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Chloride-ion stimulation of the tonoplast H^+-translocating ATPase from *Hevea brasiliensis* (rubber tree) latex

A dual mechanism

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The effect of Cl^- and other anions on the tonoplast H^+-translocating ATPase (H^+-ATPase) from *Hevea brasiliensis* (rubber tree) latex was investigated. Cl^- and other anions stimulated the ATPase activity of tightly sealed vesicles prepared from *Hevea* tonoplast, with the following decreasing order of effectiveness: $Cl^- > Br^- > SO_4^{2-} > NO_3^-$. As indicated by the changes of the protonmotive potential difference, anion stimulation of tonoplast H^+-ATPase was caused in part by the ability of these anions to dissipate the electrical potential. This interpretation assumes not a channelling of these anions against a membrane potential, negative-inside, but a modification of the permeability of these ions through the tonoplast membrane. In addition, Cl^- and the other anions stimulated the ATPase activity solubilized from the tonoplast membrane. Consequently, the tonoplast H^+-pumping ATPase can be considered as an anion-stimulated enzyme. These results are discussed in relation to various models described in the literature for the microsomal H^+-ATPase systems claimed as tonoplast entities.

It was reported fairly recently that the H^+-translocating ATPase in corn (*Zea mays*) root homogenates is stimulated by Cl^- (Du Pont *et al.*, 1982; Bennett & Spanswick, 1983*a*,*b*). As this activity catalyses an electrogenic influx of H^+ and as the basal $\Delta\psi$ is always positive-inside, it is likely that the stimulation by Cl^- is due to an electrophoretic inflow of Cl^-, which reduces the membane potential and thus releases any electrical restraint on the H^+-ATPase.

In the present paper, the effects of Cl^- and other anions on *Hevea* tonoplast H^+-translocating ATPase are reported. The data are compared with the results described with the H^+-ATPase found in the microsomal membrane fraction isolated from higher-plant tissues (Hager & Helmle, 1981; Mandala *et al.*, 1982; Mettler *et al.*, 1982; Stout & Cleland, 1982; Bennett & Spanswick, 1983*a*,*b*; Churchill & Sze, 1983; O'Neill *et al.*, 1983).

Our results support the idea that Cl^- has a direct effect on *Hevea* tonoplast H^+ translocation. Studies on ATPase activity released from tonoplast membranes by organic solvent indicate that Cl^- activates it and that this activation is abolished by the anion-channel blocker DIDS. In addition to this direct effect, Cl^- also stimulates the tonoplast ATPase by decreasing the membrane potential ($\Delta\psi$).

Abbreviations used: ΔpH, transmembrane pH gradient; $\Delta\psi$, electrical transmembrane potential; Bistris IDA, Bistris iminodiacetate; DCCD, *NN'*-dicyclohexylcarbodi-imide; DIDS, 4,4'-di-isothiocyano-2,2'-stilbenedisulphonic acid; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; $TPMP^+$, triphenylmethylphosphonium ion; H^+-ATPase, H^+-translocating ATPase.

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Experimental

Materials

All reagents were of analytical grade. Except where mentioned below, they were obtained from (i) Labosi, Paris, France, (ii) Fluka Feinchemika-

lien GmbH, Ulm, Germany, (iii) Merck, Darmstadt, Germany, and (iv) Sigma Chemical Co., St. Louis, MO, U.S.A. ATP (sodium salt) was from Boehringer-Mannheim GmbH Biochemica, Mannheim, Germany.

[^{14}C]Dextran (1.30–5.18 TBq·mol^{-1}) was obtained from New England Nuclear Chemicals, Dreieich, Germany; $^{3}H_2O$ (3.33 GBq·mol^{-1}) from Amersham Buchler, Braunschweig, Germany; [^{14}C]TPMP^{+} iodide (1.84 Tbq·mol^{-1}) and [^{14}C]-methylamine hydrochloride (1.52 TBq·mol^{-1}) were both obtained from Commissariat à l'Energie Atomique, Gif-sur-Yvette, France.

Plant material

Latex was obtained from trees of *Hevea brasiliensis* Müll. Arg. (clone Prang Besar 86) growing on the experimental plantation of I.R.C.A. (Institut de Recherches sur le Caoutchouc en Afrique, Abidjan, Ivory Coast). The fluid cytoplasm was harvested in ice-cooled flasks as described previously (Marin & Trouslot, 1975).

Vacuole isolation and purification

Vacuoles were isolated, purified and freeze-dried as described elsewhere (Marin *et al.*, 1981*b*; Marin, 1982).

Preparation of tonoplast membranes

Tonoplast membranes were dispersed in 25 mM-Mes/25 mM-Hepes/5 mM-β-mercaptoethanol, adjusted to pH 6.0 with Tris base. As previously described (Marin *et al.*, 1981*b*; Marin, 1982), the procedure used allowed the preparation of a highly purified tonoplast membrane fraction (D'Auzac *et al.*, 1982) that corresponded to a population of tightly sealed vesicles, as shown elsewhere (Marin, 1982).

Standard incubation conditions

Experiments were conducted at 30°C in 50 mM-Mes/50 mM-Hepes/5 mM-β-mercaptoethanol, pH 6.8. Ammonium molybdate was added at a final concentration of 0.1 mM. ATP and Mg^{2+} (as $MgSO_4$ or $MgCl_2$) were used at a final concentration of 5 mM. Further details are given below where appropriate.

