Contribution of Lutoidic Tonoplast in Regulation of Cytosolic pH of Latex from *Hevea brasiliensis*: Effect of Ethephon

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Abstract in Bahasa Malaysia

Pengeluaran getah didapati mempunyai korelasi positif dengan pH sitosol lateks dan kecerunan pH transtonoplas[1] (sitosol/lutoid). Seterusnya didapati satu perhubungan songsang yang sangat ketara di antara pH kompartmen sitosol dan perubahan-perubahan di dalam pH intra-lompang (pH intra-lutoid). Korelasi-korelasi ini boleh dijelaskan dengan menunjukkan fungsi dua pam H^+ bersetentangan yang bertempat pada tonoplas lutoid.

Pertama ialah ATPase tonoplas bersandaran kepada Mg yang dibuktikan oleh d'Auzac[2,3]. Aktivitinya mengakibatkan aliran masuk elektrogenik yang aktif proton-proton di dalam lompang. Apabila direndam di dalam bahantara fisiologi (sitosol ultraturas), dengan Mg-ATP eksogenus, lutoid-lutoid yang baru tersisih menunjukkan pengasidan lompang yang membawa kepada kecerunan pH transtonoplas yang mendekati dua unit, pengawakutuban membran (di dalam kurang negatif), dan satu kecerunan elektrokimia proton ($\triangle \mu H^+$) hampir 150 mV. ATPase yang mengepam proton dari lutoid-lutoid segar telah dicirikan: ia tidak peka kepada perencat-perencat biasa ATPase plasmalema dan mitokondria, tetapi direncat oleh DCCD, DIDS, NEM, kuersetin, NO_3^- dan CU^{++}; K_m untuk ATP ialah kira-kira 0.5 mM.

Dalam 12 jam hingga 36 jam selepas penggunaannya pada kulit *Hevea*, etefon mengaruh peningkatan dalam aktiviti ATPase tonoplas, meningkatkan kesedia-adaan substratnya (ATP sitosol lateks), dan pengasidan lompang beserta dengan pengalkalian sitosol lateks yang nyata.

Kedua ialah sistem redoks tonoplas yang menggunakan NADH dan *c*. Sitokrom sebagai penerima eksogenus (penerima fisiologi masih belum diketahui). Ia tidak peka kepada perencat-perencat biasa rantaian-rantaian redoks bakteria dan mitokondria (Antimisin A, KCN, PG, SHAM dll.). Apabila dianalisis dengan lutoid-lutoid yang baru tersisih di dalam sitosol ultraturas, fungsi sistem redoks ini mengaruh aliran keluar H^+ elektrogenik dari lompang kepada sitosol. Ini mengakibatkan pengalkalian lompang, runtuhan kecerunan pH transtonoplas, hiperpengkutuban

membran (di dalam lebih negatif) dan penurunan berkorelasi kecerunan elektrokimia proton.

Operasi pam-pam H^+ bersetentangan ini, seperti yang ditentukan dalam keadaan-keadaan fisiologi (sitosol ultraturas) menunjukkan bahawa ATPase mempunyai pH optimum antara 6.5 dan 7.0, manakala pH sistem redoks mengekstrud H^+ pula ialah kira-kira 7.5. Oleh kerana pH fisiologi sitosol lateks boleh berubah dari pH 6.5 kepada 7.3, kesimpulan dibuat bahawa kedua-dua pam ini boleh beroperasi sebagai pH-Stat biofizik. Ia terlibat dalam pengawalan berbagai enzim penunjuk sitosol di dalam lateks yang menunjukkan persandaran pH yang tinggi, dan juga di dalam pengaturan regenerasi dan pengeluaran getah.

Abstract

Rubber production has been shown to be correlated positively with the cytosolic pH of the latex and the transtonoplastic (cytosol/lutoids) pH gradient[1]*. Further, a highly significant inverse relationship between the pH of the cytosolic compartment and the changes in the intra-vacuolar pH (intra-lutoidic pH)*[1] *has been demonstrated. These correlations could be explained by the demonstration of the function of two opposite* H^+ *pumps localised on the lutoidic tonoplast.*

The first is the Mg dependent tonoplastic ATPase, initially evidenced by d'Auzac[2,3]*. Its activity results in an active electrogenic inflow of protons inside the vacuole. When immersed in a physiological medium (ultra-filtered cytosol), with exogenous Mg-ATP, freshly isolated lutoids exhibit a vacuolar acidification leading to a transtonoplastic pH gradient approaching two units, a membrane depolarisation (interior less negative), and a resulting electrochemical gradient of proton (*$\triangle \mu H^+$*) near 150 mV. This proton pumping ATPase from fresh lutoids has been exhaustively characterised: it is insensitive towards the classical inhibitors of the plasmalemma and mitochondrial ATPase, but inhibited by DCCD, DIDS, NEM, quercetin,* NO_3^- *and* CU^{++}*; its* K_m *for ATP is about 0.5 mM.*

Within 12 h to 36 h after its application on Hevea *bark, ethephon induces an increase in the tonoplastic ATPase activity, a wide enhancement in its substrate availability (latex cytosolic ATP), and a vacuolar acidification concomitant with a marked alkalinisation of the latex cytosol.*

The second is a tonoplastic redox system which consumes NADH and c. *Cytochrome as exogenous acceptor (physiological acceptor still unknown). It is insensitive towards the classical inhibitors of the bacterial and mitochondrial redox chains (Antimycin A, KCN, PG, SHAM, etc). When assayed with freshly isolated lutoids in ultra-filtered cytosol, the functioning of this redox system induces an outflow of electrogenic* H^+ *from the vacuole to the cytosol. This results in a vacuolar alkalinisation, a collapse of the*

transtonoplastic pH gradient, a membrane hyperpolarisation (interior more negative) and a correlative decrease of the electrochemical gradient of protons.

The operation of these two opposite H^+ pumps, as determined under physiological conditions (ultra-filtered cytosol), shows that the ATPase has an optimum pH of between 6.5 and 7.0, whereas that of the H^+-extruding redox system is about 7.5. As the physiological pH of the latex cytosol can vary from pH 6.5 to 7.3, it is concluded that these two pumps can operate as a biophysical pH-Stat. It is involved in the control of numerous cytosolic key-enzymes in the latex that exhibit high pH-dependency, hence in the regulation of the regeneration and production of rubber.

