J. Membrane Biol. 65, 175–184 (1982)

The Journal of
Membrane Biology

The Proton Gradient across the Vacuo-Lysosomal Membrane of Lutoids from the Latex of *Hevea brasiliensis*. I. Further Evidence for a Proton-Translocating ATPase on the Vacuo-Lysosomal Membrane of Intact Lutoids

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Summary. Lutoids (vacuo-lysosomal particles) were isolated from the latex of *Hevea brasiliensis*. Using flow dialysis with ^{14}C-methylamine uptake as a ΔpH probe and ^{86}Rb rubidium + valinomycin distribution for estimations of transmembrane electrical potential, intact lutoids exhibited a ΔpH of 1 unit (interior more acid) and a $\Delta\Psi$ of -70 mV (interior negative), when suspended in an isotonic medium at physiological concentration of potassium (30 mM) and pH 7.0, in the absence of ATP. In most cases, the Donnan potential was shown to fully account for ΔpH in nonenergized lutoids. The addition of Mg-ATP (5 mM) resulted in a marked acidification of the lutoidic internal space (0.7 to 1 pH unit) depending on the composition of the medium, and in a membrane depolarization by 60 mV (interior becoming less negative). The resulting electrochemical potential of protons ($\Delta\tilde{\mu}$H) increased by a hundred millivolts when lutoids were energized by ATP. These data strongly support an inward electrogenic proton translocating function for the ATPase of the vacuo-lysosomal membrane of lutoids. Results are discussed in terms of the *in vivo* maintenance of large "lutoids/cytoplasm" proton gradients, and of the rôle of these vacuo-lysosomes in the homeostasis of the cytoplasmic metabolism.

Key words tonoplast · ATPase · proton pump · electrochemical proton gradient

Introduction

The latex from *Hevea brasiliensis* is a specialized fluid cytoplasm which is expelled from wounded latex vessels (McMullen, 1962; Archer, Barnard, Cockbain, Dickenson & McMullen, 1963). Besides rubber particles, the latex contains various organelles which are essentially lutoids. The lutoids are single-membrane microvacuoles with lysosomal characteristics (Ruinen, 1950; Pujarniscle, 1968; Dickensen, 1969; Ribailler, Jacob & d'Auzac, 1971). They make up about 12% of the total latex.

Like all plant vacuoles, lutoids have a lower internal pH (about 5.5) than that of their cytoplasmic environment (about 7). Also they accumulate *in vitro* as *in vivo* numerous mineral and organic cations such as Mg^{2+}, Ca^{2+}, Cu^{2+}, and basic amino acids, as well as anions such as citrate and phosphate, and alkaloids (Ribailler et al., 1971; d'Auzac & Lioret, 1974; Brzozowska, Hanower & Chezeau, 1974). Pujarniscle (1968) reported latent acidic hydrolases in the lutoids, and pointed out their similarity to animal lysosomes.

Intact lutoids (easily isolated by centrifugation), can absorb citrate and basic amino acids in an isotonic and nonenergetic medium. These absorption processes, which operate against a large concentration gradient, show saturation and stereospecificity. They are also temperature and pH dependent (d'Auzac & Loiret, 1974; d'Auzac, Brzozowska, Hanower, Lambert, Lioret & Niamien N'Goran, 1977*a*; Hanower, Brzozowska & Niamien N'Goran, 1977).

The increase in the absorption rate by the addition of Mg^{2+}-ATP in the medium (d'Auzac et al., 1977*a*; Hanower et al., 1977) led d'Auzac (1975, 1977) to characterize a Mg^{2+}-dependent ATPase bound to the vacuo-lysosomal membrane of lutoids. Further studies on the characteristics of these particles demonstrated the presence of an antimycin-insensitive NADH cytochrome *c* reductase closely associated with their membrane (Moreau, Jacob, Dupont & Lance, 1975; d'Auzac, Dupont, Jacob, Lance, Marin & Moreau 1977*b*).

Recent findings have demonstrated a direct relationship between the *in vivo* pH gradient (lutoids/cytoplasm) and latex production, on the one hand, and ^{14}C-citrate absorption by lutoids on the other (Coupe & Lambert, 1977; Brzozowska-Hanower, Cretin, Hanower & Michel, 1979). These results suggest a direct role of lutoids in the homeostasis of the cytoplasmic compartment.

0022-2631/82/0065-0175 $02.00

Some early results have been found indicating an acidification of the lutoids internal space in the presence of Mg-ATP, linked to enhanced substrate absorptions (Lambert, 1975; Hanower et al., 1977). More recently, preliminary results revealed some good evidence in favor of a Mg-dependent proton pump in *Hevea* vacuo-lysosomes (Marin, 1980).

Much data are available on lysosomes from animal origin, (Schneider, 1977, 1979; Dell'Antone, 1979; Hollemans, Donker-Koopman & Tager, 1980) but little is known about the occurrence of membrane-bound ATPase on plant vacuo-lysosomes (d'Auzac, 1977; Lin, Wanger, Siegelman & Hind, 1977) and even less about their proton translocative function.

Using ^{14}C-methylamine as a ΔpH probe, and the distribution of ^{86}Rb in the presence of valinomycin to estimate transmembrane potentials, we show further definite evidence for a proton-translocating ATPase associated with the vacuo-lysosomal membrane of lutoids. The enzyme is involved in generating a high proton gradient and $\Delta\tilde{\mu}H$ across the lutoidic membrane.