Measurement of tracer uptake

Uptake of radioactively labelled compounds by tonoplast vesicles was used to determine $\Delta\psi$, ΔpH and the osmotic volume of the vesicles by the procedure described previously (Marin *et al.*, 1981*a*; Marin, 1982). [^{14}C]TPMP^{+} was used for the determination of $\Delta\psi$. [^{14}C]Methylamine distribution was used to measure ΔpH across the tonoplast. Intravesicular volumes were determined with $^{3}H_2O$ and [^{14}C]dextran.

The experiment was started after the preincubation period (10 min) by the addition of 5 mM-ATP, in the presence of 5 mM-$MgSO_4$ or 5 mM-$MgCl_2$. Assays were performed so that each series of vials contained $^{3}H_2O$ and a ^{14}C-labelled compound with a $^{14}C/^{3}H$ ratio of 0.2. At the appropriate time, the vesicles were sedimented by centrifugation in an Eppendorf model 3200 desk Microfuge for 3 min at 10000*g*.

Further experimental details were as described previously (Marin, 1982, 1983*a*).

The distribution of the labelled compounds was determined from their relative activities in the pellet and supernatant (Marin *et al.*, 1981*a*; Marin, 1982). From these values, $\Delta\psi$ and ΔpH were calculated.

ATPase activity

Assays were performed in a final volume of 1 ml. Reactions were started by the addition of a portion of tonoplast membranes containing about 300 μg of protein. Unless indicated otherwise, incubation proceeded for 30 min after the addition of ATP.

Enzymic hydrolysis of ATP was stopped with the addition of 250 μl of ice-cooled 20% (w/v) trichloroacetic acid. Then the mixtures were centrifuged at 7500*g* for 5 min at ambient temperature. The liberated phosphate present in the supernatant was determined by the method of Taussky & Shorr (1953) or Heinonen *et al.* (1981).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with the modifications described by Marin (1983*a*). Bovine serum albumin was used as standard. These modifications eliminate any interference from components of the incubation medium.

Results

Effect of Cl^- on Hevea tonoplast ATPase

As shown in Fig. 1, the *Hevea* tonoplast ATPase, measured after 1 h incubation, was stimulated by 32% by an addition of 10 mM-KCl. This stimulation was probably due to the Cl^- rather than the K^+, since K_2SO_4 had no significant effect on the rate of ATP hydrolysis (Fig. 1), and, moreover, when 5 mM-$MgSO_4$ ws replaced with 5 mM-$MgCl_2$ or when 5 mM-$MgSO_4$ was supplemented with 10 mM-Cl^- as either potassium, sodium, lithium or rubidium salts, the rates of ATP hydrolysis were increased in a somewhat similar manner. Highest rates of hydrolysis were obtained with 50 mM-KCl (Fig. 2), where the stimulation was 70–80%.

ATPase activity was relatively insensitive to the accompanying univalent cation, since when the anion was kept constant at 50 mM-Cl^- and the

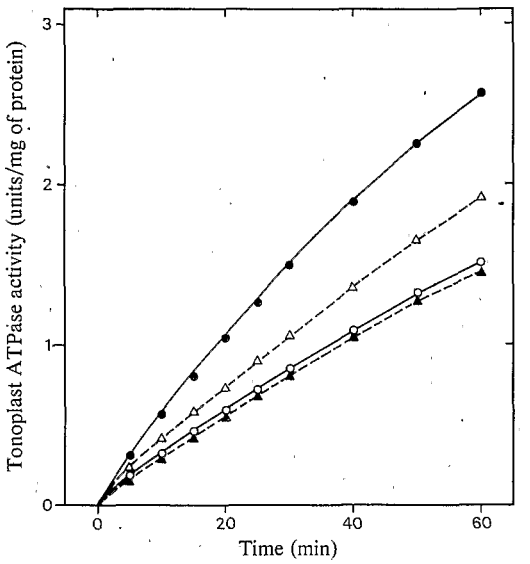

Fig. 1. *Effects of K_2SO_4 and different chloride salts on the hydrolysis of ATP by Hevea tonoplast ATPase*
Tonoplast vesicles were incubated at pH 6.8 in the presence of 5 mM-ATP, 5 mM-$MgSO_4$ and the different salts as indicated. The addition corresponded to 10 mM-KCl (△), 50 mM-KCl (●) and 5 mM-K_2SO_4 (○); ▲, without addition (control).

Table 1. *Effect of different chloride salts on ATPase activity from Hevea tonoplast*
ATPase activity was assayed at pH 6.8 as described in the Experimental section. The final concentration of each salt was 50 mM. The ATPase activity of tightly sealed tonoplast vesicles was 1.62 ± 0.10 units/mg of protein. When the enzyme was solubilized from tonoplast membrane, the activity was 125.2 ± 0.3 units/mg of protein. These values were set to 100%. For the values in parentheses the activity in the presence of 50 mM-KCl was set to 100%. All these values represent the means for two to five experiments.