Latex from *Hevea brasiliensis* is a fluid specialised cytoplasm which is expelled from the wounded latex vessels[4]. As a plant cellular content, the latex contains various organelles, and especially a vacuolar compartment – the so-called 'lutoids'. The lutoids are single membrane micro-vacuoles[5,6] with lysosomal characteristics[7]. They make up about 15% of the total latex and can be easily isolated and purified by simple differential centrifugations[6,7]. Like all plant vacuoles, the lutoids exhibit a lower internal pH (about 5.5) than that of their cytosolic environment (about 7.0). Also they accumulate *in vitro* as *in vivo*, numerous mineral and organic cations such as Mg^{++}, Ca^{++}, CU^{++}, *etc*, and basic amino acids, as well as anions such as citrate and phosphate[6,8,9]. When there is no major difficulty in the latex flow (abnormally high plugging index), one can consider that the production of rubber reflects the intensity of metabolism within the laticiferous cells. Indeed, this regenerative metabolism must be sufficiently active to compensate for the loss of latex between each tapping (generally twice a week).

Rubber production has been shown to be correlated negatively with the intra-vacuolar pH, and positively with the cytosolic pH and the transtonoplastic pH gradient (cytosol-lutoids) in the latex[1,10]. Further, we have shown[1,11] a highly significant inverse relationship between the pH of the cytosolic compartment and the changes in intra-vacuolar pH (intra-lutoidic pH) in the latex, suggesting the occurrence of some vectorial H^+ fluxes controlled at the level of the lutoidic membrane (tonoplast).

As early as 1975, d'Auzac[2,3] reported, for the first time in the higher plant kingdom, the occurrence of a typical ATPase activity, located on, and constitutive of, the lutoidic tonoplast (vacuolar membrane). At the same time, Moreau *et al.*[12] demonstrated the presence of an antimycin-insensitive NADH – *c*. Cytochrome-oxidoreductase, closely associated with the lutoidic membrane.

By analogy with the well-demonstrated ability of the plasmalemma or mitochondrial ATPase to convert the energy derived from ATP-splitting in trans-membrane vectorial fluxes of H^+ as well as the ability of the mitochondrial, chloroplastic and

bacterial membrane electron-transporting chains to convert redox power in transmembrane proton fluxes as well, we attempted to see if the ATPase and the redox chain activities located on the lutoidic tonoplast could be involved in some analogous transtonoplastic flux of protons, and then involved in the regulation of the pH of the two major compartments in the latex (cytosol and lutoids).

MATERIAL AND METHODS

Plant Material

Twelve- to fourteen-year-old *H. brasiliensis* (clone GT 1) were selected for their growth homogeneity and high latex production, at the IRCA Experimental Plantation, Languededou (Ivory Coast).

For the experiment attempting to follow the kinetic actions of ethephon treatments on the biochemical parameters of the latex studied here, twenty-one trees ($\frac{1}{2}$S d/3 6d/7) were selected for their growth, production and latex biochemical parameter homogeneity, and left untapped for ten days to allow their latex to reach a state of metabolism equilibrium *in situ*. Six batches of three homogenous trees wre selected and treated with ethephon (ET 5% ba 2/2) below their tapping-cut: 2, 13, 21, 33, 48 and 78 h respectively before the 'post-stimulation tapping'. Three control trees were treated with palm oil alone (no ethephon). All (treated and control) trees were tapped at the same time on the same day, in order to eliminate any daily variability.

Latex Collection

The fresh latex was collected in glass vessels held in melting ice, from rubber trees, tapped as indicated in the experimental schemes, twice a week. The first 10 ml were discarded, because of possible bacterial contamination and damaged particles.

Preparation of Lutoid Fractions

The fresh latex was immediately centrifuged at 35 000 × g for 20 min at 8°C. The supernatant serum (cytosol) was sucked-off and kept cold for eventual further utilisation, while the rubber phase was discarded. The pellet, resuspended in ten volumes of a Hepes-Mes (50 mM), mannitol (320 mM) buffer, adjusted to pH 7.0 with Trizma base, formed the crude lutoid fraction. This fraction was washed three times with the same buffer to provide the purified lutoid fraction. The sediment obtained from the last washing-centrifugation at 35 000 × g for 10 min at 10°C, was resuspended either in the same buffer or in deproteinised cytosol adjusted to the desired pH with Tris or Mes (powder), and eventually complemented with the desired effectors.

Preparation of Deproteinised Cytosol

When necessary, after centrifugation, the latex cytosol was deproteinised by ultrafiltration in an Amicon cell equipped with a PM 10 membrane, under 4 bars nitrogen gas pressure at 4°C.

Estimations of Transmembrane $\triangle$pH and $\triangle\psi$

Most of the results presented in this paper were obtained by means of flow dialysis[13, 14]. This technique enables a rapid and continuous determination of changes in the concentration of (labelled) solutes in the external medium of a suspension, without further manipulation of the organelles in suspension. This is of particular interest when these organelles are relatively instable as the lutoids.

The suspension of intact fresh lutoids, generally pre-incubated with the labelled probes, was introduced in the upper 'incubation' chamber. Substrates, adjusted to the same pH as the incubation medium, were added as indicated by the arrows in *Figures 1, 2* and *3*. The flow dialysis buffer was pumped from the lower 'flow' chamber (separated from the upper chamber by a 4.5 cm^2 piece of dialysis tube), at a constant rate of 3 ml per minute. Fractions were collected every minute and their radioactivity determined by liquid scintillation spectrometry.

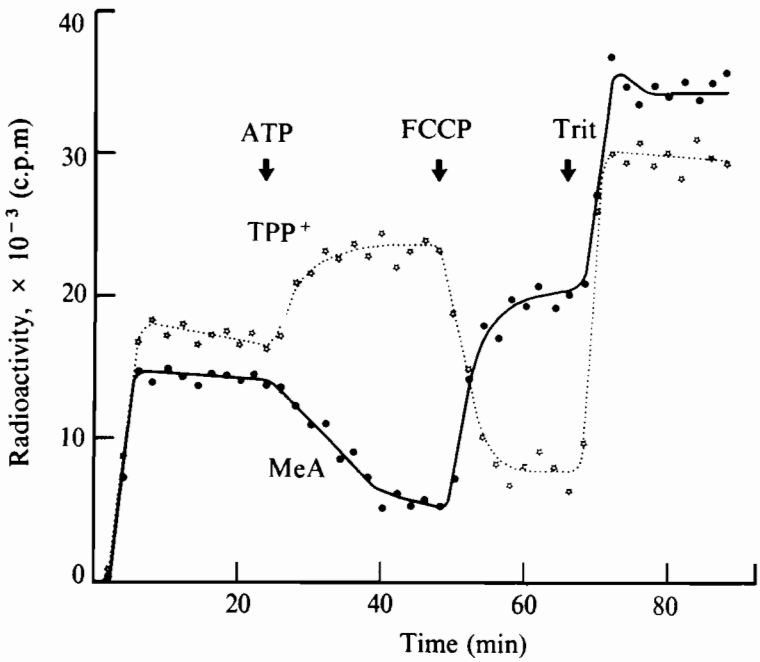

Figure 1. Effects of Mg-ATP and of the protonophore FCCP on the simultaneous fluxes of the $\triangle$pH probe ^{14}C-methylamine (MeA) and of the cationic lipophilic $\triangle\Psi$-probe ^{14}C-tetraphenyl phosphonium (TPP^+). (The assay was performed on a fresh lutoidic suspension by the flow dialysis method at pH 7.1 with 2.5 mM Mg-ATP and 25 µM FCCP.)

