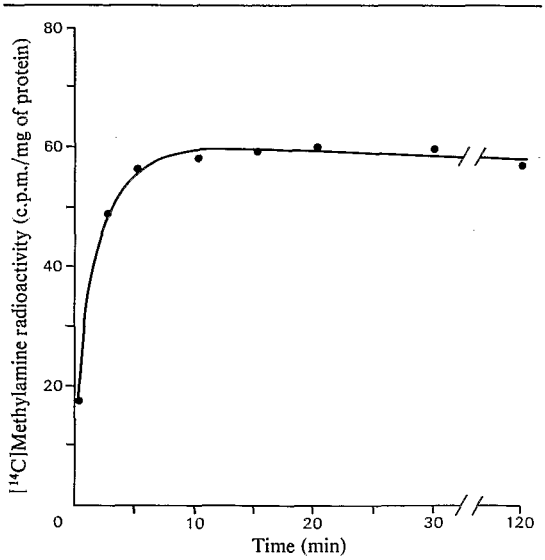

Fig. 1. *Time course of uptake of methylamine by Hevea vacuo-lysosomes*

Hevea vacuo-lysosomes (110 mg of protein) were incubated at 30°C in 18 ml of buffer, pH 7.5, containing [^{14}C]methylamine (0.1 μCi). At the times indicated, 1 ml samples were removed from the medium and processed as described in the text.

relatively constant value during variation of external pH, whereas the internal pH value of some organelles, such as mitochondria or chloroplasts, changes with external pH, so that the ΔpH stays fairly constant (Rottenberg *et al.*, 1972; Kashket & Wilson, 1973; Schuldiner *et al.*, 1974; Komor & Tanner, 1974).

In vacuo-lysosomes of *Hevea*, the ΔpH and the internal pH value change as the external pH is varied (Fig. 2), so that the pH gradient is nearly zero at pH 5.5 outside, and 1.2 at pH 8.5 outside. This feature is remarkably similar to the situation in animal lysosomes (Reijngoud *et al.*, 1976; Henning, 1975) and *Chara* vacuoles (Walker & Smith, 1975). At the pH of the latex cytoplasm, pH 7.2 (Brzozowska-Hanower *et al.*, 1979), the pH gradient would be about 0.7 unit (without involvement of the ATPase, as will be explained below).

The origin of the internal acidification can be only a matter of speculation. Either it is due to Donnan equilibrium of intravesicular macromolecules as suspected for liver lysosomes (Reijngoud *et al.*, 1976; Henning, 1975), or it is due to an internally negative transmembrane potential under the experimental conditions. (It will be shown below that *Hevea* vacuo-lysosomes indeed have a negative $\Delta\psi$.)

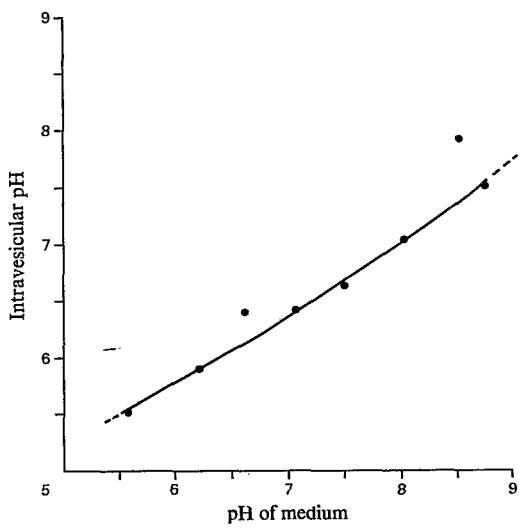

Fig. 2. *Effect of medium pH on the internal pH of Hevea vacuo-lysosomes*
The reaction mixture consisted of 1 ml of buffer adjusted with sufficient Tris base to the indicated pH and *Hevea* vacuo-lysosomes (5 mg of protein). Parallel experiments were performed under the same conditions in the presence of 0.5 μCi of ^3H_2O and either [^{14}C]dextran (0.1 μCi) or [^{14}C]methylamine (0.1 μCi). The incubation was conducted for 30 min at 30°C. The internal pH of organelles was determined by the distribution of ^{14}C-labelled methylamine through the *Hevea* vacuo-lysosomal membrane.

The pH gradient appeared relatively independent of the nature of the external medium used, such as mannitol, sucrose or choline chloride; NaCl and KCl caused a slight decrease of ΔpH (Table 1), as did the addition of nigericin in the presence of external K^+, presumably by exchange of external K^+ with internal H^+. Nigericin without the addition of external K^+ had no effect. The addition of Ca^{2+} in the presence of the ionophore A23187 had the most severe effect, nearly abolishing the pH gradient.

