ACKNOWLEDGEMENT

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Cooperation of a "Davies Type" Biochemical pH-Stat and the Tonoplastic Bioosmotic pH-Stat in the Regulation of the Cytosolic pH of *Hevea* Latex

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Introduction

The latex from Hevea brasiliensis is a fluid cytoplasm which is expelled from wounded laticiferous vessels (i.e.: articulated, anastomosed cells) (Archer et al, 1963). It contains a vacuolar compartment: the so-called "lutoids", consisting of micro-vacuoles with lysosomal characteristics, which can be easily isolated and purified by simple centrifugation (Pujarniscle, 1968; Ribaillier et al, 1971; d'Auzac et al, 1982).

Hevea latex is collected for its high natural rubber (cis-poly-isoprene) content. Production of latex (rubber) reflects the intensity of metabolism within these specialized cells. It must be sufficient to regenerate and compensate for the loss of latex upon each tapping (generally twice a week).

Rubber production has been shown to be correlated, positively and with very high significance to the pH of the cytosol of the latex cell (Coupé and Lambert, 1977; Brzozowska - Hanower et al, 1979). This could be satisfactorily explained, as far as numerous key enzymes of latex cytosolic metabolism are extremely pH-sensitive in the physiological pH range. This is, in particular, the case of invertase (Tupy, 1973 a), which controls the entry of sucrose catabolism and latex regeneration (Tupy, 1973 b). It is also the case of pyruvate-decarboxylase (Jacob, 1970; Tupy and Primot, 1976) which converts pyruvate into acetate, the obligatory precursor of rubber synthesis. In the same way, Jacob et al (1979 - 1983) demonstrated that phosphoenol-pyruvate-carboxylase (PEPcase), which can divert glycolysis from the "isoprenoid pathway" to Krebs cycle acid synthesis, is very sensitive to physiological pH change.

Insofar as extreme values of the latex cytosolic pH vary from 6.5 to 7.3 (Brzozowska-Hanower et al, 1979) which correspond precisely to the pH range controlling the activity of these key enzymes, the understanding of the mechanism likely to regulate the cytosolic pH in the latex cells became of capital interest. The characteristics and the pH sensitivity of the latex PEPcase led Jacob et al (1979) to

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postulate and study its role as a possible "Davies type" pH-stat (Davies, 1973). Main results on these studies will be summarised in this report.

Further investigations established that latex production was not only linked to the pH of the latex cytosol but positively as well to the transtonoplastic pH gradient, and negatively (high significance) to the intravacuolar pH (Coupé and Lambert, 1977; Brzozowska-Hanower et al, 1979). Moreover, we demonstrated a highly significant inverse relationship between the pH of the cytosolic compartment and the changes in intravacuolar pH (lutoidic pH), suggesting the existence of some vectorial H^+ fluxes at the level of the lutoidic tonoplast (Brzozowska-Hanower et al, 1979). Supporting such an hypothesis, it has been shown that treatments of Hevea bark with Ethrel, an ethylene generator which "stimulates" latex production (d'Auzac and Ribaillier, 1969), induce a wide alkalinization of the latex cytosol (Tupy, 1969; Coupé et al, 1976; Brzozowska-Hanower et al, 1979) concomitant with a marked vacuolar acidification (Coupé et al, 1976, Brzozowska-Hanower et al, 1979). This led to looking for possible mechanisms able to control transtonoplastic protons fluxes, and then regulate the cytosolic pH. ATPase activity evidenced by d'Auzac (1975, 1977) as well as the redox system demonstrated by Moreau et al, (1975), both located on the lutoidic tonoplast, were suspected as good candidates for controlling such protons exchanges between the cytosolic and the vacuolar compartment within the latex cells.

We report here the present state of knowledge of the functionning of the bioosmotic tonoplastic pH-stat.