The value of $\triangle$pH was estimated by following the ^{14}C-methylamine level in the external medium (dialysate), assuming that this amine was accumulated in the most acidic space[15,16,17]. The membrane potential ($\triangle\psi$) changes were estimated by measuring the changes in the level of the cationic lipophilic probes ^{14}C-methyl-triphenyl phosphonium ($MTPP^+$) or ^{14}C-tetraphenyl phosphonium (TPP^+)[16,17]. $\triangle$pH and $\triangle\psi$ were calculated, after estimation of the intra-lutoidic volume, from the extent of the labelled probes uptakes before lutoid lysis. The difference between the radioactivity 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[13–16].

Enzyme Assays

ATPase activities were performed in the presence of 0.1 mM ammonium molybdate in order to inhibit the residual acidic phosphatase activities[2,3]. ATP hydrolysis was estimated by enzymatic assays of ADP and AMP according to Adams[18]. The NADH-*c*. Cytochrome-oxidoreductase was assayed spectrophotometrically as described by Moreau *et al*[12].

Adenine Nucleotide Concentrations in Latex

For the determination of adenine nucleotide concentration, the first 10 ml of the latex flowing immediately after the trees had been tapped, were discarded. Subsequently, the following twenty-five drops of latex were collected in 2.5 ml of an isotonic alkaline buffer (300 mM mannitol, 25 mM Tris, 0.1 mM ammonium heptamolybdate, pH 9.8) in an ice bath, under continuous manual shaking, in order to obtain immediate latex dilution and to stop its metabolism. As soon as the twenty-five drops had been collected, the diluted latex was fixed (on the field) by vigorous shaking with trichloracetic acid (0.5 *N* final concentration), and kept cool until analysis.

The coagulum obtained from TCA treatments was stirred and triturated with a glass rod in order to release any extractable adsorbed solutes. Coagulated rubber was rinsed twice with TCA, then dried (24 h at 105°C) to serve as reference. The TCA latex extracts were centrifuged (27 000 × g for 10 min at 4°C), and TCA was removed by three successive extractions with cold ether. The residual ether was evaporated by bubbling with nitrogen gas. The aqueous samples were neutralised with diluted KOH under pH control, and then made up to 10 ml with a buffer (30 mM Hepes-Tris, pH 7.4). ATP was assayed by the bioluminescence method using the luciferin-luciferase complex[19,20]. An LKB-Wallack 1250 luminometer, equipped with an injection module and a digital display unit was used. ADP and AMP were assayed with the same method, after their phosphorylation to ATP with commercial pyruvate-kinase and adenylate-kinase[21]. The concentrations of adenine nucleotides in latex were determined from standard curves with nucleotides of the highest grade.

RESULTS

Evidence for Proton-Pumping Function of Lutoidic Membrane ATPase

Electrogenic proton-pumping ATPase. Figure 1 depicts a typical flow dialysis experiment carried out as described under 'Material and Methods'. The experiment was started by adding, in the upper chamber of the dialysis cell, a suspension of fresh intact lutoids previously incubated (20 min) in the presence of the ΔpH-probe ^{14}C-methylamine (MeA) or of the $\Delta\psi$ probe ^{14}C-tetraphenyl phosphonium (TPP^+). Within 3 min, a steady-state distribution of the labelled probes was reached across the dialysis membrane. In subsequent fractions, the amount of radioactivity in the outflow decreased slightly, because of continuous evacuation of the labelled probes from both chambers (in equilibrium) through the dialysis membrane and continuous flow. Addition of Mg-ATP (2.5 mM) to the lutoidic suspension induced a wide increase in the accumulation of ^{14}C-methylamine inside the lutoids, as indicated by the dramatic decline of the radioactivity in the dialysate, which proportionally reflected the changes of solutes concentrations in the external medium of the lutoidic suspension. As the amine accumulated in the most acidic space, proportionally to the amplitude of the trans-membrane pH gradient[14-17], we concluded that the presence of Mg-ATP induced a wide acidification of the intra-lutoidic space. It was also shown that more than 90% of the added ATP had been hydrolysed after 20 min incubation, whereas the acidic phosphatase activities were virtually completely inhibited in our conditons (pH 7.1, presence of Mg^{++} and molybdate). Furthermore, the pH of the external well-buffered medium did not significantly change. Moreover, the addition of Mg-ADP was shown to be without effect[16,17]. It could be concluded therefore that the lutoidic acidification, in the presence of Mg-ATP, was attributable to the working of the tonoplastic ATPase itself as a H^+ pump. The addition of the electrogenic protonophore FCCP caused the dissipation of the only part of the proton gradient generated in the presence of ATP *(Figure 1)*, indicating that the functioning of the tonoplastic ATPase brought about a charge uncompensated vectorial flux of protons[22] across the lutoidic membrane, thus suggesting that the Mg-ATP-induced uptake of ^{14}C-methylamine is associated with the working of a H^+ pumping-ATPase that operates electrogenically.

From *Figure 1*, it can be seen that the lutoids pre-incubated in the presence of ^{14}C-tetraphenyl phosphonium, had accumulated significant amounts of the cationic $\Delta\psi$ probe, thus indicating that the lutoids (in the 'resting state') exhibited a trans-membrane potential (interior more negative) at least when suspended in an artificial medium. The addition of Mg-ATP induced a rapid efflux of the $\Delta\psi$ probe out of the lutoids, symmetrically with the influx of the ΔpH probe, suggesting a membrane depolarisation (interior less negative) upon the functioning of the H^+ pumping-ATPase. The presence of the electrogenic protonophore FCCP resulted in an immediate back-influx of TPP^+ inside the lutoids, correlative with the protonophore induced efflux of ^{14}C-methylamine. All the labelled probes accumulated, or remaining inside the lutoids after the successive additions of effectors, could be completely freed in the medium, only after the lutoid lysis caused by the addition of a lytic amount of the neutral detergent Triton X-100.

All these data clearly indicate that the proton-pumping function of the lutoidic membrane ATPase is electrogenic in nature, *i.e.* it brings about a charge uncompensated influx of H^+ inside the vacuolar compartment, resulting in the accumulation of free protons, then in a vacuolar acidification. Its working generates a transtonoplastic electrochemical gradient of protons ($\triangle \mu H^+$), able to energise transport of solutes through the tonoplast[16,17,23,24].