These data show that the ΔpH is quite constant, despite large variation of the external solution, as long as ion permeation is kept low. The membrane appears to be relatively impermeable or, at least, very selectively permeable with respect to ions, a prerequisite for establishing a protonmotive potential difference across the vacuo-lysosomal membrane.

Determination of $\Delta\psi$

Since the vacuo-lysosomal membrane seems suited for maintaining a ΔpH, attempts were made to estimate the electrical part of the protonmotive force, $\Delta\psi$. The method employed was to measure the distribution of the lipophilic cation TPP^+, a method that has proved to be suitable for systems that cannot be punctured by micro-electrodes, such as small unicellular organisms or organelles (Komor & Tanner, 1976; Heinz *et al.*, 1976; Harold & Papineau, 1972).

The time course of TPP^+ uptake reveals a rapid

Table 1. *pH gradient through the Hevea vacuo-lysosomal membrane under different experimental conditions*
Hevea vacuo-lysosomes (3.8 mg of protein) were suspended in 1 ml of modified buffer (mannitol was replaced by salt in the form of choline chloride, NaCl or KCl), with the pH values as indicated. Parallel experiments were performed under the same conditions in the presence of 0.5 μCi of ^3H_2O and either [^{14}C]dextran (0.1 μCi) or [^{14}C]methylamine (0.1 μCi). Additions were made before incubation at the indicated concentration. After 20 min at 30°C the organelles were rapidly centrifuged. The distribution of the tracers were measured as described in the Materials and methods section.

Medium	pH_{out}	pH_{in}	ΔpH
300 mM-Mannitol	7.50	6.41	1.09
+Nigericin (10 μg/ml)	7.48	6.38	1.10
+30 mM-KCl	7.50	6.75	0.75
+nigericin (10 μg/ml)			
+1 mM-$CaCl_2$	7.43	7.34	0.09
+ionophore A23187 (10 μg/ml)			
300 mM-Sucrose	7.45	6.47	0.98
150 mM-Choline chloride	7.45	6.42	1.03
150 mM-NaCl	7.48	6.56	0.92
150 mM-KCl	7.48	6.59	0.89

uptake phase with constant plateau value for at least 30 min (Fig. 3). The calculated distribution shows more than a 100-fold accumulation in the intravesicular space, corresponding to −130 mV, internal negative. The non-specific adsorption of the lipophilic cations seems to be less than 10%, as seen by osmotic rupture of the vacuo-lysosomes, or heating of the sample, which sets free more than 90% of the previously accumulated radioactivity. The remaining radioactivity might merely be adsorbed, and it is not important for calculation at high potential. The correction becomes significant, however, at low membrane potentials. Variation of TPP^+ or protein concentration by a factor of 2 did not change the distribution ratio.

Change of $\Delta\psi$ by various agents

The magnitude of $\Delta\psi$ was moderately decreased by the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Table 2), a result that was expected, since the protons are nearly at the electrochemical equilibrium. Valinomycin was without effect. Nigericin decreased the membrane potential more markedly, which might be explained by a rearrangement of the H^+ and K^+ gradients. The most prominent depolarization is obtained by increasing the external K^+ concentration (irrespective of the presence of valinomycin), which causes strong depolarization (Table 2 and Fig. 4).

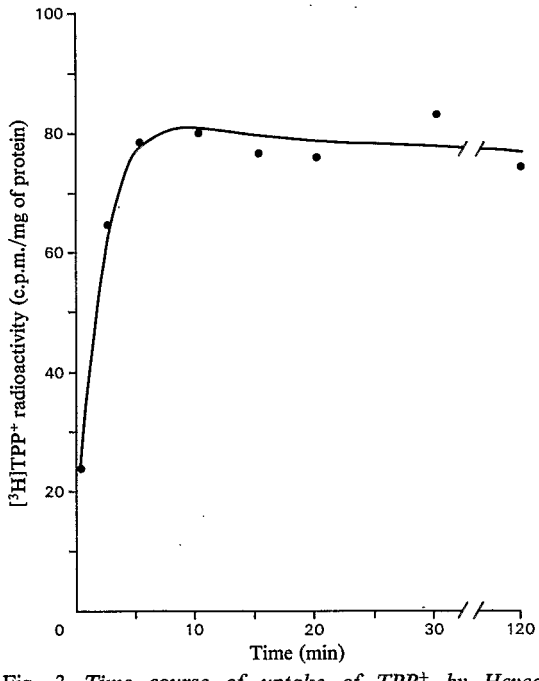

Fig. 3. *Time course of uptake of TPP^+ by Hevea vacuo-lysosomes*

Hevea vacuo-lysosomes (118 mg of protein) were incubated at 30°C in 18 ml of buffer, pH 7.5, containing [^{3}H]TPP^+ (0.1 μCi). At the time indicated, 1 ml samples were removed from the mixture and processed as described in the text.