Materials and Methods

The latex was collected and immediately centrifuged either at 35,000 g for lutoid purification, or at 80,000 g to obtain the clear cytosol. The pelleted lutoids were washed in an isotonic buffer at pH 7 (mannitol 320 mM, Hepes-Mes 50 mM adjusted to the desired pH by Tris, KCl 30 mM, $MgSO_4$ 2.5 mM and, according to d'Auzac (1975), ammonium molybdate 0.1 mM in order to inhibit acid phosphatase activities). The purified lutoids were resuspended either in the same isotonic buffer, or in ultrafiltered latex cytosol, complemented with $MgSO_4$ 2.5 mM and ammonium and molybdate 0.1 mM, then adjusted to the desired pH by the zwiterionic buffers.

The latex cytosol was deproteinized by ultrafiltration of the clear centrifuged cytosol on AMICON molecular filter PM 10, under 3.5 bars N_2, at 4 °C. This ultrafiltered serum is a quasi-physiological medium to test enzymatic activity.

The vacuolar pH changes were monitored using ^{14}C-methylamine as a ΔpH probe, transtonoplastic potentials were estimated using either ^{3}H-TPP$^+$ or ^{14}C-MTPP$^+$ or ^{86}Rb$^+$ + valinomycin, as Δφ probes. (Marin, 1981; Marin et al, 1981; Crétin, 1982; Chrestin, 1984).

ATPase activities were performed enzymatically according to d'Auzac (1975 - 1977), NADH-cytochrome c-reductase as described by Moreau et al (1975), and acid phosphatases according to Pujarniscle (1968). Latex ATP and adenine nucleotides were measured using the luciferin-luciferase method, according to Pradet (1967).

The Cytosolic Biochemical pH-Stat

PEP is located at a metabolic "crossroads" in Hevea. It either directs extremely active glycolysis (Jacob, 1970) to the production of acetate and isoprenic anabolism, or enables the synthesis of oxaloacetate (OAA) and strong acids together with anaplerotic reactions; some of these substances may be involved in energy-generating mitochondrial oxidation processes. Study of the enzymes which constitute the phosphoenolpyruvic crossroads - PEPcase (Jacob and d'Auzac, 1969) and pyruvate-kinase (Jacob et al, 1981) made it possible to demonstrate the existence of regulation mechanisms playing a role in the control of the cytosolic pH.

In the laticiferous cytosol, PEPcase leads via OAA to the rapid formation of malate and also citrate (Jacob, 1970). It has been shown that malate, and citrate to a lesser extent, are inhibitors of PEPcase and their effectiveness depends on the pH (Jacob et al, 1979). At a sufficiently high concentration, malate inhibits PEPcase much more in a slightly acid medium than in a slightly alkaline one. The result is that the presence of malate makes the functioning of carboxylase sensitive to pH variations. In this case, slightly alkalinization induces strong activity on the part of the enzyme ; slightly acidification inhibits it effectively. Malate contents (< 7mM) in the cytosol and the natural variations of the cytosolic pH (6.5 - 7.3) can effectively modulate carboxylation of PEP. As the functioning of PEPcase produces strong organic acids (malate and citrate) which can accumulate and acidify the medium (self-regulation follows tending to inhibit the enzyme (Fig. 1). Thus, in relatively acid cytosol (pH 6.6 - 6.8) the production of OAA, malate and citrate from PEP and $^{14}CO_2$ is extremely small. On the other hand, from pH 6.8 carboxylation of PEP in the cytosol and in the latex in toto leads to the formation of relatively large quantities of malate and citrate (Jacob et al, 1970 ; Jacob et al, 1983) tending to limit the alkalinization of the medium.

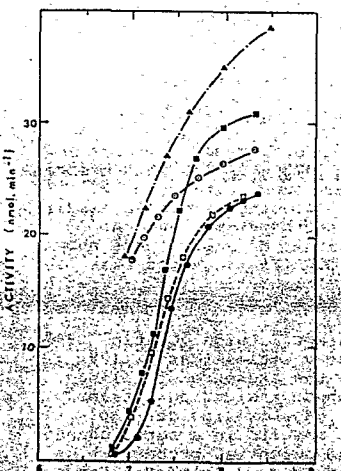


Fig. 1 . Influence of the pH on the activity of the purified PEPcase of the Hevea brasiliensis latex.