	ATPase activity	
	Native	Solubilized
No addition ($MgSO_4$ only)	100 (–)	100 (–)
KCl	169 (100)	146 (100)
NH_4Cl	238 (141)	143 (98)
NaCl	175 (104)	151 (103)
RbCl	171 (101)	149 (102)
CsCl	170 (101)	146 (100)
LiCl	169 (100)	145 (99)
Choline chloride	141 (83)	140 (96)
Lysine hydrochloride	188 (111)	143 (98)
Arginine hydrochloride	178 (105)	145 (99)
Tris chloride	135 (79)	123 (84)
Bistris chloride	165 (98)	141 (97)

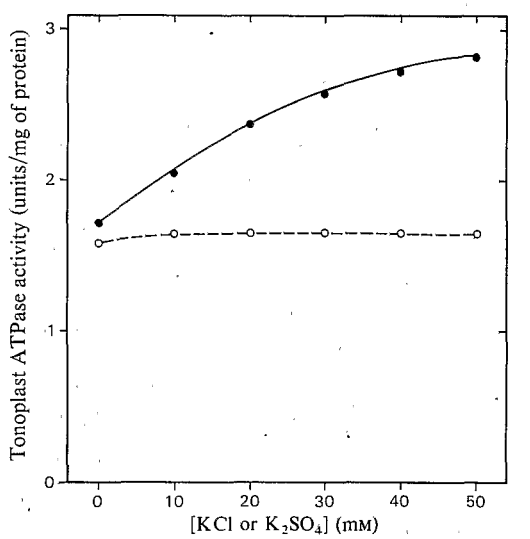

Fig. 2. *Cl^- stimulation of native ATPase in tightly sealed tonoplast vesicles from Hevea latex*
Assay conditions were as described in the Experimental section and in the legend to Fig. 1, except that KCl (●) and K_2SO_4 (○) concentrations ranged between 0 and 50 mM.

nature of the cation was varied, most of the salts tested were stimulatory (Table 1). In addition, lysine and arginine, when added as monohydrochlorides, were as effective as KCl in stimulating the ATPase activity.

There are two possibilities to explain this Cl^--stimulating effect, as suggested by Bennett & Spanswick (1983*a*) for the Cl^--sensitive H^+ ATPase from corn roots. Firstly, Cl^- could stimulate H^+ transport by decreasing the depolarization effect of the H^+-ATPase. Secondly, Cl^- could directly activate the H^+-ATPase. Consequently, these two possibilities were tested on the *Hevea* tonoplast H^+-ATPase.

Effect of Cl^- on the protonmotive potential difference across the tonoplast membrane

Fig. 3(*a*) shows that the ΔpH increased when ATP was added to a suspension of tonoplast vesicles in the presence of Mg^{2+}, and this increase was greater in the presence of Cl^- than in the presence of SO_4^{2-}. Routinely it was observed that acidification of the vacuoles by tonoplast ATPase was stimulated by anions in the following order of effectiveness: $Cl^- > Br^- >$ iminodiacetate $\geqq SO_4^{2-} > NO_3^-$ (Table 2). Sulphate, benzenesulphonate and Bistris IDA had little or no effect on H^+ pumping. In addition, the magnitude of the

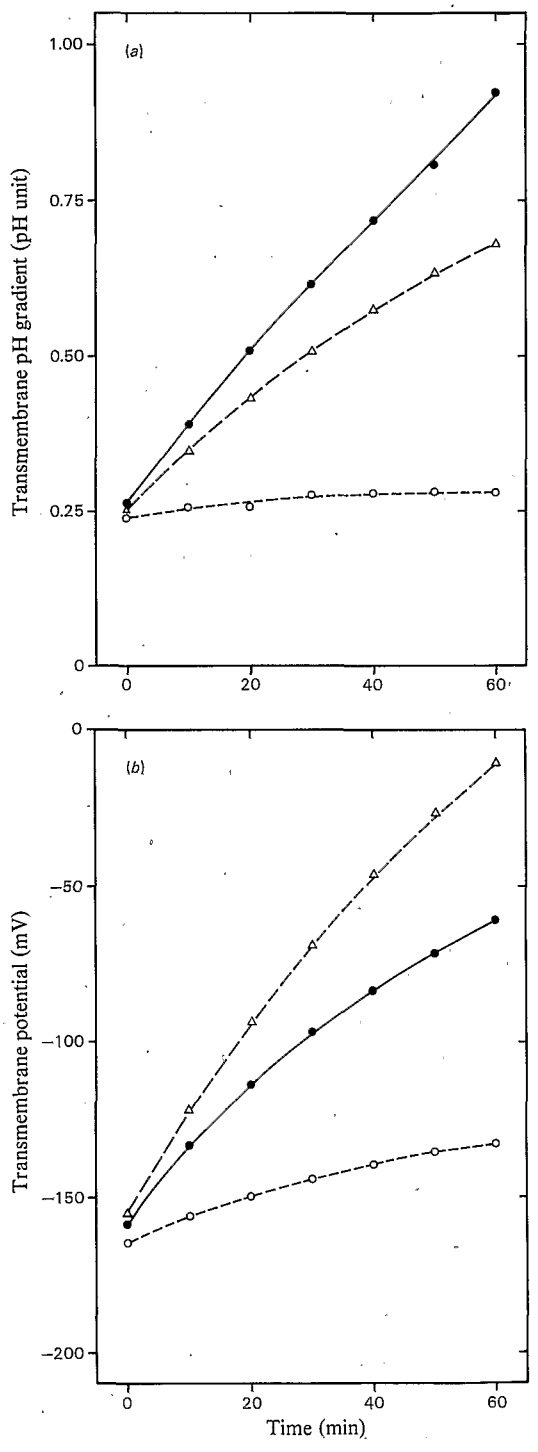

Fig. 3. *Effects of different anions on the magnitude of the electrochemical proton gradient in Hevea tonoplast vesicles*

Tonoplast vesicles were incubated at pH 6.8. Each 1 ml of incubation medium contained ^3H_2O (18.5 kBq) and either [^{14}C]TPMP$^+$ (3.7 kBq), [^{14}C]-methylamine (3.7 kBq) or [^{14}C]dextran (3.7 kBq). The reaction was started by an addition of ATP to a final concentration of 5 mM, after 10 min pre-incubation, in the presence of 5 mM-$MgCl_2$ (●) or 5 mM-$MgSO_4$ (△); ○, control in the presence of 5 mM-$MgSO_4$ without nucleotide (the control in the presence of 5 mM-$MgCl_2$ was no different from this). The transmembrane pH gradient (ΔpH) (*a*) and the electrical potential difference ($\Delta\psi$) (*b*) across the tonoplast were respectively calculated from the transmembrane distribution at equilibrium of methylamine and TPMP$^+$.

pH gradient formed from the proton translocation depended on the Cl^- concentration (Fig. 4).