The origin of the highly negative membrane potential under the experimental conditions might be largely governed by a diffusion potential, since the internal concentration of K^+ (50 mM) and Mg^{2+} (60 mM) (Ribaillier *et al.*, 1971) is much higher than outside in the experimental medium, and the diffusion potential due to K^+ could be −130 mV. Under natural conditions, i.e. at a K^+ concentration in the latex serum of 50 mM, the membrane potential is expected to be about −70 mV (according to Fig. 4).

Role of ATPase on the protonmotive potential difference

Since the discovery of an ATPase bound to the membrane of the vacuo-lysosomes (d'Auzac, 1975, 1977), it has been argued that the ATPase might energize the membrane of this compartment (d'Auzac *et al.*, 1977*a*). Because it is possible to quantify the protonmotive potential difference, the intervention of ATPase on the ΔpH and $\Delta\psi$ should be measurable if this enzyme works as a proton pump [indications for an ATP-caused intravesicular acidification had been already obtained by Lambert (1975)].

Indeed, it was found that the internal pH decreased, i.e. the ΔpH was increased 0.4–0.5 unit by addition of ATP (Fig. 5). More importantly, the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which only slightly inhibits the rate of ATP hydrolysis (d'Auzac, 1977), prevented ATP-induced acidification completely. ATP-induced acidification of the intravesicular

Table 2. *Change of membrane potential under different incubation conditions*

The membrane potential was determined as described in the Materials and methods section. Parallel experiments were performed under the same conditions in the presence of [^{14}C]dextran (0.1 μCi) and either 0.5 μCi of ^3H_2O or [^{3}H]TPP^+ (0.5 μCi). *Hevea* vacuo-lysosomes (3.7 mg of protein) were suspended in 1 ml of buffer, pH 7.5. Additions were made before incubation at the indicated concentrations. After 20 min incubation at 30°C, samples were removed and processed as described in the text. Abbreviation used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Conditions of incubation	$\Delta\psi$ (mV, inside negative)
Control	−130
+FCCP (10^{-5} M)	−73
+30 mM-KCl + nigericin (10 μg/ml)	−51
+150 mM-KCl	⩽0
+150 mM-KCl and valinomycin (10 μg/ml)	⩽0

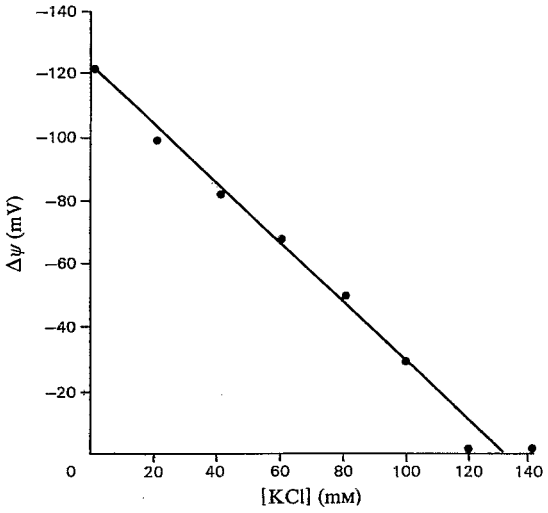

Fig. 4. *Variation of membrane potential as a function of external concentration of KCl*

The membrane potential was determined by the distribution of [^{3}H]TPP$^+$ through the *Hevea* vacuo-lysosomal membrane. The incubation mixture consisted of 1 ml of buffer, pH 7.5, [^{3}H]TPP$^+$ (0.5 μCi) and *Hevea* vacuo-lysosomes (5 mg of protein). After 10 min incubation at 30°C, samples were removed from the medium and processed as indicated in the text.

space showed a similar dependence on external pH as did the activity of the ATPase (Fig. 6), which is optimal at about pH 7.7 (d'Auzac, 1977). These data therefore suggest that the ATPase of the vacuo-lysosomes functions as a proton-translocating enzyme.

The membrane potential of vacuo-lysosomes was also changed by the addition of ATP. Again, Mg^{2+} and ADP had no effect and the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone prevented the ATP-induced change of membrane potential (Fig. 7). The value of $\Delta\psi$ became more positive by more than 60 mV as would be expected for an ATPase pumping protons into the vacuo-lysosomes electrogenically. Thus there is strong evidence that the ATPase, located at the outside of the vacuo-lysosomal membranes and accessible to cytoplasmic ATP, catalyses electrogenic proton transport into the vesicular space. It is easily visualized that this energization of the vacuo-lysosomes might be used for transport.