Supply of ATP for maintaining high transtonoplastic pH-gradient. When fresh lutoids were pre-incubated with ^{14}C-methylamine in the presence of glucose (10 mM), they also accumulated the $\triangle$pH probe, according to their initial transtonoplastic pH gradient *(Figure 2)*. As described above, the addition of Mg-ATP (3 mM) induced a rapid influx of methylamine indicating a vacuolar acidification, attributable to the operation of the tonoplastic H^+ pumping ATPase. When, after 30 min incubation, the same lutoidic suspension was provided with a supplementary but limited (1.5 mM) amount of Mg-ATP (without exogenous Hexokinase), it could be seen that the lutoids maintained and even slightly amplified their high ATP-dependent transtonoplastic pH gradient.

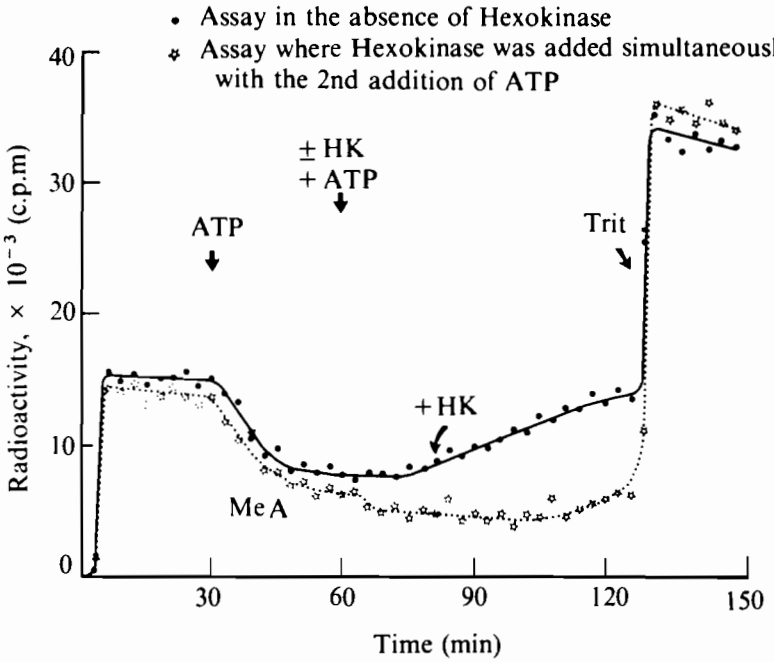

Figure 2. Efflux of the free protons from lutoids in a medium lacking ATP after its complete consumption by Hexokinase + glucose. (The assay was performed in the same conditions as described in Figure 1, *but in the presence of 10 mM glucose.)*

Figure 2 depicts a similar experience, but where the second addition of ATP was accompanied by a concomitant addition of a saturating amount of Hexokinase (10 mM glucose present) in order to consume, then eliminate as rapidly as possible all the ATP present in the medium. It could be seen that under these conditions the

lutoids progressively lost the proton pool they had previously accumulated according to the working of the tonoplastic H^+ pumping-ATPase, when ATP was still available in the medium (*i.e.* before the addition of Hexokinase). This clearly indicates that the pool of electrogenic-free protons accumulated inside the vacuolar compartment (against the thermodynamic equilibrium) during the functioning of the H^+ pumping-ATPase tends to self-dissipate with time, in case of unavailability of sufficient amounts of ATP.

We conclude that high transtonoplastic pH gradient, then intra-vacuolar acidity, hence cytosolic (external medium) pH are actively controlled by a continuously energy-consuming proton-pump. This leads to the proposal of a 'cinetic control' of the H^+ pumping-ATPase, essentially by its own substrate availability, *i.e.* ATP.

Characterisation of the proton-pumping ATPase. The lutoidic H^+ pumping ATPase has been exhaustively characterised either from fresh intact lutoids[14,16,25-28] or from tonoplast vesicles reconstituted from lyophilised lutoids[17,26,29]. The Mg-dependent ATPase activity, as well as the ATP-dependent H^+ pumping activity, are insensitive towards the classical inhibitors of the bacterial, mitochondrial and plasmalemma H^+ pumping ATPase (olygomycin, NaN_3, *o*-Vanadate, Nystatine), but powerfully inhibited by DCCD, DIDS, NEM or mersalyl, flavonols, NO_3^-, and Cu^{++}. The ATPase activity is significantly activated, whereas the ATP-dependent-H^+ pump is powerfully inhibited by ionophores such as gramicidin, nigericin, A-23187, FCCP and 2-4 DNP, confirming the electrogenic H^+ pumping function of the lutoidic membrane ATPase, and that the H^+ pumping ATPase is also under the thermodynamic control[30] of the transtonoplastic $\triangle\mu H^+$.

Evidence for Occurrence of a H^+ Outward-Translocating Redox System on Lutoidic Tonoplast

Using the same method and experimental scheme as described in the previous figures, *Figure 3* shows that the successive additions of *c*. Cytochrome then of NADH to a suspension of fresh intact lutoids pre-incubated with the $\triangle$pH probe ^{14}C-methylamine, induced a wide increase in the radioactivity in the dialysate, reflecting a significative efflux of H^+, outward the lutoids (decrease of the $\triangle$pH). The phenomenon was partially reversed in the presence of the protonophore FCCP. The remaining radioactivity could only be liberated in the medium after the addition of a lytic amount of Triton X-100. Further it was shown that the addition of NAD was without detectable effect, or that the addition of protonophores, prior to the substrates of the redox chain, completely inhibited the transtonoplastic ^{14}C-methylamine movements[16,31].

From *Figure 3*, it can be seen that the functioning of the lutoidic redox system induced, in the same time, a significative accumulation of the $\triangle\psi$-probe TPP^+ inside the lutoids, reflecting a trans-membrane hyperpolarisation (interior more negative). Here again the phenomenon was partially reversed by the electrogenic protonophore FCCP.

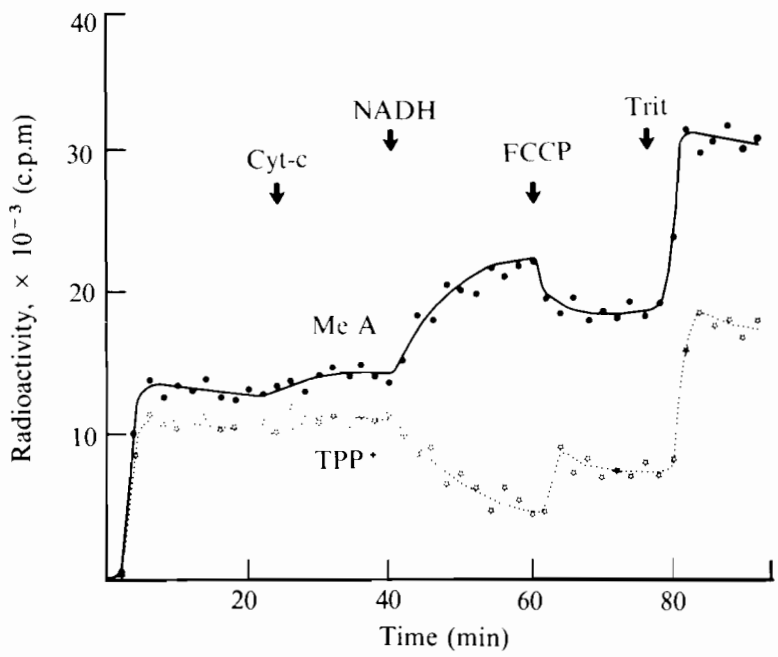

Figure 3. Efflux of the $\triangle pH$ probe ^{14}C-methylamine and influx of the potential probe TPP^+ during the working of the lutoidic NADH-c. Cytochrome-oxidoreductase. (The assays were performed with 'ultra-fresh' lutoids at pH 7.0 with 2 mM NADH and 0.5 mM c. Cytochrome.)