In parallel, as shown in Fig. 3(*b*), the presence of MgATP in the incubation medium caused a decrease of the initial negative $\Delta\psi$ (estimated routinely between −160 and −140 mV). In the data reported in Fig. 3(*b*), after 1 h incubation, the depolarization amounted to 122 mV. It was greater in the presence of SO_4^{2-} than in the presence of Cl^-.

Certainly these results suggest that the electrogenic nature of tonoplast H^+-ATPase can be decreased in the presence of Cl^-. Moreover, it has been observed that anions which dissipate $\Delta\psi$, ΔpH or both, stimulate tonoplast ATPase activity (Gidrol *et al.*, 1984; Marin, 1984*a*). This depolarization effect does not seem to be specific to Cl^-, but general to permeant anions. This decrease in the electrogenic effect of the proton pump may well explain how the rate or ATP hydrolysis by the tonoplast ATPase is increased by Cl^- and by permeant anions.

Table 2. *Effect of different anions on the proton translocation catalysed by Hevea tonoplast ATPase*

The effect of different salts was tested on the ATP-dependent ΔpH changes in tonoplast vesicles, incubated under the conditions outlined in the Experimental section. The final concentration of salts was 50 mM. These data are from a typical experiment conducted on the same membrane vesicle preparation. For the values in parentheses, the change measured in presence of KCl was set to 100%.

Anion source	ATP-dependent ΔpH change
Control (no addition of salt)	0.62 (56)
KCl	1.10 (100)
KBr	0.95 (86)
KNO_3	0.30 (27)
K_2SO_4	0.65 (59)
IDA (potassium salt)	0.65 (59)
Benzenesulphonate (potassium salt)	0.60 (55)
Bistris chloride	0.98 (89)
Bistris nitrate	0.32 (29)
Bistris IDA	0.62 (56)
NH_4Cl	0.01 (1)

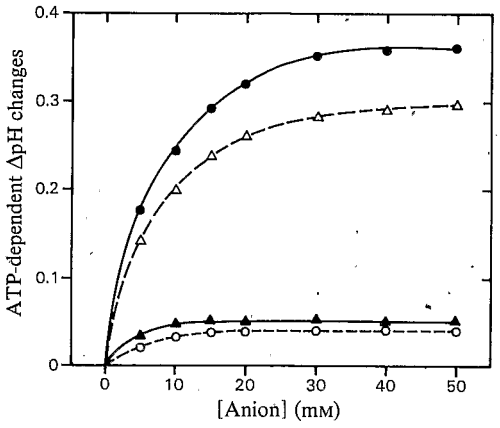

Fig. 4. *Cl^- stimulation of ATP-dependent ΔpH changes in tonoplast vesicles from Hevea latex*
Assay conditions were as described in the Experimental section and in the legend to Fig. 1, except that KCl (●), Bistris chloride (△), K_2SO_4 (▲) and Bistris IDA (○) concentrations ranged between 0 and 50 mM.

Table 3. *Effect of gramicidin on the Hevea tonoplast ATPase in presence of different cations*
ATPase activity was assayed at pH 6.8 as described in the Experimental section with the indicated salts added at a final concentration of 50 mM. When present, gramicidin was used at a concentration of 2 μM. As gramicidin is used as an ethanolic solution, an equivalent volume of ethanol (0.5%) was added to the control. The S.E.M. never exceeded 0.1 unit/mg of protein. Values in parentheses are based on the 'non-salt' value being set to 100%.

	Tonoplast ATPase activity (units/mg of protein)	
	−Gramicidin	+Gramicidin
No salt	1.6 (100)	2.9 (100)
KCl	2.6 (163)	4.6 (159)
NaCl	2.7 (169)	4.8 (166)
LiCl	2.8 (175)	4.9 (169)
RbCl	2.9 (181)	5.1 (176)
Tris chloride	2.1 (131)	3.9 (134)
Bistris chloride	2.5 (156)	4.5 (155)

Effect of gramicidin on the Cl^- stimulation of Hevea tonoplast ATPase activity

The stimulation of *Hevea* tonoplast ATPase activity by Cl^- was determined both in the absence and in the presence of 2 μM-gramicidin (Table 3).

Gramicidin is a channel-forming ionophore that relieves both the two components of the protonmotive potential difference across the tonoplast membrane from *Hevea* vesicles. Under the conditions used in the experiments reported in the present paper, it decreases the ΔpH component from 0.8 to approx. 0.05 pH unit and the $\Delta\psi$ component from −160 to 0 mV. Consequently, this ionophore was used to abolish any effect of the electrochemical proton gradient on the tonoplast ATPase.