Discussion

There is very limited information about the protonmotive potential difference of isolated plant lysosomes and vacuoles, possibly because isolation procedures are laborious and delicate and yields are generally low. *Hevea* vacuo-lysosomes can be

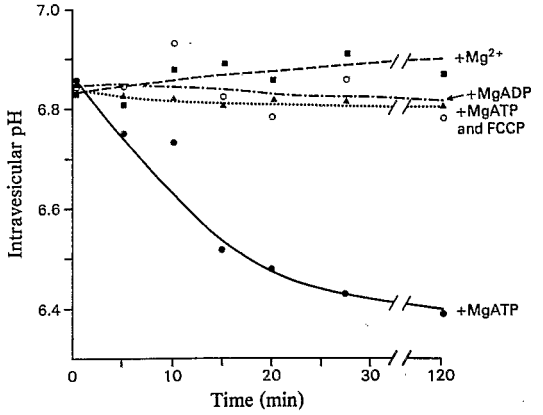

Fig. 5. *Time course of the influence of ATP on the evolution of transmembrane ΔpH*

The assay mixture contained *Hevea* vacuo-lysosomes (41 mg of protein) in 8 ml of buffer [^{14}C]methylamine (0.9 μCi) and the indicated additions (e.g. ATP). At the indicated time, 1 ml samples were removed from the medium and processed as indicated in the Materials and methods section. The concentrations were 5 mM for Mg^{2+}, MgADP or MgATP, and 0.1 mM for carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. In each case 0.1 mM-ammonium molybdate was added to inhibit acid phosphatase. ■, +Mg^{2+}; ○, +MgADP; ▲, +MgATP and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); ●, +MgATP.

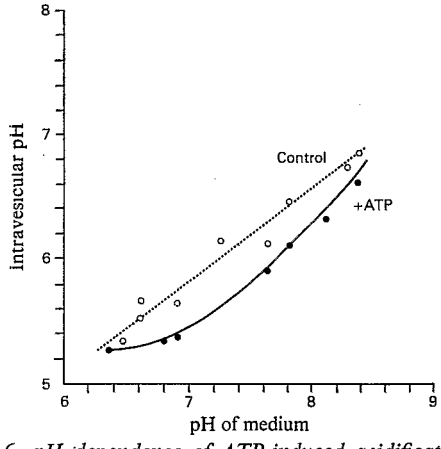

Fig. 6. *pH dependence of ATP-induced acidification of the intravesicular space*

Hevea vacuo-lysosomes (10.5 mg of protein) were incubated for 30 min at 30°C in 2.5 ml of buffer adjusted with sufficient Tris base to the expected pH with or without 5 mM-MgATP. Samples (1 ml) were removed from the medium and processed as noted in the text. The medium contained 2.5 μCi of ^3H_2O and either [^{14}C]dextran (0.5 μCi) or [^{14}C]-methylamine (0.5 μCi). ○, Control; ●, +ATP.

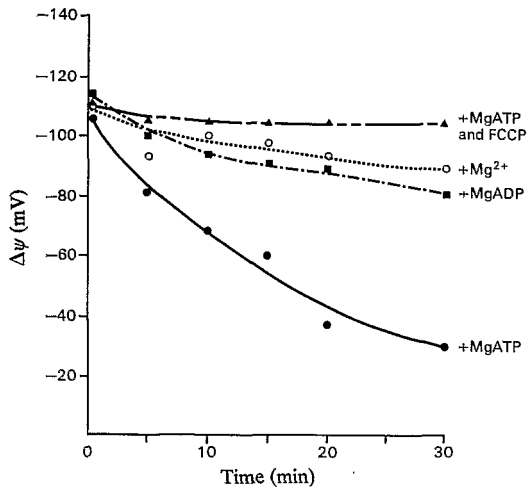

Fig. 7. *Time course of the influence of ATP on the evolution of membrane protential*
The reaction mixture contained *Hevea* vacuo-lysosomes (38.5 mg of protein) in 8 ml of buffer, pH 7.5, with $[^3H]TPP^+$ (4 μCi), 0.8 μCi of $[^{14}C]$dextran or 4 μCi of ^3H_2O, and the indicated additions 5 mM-Mg^{2+}, or 5 mM-Mg-ATP, and 0.01 mM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)]. At the indicated time, 1 ml samples were removed and processed as described in the text. ▲, +MgATP + FCCP; O, +Mg^{2+}; ■, +MgADP; ●, +MgATP.