It was shown that this lutoidic redox activity, and the oxidoreductase dependent transtonoplastic movements of the $\triangle$pH, and $\triangle\psi$ probes as well, were insensitive towards the classical inhibitors of the mitochondrial or bacterial redox chains (KCN, Antimycin A, SHAM)[16,31].

We conclude from these data that a redox system located on the lutoidic membrane, perhaps the same as evidenced by Moreau *et al.*[12], can function as an outward-translocating H^+ pump. Its working induces a charge uncompensated efflux of protons from the lutoids to the external medium. This results in an intra-vacuolar alkalinisation and a symmetrical medium acidification, leading to a collapse of the transtonoplastic pH gradient and a decrease of the trans-membrane $\triangle\mu H^+$.

Further Evidence For Lutoidic Localisation of Both H^+ Pumps

Whereas there was good evidence demonstrating that the two opposite working H^+ pumps were not from bacterial, mitochondrial or plasmalemmic origin, one could have thought that one or the other proton-pumping system might have been located on different sedimentable organelles within the latex (Frey-Wyssling

particles, microsome-like entities, vesiculated endoplasmic reticulum, *etc.*). From sucrose density gradient profiles *(Figure 4)*, it can be seen that the ATPase activity and the ATP-dependent H^+ pump, the NADH-*c*. Cytochrome – oxidoreductase and its associated H^+ translocating system as well, were all superposable to the fractions displaying the acidic phosphatase activity (molybdate inhibitable), a typical marker for the lutoids[6, 7, 12, 32]. Neither of the proton-pumps, which necessarily characterised only the intact organelles (obligatory membrane integrity to detect trans-membrane fluxes) could be associated with *o*-diphenol-oxidase (a marker for the Frey-Wyssling particles) nor with 'sedimentable' malate-dehydrogenase (a marker of a still unidentified mid-dense membrane particle in fresh latex)[16, 32]. So we conclude that both opposite working proton-pumps are located on the lutoidic tonoplast.

The Two Tonoplastic H^+ Translocating Systems can be Functional *In Vivo*

The results presented above, reporting the occurrence of two opposite working proton-pumps located on the lutoidic membrane, were obtained from assays where the lutoids were suspended in totally artificial medium. In order to get some confirmation on their ability to function *in vivo*, similar experiments were realised with lutoids immersed in deproteinised cytosol (ultra-filtered cytosol), complemented with the desired effectors, buffered and pH-adjusted with known amounts of Hepes-Tris buffers.

The results presented in *Tables 1* and *2*, reporting the quantified data obtained from lutoids incubated either in totally artificial or in latex cytosolic medium, show that both the lutoidic proton-pumping ATPase and redox chain are functional in *quasi-in vivo* medium. Thus we conclude that the two lutoidic H^+ pumps can function in the latex vessels, as far as their respective substrates are supposed to exist in the latex cytosol. It must be noticed that if there is no doubt about the presence of ATP and NADH in latex, there is no evidence about the existence of *c*. Cytochrome, neither in the latex cytosol nor in the lutoids. So presently, the physiological electron acceptor of the tonoplastic redox system remains to be identified in the latex.

Two Tonoplastic H^+ Pumps Working as a Biophysical pH-STAT

Plotting the activity of these two H^+ pumps as a function of the pH of the medium (ultra-filtered cytosol) clearly shows that the ATPase remains at its maximal potential activity over the physiological range of pH (6.5 to 7.3), while the tonoplastic e transport chain, being pH-sensitive in the same pH range, becomes more efficient at slightly alkaline pH *(Figure 5)*. This suggests that any excessive alkalinisation of the latex cytosol, eventually through excessive working of the H^+ pumping ATPase, will be efficiently counteracted by the activation of the redox chain-dependent efflux of protons from the lutoids. From this, the cytosolic pH will theoretically tend to self-stabilise in the range of pH comprised between the optimum pH of each proton-pump, as far as their respective substrate availability would not be limiting in the latex.

This system consists in a typical biophysical (bio-osmotical) pH-STAT, based on controlled (energy-dependent) trans-membrane fluxes of free H^+. Its working will actively participate in the fine regulation of the cytosolic pH and will be highly implicated in the control of the cytosolic metabolism in the latex.