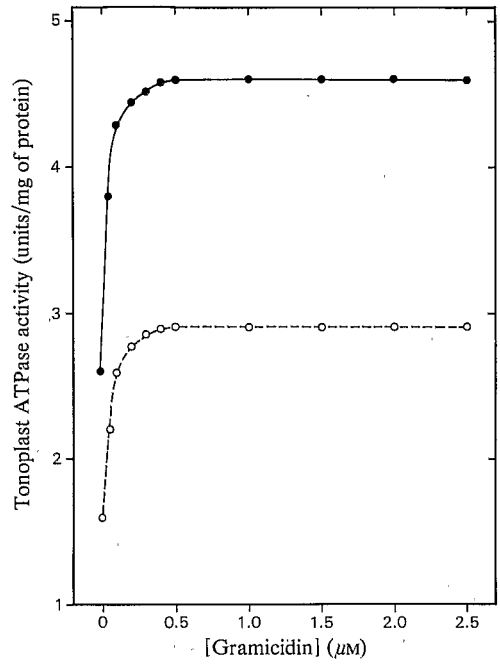

Fig. 5. *Effect of gramicidin on the Cl^- stimulation of native ATPase in Hevea tonoplast vesicles*
Assay conditions were as described in the Experimental section and in the legend to Fig. 1, in the absence (○) or presence (●) of 50 mM-KCl. Gramicidin concentrations ranged between 0 and 1.5 μM.

As shown in Table 3 and Fig. 5, when it is used at a sufficient concentration (0.5–2.5 μM), gramicidin stimulates the tonoplast ATPase activity by a factor of approx. 1.8, both in the presence and in the absence of Cl^-. As gramicidin has no effect on the solubilized form of tonoplast-bound ATPase, it could be suggested that, in native vesicles in the presence of Cl^-, the collapse of $\Delta\psi$ (which corresponds to a depolarization of tonoplast) and the dissipation of ΔpH activate the rate of ATP hydrolysis. Tonoplast ATPase could be regarded as regulated by the magnitude of the protonmotive force. In addition, independently of this effect of gramidicin, Cl^- stimulates ATPase activity by 60–80%, the extent depending on the nature of accompanying cation. When the cation used was Tris or Bistris, the extent of Cl^- stimulation

decreases slightly, as shown in Table 3. Consequently, the depolarizing effect of Cl^- is not the only factor involved in the stimulation of tonoplast ATPase activity. From such an experiment, where any effect of the electrochemical proton gradient on the tonoplast ATPase is abolished, there appears to be a direct effect of Cl^- on the *Hevea* tonoplast ATPase.

Cl^- effect on the ATPase solubilized and purified from tonoplast of Hevea latex

Tonoplast ATPase from *Hevea* latex was recently solubilized and purified (Marin & Komor, 1984*a*), and its properties described (Marin & Komor, 1984*a,b,c*). Some characteristics of the solubilized ATPase are similar to those of the membrane-bound enzyme. Thus, like the membrane-bound ATPase, it is also stimulated by Cl^-, and this stimulation depends on the Cl^- concentration (Fig. 6). KCl at 50 mM stimulated by 45–50%. The Cl^- stimulation of *Hevea* tonoplast ATPase was slightly different according to whether it was integrated in the tonoplast membrane or solubilized (in Table 1). NH_4^+ had an additional stimulatory effect that was shown only with tonoplast vesicles, which is consistent with the idea that tonoplast ATPase activity was regulated by the electrochemical proton potential (Marin, 1984*a,b*; Gidrol *et al.*, 1984), the ΔpH component of which is abolished by NH_4^+.

The K_m value for $MgATP^{2-}$ of 0.29 mM was not changed in the presence of 50 mM-KCl (Fig. 7), and the stimulation of the tonoplast ATPase activity by KCl was clearly due to an increase in $V_{max.}$, as suggested previously for the *Beta* (beet) tonoplast (Walker & Leigh, 1981).

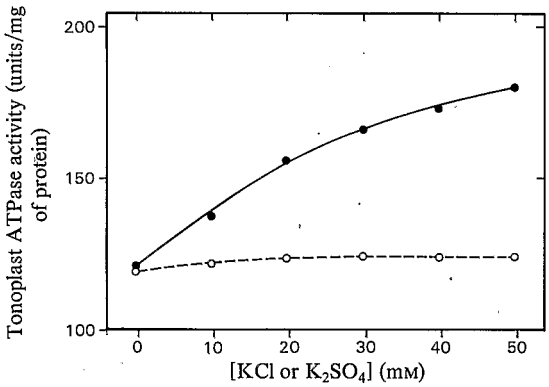

Fig. 6. *Cl^- stimulation of solubilized tonoplast ATPase from Hevea latex*
Experiments were conducted as described in the legend to Fig. 2, in the presence of KCl (●) or K_2SO_4 (○).

From all these data, it can be concluded that Cl^- has a direct effect on the *Hevea* tonoplast ATPase, which is seen as an increase in the $V_{max.}$

Inhibitors

Among the different potential ATPase inhibitors, reviewed by Goffeau & Slayman (1981) and tested, DCCD and EEDQ have been shown to be effective on the *Hevea* tonoplast ATPase (Marin, 1983*b*).

As shown in Table 4, the same percentage inhibition of tonoplast ATPase was obtained both in the presence and in the absence of KCl. The extent of inhibition was identical both in the presence and in the absence of 2 μM-gramicidin. From the data of Table 4, it can be concluded that the same enzyme was concerned in the presence and in the absence of Cl^-.