obtained from the latex in very high yield and in fair purity. Since conventional ΔpH and $\Delta\psi$ measurements are difficult to perform with small organelles, the use of lipophilic pH indicators and lipophilic ions offers an opportunity to determine the components of protonmotive potential difference. Similar studies have already been performed with chromaffin granules, membrane vesicles, small cells etc. (e.g. Harold & Papineau, 1972; Maron *et al.*, 1979; Phillips & Allison, 1978). The non-specific adsorption of the lipophilic probes on membranes and proteins has to be subtracted to obtain the distribution ratios of dissolved probes on both sides of the membrane. In the present study the amount of lipophilic probe that stayed with the vacuo-lysosomes after membrane rupture by heating, osmotic lysis or sonication was regarded as adsorbed. Having this correction in mind, the absolute values that are obtained for ΔpH and $\Delta\psi$ are only approximate values, especially if they are low, e.g. −30 mV. Much more significant, therefore, is the change of ΔpH and $\Delta\psi$ that is observed after addition of small amounts of different reagents such as ATP, uncoupler etc. under constant conditions.

Under experimental conditions, the *Hevea* vacuo-lysosomes have an internally negative membrane potential and a pH value that is internally more acid than outside. These features are most probably due to the experimentally imposed K^+ gradient between the vacuo-lysosomes, which have retained their internal ions (e.g. 50 mM-K^+), and the buffer (0.5 mM-K^+). The K^+-caused diffusion potential might cause H^+ inflow, or at least prevent outflow of H^+ ions that had accumulated in the vacuo-lysosomes *in situ*. Consistent with this explanation is the fact that $\Delta\psi$ and ΔpH are more or less independent of medium pH value [in contrast with cells with an active proton-pump (Komor & Tanner, 1974, 1976)], are only slightly changed by uncoupler, but decreased by increase of K^+ concentration in the medium.

The addition of ATP to the vacuo-lysosomes causes a further acidification of the internal space and a significant shift of $\Delta\psi$ to a more positive value. This effect of ATP is not brought about by ADP and does not occur in the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; furthermore, the pH-dependence of the ATP effect is similar to the pH-dependence of the membrane-bound ATPase characterized by d'Auzac (1975, 1977). Therefore the shift of the protonmotive potential difference by about 90 mV is most probably brought about by the membrane-bound ATPase, which obviously works as an electrogenic proton pump by transferring H^+ from the external medium into the internal space. The magnitude of the change of ΔpH plus $\Delta\psi$ would be sufficient to explain the more-than-10-fold accumulation of citrate in the vacuo-lysosomes *in vivo*. It must be kept in mind, however, that the experiments were performed under conditions that were different from the situation *in vivo*, especially with respect to K^+. Thus the ΔpH and $\Delta\psi$ of vacuo-lysosomes *in situ* at the 'resting stage' (i.e. without ATP) is expected to be smaller than under the applied experimental conditions (Table 3). The change of protonmotive potential difference by the action of ATPase *in situ* could be different in quantity if the electric field through the membrane has some regulatory role.

The observed acidification of vacuo-lysosomal space and the positive polarization of the membrane by ATP are consistent with other reports from plant vacuoles which indicate an internally acid medium and a slightly positive $\Delta\psi$ (reviewed by Raven, 1976). The protonmotive potential difference might energize uptake of solutes such as sugar or organic acids into vacuoles by a mechanism corresponding to proton antiport.

Note added in proof

Qualitatively similar effects of ATP on proton motive potential difference of vacuo-lysosomes, measured with a different technique, have been obtained by Cretin (1981).

Table 3. *Protonmotive potential difference of Hevea vacuo-lysosomes under experimental conditions and supposed values for conditions existing in vivo*

Representative values for ΔpH and $\Delta\psi$ were taken to calculate the protonmotive potential; the state *in vivo* was deduced for conditions of pH 7.2 and 50 mM-K^+ with the assumption that the change of ΔpH and $\Delta\psi$ brought about by ATP was the same as that in the experiments. Abbreviation used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

	ΔpH	$\Delta\psi$ (mV)	Protonmotive force* (mV)
Experimental conditions			
Control	1.0	−120	−60
+FCCP	1.0	−73	−13
+ATP	1.5	−40	+50
In vivo			
Without ATP	0.7	−70	−28
+ATP	1.2	−10	+62

* A negative value of protonmotive force indicates that internal proton concentration is below electrochemical equilibrium; a positive value indicates that it is above electrochemical equilibrium.

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