The results presented in this paper were obtained by means of flow dialysis (Collowick & Womack, 1969; Ramos, Schuldiner & Kaback, 1976; Kell, John & Ferguson, 1978; Sorgato, Ferguson, Kell & John, 1978). This technique enables a rapid and continuous determination of changes in the concentration of solutes in the external medium, without further manipulation of lutoids, which are known to be relatively unstable (Pujarniscle, 1969).

Materials and Methods

Latex Collection

Twelve-year-old *Hevea brasiliensis* (clone GT1) were selected for their growth and high latex production, at the I.R.C.A. experimental plantation, Languededou (Ivory Coast).

Fresh latex was collected in glass vessels held in melting ice, from *Hevea* trees, tapped in a full spiral on virgin bark twice a week. The first 20 ml were discarded, because of possible bacterial contamination (Taysum, 1960) and damaged particle content (Ribailler, 1972).

Preparation of the Crude Lutoid Fraction

The fresh latex was immediately centrifuged at $35{,}000 \times g$ for 20 min at 10 °C. The supernatant serum (cytoplasm) and the polyisoprenoid particle fractions were discarded. The pellet, resuspended in 5 volumes of a Hepes-Tris (10 mM) mannitol (320 mM) pH 7 or 7.45 buffer, formed the crude lutoid fraction.

Removing Contamination by Successive Washings

The crude lutoid fraction was washed 3 times with the same Hepes-Tris buffer. The sediment obtained by centrifugation of the lutoidic suspension at $35{,}000 \times g$ for 10 min at 15 °C, was resuspended in the "dialysis-incubation" medium (320 mM mannitol, 50 or 100 mM Hepes-Tris, 3 mM $MgCl_2$ and 25 µM ammonium molybdate in order to inhibit acid phosphatase activities) (d'Auzac, 1975), at room temperature (26 °C) and adjusted to the desired pH by Hepes or Tris additions. This suspension is the "purified" vacuo-lysosomal fraction.

Transport Assays

Flow dialysis was performed with a dialysis cell entirely made in our laboratory, consisting of two Plexiglass blocks with a central cylindrical hole.

The upper "incubation" compartment (6 ml) was completely open, and separated from the lower "flow" chamber (2.5 ml) by a 4.5 cm^2 piece of dialysis tubing. Both chambers were stirred continuously by magnetic microbars.

The suspension of intact fresh luoids (with or without preincubation with labeled solutes) was introduced into the upper chamber. Substrates, adjusted to the same pH as the incubation medium, were added as indicated by arrows (*see* Fig. 1, 4, 5, 6). Parallel experiments were performed without lutoids in order to determine the effect of substrate introductions on the transfer of labeled solutes across the dialysis membrane.

The flow dialysis buffer (the same as the incubation medium), was pumped from the lower chamber at a constant rate of 3 ml/min. Fractions were collected every minute and their radioactivity level was tested by liquid scintillation spectrometry (Intertechnique SL 30).

All results and figures were adjusted to account for eventual quenching and dilution resulting from the additions of various solutes.

Estimation of ΔpH and $\Delta\Psi$

ΔpH was estimated by following the changes in the ^{14}C-methylamine level in the external medium (dialysate), assuming that this amine was accumulated in the most acidic space (Rottenberg, Grunwald & Avron, 1972; Reijngoud & Tager, 1973).

The membrane potential ($\Delta\Psi$) changes were estimated by measuring the changes in the level of ^{86}Rb in the external medium in the presence of valinomycin (Padan & Rottenberg, 1973; Altendorf, Hiarata & Harol, 1975).

ΔpH and $\Delta\Psi$ were calculated from the extent of the labeled probe uptake before lutoid lysis: the difference between the radioactivity level in a given fraction of the outflow during the steady state before adding a lytic amount of Triton X-100, and the amount of radioactivity that would have been in the same fraction had triton been present throughout (estimated by extrapolation of the radioactivity in evidence after lysis), was used to estimate the absorption of the radioactive probes by the lutoids (Ramos et al., 1976; Elema, Michels & Konings, 1978; Sorgato et al., 1978).

Estimation of the Intravacuo-Lysosomal Volume

Lutoids suspended in the medium were incubated simultaneously in the presence of ^{14}C-inulin and ^{3}H-water until a steady state was reached. Then 2-ml aliquots were sampled. Lutoids were pelleted by centrifugation at $30{,}000 \times g$ for 5 min and the radioactivity of each supernate and pellet was determined. Internal volumes of each sample were calculated by subtracting the ^{14}C-inulin external space from the total water space as described by Reijngoud and Tager (1973).

Enzyme Assays

ATP hydrolysis was estimated by enzymic assays of ADP and AMP. After incubation of the lutoidic suspension with Mg-ATP (5 mM), in the presence of 25 µM ammonium molybdate (in order