All media contain : 5.1 mM Mg^{2+}, 2 mM HCO_3^-, 0.75 mM PEP and 10 µl of purified enzyme solution. The pH has been checked before and after each measuring.
(▲) 0.1 M tris-HCl buffer
(⊙) 0.1 M tris-HCl buffer + 1.5 mM citrate
(■) 0.1 M tris-HCl buffer + 13 mM malate
(□) 0.1 M tris-HCl buffer + 1.5 M citrate + 13 mM malate
(●) filtered cytoplasmic serum (containing 1.5 mM citrate and 13 mM malate)

The small buffer capacity of the cytosol should be stressed ; small variations of H^+ and OH^- concentrations cause physiologically important modifications of pH.

The first part of a pH-stat mechanism mentioned by Davies (1973) and then by Smith and Raven (1979) thus appears to be physiologically functional in latex. The second part of this mechanism implies the irreversible decarboxylation of malate by the malic enzyme into a weaker acid, pyruvate and CO_2 ; this is followed by re-alkalinization of the medium. The malic enzyme is present in the laticiferious cytosol and functional within the limits of the available $NADP^+$ (Jacob and Prévot, 1981). All the elements are therefore present for the functioning of a biochemical pH-stat in latex.

Nevertheless, the deviation of glycolysis at PEPcase level is under strict metabolic control and, under normal conditions, its functioning is never very intense compared to the pathway using pyruvate kinase and leading to isoprenic synthesis. It should also be noticed that the functioning of the malic enzyme leads to the production of NADPH, an indispensable cofactor in isoprenic synthesis.

The Functioning of a Bioosmotic pH Stat at Tonoplast Level

Like all plant vacuoles, lutoids have a lower internal pH (about 5.5) than that of their cytosolic environment (about 7). H^+ accumulation within intact lutoids has been shown to originate from two complementary processes (Crétin, 1982 ; Chréstin, 1984).

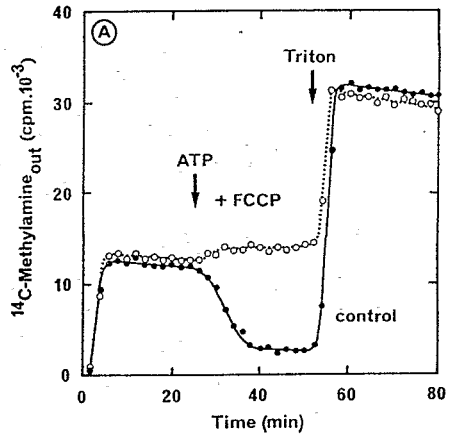
A large pool of protons remains sequestered at the thermodynamic equilibrium within the vacuolar compartment, owing to the existence of a transtonoplastic Donnan potential (Crétin, 1982).

Transtonoplastic H^+ fluxes, which determine the cytosolic as well as the vacuolar pH changes, are shown to be under the control of two opposing H^+ translocating systems located at the level of the lutoidic tonoplast (Chréstin, 1984).

The Inwards Proton Pumping Activity of the Tonoplast ATPase

The first is the tonoplastic ATPase dependent on Mg^{++}, evidenced and partially characterized by d'Auzac (1975 - 1977). This membrane constitutive ATPase functions as a proton pump, catalysing H^+ influx into the vacuole (acidification), causing cytosolic alkalinization and an increase in transtonoplastic pH gradient. As expected, the ATP dependent transtonoplastic H^+ fluxes were shown to be inhibitable by the protonophore FCCP (Fig. 2 A and B ; Table 1) (Marin, 1981 ; Marin et al, 1981 ; Crétin, 1982 ; Crétin et al, 1982 ; Chréstin, 1984, Marin, 1984 a - c).