Effect of DIDS on the Cl^- stimulation of Hevea tonoplast ATPase

The ATPase activity from tonoplast vesicles was inhibited by an anion-transport inhibitor, DIDS, even in the absence of Cl^- (Fig. 8*a*). However, the inhibition of the native ATPase (present in tightly sealed tonoplast vesicles) was slightly greater in

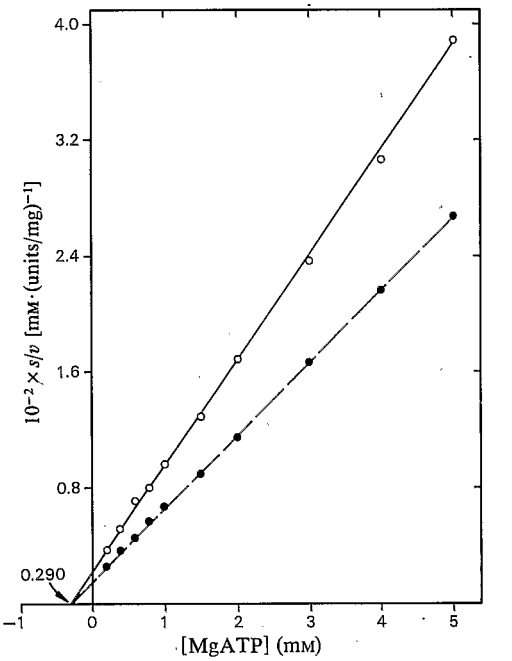

Fig. 7. *Hanes plot of MgATP-concentration-dependence of Hevea solubilized tonoplast ATPase*
Experiments were conducted as described in the legend to Fig. 2, in the absence of (○) or presence (●) of 50 mM-KCl. The Mg/ATP ratio was held constant at 1.0.

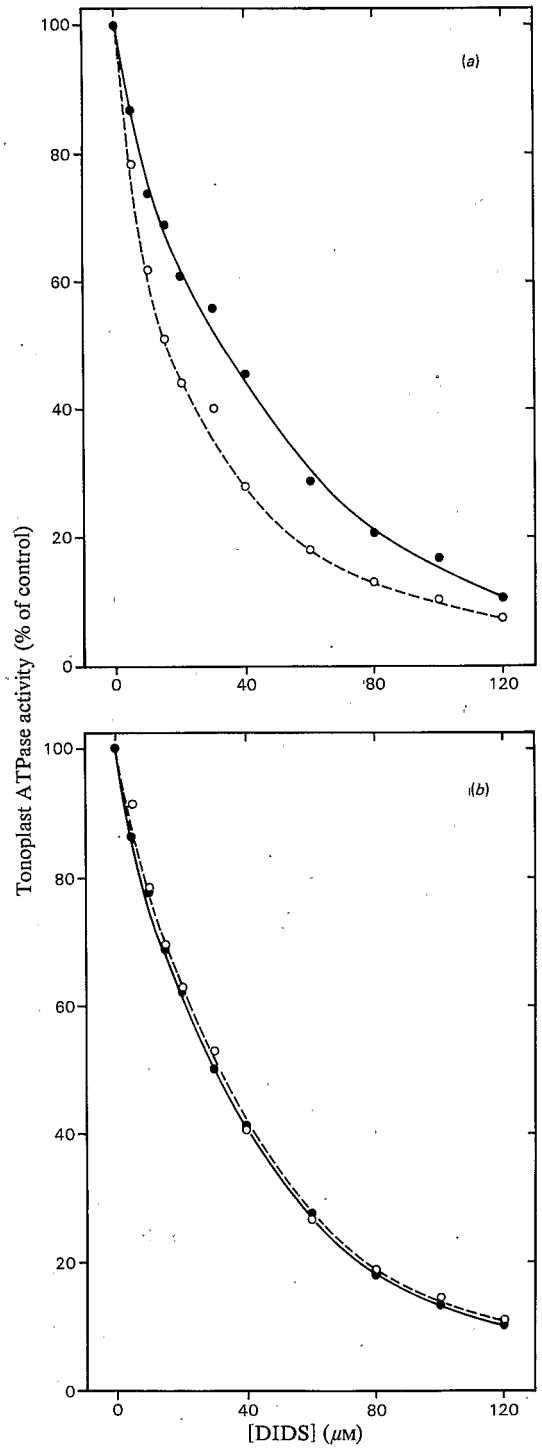

Fig. 8. *Effect of DIDS on Hevea tonoplast ATPase*
ATPase activity was assayed as described in the legend to Fig. 1, except [DIDS] ranged between 0 and 120 μM, in the absence (●) or presence (○) of 50 mM-KCl. (*a*) Native ATPase; (*b*) solubilized ATPase.

Table 4. *Effect of various inhibitors on the Hevea tonoplast ATPase activity*
The ATPase activity of tonoplast vesicles was measured as indicated in the Experimental section. It was conducted at pH 6.8, with 5 mM-$MgSO_4$ and 5 mM-ATP in the presence or absence of 50 mM-KCl. In absence of KCl, the activity amounted to 1.6 units/mg of protein. In its presence, it increased to 2.6 units/mg of protein. These values were set to 100%. Extra abbreviation used: TMT, trimethyltin chloride.

Additions	Concn. (μM)	Relative ATPase activity (as % of control) −KCl	+KCl
Control		100	100
DCCD	10	38	35
EEDQ	10	28	25
EDAC	10	88	90
TMT	5	25	21

Table 5. *Effect of DIDS on the Hevea tonoplast ATPase activity*
The ATPase activity of tonoplast vesicles and the solubilized tonoplast ATPase were measured as indicated in the Experimental section, at pH 6.8, with 5 mM-$MgSO_4$ and 5 mM-ATP in the presence or absence of 50 mM-KCl. The activity was expressed in units/mg of protein. The S.E.M. never exceeded 0.1 unit/mg of protein.