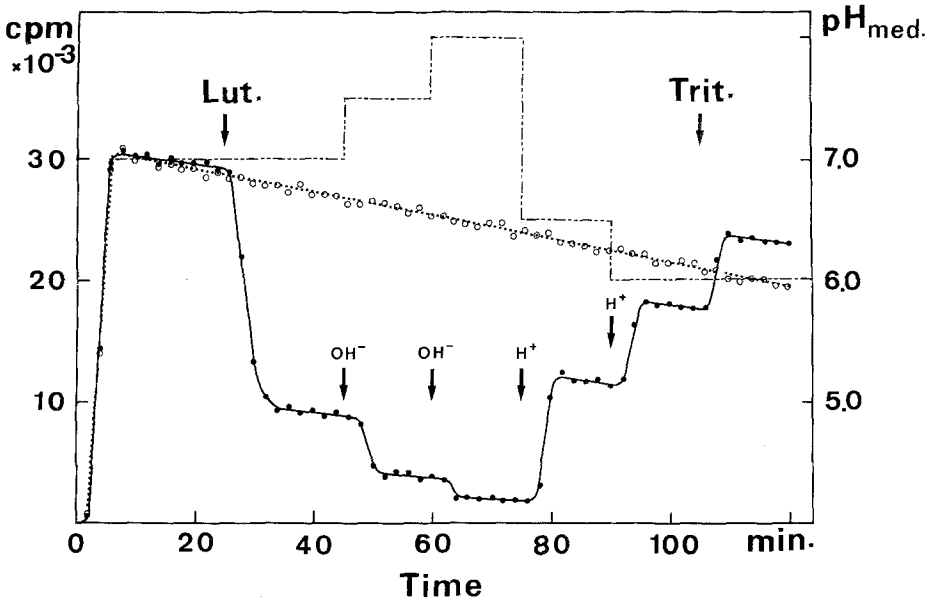

Fig. 1. Absorption of ^{14}C-methylamine by vacuo-lysosomes (lutoids) in "nonenergetic" medium. pH probe movements induced by pH variations of the external medium. The experiment was started by the addition of 15 µCi ^{14}C-methylamine in the upper chamber containing 2.5 ml of the "dialysis incubation" medium (*see* Materials and Methods) at pH 7.45. As indicated by arrows, 0.5 ml of a concentrate lutoidic vacuo-lysosomes suspension (internal volume: 347 µl) in the same emdium and at the same pH was added (Lut.): closed symbols (-●-●-●-). External pH was modified by successive addition of NaOH (0.5 N) then Tris buffer (0.5 M) for alkalinization (as indicated by OH^-), and HCl 0.5 N then Hepes 0.5 M for acidification (H^+), under direct external pH control measurement (dashed lines -·-·-). At the end of the experiment 100 µl Triton X-100 1% were added. Open symbols (··o··o··o··): no vacuo-lysosomes added. All data were corrected for successive dilutions according to Fig. 2

to inhibit the residual acidic phosphatase activity), a 500-µl sample was pipetted from the upper chamber, precipitated with $HClO_4$ 1 M, and then neutralized by KOH. After centrifugation, ADP and AMP formed were estimated from the supernate, in the presence of phosphoenolpyruvate, pyruvatekinase, lactate dehydrogenase and myokinase (Adams, 1965).

The phosphatase activities were assayed with nitrophenyl phosphate (10 mM), with or without 25 µM ammonium molybdate, in order to determine the residual accessible phosphatase activities (Pujarniscle, 1968), relative to ATPase activities.

Estimation of Proteins

Proteins were assayed according to Lowry, Rosebrough, Farr and Randall, (1951).

pH Measurements

Direct pH measurements of the suspending medium or solutes were performed using an ORION model 701-A digital pH meter with a semimicro combination pH electrode ("research quality").

Chemicals

All isotopically labeled solutes were the generous gifts of the Commissariat à l'Energie Atomique (C.E.A.), France.

Other materials were from Sigma Chemical Company, St. Louis, Mo.

Abbreviations: ADP, adenosine-diphosphate; AMP, adenosine monophosphate; ATP, adenosine-triphosphate; ATPase, adenosine-triphosphatase; ΔpH, transmembrane pH gradient; $\Delta\Psi$, transmembrane potential; $\Delta\tilde{\mu}$H, electrochemical proton gradient across the membrane where $\Delta\tilde{\mu}H = \Delta\Psi - Z\ \Delta pH (Z=2,\ 3RT/F = 59$ at 25 °C when expressed in mV); FCCP, carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; Tris, tris-(hydroxymethyl)-aminomethane; *vi*, intravacuolar volume.

Results

^{14}C-Methylamine as a Suitable ΔpH Probe for Lutoids

Figure 1 depicts a typical flow dialysis experiment carried out as described in Materials and Methods. The open symbols represent a control experiment without lutoids in the upper chamber.

The experiment was started by adding about 15 µCi (20 µM) ^{14}C-methylamine to the upper chamber of the dialysis cell with no lutoid present. Within 3 min, a steady-state distribution of ^{14}C-methylamine across the dialysis membrane was reached (Fig. 1). In subsequent fractions, the amount of radioactivity in the outflow decreased slightly. Dilution of the ^{14}C-methylamine in the upper chamber resulted in a proportional decrease of the radioactivity level in the outflow (Fig. 2). Thus the concentration of unbound amine in the upper chamber could be reliably determined from the concentration of the amine in the outflow in the lower chamber. These characteristics also allowed for corrections of

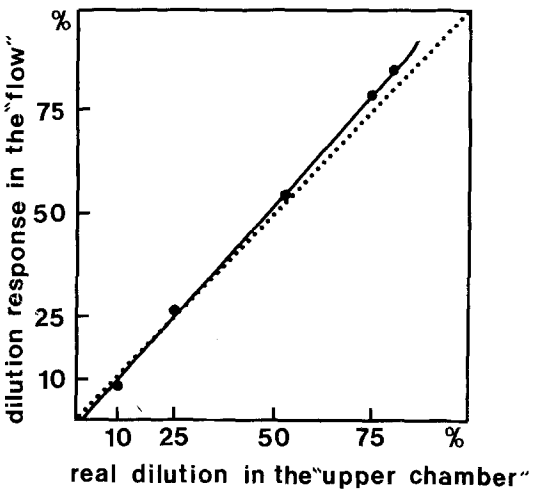

Fig. 2. Characteristics of the "laboratory-made" flow dialysis apparatus: plotting dilution responses in the dialysate as a function of the real dilution performed in the upper chamber by successive buffer addition, expressed in % of radioactivity recovery and % dilution of initial ^{14}C-methylamine dilution (dotted line: ideal dilution response)