In the absence of any energy supply, intact lutoids (Crétin, 1982, 1984) as well as reconstituted tonoplast vesicles (Marin, 1981 ; Marin et al, 1981) accumulate $^{86}Rb^+$ (in the presence of valinomycin) and the cationic phosphonium probes, indicating a transmembrane electric potential difference, negative inside (Table 1). The addition of MgATP to both materials results in a rapid change in the transmembrane distribution of the potential probes, which corresponds to a membrane depolarization (Fig. 3 A and B ; Table 1).



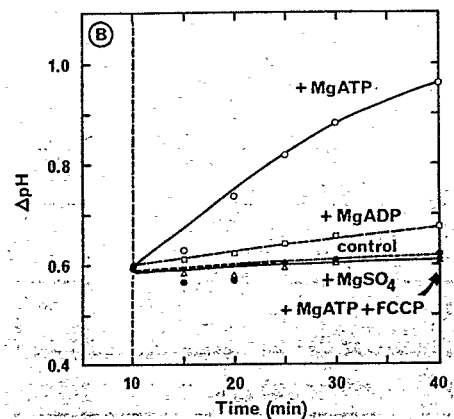


Fig. 2. Time course of ΔpH changes across the tonoplast (inside acid) and the effect of ATP and FCCP as measured by accumulation of methylamine.
A: a typical flow dialysis experiment (Crétin, 1982) performed with intact fresh lutoids at pH 7 in the absence (●——●) or presence (O·····O) of FCCP 3.3 µM (Mg-ATP 5 mM; Triton X-100 0.1 %).
B: experiments performed with reconstituted tonoplast vesicles by the centrifugation technic (Marin et al, 1981) at pH 7.5 (concentration of effectors 5 mM; FCCP 1 µM).

Table 1. Comparative study of the transtonoplastic ΔpH, Δφ and $\Delta\tilde{\mu}H^+$ of intact lutoids loaded in artificial buffer of ultrafiltered latex cytosol in the presence or absence of Mg-ATP

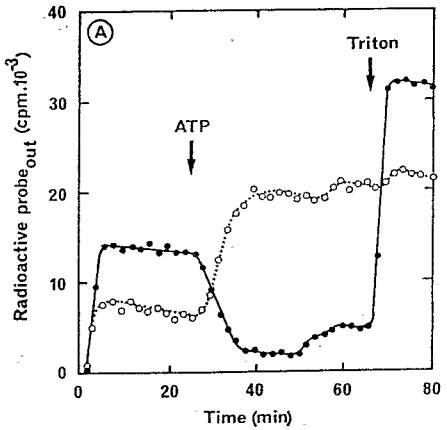
	− Z · ΔpH (mV)		Δφ(mV)		$\Delta\tilde{\mu}H$ (mV)	
Medium	none	+ ATP	none	+ ATP	none	+ ATP
Buffer	70	101	− 68	− 20	+ 2	+ 81
Cytosol	82	145	− 28	− 2	+ 54	+ 143
Cytosol + FCCP	40	33	− 87	− 97	− 47	− 59

Fresh intact lutoids were preloaded in the isotonic buffer or in ultrafiltered latex cytosol, at pH 7.0. ΔpH (^{14}C-MeA) and Δφ ($^{86}Rb^+$ + Valinomycin) were estimated by the centrifugation technic, after 20 min incubation in the presence or absence of ATP 5 mM (Mg^{++} 5 mM present; ± FCCP 50 µM). The values are expressed in mV.

Table 2. Comparative study of the transtonoplastic ΔpH and $\Delta\tilde{\mu}H^+$ of intact fresh lutoids loaded in artificial buffer of ultrafiltered latex cytosol, in the presence or absence of NADH + cytochrome c

	− Z · ΔpH (mV)		Δφ (mV)		$\Delta\tilde{\mu}H^+$ (mV)	
Medium	none	+ NADH + cyt.c.	none	+ NADH + cyt.c	none	+ NADH + cyt.c
Buffer	68	47	− 80	− 102	− 12	− 55
Cytosol (*)	73	42	− 37	− 63	+ 31	− 11
Cyt. (*) + FCCP	41	36	− 100	− 93	− 59	− 57

The experiments were performed as described in Table 1, except that NADH (2 mM) + c cytochrome (0.5 mM) were substituted for ATP.
(*) : the cytosol was pretreated with ascorbate oxydase, then ultrafiltered, in order to avoid rapid chemical reduction of c cytochrome, and consecutive lack of the appropriate electron acceptor.