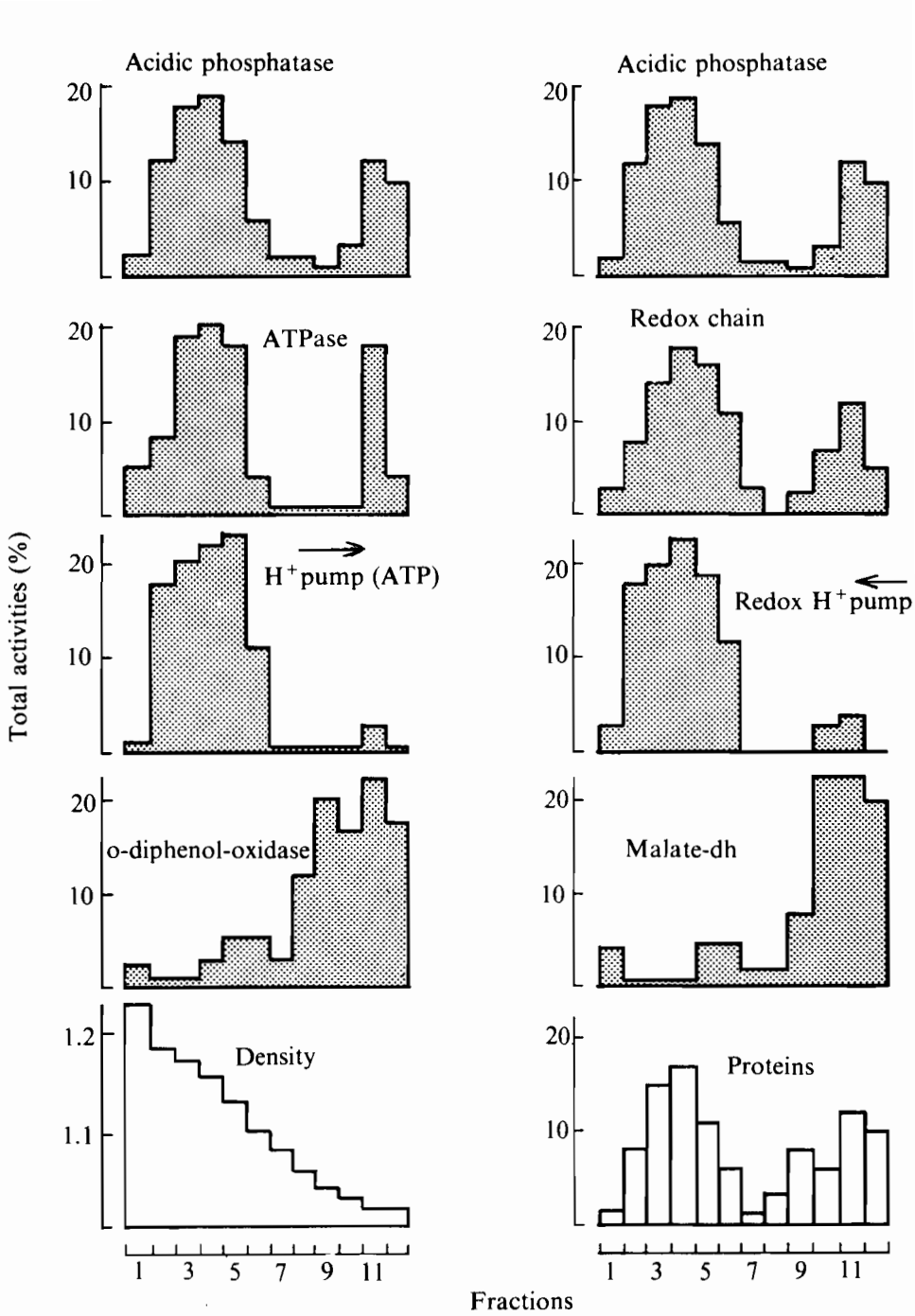

Figure 4. Localisation of the proton-pumping activities by density gradient centrifugation of the latex organelles. [An isotonic suspension of the bottom fraction from freshly centrifuged latex was layered on the top of the continuous sucrose gradient (0.6 M to 1.8 M sucrose) containing 0.3 M mannitol, 50 mM Hepes-Tris at pH 7.0, and centrifuged 75 min at 95 000 × g at 8°C. The different fractions were sucked off, then analysed for their enzymatic and proton-pumping activities, as described under 'Methods'].

TABLE 1. COMPARATIVE STUDIES OF THE TRANSTONOPLASTIC $\triangle$pH, $\triangle\psi$ AND $\triangle\mu H^+$, ENERGISED BY EXOGENOUS ATP IN A TOTALLY ARTIFICIAL MEDIUM OR IN ULTRA-FILTERED CYTOSOL

Medium (pH 7.0)	$\triangle$pH ($-Z\triangle$pH) (mV)		$\triangle\psi$ (mV)		$\triangle\mu H^+$ (mV)	
	Initial	+ ATP	Initial	+ ATP	Initial	+ ATP
Artificial buffer	1.13 (70)	1.68 (101)	−68	−20	+ 2	+81
Ultra-filtered cytosol	1.37 (82)	1.90 (145)	−28	− 2	+54	+143

The lutoids were incubated at pH 7.0 (ambient temperature) in the presence of Mg-ATP (3 mM) with ^{14}C-methylamine or ^{14}C-TPP$^+$

$\triangle\mu H^+ = \triangle\psi - Z\triangle$pH

TABLE 2. COMPARATIVE STUDIES OF THE TRANSTONOPLASTIC ΔpH, $\Delta\psi$ AND $\Delta\mu H^+$ OF LUTOIDS INCUBATED IN THE PRESENCE (OR ABSENCE) OF NADH (2 mM) + *c.* CYTOCHROME (0.5 mM) IN ARTIFICIAL BUFFER OR IN ULTRA-FILTERED CYTOSOL AT pH 7.0

Medium (pH 7.0)	ΔpH ($-Z\Delta pH$) (mV)		$\Delta\psi$ (mV)		$\Delta\mu H^+$ (mV)	
	Initial	+ NADH + *c.* Cytochrome	Initial	+ NADH + *c.* Cytochrome	Initial	+ NADH + *c.* Cytochrome
Artificial buffer	1.13 (70)	0.78 (47)	−80	−102	−12	−55
Ultra-filtered cytosol	1.22 (73)	0.70 (42)	−37	− 63	+31	−11

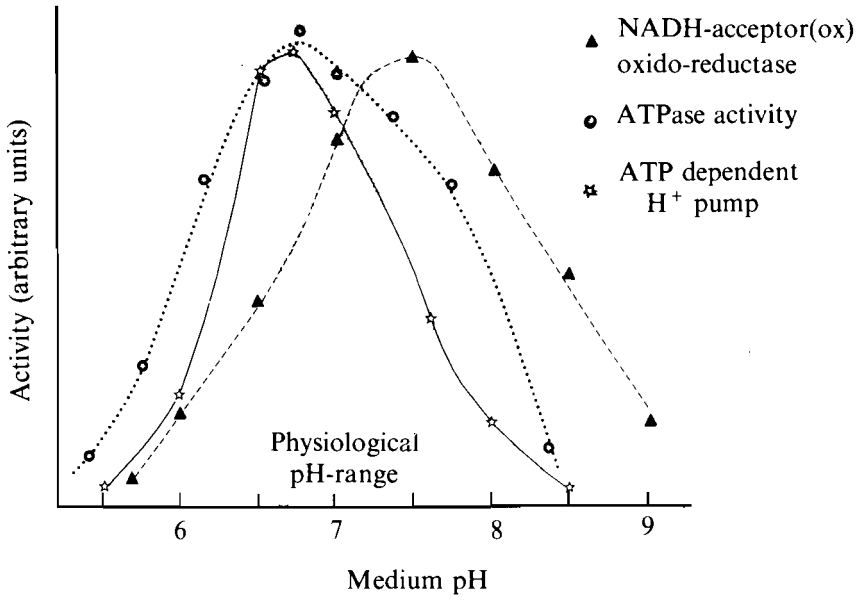

Figure 5. Dependence on pH of the lutoidic ATPase and NADH-c. Cytochrome-oxidoreductase activities, and of their proton-pumping efficiency, measured in buffered ultra-filtered cytosol from Hevea *latex.*

Activation of Lutoidic H^+ Pumping ATPase by Bark Treatment with Ethephon

Compartmental pH changes. The stimulation of rubber production by treatment of *Hevea* bark with ethephon, is known to induce an early alkalinisation of the latex cytosol, and a more or less delayed acidification of the lutoidic compartment[1,10,33]. We attempted to see if the function of the tonoplastic proton-pumps was involved in these compartmental pH changes.