	ATPase activity	
	Tightly sealed tonoplast vesicles	Solubilized
Control		
−KCl	1.7 (100)	116.5 (100)
+KCl	3.0 (100)	161.0 (100)
+DIDS (15 μM)		
−KCl	1.2 (71)	80.0 (69)
+KCl	1.5 (50)	112.5 (70)

the presence of Cl^- than in its absence (Table 5). Furthermore, the mode of inhibition by DIDS was different in the absence and in the presence of Cl^-. It was competitive in its absence (X. Gidrol, B. P. Marin & H. Crétin, unpublished work). It became often mixed in its presence (Fig. 9*a*). Consequently DIDS could be involved in a second mechanism, where it reacts non-competitively with $MgATP^{2-}$.

But the solubilized tonoplast ATPase showed the same responses to DIDS in the absence and in the presence of Cl^-. No difference was observed in the sensitivity of DIDS (Fig. 8 and Table 5), and the mode of inhibition was identical (Fig. 9).

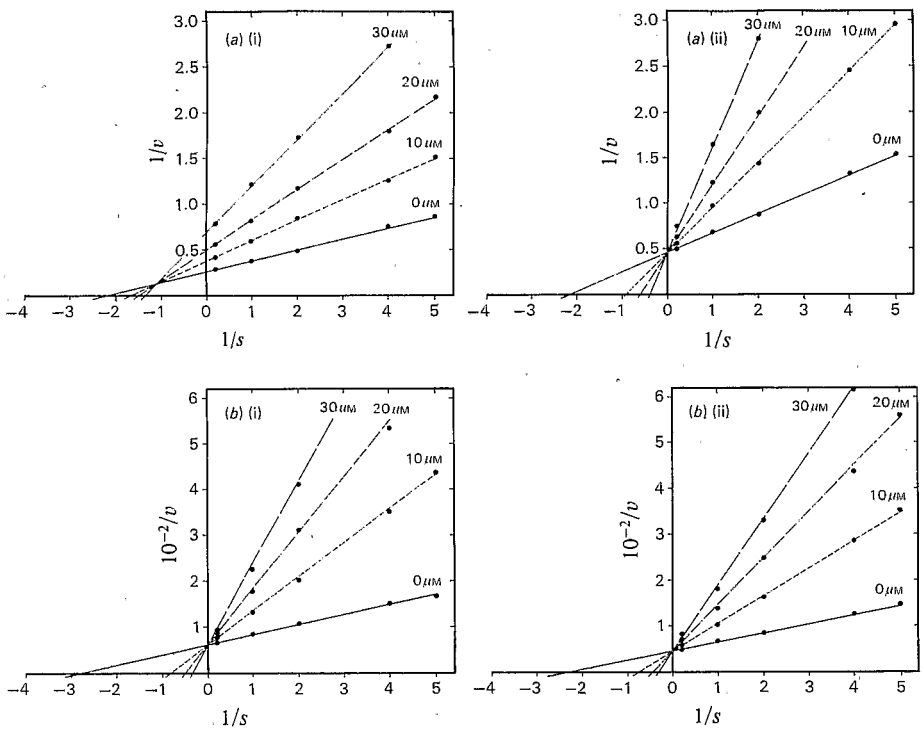

Fig. 9. *Double-reciprocal plot of MgATP-dependence of Hevea tonoplast ATPase, at various concentrations of DIDS* Experiments were conducted as described in the legend to Fig. 2, in presence of DIDS, added at the indicated concentration. The Mg/ATP ratio was held constant at 1.0. (*a*) Native ATPase; (*b*) solubilized ATPase, in the presence (i) or absence (ii) of 50mM-KCl.

In addition, in either cases, DIDS reacts non-competitively with Cl^- (results not shown).

The differences observed between the native ATPase and the solubilized ATPase could be explained by the occurrence of a dual mechanism, as discussed below.

Discussion

Vacuoles from *Hevea* latex were shown previously to contain an H^+-translocating ATPase bound to the tonoplast (Marin *et al.*, 1981*a*,*b*; D'Auzac *et al.*, 1982; Marin, 1983*a*; Crétin, 1982). Recently the properties of the membrane-bound activity have been compared with those of the activity solubilized and purified from *Hevea* tonoplast membrane (Marin & Komor, 1984*a*,*b*,*c*).

If these results could be extended to other higher plants, with this reservation in mind, it could be likely concluded that a proportion of the microsomal vesicles which bears a H^+-translocating ATPase originates from vacuoles. It was possible to compare the properties of this microsomal H^+-translocating ATPase with those for the *Hevea* tonoplast ATPase, especially their Cl^- stimulation.

A direct interaction of Cl^- with the Hevea tonoplast ATPase

Cl^- clearly stimulated the *Hevea* tonoplast H^+-ATPase, essentially by a direct interaction at the enzymic level. This conclusion was based on several properties evidenced for tonoplast vesicles and for the solubilized enzyme. Thus, when the ATPase activity was integrated in the tonoplast membrane, the collapse of any electrochemical proton gradient (capable of regulating the ATPase activity) being obtained in the presence of gramicidin for example, the rate of ATP hydrolysis was stimulated by 59% (Fig. 5 and Table 3). Moreover, Cl^- stimulated by 45–46% the ATPase solubilized and purified from tonoplast membranes (Fig. 6 and Table 1).

Actually the mode of this direct action of Cl^- on the *Hevea* tonoplast-bound ATPase activity is not known. It corresponds to an increase in $V_{max.}$, K_m not being affected (Fig. 7).