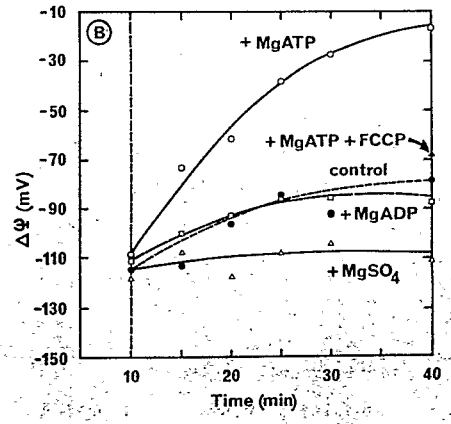


Fig. 3. Time course of Δφ changes across the tonoplast (inside negative) and the effect of ATP and some effectors, as measured by accumulation of Rb^+ and $MTPP^+$.
A: a flow dialysis experiment performed with intact fresh lutoids in the presence of valinomycin (10 µM·ml^{-1}) in the presence of KCl 30 mM at pH 7, either with $^{86}Rb^+$ or ^{14}C-methylamine (effectors added Mg ATP 5 mM; KCl 120 mM; Triton 0.1 %).
Bi: experiments performed with reconstituted tonoplast vesicles: same conditions as in Fig. 1.B, except that ^{14}C-$MTPP^+$ was substituted for ^{14}C-methylamine

All these results led to the conclusion that the Mg-dependent ATPase located on the lutoidic tonoplast works as an electrogenic proton pump, building a high (inside positive) electrochemical proton gradient ($\Delta\mu H^+$) accross the lutoidic tonoplast.

Moreover, the electrogenic proton pumping ATPase has recently been shown (Chréstin, 1984 ; Gidrol, 1984) to function, at least as well, in "quasi" in vivo media (i.e.: in fresh latex cytosol deproteinized by ultrafiltration) (Table 1), suggesting that this ATP-dependent H^+ pump may indeed function in vivo.

Finally, the proton-pumping ATPase of the lutoidic tonoplast has recently been fully characterized (see : Gidrol et al, in this volume ; Marin and Komor, 1984 a and b).

The Outward H^+-Translocating Activity of the Tonoplast NADH Cytochrome c Reductase

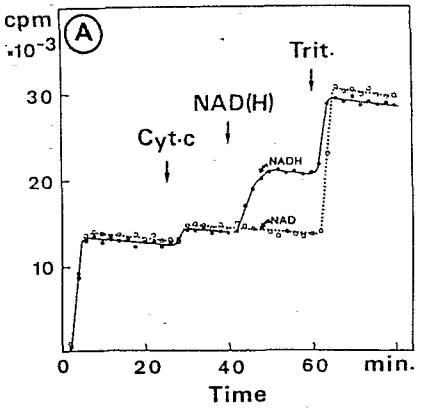
A second H^+-translocating system has recently been evidenced on the lutoidic tonoplast. It consists of a NADH-cytochrome c (artificial acceptor)-oxido-reductase, perhaps the same as the one, including b type cytochrome, discovered by Moreau et al (1975). Isopycnic centrifugation experiments did confirm the tonoplastic location of this H^+-pumping redox system, the activity of which closely followed the distribution of the typical lutoidic phosphatase and ATPase activities all along the density gradient profile (Chréstin, 1984). The functioning of this redox chain induces H^+ efflux from intact fresh lutoids resulting in a cytosolic acidification, and a collapse of the transtonoplastic pH gradient (Fig. 4 ; Table 2) (Crétin, 1983).