Figure 6 shows some kinetic aspects of the effects of ethephon on the cytosolic and lutoidic pH changes within the latex vessels. It can be seen that the response to ethephon treatment could be subdivided into two distinct stages:

- An initial stage (lasting about 21 h) which was characterised by a slow and slight alkalinisation of the cytosol and a transient slight rise in the intra-vacuolar pH (maximum after 13 h). During this early stage the response of the pH of the two compartments varied in the same way, and with a similar amplitude. Thus, The transtonoplastic pH gradient ($\triangle$pH) remained stable for at least 13 h.
- A delayed wide response which exhibited a simultaneous alkalinisation of the cytosol and acidification of the lutoids. The pH of the cytosol increased 0.42 units compared with the control (maximum at 33 h) and then remained 0.3 units higher for more than two days. During the same time, the intra-vacuolar pH decreased 0.2 to 0.3 units below the control *(Figures 6* and *7)*.

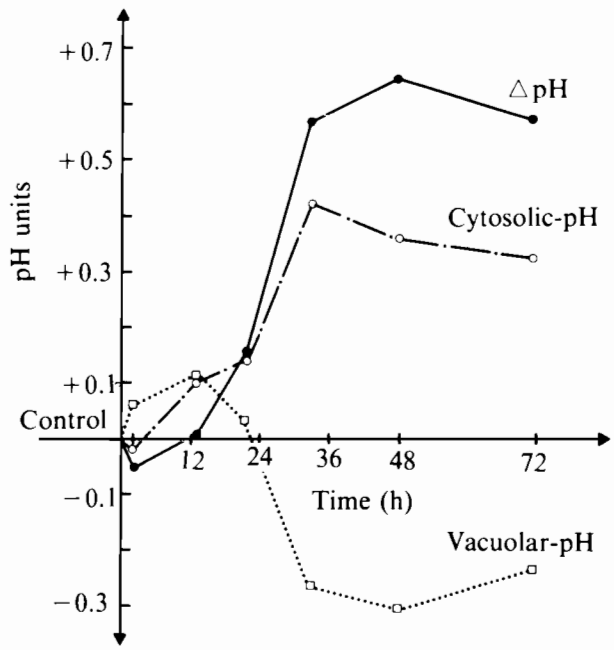

Figure 6. Kinetics of ethephon effects on the cytosolic and vacuolar pH, and on the resulting transtonoplastic △pH within the latex from Hevea. *(The results are expressed as mean differences of the latex characteristics from stimulated trees, compared with the control.)*

The resulting transtonoplastic △pH increased 0.57 units (33 h) and then remained 0.55 units over the control. Although the pH changes of the two cellular compartments moved in opposite symmetrical ways, the cytosolic pH was shown to be more affected (+ 0.42 units after 33 h) than the intra-vacuolar pH (−0.26 units). This could be explained by the greater buffering capacity of the intra-lutoidic serum[16].

Increase in tonoplastic ATPase activity. From *Figure 7* it can be seen that, 21 h after the treatment, ethephon induced a sudden increase in the tonoplastic ATPase activity (measured *in vitro*). The activity of the H^+ pumping ATPase was shown to increase by 37% compared with the control after 21 h, and then by 55% to 60% after 48 h. Kinetic studies indicated that the K_m of the enzyme for Mg-ATP remained unchanged[27,28], whereas increase in V_{max} could be attributed to an increase in the number of catalytic sites on the tonoplast, owing to an activation of the *de novo* synthesis of the enzyme[27,28].

Increase of adenine nucleotide content in latex. As early as 2 h after stimulation with ethephon, the ATP content of the latex from stimulated trees fell by 30% below the control; it fell by 47% after 13 h. Following this transient decrease, the ATP content increased rapidly, exceeding the control level about 24 h after treatment.

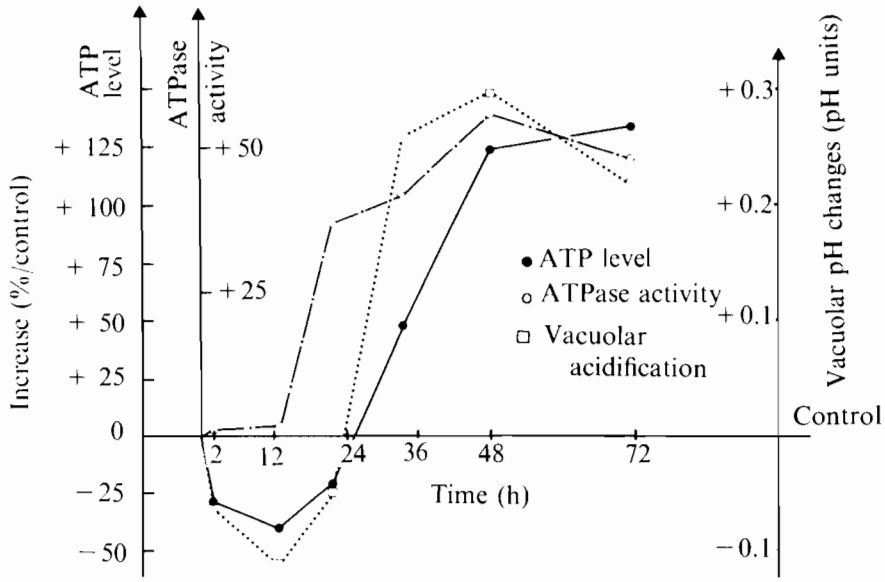

Figure 7. Kinetics of the ethephon effects on the lutoidic ATPase potential activity, ATP level, expressed in percentage variations compared with the control, and on the vacuolar acidification within the latex from stimulated trees.

Later, the ATP content in the latex from stimulated trees reached values of 225% (after 33 h) and then 240% (after 71 h) over the control. In addition, we found that, in fact, the total adenine nucleotide pool (ATP + ADP + AMP) closely followed the kinetic variations of ATP contents alone, resulting in scarcely detectable changes in the energy charge[20,21] in the latex from stimulated trees[16]. This suggests that the early collapse of ATP level, after stimulation, was not attributable to the simple dephosphorylation of ATP, but to some true consumption of the total nucleotide, under an unmeasurable form by the method used here (for instance, immobilisation in ARN because of the activation of protein synthesis, as an early response to stimulation with ethephon[27,28,33]). For the same reasons (energy charge unchanged), the wide increase in the ATP content, as a delayed response to stimulation, could not be explained by ADP rephosphorylation alone, suggesting the occurrence of a *de novo* synthesis of adenine nucleotide entities induced by ethylene, then re-equilibration of the energy charge through adenylate kinase and metabolism activities[34].