However, such data constitute the first demonstration of a hypothesis recently suggested for the anion-sensitive H^+-pumping ATPase in membrane vesicles from corn roots (Bennett & Spanswick, 1983*a*) and from oat (*Avena sativa*) roots

(Churchill & Sze, 1983), supposedly from tonoplast membrane. Here we demonstrate that Cl^- has a direct effect on a typical tonoplast H^+-pumping ATPase insensitive to vanadate (cf. Marin, 1984*a*).

Effect of Cl^- on the electrogenicity of Hevea tonoplast H^+-pumping ATPase: problem of the occurrence of a Cl^- channel with the H^+ pump

According to the material studied, either the native ATPase (present in tonoplast vesicles) or the solubilized ATPase, the extent of the Cl^- stimulation was different: 59–65% for the native activity and 45–46% for the solubilized activity. Consequently even if the difference observed was small, it amounts significantly to 14–20%. In addition, their sensitivity to DIDS was different: DIDS inhibited the ATPase activity of tonoplast vesicles more than it did that of the solubilized tonoplast activity. The difference was also significant: it amounted to 19% (Table 5). In addition, the mode of inhibition was different only when the ATPase was integrated with the membrane (Fig. 9). Such results suggest that there may be an additional effect linked to the status of tonoplast ATPase in a vesicular compartment.

On the basis of the changes of $\Delta\psi$ induced by the addition of Cl^- and SO_4^{2-} to an incubation medium containing tightly sealed tonoplast vesicles, the former anion can be considered as a permeant anion and the latter as an impermeant anion (Marin, 1982; B. Marin, unpublished work). The present results show that permeant anions, such as Cl^-, stimulated ΔpH generation at the expense of $\Delta\psi$, whereas impermeant anions, like IDA, benzenesulphonate or sulphate did not dissipate, and could not stimulate, ΔpH formation. Consequently, Cl^- stimulation of the *Hevea* H^+-ATPase can be attributed in part to the dissipation of the electrical potential. This effect has been invoked to explain the stimulation effects of Cl^- obtained with membrane vesicles from corn (Bennett & Spanswick, 1983*a*) and oat (Churchill & Sze, 1983) roots. Thus, as suggested for the Cl^--sensitive H^+-pumping ATPase from oat roots (Churchill & Sze, 1983), the Cl^- effects could be explained as follows: (*a*) Cl^- stimulates the H^+-pumping ATPase and is not transported itself; (*b*), Cl^- is co-transported via a specific anion channel indirectly or by the ATPase complex directly; or (*c*), H^+/Cl^- are co-transported out of the vesicles driven by a Cl^- or H^+ gradient or both. The present results rule out these explanations. The movement of Cl^- across the *Hevea* tonoplast membrane could be explained differently. The occurrence of a Cl^- channel, associated with the proton pump or not, was questionable.

DIDS has been described as an anion-transport inhibitor in red blood cells (Shami *et al.*, 1978) and in corn root tonoplasts (Lin, 1981). The increase of inhibition of tonoplast ATPase by DIDS in the presence of Cl^- only with tightly sealed vesicles, and the modification of the mode of its inhibition in its presence, could suggest the involvement of some Cl^- channel, coupled or not with the proton pump, when it operates as described for the chromaffin H^+-translocating ATPase (Pazoles *et al.*, 1980; Pazoles, 1982; Cidon *et al.*, 1983) or for similar systems found in the microsomal membrane fraction from higher plants (Hager & Helmle, 1981; Mandala *et al.*, 1982; Mettler *et al.*, 1982; Stout & Cleland, 1982; Churchill & Sze, 1983; Bennett & Spanswick, 1983*a*,*b*; O'Neill *et al.*, 1983). But, as described previously, even in the absence of any Cl^- from the medium, DIDS inhibited the *Hevea* tonoplast H^+-ATPase (Gidrol *et al.*, 1984). It was verified that membrane preparations do not contain any trace of native Cl^- (results not shown). Consequently, the effect of DIDS on *Hevea* tonoplast H^+-ATPase may be due to a direct interaction with some sites present in the ATPase system, i.e. basic proteins of the enzyme, which modified only the affinity of the substrate for its catalytic site without altering the rate of the reaction. This interaction was not modified by Cl^-. Consequently, this type of argument could not be used to favour the Cl^--channel hypothesis, as often seen in the literature (cf. Bennett & Spanswick, 1983*a*).

In addition, the membrane vesicles from corn (Bennett & Spanswick, 1983*a*) and from oat (Churchill & Sze, 1983) roots had a positive-inside membrane potential. Consequently, Cl^- could move electrophoretically inwards to neutralize $\Delta\psi$ and, indirectly, contribute to increase ΔpH. The situation is somewhat different with *Hevea* tonoplast vesicles: the membrane potential is negative-inside (Fig. 3*b*). Thus it was difficult to understand clearly how Cl^- could decrease the tonoplast depolarization by a simple electrical compensation of the electrogenic proton pump when working. Thereby, as clearly shown in Fig. 3(*b*), the involvement of some mechanism which corresponds to a Cl^- pump functioning as a symporter with the proton pump (as proposed by Hager & Helmle, 1981), was impossible, especially if these two processes were strictly coupled. Consequently, when all the data are taken into consideration, we favour the following hypothesis: the H^+-translocating ATPase bound to the tonoplast from *Hevea* latex is capable of modifying the permeability of this membrane to Cl^-. Thus an electrical compensation of the H^+ pump is possible if the basal membrane potential is negative.

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