This H^+-translocating redox system works electrogenically and brings about membrane hyperpolarization (Table 2), leading to the collapse of the transtonoplastic ΔpH (Chréstin, 1984). Furthermore, the H^+-pumping NADH-c cytochrome-oxidoreductase was recently shown to function in ultrafiltered cytosol (Table 2) suggesting that it may indeed function in vivo as well.

The partial characterization of this redox H^+ pump showed that it is insensitive towards the classical inhibitors of the cytochromic respiratory chains (KCN, antimycin A, ...) and of the mitochondrial alternate pathway as well (Chréstin, 1984).

Regulation of and by pH: The Tonoplastic Double pH-Stat

Plotting the activity of these two opposing H^+ pumps as a function of the pH of the medium (buffered cytosol) shows that the H^+-translocating ATPase remains near its maximal potential activity over the physiological range of pH (6.6 - 7.2), while the tonoplastic electron transport chain, being pH sensitive in the same pH range, becomes more efficient at a slightly alkaline pH (over pH 7.3) (Fig. 5). This suggests that excessive cytosolic alkalinization, possibly resulting from some ATPase hyperactivity, will be immediately counteracted by the activation of the NADH-dependent-H^+ efflux, as long as all the substrate is not in limiting concentrations (Chréstin, 1984).



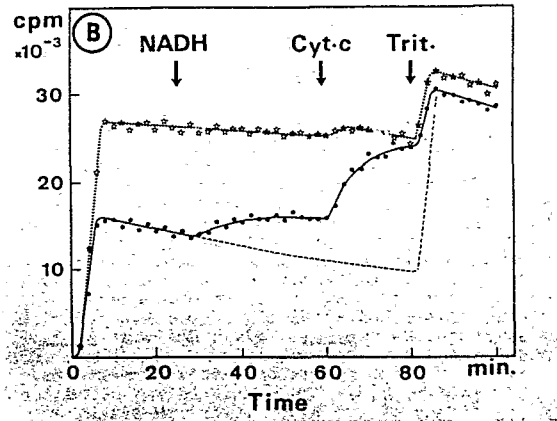


Fig. 4: Transtonoplast efflux of ^{14}C-methylamine (ΔpH probe) during the lutoidic NADH-cytochrome c-oxidoreductase working. Flow dialysis experiments performed with intact fresh lutoids: A: addition of c-cytochrome 0.5 mM and then NADH (●——●) or NAD (O···O) 2 mM; pH 7. B: addition of NADH (2 mM) prior to c-cytochrome (0.5 mM) in the presence (▼···▼) or absence (●——●) of FCCP (50 µM).

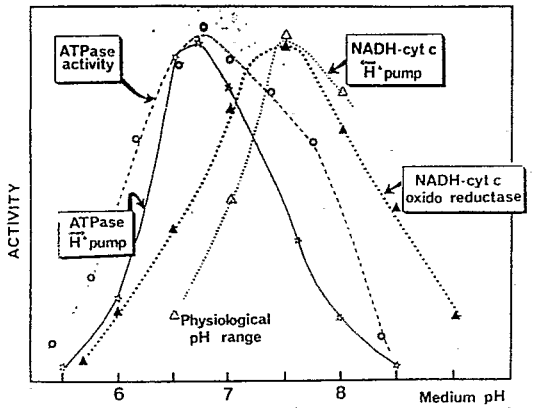


Fig. 5 . Dependence on pH of the tonoplastic ATPase and NADH-c-cytochrome-oxidoreductase activities, and of their proton pumping efficiency measured in buffered ultrafiltered cytosol from Hevea latex

Activation of the ATPase Moiety of the Tonoplastic pH-Stat by Ethylene

Treatment of Hevea bark with ethrel, an ethylene generator commonly used to "stimulate" latex production (d'Auzac and Ribaillier, 1969) induces a marked increase in the activity of the H^+ pumping ATPase, partly owing to activation of specific protein synthesis at the tonoplast level (Gidrol, 1984 ; Chrestin et al, 1984 a). The major part of this ATPase activation, in situ, results from a great increase in the concentration of the substrate ATP in the cytosol (Fig. 6), and the appearance of some low molecular weight activators in the cytosol (Gidrol, 1984). This activation of the lutoidic H^+ pumping ATPase induces a great increase of transtonoplastic ΔpH, and then a marked cytosolic alkalinization (Tupy, 1973 ; Coupé et al, 1986 ; Brzozowska-Hanower et al, 1979 ; Chrestin et al, 1984), resulting in the activation of the metabolism within the laticiferous vessels, hence in an enhancement of rubber production.