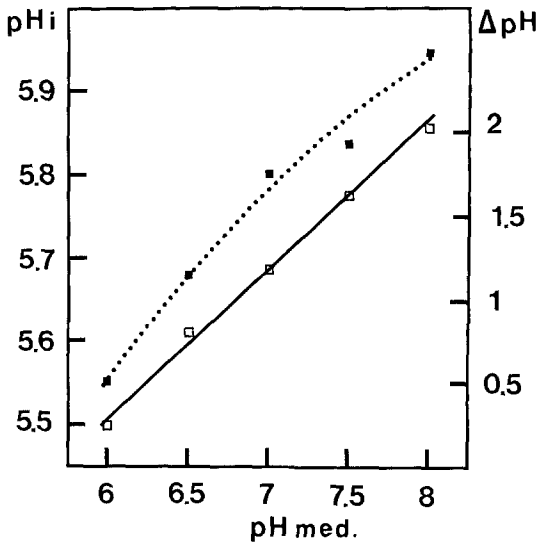

Fig. 3. Effect of varying medium pH on the internal pH and the ΔpH of latex vacuo-lysosomes. The internal pH of particles was estimated by the distribution of ^{14}C-methylamine and determination of internal volume as described in Materials and Methods. Data were plotted from Fig. 1 and Table 1. (···■···■···) pH*i*, and (—□—□—) ΔpH

radioactivity measures following the addition of various solutes.

The closed symbols (Fig. 1) show an experiment in which a concentrated lutoidic suspension was added to the upper chamber after the steady state was reached. The presence of the lutoids induced a sharp decrease in the concentration of the labeled amine in the outflow. Changes in the labeled amine/lutoid concentration ratio had no significant effect on the amine accumulation ratio (not shown). This is consistent with our assumption that the amine is accumulated inside the lutoids rather than bound to the membranes which would be a saturable process.

We conclude that the decrease in the concentration of the labeled amine in the outflow reflects an accumulation inside the lutoids caused by their low internal pH.

Magnetic stirring increased by less than 6% by hour the bursting index of lutoids relative to control experiments (magnetic microbar absent).

Effect of Varying External pH on ΔpH and Internal pH of the Vacuo-Lysosomes

It was found, as shown in Fig. 1, that alkalinization of the external medium by addition of small amounts of concentrated mineral base or "nonpermeant" buffer (Tris), led to an increased accumulation of ^{14}C-methylamine in the intravacuo-lysosomal space.

In contrast, the addition of HCl or concentrated Hepes, increased the radioactivity level in the outflow, indicating the liberation of the amine previously entrapped in the lutoids.

The pH gradient was found to be much more sensitive to changes in the external pH than was the internal pH. Indeed when the external pH varied 2 points the pH gradient was found to vary 1.6 points while internal pH only changed by 0.4 (Fig. 3; Table 1). All variations were found to be in the same direction.

These data are in good agreement with the "*in vivo*" pH gradient measured from centrifuged fresh latex and sonicated lutoids. In this last case the ΔpH of about 1.3 at a cytoplasmic pH of about 7 corresponds to the value predicated in Fig. 3 (Brzozowska-Hanower et al., 1979).

As ΔpH varied markedly with changes in external pH, it can be supposed that the vacuo-lysosomal membrane is not freely permeable to protons, and that there must be a "nonenergetic" process for maintaining the acidic pH within the lutoids.

The Donnan Potential and the "Nonenergized Proton Gradient"

When the lutoids were preincubated in the presence of ^{14}C-methylamine together with ^{86}Rb + valinomycin until a steady-state was reached, normal physiological concentrations of potassium (about 30 mM) in the medium resulted in a ΔpH of about 1 unit and a $\Delta\Psi$ of -70 mV (interior negative). The

Table 1. Estimation of internal pH and ΔpH across the vacuo-lysosomal membrane under different conditions of incubation medium[a]

Reference Figures	External pH	Initial preincubation medium modification	Effectors added	Further additions	pH*i*	ΔpH
Fig. 1	6.0	–	H^+	$+H^+$ (Hepes)	5.55	0.45
	6.5	–	H^+ (HCl)	–	5.68	0.82
	7.0[b]	–	–	–	5.805	1.19
	7.5	–	OH^- (NaOH)	–	5.835	1.66
	8.0	–	OH^-	$+OH^-$ (Tris)	5.95	2.05
Fig. 4	7.45	+KCl 30 mM	–	–	6.12	1.33
	7.45	+KCl 30 mM	ADP-Mg 5 mM	–	6.06	1.39
	7.45	+KCl 30 mM	ATP-Mg 5 mM	–	5.43	2.02
	7.45	+KCl 30 mM	ATP-(or) ADP-Mg	$+NH_4Cl$ 30 mM	7.32	0.13
	7.45	+KCl 30 mM	NH_4Cl 30 mM	+Triton	7.45	0
Fig. 5	7.0	–	–	–	5.84	1.16
	7.0	–	ATP-Mg 5 mM	–	5.0	2
	7.0	+FCCP 10 µM	–	–	5.89	1.11
	7.0	+FCCP 10 µM	ATP-Mg 5 mM	–	5.97	1.03
not shown	7.0	–	–	–	5.93	1.07
	7.0	–	ATP-Mg 5 mM	–	5.15	1.85
	7.0	–	ATP-Mg 5 mM	+FCCP	6.05	0.95
	7.0	–	FCCP 5 µM	$+NH_4Cl$ 30 mM	6.68	0.32
Fig. 6	7.0	+valinomycin 10 µg/ml + 10 mM KCl	–	–	5.89	1.11
	7.0	+valinomycin 10 µg/ml + 10 mM KCl	ATP-Mg 5 mM	–	4.89	2.11
	7.0	+valinomycin 10 µg/ml + 10 mM KCl	ATP-Mg 5 mM	+KCl 120 mM	5.35	1.65