From *Figure 7*, it can be seen that the intra-vacuolar pH changes closely followed the variations in ATP content in the latex. Lower concentrations of ATP were associated with a less acid vacuolar serum, and higher ATP concentrations were associated with a considerable acidification of the vacuolar compartment. Data clearly show that the early marked activation of the tonoplastic H^+ pumping ATPase (between 13 h and 21 h) did not immediately result in any expected vacuolar acidification. This might be attributed to a transient relative lack in the availability of the substrate for the H^+ pumping ATPase (*i.e.* ATP) as an early side-effect of ethephon treatment.

CONCLUSION

All the results reported here demonstrate the presence of two proton-pumping systems, definitively located on the lutoidic tonoplast and able to control opposite transtonoplastic fluxes of H^+. Their characteristics, and in particular their different sensitivities towards the pH of the medium, are in good agreement with their functioning as a biophysical pH-STAT, controlling, through an energy-consuming way, adverse proton fluxes across the lutoidic tonoplast. These two systems work electrogenically, and thus modulate the amplitude of the transtonoplastic electrochemical gradient of protons ($\triangle\mu H^+$) which has been shown to energise transport of various solutes across the lutoidic membrane[16,17,23,24].

Consistent with the functioning of the two opposing H^+ pumps at the lutoidic tonoplast, we propose that the lutoids: the vacuolar compartment of the latex cells, play a triple role as a 'biophysical pH-STAT', a 'detoxicating trap' and a 'storage compartment', directly involved in the control of the latex cytosolic homeostasis. Its adequate function favours an active metabolism within the latex cells, which has clearly been shown to be highly pH-dependent and modulated by the ionic composition of the cytosol[11,16,35,36]. *Figure 8* schematises the present status of the knowledge about the role of lutoids in the control of cytosolic homeostasis within the latex cells.

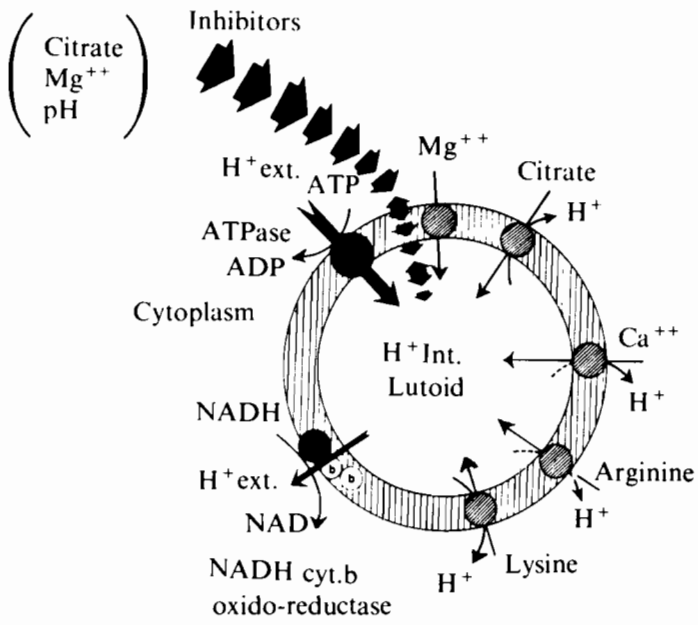

Figure 8. The dual role of the lutoidic tonoplast in the control of the cytosolic homeostasis within Hevea *latex: as a 'biophysical pH-STAT' and a 'detoxicating trap' compartmentalising inhibitory ions of the cytosolic metabolism.*

One of the components of this tonoplastic pH-STAT *i.e.* the H^+ pumping ATPase is activated by bark treatments with ethephon (ethylene generator). This activation consists both in an early direct increase of the ATPase activity itself (measured *in vitro*), probably through *de novo* synthesis of new catalytic sites at the tonoplast level[27,28], and in a wide increase in the availability of its substrate (ATP) in the latex, after stimulation with ethephon. The simultaneous opposite wide changes in the pH of the cytosol and of the vacuole suggest some stimulation of tonoplastic H^+ pumps by ethylene. The fact that the intra-vacuolar acidification parallelly followed the changes in the cytosolic ATP content leads to the conclusion that these transtonoplastic proton fluxes depend on the activity of the lutoidic ATPase, which appears to be essentially under the control of the ATP availability within the latex. Indeed the K_m of the lutoidic ATPase for ATP was shown to be about 0.5 mM (determined in ultra-filtered cytosol)[27,28], while the mean ATP content in the latex cytosol remains less than 0.2 mM. Thus the tonoplastic H^+ pumping ATPase always operates at far less than its maximum potential *in vivo*. The initial decrease in the ATP content of latex (30% to 40% below the control) is supposed to decrease the ATPase activity, and cause some vacuolar alkalinisation (according to the phenomenon shown in *Figure 2*). In contrast, later, the increase in the potential ATPase activity was followed by an increase in cytosolic ATP. The addition of these two biochemical events brought about, *in vivo*, an efficient activation of the H^+ pumping ATPase, accounting for the rise in the transtonoplastic $\triangle$pH and for the alkalinisation of the cytosol. Both these phenomena favour an active metabolism and the regeneration of latex in the latex cells, then 'stimulate' rubber production.

ACKNOWLEDGEMENT

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DISCUSSION

S. SIVAKUMARAN (Rubber Research Institute of Malaysia, Kuala Lumpur, Malaysia)

I refer to a statement in the paper on overdose of ethylene constantly sustained within the latex vessels and autocatalytic biosynthesis of endogenous ethylene.

It is now very evident that plant tissues can readily oxidise ethylene to carbon dioxide or ethylene oxide and one could consider this as an ethylene detoxification system. So, why cannot these mechanisms be operative in *Hevea* tissues?

At the RRIM, time-course studies of ethylene release by excised *Hevea* bark shavings following ethephon stimulation show that by the twelfth tapping, the ethylene levels in stimulated trees have actually declined to ethylene levels detectable in control bark tissues.

Attempts to induce ethylene synthesis in excised *Hevea* bark tissues by exogenous feeding of ethylene have not been successful.

H. CHRESTIN

I agree with you. It has effectively been shown elsewhere that ethylene (from ethephon) can be metabolised in *Hevea* leaf tissues. We have also demonstrated in the latex the presence of the glyoxalase system which is able to detoxify ethylene oxide.

D. OSBORNE (University of Oxford, UK)

The maintenance of the proper electrode potential (and pH-STAT) across the lutoid membrane and the proposed two-mechanism controls that could be exerted by the regulation of proton secretion inwards

(ATPase involvement) and oxido-reductase NADH outwards suggests the possibility of controlling one reaction but not the other and hence an opportunity for maintaining the optimum electrochemical potential (internal pH) for membrane stability. In view of the known effects of fusiccocin (FC) in enhancing membrane ATPase, does FC offer possibilities for such control?

H. CHRESTIN

Fusiccocin is a typical activator of the plasmalemma ATPase. We tested FC on the lutoidic ATPase, and we did show that it was without effect.

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