Cooperation of the Biochemical and of the Bioosmotical pH-Stats in the Regulation of the Cytosolic pH

The strong specific activity of PEPCase, the very weak buffer capacity of latex cytosol (Jacob et al, 1978 ; Chrestin et al, 1984) and the existence of a malic enzyme within latex leaves very little doubt about the active role of the alternative phophoenol-pyruvate pathway as a biochemical pH-stat within the latex cytosol (Jacob et al, 1983).

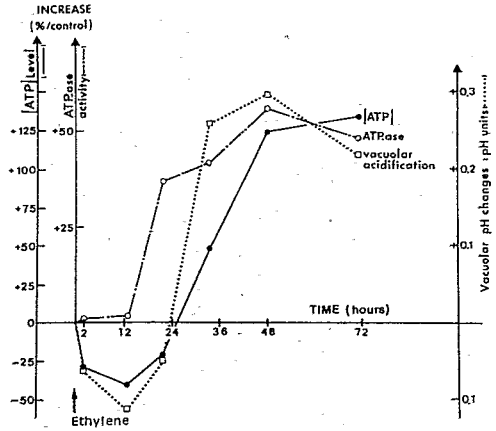


Fig. 6 . Kinetics of effects of ethrel on tonoplastic ATPase activity (·—O—·—O—·), the cytosolic ATP level (-●—●-) as expressed in % compared with the control, and on the vacuolar acidification (□·····□) in the latex from ethrel-treated Hevea bark (base line=control)

The central role of malate, the major acidic product of the PEPC pathway through the NADH-dependent-malate-dehydrogenase (mdh), is to be noted. Malate ensures permanent sensitization of PEPCase with respect to pH so that acidification of the cytosol (possibly caused by malate accumulation through the PEPC pathway), in the presence of this diacide will considerable inhibit PEPCase, and hence further malate-dependent acidification. Furthermore, malate has been shown to behave as an efficient activator of the lutoidic tonoplastic ATPase (d'Auzac, 1977 ; Gidrol, 1984). One can consider that a slight acidification of the cytosol (physiological range up to pH 6.75 - 6.5 which corresponds to the optimal pH for the H^+-pumping-ATPase activity) in the presence of (or caused by) malate, will activate the tonoplastic H^+-pumping-ATPase. This will lead, on the one hand, to realkalinization of the cytosol, and, on the other hand, to an accumulation of ADP : the prime regulator of pyruvate-kinase, hence favouring the relative activities of PEPCase and PK in the direction of the PK pathway.

Articulation of the two pH-stats is also thought to occur at their respective NADH consuming moiety. The functioning of the malate pathway through PEPCase and mdh will bring about NADH consumption (mdh) and cytosolic acidification, so that the redox moiety of the tonoplastic bioosmotical pH-stat will be inhibited because of the acidic pH (malate accumulation) and of probable lack of its electron donor (NADH). We conclude that latex cytosol is effectively protected against excessive cytosolic acidification.