[a] pH*i* and ΔpH of vacuo-lysosomes were calculated as described in Materials and Methods from the accumulation ratio of methylamine and internal volume of the particles. Data were plotted from reference Figures.
[b] Initial pH of the incubation medium (molarities are expressed in final molarities in the upper chamber of the flow dialysis cell).

resulting $\Delta\tilde{\mu}H$ was shown to be near zero or slightly negative. Increasing the concentration of KCl in the external medium led to a $\Delta\Psi$ collapse (interior less negative) and a corresponding ΔpH decrease, $\Delta\tilde{\mu}H$ remaining about zero. More than 200 mM KCl were necessary to abolish the Donnan potential. At these very high potassium concentrations ΔpH was, in most cases, completely discharged (*see* Table 2).

When valinomycin was omitted, a far longer preincubation period (more than 60 min), with large amounts of KCl, was necessary to bring about the same ΔpH and $\Delta\Psi$ collapse (*not shown*). This indicates that the lutoidic membrane is not completely impermeable to potassium and that the lutoids equilibrate very slowly with external potassium. Valinomycin accelerated the potassium transfer through the lutoidic membrane. These results indicate clearly that the Donnan potential can fully account for ΔpH in nonenergized lutoids.

Table 2. Effects of increasing concentration of potassium on the initial ΔpH, $\Delta\Psi$ and the resulting $\Delta\tilde{\mu}H$ of nonenergized lutoids

KCl (mM) Ext. medium	ΔpH	$-Z\Delta$pH (mV)	$\Delta\Psi$ (mV)	$\Delta\tilde{\mu}H$
10	-1.19 ± 0.11	71 ± 7	-84 ± 8	-13 ± 15
30	-0.98 ± 0.12	58 ± 7	-70 ± 6	-12 ± 13
120	-0.53 ± 0.10	31 ± 6	-40 ± 5	-9 ± 11
220	-0.20 ± 0.08	12 ± 5	-10 ± 5	$+2\pm10$

The lutoids (internal volume = 307 ± 22 µl) were preloaded for 30 min in the dialysis-incubation medium + 10 mM KCl at pH = 7.0 in the presence of 20 µCi ^{14}C-methylamine together with 15 µCi ^{86}Rb + 30 µg valinomycin. When the steady state was reached in the upper chamber, various amounts of KCl 3 M were added in the medium. Vi, ΔpH, $\Delta\Psi$ and $\Delta\tilde{\mu}H$ were calculated as described in Materials and Methods (a mean of triplicate experiments).

The "Proton Pump" Function of the ATPase Bound to the Vacuo-Lysosomal Membrane

Figure 4 shows that the lutoids accumulated the amine during the 20-min preincubation period.

Amine accumulation was found to increase sharply, as indicated by the dramatic decline in the radioactivity of the external medium, after the addition of 5 mM Mg-ATP. The addition of 5 mM Mg-ADP had no effect.

In our experimental conditions, acidic phosphatases are largely inhibited by alkaline pH, by the presence of 5 mM Mg^{2+} and by 25 µM molybdate (Jacob & Sontag, 1974; d'Auzac, 1975). Assuming an

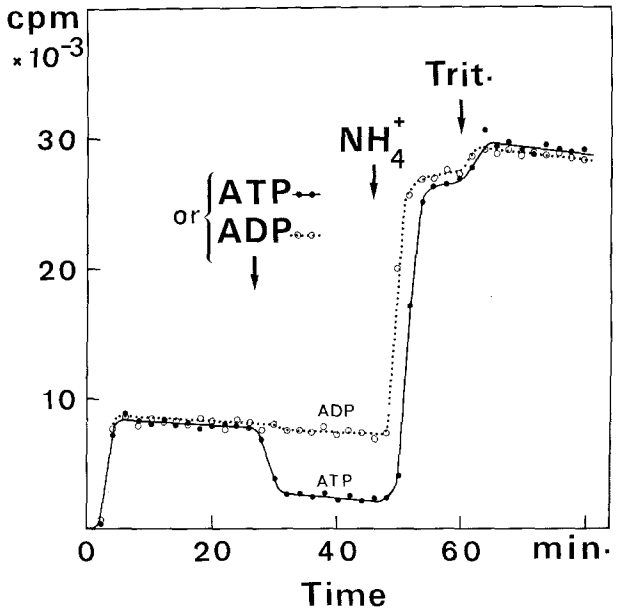

Fig. 4. ATP-Mg-dependent ^{14}C-methylamine uptake by vacuo-lysosomes: The proton-translocating ATPase. Fresh vacuo-lysosomes (internal volume = 360 µl) were suspended in the "dialysis incubation" medium, pH = 7.45, in the presence of 30 mM KCl and 20 µCi ^{14}C-methylamine (3 ml total volume) at 26 °C. After a 20-min preloading period, the vacuo-lysosomal suspension was transferred to the upper chamber of the flow-dialysis cell. After 25-min dialysis 5 mM ATP-Mg (-●-●-●-) or ADP-Mg (··○··○··○··) carefully adjusted to the pH of the incubation medium were added. As indicated by the arrow 30 mM NH_4Cl (final concentration) were added. At the end of the experiment organelles were lysed by 100 µM Triton X-100 (1%). pH evolution of the external medium was periodically controlled

ATP/PNPP activity ratio of about 0.4 for acidic phosphatase activity (J.L. Jacob, *personal communication*), we estimate that the ATPase is about 28 times more active than the phosphatase with Mg-ATP as substrate (Table 3). Then we can consider that nearly all of the hydrolyzed ATP (91.3%) is ascribable to the ATPase activity. The ATP-dependent amine uptake was oligomycin-insensitive (10 µg oligomycin) discarding any possibility of mitochondrial contamination (*not shown*).

As no marked change in external pH (100 mM buffer) could be detected by direct pH measurements, we conclude that the amine decrease in the external medium reflects a marked acidification of the lutoids during ATP hydrolysis.

Addition of NH_4Cl, at uncoupling concentration, caused the efflux of the amine accumulated in the lutoids that were incubated with Mg-ATP or Mg-ATP.

The subsequent addition of a lytic amount of Triton X-100 caused only a very slight increase in the external radioactivity. This indicates that the addition of the protonophore NH_4^+ had nearly completely dissipated the pH-gradient, and therefore caused the total efflux of the amine which was entrapped in the vacuo-lysosomes.

Table 3. Relative accessible phosphatase and membrane-bound ATPase activities with ATP-Mg^{2+} (5 mM) as substrate

ATP hydrolyzed after 20 min	91.3% or 4.56 mM
ATPase activity with ATP-Mg as substrate	0.311
Residual accessible phosphatase with PNPP as substrate	0.027
Residual accessible phosphatase with ATP-Mg as substrate (assuming an ATP/PNPP activity = 0.4)	0.011
Membrane-bound ATPase/residual accessible phosphatase activity ratio (ATP as substrate)	28.3

ATPase and acid phosphatase activities were determined as described in methods from parallel experiments, in the same condition as described in Fig. 4.

Initial incubation medium: Mannitol 320 mM; Hepes-Tris buffer 100 mM; $MgCl_2$ 3 mM; KCl 30 mM; ammonium molybdate 25 µM, pH = 7.45; vacuolysosomes (internal volume = 360 µl for 3 ml total). Assays were started by 5 mM ATP-Mg or 10 mM PNPP. Enzyme activities are expressed in arbitrary units: mM hydrolyzed substrates/min/3 ml of the total suspension with 360 µl lutoids.

While the pH of the medium remained constant, internal pH and therefore ΔpH were observed to shift 0.7 unit when Mg-ATP was added, energizing the lutoids. Subsequent addition of NH_4Cl caused the proton gradient to drop from 2.02 to 0.13 (Table 1).

From these results, it can be concluded that the ATP-induced acidification of the lutoids is attributable to the membrane-bound ATPase functioning as a proton pump.

Effects of Ionophores

Preincubation of lutoids in the presence of FCCP (5 to 15 µM) together with ^{14}C-methylamine, slightly lowered the initial proton gradient, but inhibited completely further accumulation of the amine when Mg-ATP was added. On the contrary, when FCCP was present prior to any addition of ATP, the ATPase activity caused a slight efflux of the amine previously accumulated by the lutoids (*see* Fig. 5). Addition of FCCP after the internal acidification resulting from the ATPase activity, caused a corresponding efflux of the amine. The total amine could be liberated by subsequent addition of NH_4Cl or a lytic amount of Triton X-100.

It is concluded that the Mg-ATP-induced uptake of methylamine is more sensitive to FCCP than

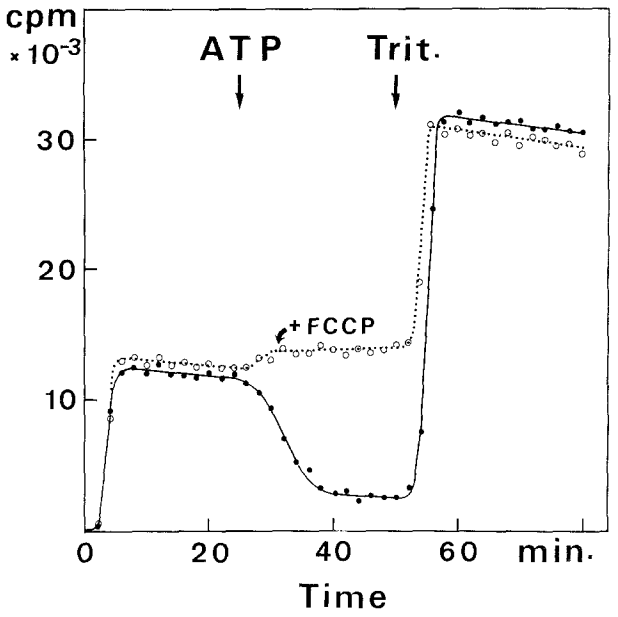

Fig. 5. Effect of preincubation of the vacuo-lysosomes with FCCP on initial and ATP-induced methylamine uptake (pH medium = 7.0). Vacuo-lysosomes (internal volume = 320 µl) were preloaded 20 min with ^{14}C-methylamine in the presence (··o··o··o··) or absence (-●-●-●-) of 10 µM FCCP, pH medium = 7.0 (KCl absent). Then the vacuo-lysosomal suspension was transferred to the upper chamber of the flow-dialysis cell. As indicated by arrows ATP-Mg^{2+} (5 mM final; pH 7.0) was added in both assays. The experiments were ended by a 100-µl Triton X-100 (1%) addition