Discussion - Conclusion

The regulation of intracellular pH in plant cells has attracted more and more interest during the past few years. It is now well accepted that different metabolic activities of plant cells such as the conversion of CO_2, NH_4^+, NO_3^-, sugars, etc. into cell material cause large production or consumption of H^+ (Raven and Smith, 1974 ; Smith and Raven, 1979). The homeostasis of the cytoplasm in terms of H^+ concentration cannot be maintained without efficient regulation of pH. Classically one considers that two processes have been developed by plant cells to counteract these ineluctable pH changes: (a) the regulation of H^+ transport between the cells and the surrounding medium and (b) the biochemical production or consumption of H^+. Davies (1973, 1979) proposed that the regulation of cytoplasmic pH might result from the opposite action of proton concentration on the activities of PEPCase and malic enzyme. There is no doubt that the highly pH dependent latex PEP-carboxylase is involved in the regulation of intracellular pH of plant cells. However, there exist controversial arguments about the real effectiveness of such a Davies type pH-stat in fine control of the latex cytosolic pH because of a relatively low PEPCase/PK ratio activity in the latex cell, and of the parallel (not opposite) sensitivity of the latex PEPCase and malic enzyme towards pH changes in quasi in vivo conditions.

The regulation of intracellular pH through the control of transplasmalemna H^+ transports is very likely in the latex cells, though no experimental data could be obtained, because of the inadequacy of mature Hevea material for such studies. However, transplasmalemna H^+ transports are at least supposed to occur via energized solute symports or antiports, in particular via a sucrose-H^+ symport which is very likely to occur at the plasmalemna, as no plasmodesmata or analogous structure able to ensure the lading of the latex cells could be detected (Hébant and de Fay, 1980).

We report here a new aspect concerning the fine regulation of the cytosolic pH within the latex cell. It consits in the active regulation of transtonoplastic H^+ fluxes, remaining under the control of two opposing H^+-translocating systems located on the lutoidic tonoplast. The pH-dependence of the two operating moieties makes this system function as a true tonoplastic bioosmotic pH-stat, cooperating with the Davies type biochemical pH-stat in the regulation of the pH of the latex cell cytosol. Moreover, as these H^+-translocating systems operate electrogenically, they build up and control the amplitude of a transtonoplastic electrochemical proton gradient. The protonmotive force has been shown to energize the transport and accumulation of numerous solutes within the latex cell vacuoles (see : Marin et al, in this issue ; Marin et al, 1982), some of them (citrate, Mg^{++}, Ca^{++}, ...) being potent inhibitors of numerous key enzymes of the cytosolic metabolism (Chrestin et al, 1984 b).

Consistent with the functioning of the two opposing H^+ pumps at the tonoplast, we propose that the vacuolar compartment of the latex cells plays a double role as a "bioosmotic pH-stat" and a "detoxicating trap", thus controlling homeostasis of the cytosol, and favouring active metabolism within the cells, thus resulting in high latex (rubber) production (Fig. 7).

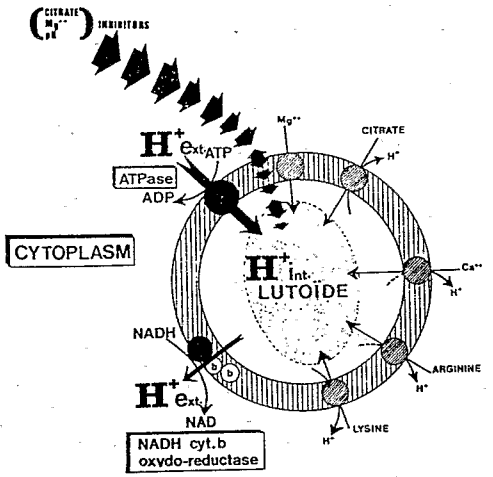


Fig. 7. The dual role of the lutoidic tonoplast in the control of the cytosolic homeostasis in Hevea latex: as a "biophysical pH-stat" and a "detoxicating TRAP" compartmentalizing inhibitory ions of the cytosolic metabolism. The figure shows the present state of knowledge about the energization of transtonoplastic H^+ fluxes and transport of solutes in the lutoids. The tonoplast ATPase and "redox system" function as opposing proton pumps. Data obtained from citrate, lysine and Ca^{++} fluxes across the lutoidic tonoplast agree with mechanisms of sulutes/proton(s) antiporter. The existence of two kinetic pools for accumulated soluted is proposed

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