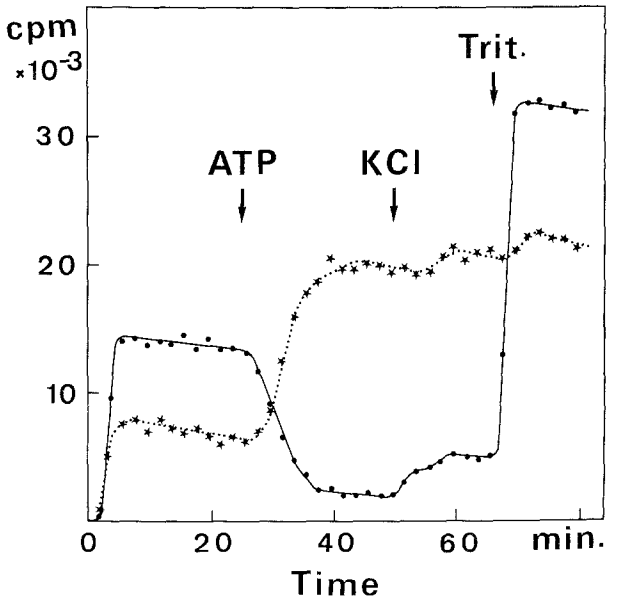

Fig. 6. Effect of ATP−Mg on the simultaneous movements of ^{14}C-methylamine and ^{86}Rb in the presence of valinomycin. Vacuo-lysosomes (internal volume = 318 µl) were preloaded 30 min in the dialysis-incubation medium + 10 mM KCl at pH = 7.0, in the presence of 20 µCi ^{14}C-methylamine and 15 µCi ^{86}Rb + 30 µg valinomycin. 25 min after the suspension had been transferred to the upper chamber, ATP-Mg^{2+} (5 mM final, pH = 7.0) then KCl (120 mM, pH = 7.0) and finally 100 µl Triton X-100 (1%) were added. (^{14}C-methylamine evolution: -●-●-●-; ^{86}Rb evolutions: ···*···*···

is that depending on the initial proton gradient. Because FCCP brings about a charge uncompensated transport of protons across the membranes (Mitchell & Moyle, 1968), these data suggest that the Mg-ATP-induced uptake of the amine is associated with a proton pump operating electrogenically.

When the lutoids were preincubated in the presence of ^{14}C-methylamine together with ^{86}Rb + valinomycin, addition of Mg-ATP caused the same amine influx in the lutoids as described above, associated with a rapid efflux of the ^{86}Rb accumulated during the preincubation period in the presence of valinomycin (*see* Fig. 6). Here again valinomycin strongly facilitated the transfer of ^{86}Rb (and K^+) though the membrane. When valinomycin was omitted, the efflux of ^{86}Rb was found to be very slow and less, even though triton lysis did show that an appreciable amount of ^{86}Rb had been accumulated during a 75-min preloading period (*not shown*). From this, it seems evident that, in the absence of valinomycin, proton uptakes are not directly compensated by corresponding efflux of ^{86}Rb or potassium. So we could expect a corresponding change in $\Delta\Psi$, indicating electrogenicity.

Addition of KCl (120 mM) in the presence of valinomycin caused only a very slight additional efflux of ^{86}Rb, and subsequent addition of a lytic amount of Triton X-100 revealed that very little ^{86}Rb remained in lutoids. This indicates that Mg-ATP had induced a nearly complete depolarization of the vacuo-lysosomal membrane.

With the addition of Mg-ATP, ΔpH was estimated to change one unit from 1.11 to 2.11 (Table 1; Fig. 6) and $\Delta\Psi$ shifted from −83 mV to −19.4 mV with addition of ATP, corresponding to a depolarization of 63.6 mV (interior less negative).

The estimated values of the corresponding electrochemical $\Delta\tilde{\mu}H$ shifted from −17.5 mV, when lutoids were in a nonenergized state, to +105 mV when they were energized by Mg-ATP. These data are in good agreement with the concept that the ATPase located on the vacuo-lysosomal membrane functions as an electrogenic proton pump.

Effects of KCl on the Proton Pump Activity and the Resulting $\Delta\tilde{\mu}H$

When the lutoids were incubated in the presence of ^{14}C-methylamine together with ^{86}Rb + valinomycin

Table 4. Effects of increasing concentration of KCl on the ATP-dependent proton pump activity and the resulting $\Delta\tilde{\mu}H$[a]

KCl (mM) external incubation medium	$-Z\Delta pHi$ (mV)	$-Z\Delta pH$ (ATP) (mV)	$\Delta\Psi i$ (mV)	$\Delta\Psi$ (ATP) (mV)	$\Delta\tilde{\mu}Hi$ (mV)	$\Delta\tilde{\mu}H$ (ATP) (mV)
10	74	124	−92	−28	−18	+96
30	62	106	−73	−17	−11	+89
120	32	88	−44	− 8	−12	+80
220	11	78	−12	> 0	− 2	>78

[a] The lutoids were preincubated as described in Table 2, in the presence of various concentrations of KCl before their transfer in the flow dialysis cell. After the steady state was reached in the outflow, 5 mM Mg−ATP were added. *Vi*, ΔpH, $\Delta\Psi$ and resulting $\Delta\tilde{\mu}H$ were estimated as described in Materials and Methods (a mean of duplicate experiments) (i=initial values before energization; (ATP): final value 30 min after the addition of 5 mM Mg-ATP). (Quantifications of $\Delta\tilde{\mu}H$ at very high potassium concentrations were probably underestimated because of the low accuracy of determinations at so low ΔpHi or $\Delta\Psi$ values.)

with increasing concentration of KCl (0−220 mM), there was a proportional decrease of the initial ΔpH with $\Delta\Psi$ as described above (Table 4).

The subsequent addition of Mg-ATP led to a marked increase of the ΔpH and to a significant decrease of $\Delta\Psi$ whatever the potassium concentration. After energization, the $\Delta\tilde{\mu}H$ of the lutoids increased more than 100 mV.

All results presented here suggest strongly that the proton pumping function of the ATPase is electrogenic in nature.

Discussion

Since the distribution of methylamine is directly proportional to the proton gradient through the lutoidic membrane as confirmed here (Fig. 1) and moreover by Marin (1981) we can consider that an intralutoidic accumulation of the probe, reflects indeed an acidification of the lutoidic internal space (Rottenberg et al., 1972; Reijngoud & Tager, 1973).

It is important to point out that the data presented here do show the existence of an acidic pH within the vacuo-lysosomes prior to any addition of Mg-ATP. Our estimation of the initial pH gradient by the amine distribution is in good agreement with ΔpH measured with a pH electrode from centrifuged fresh latex and sonicated lutoids (Brzozowska-Hanower et al., 1979).

The addition of large amounts of KCl in the presence of valinomycin, rapidly collapsed $\Delta\Psi$ and correspondingly reduced ΔpH. Moreover, the addition of FCCP to nonenergized lutoids did not significantly affect ΔpH indicating proton equilibration across the membrane, even in the absence of this protonophore. These results strongly support the hypothesis of a Donnan potential as the source of ΔpH in nonenergized lutoids, at low physiological potassium concentration (30 mM), hence implying some membrane permeability towards protons (Reijngoud et al., 1976*a*, *b*).

In our experimental conditions, the low sensitivity of internal pH towards external pH, and the pH dependence of ΔpH could result from at least three different phenomena: a relative slow rate of proton equilibration through the lutoidic membrane; from the dissociation of internal charges that contribute to Donnan equilibrium; and, most probably, from a high buffer power of the intra-lutoidic content in the 5.5 to 6 pH range (J.L. Jacob, *personal communication*).

The uptake of methylamine by lutoids was greatly increased by the addition of Mg-ATP reflecting an important acidification of the intra-lutoidic space during ATP hydrolysis. This ATP-dependent acidification was insensitive to oligomycin, discarding any possibility of mitochondrial contamination, and was not significantly affected by preincubation of the lutoids in the presence of various amounts of potassium (Schneider, 1979; Hollemans et al., 1980).

As Mg-ATP induced simultaneously an uptake of methylamine and a release of ^{86}Rb by lutoids (rapid only in the presence of valinomycin) we suggest that the ATPase, located on the vacuo-lysosomal membrane of the lutoids, functions as an electrogenic proton pump which generates the acidification of the intra-lutoidic space and the depolarization of the membrane.

The existence of an ATP-driven proton pump bound to the vacuo-lysosomal membrane is in good agreement with previous reports on the *in vitro* stimulation by Mg-ATP of citrate[1] and basic amino-acid uptake by lutoids (d'Auzac & Lioret, 1974; Lambert, 1975; d'Auzac et al., 1977*a*; Hanower et al., 1977). Our results strongly support the involvement of the "proton motive force" proposed by Mitchell and Moyle (1968), as the energy source for solute absorption by vacuo-lysosomal particles *in vitro* as *in vivo*. *In vitro*, the estimated values of $\Delta\tilde{\mu}H$ increased more than 100 mV when energized with Mg-ATP in the presence of a physiological concentration of KCl.

The Mg-ATP-driven proton pump allows the generation of high proton gradients despite the pres-

[1] Marin and co-workers show evidence that citrate uptake by lutoids operates through proton exchange (RH^{2-}/H^{+}) coupled to a compensating flux of charges (Probably Mg^{2+} or Ca^{2+}) with a net flux of charge equal to minus one (Marin & Grignon, 1981; Marin, Smith & Luttge, 1981).

ence of 30 mM potassium (*in vivo*) which would tend to lower the proton gradient generated by Donnan potential alone (Henning, 1975).

Consistent with the concept of an ATP-driven proton pump, we propose that the lutoids play a double role as a "biophysical pH-stat" and a "detoxifying trap", thus controlling cytoplasmic homeostasis which in turn favors the whole latex metabolism and regeneration, resulting in high rubber productions (Jacob, 1970; Ribailler et al., 1971; Tupy, 1973).

This work was partly supported by grants from the "Centre National de la Recherche Scientifique" (CNRS), France: "Thematic Programmed Actions" on physiological and genetical bases for plant yielding (51); "Thematic Programmed Actions" on energy conversion at the biological membrane level (No. 031259).

We are thankful for the constructive criticisms and encouragements of Doctors J. d'Auzac, J.L. Jacob, C. Lioret and Pr. E. Mac Robbie during the elaboration of this manuscript. We are grateful to Mr. M. Miezan and D. Khols for considerable help in English translation. We acknowledge Mr. Chefson for his large collaboration in the construction of the flow dialysis cell, and C.E.A. France for the generous gifts of radioactive solutes.

Note Added in Proof

Simultaneous with the first submission of this manuscript, the author learned that B. Marin, M. Marin-Lanza and E. Komor, using different techniques have also studied the ATP-dependent change of $\Delta\tilde{\mu}H$ across the membrane of lutoids kept for 48 hr in an ice-bath. Even though their quantified data were somewhat different, phenomenons were shown to be in the same direction as ours, and the ATPase activity was shown to increase the proton motive force by 80 mV (Martin, B., Marin-Lanza, M., Komor, E. (1981). The proton motive potential difference across the vacuolysosomal membrane of *Hevea brasiliensis* and its modification by a membrane bound adenosine-triphosphatase. *Biochem J., accepted for publication*).

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Received 1 May 1981; revised 28 July 1981