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Sensitivity of Tonoplast-Bound Adenosine-Triphosphatase from *Hevea* to Inhibitors[1]

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BERNARD MARIN[2]
Office de la Recherche Scientifique et Technique Outre-Mer, 24, rue Bayard, F-75008-Paris, France; and Université des Sciences et Techniques du Languedoc, Laboratoire de Physiologie Végétale Appliquée, Place Eugène Bataillon, F-34060-Montpellier-Cedex, France

ABSTRACT

The tonoplast-bound H^+-translocating ATPase from *Hevea* latex was found to be insensitive to vanadate, diethylstilbestrol, and octylguanidine, which are specific inhibitors of the plasma membrane ATPase. The inhibitors of the mitochondrial ATPase, oligomycin and azide, and also rotenone and antimycin A, were all without effect. In contrast, trimethyltin chloride strongly inhibited the activity of *Hevea* tonoplast ATPase.

Among the different carbodiimides tested, which strongly inhibit the *Hevea* tonoplast ATPase, *N,N'*-dicyclohexylcarbodiimide was the most inhibitory. *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline was also an efficient inhibitor.

This unique inhibitor sensitivity of the *Hevea* tonoplast H^+-translocating ATPase suggests that this enzyme differs in its mode of operation from all other known H^+-translocating ATPases.

The latex of the rubber tree (*Hevea brasiliensis* Müll.-Arg. Kunth) consists of the fluid cytoplasmic content of the lactiferous system containing lutoids which are intermediate between lysosomes and plant vacuoles (7).

A Mg-dependent ATPase has been demonstrated at the lutoid membrane of *Hevea* latex (5, 6) as well as in vacuoles from higher plant cells (12, 13). It has been argued that some vacuolar preparations might be contaminated with membranes from other organelles. The degree of contamination can be indicated by the sensitivity towards the different ATPase inhibitors. Since it is known that the lutoid preparations from *Hevea* are uncontaminated (7), it should be possible to determine the precise sensitivity of this tonoplast-bound ATPase to a variety of potential inhibitors.

Consequently, several agents, which selectively inhibit the H^+-translocating ATPases in mitochondria, chloroplasts and plasmalemma, were tested for their effect on the lutoid ATPase.

MATERIALS AND METHODS

Plant Material. Latex was obtained from trees of *Hevea brasiliensis* (Kunth) Müll.-Arg. (Clone Prang Besar 86) growing on the I.R.C.A. (Institut de Recherches sur le Caoutchouc en Afrique) experimental plantation at Bimbresso, Abidjan, Ivory Coast. The fluid cytoplasm was harvested in ice-cooled flasks as described previously (19).

Vacuole Isolation and Purification. Vacuoles were isolated and purified as previously described (18). The composition of the medium was as follows: 300 mM mannitol, 50 mM triethanolamine, 2 mM β-mercaptoethanol, adjusted to pH 7.5 with HCl. The sediment was washed twice with this medium and then lyophilized.

Preparation of Tonoplast Membranes. Tonoplast membranes were dispersed as described elsewhere (18) in the following medium: 25 mM Mes, 25 mM Hepes, 5 mM β-mercaptoethanol, adjusted to pH 6.0 with Tris-base. It was used in the ratio 100 ml buffer: 1 g lyophilized material. The membranes were centrifuged at 15,000*g* for 3 min at 4°C, which left a sediment. The sediment was then washed twice with the same medium, adjusted to pH 7.5 with Tris-base, under the same conditions. The pellet corresponds to a highly purified tonoplast membrane fraction (7, 16).

Standard Incubation Conditions. Experiments were performed at 30°C. The incubation medium contained the following: 50 mM Mes, 50 mM Hepes, 5 mM β-mercaptoethanol, adjusted to pH 7.0 with Tris-base. The buffered mixture also contained 0.1 mM ammonium molybdate to inhibit any residual acid phosphatase activity associated with the membranes (5). ATP and Mg^{2+} (as SO_4^{2-} salt) were added at a final concentration of 5 mM. Further details are given in the text where appropriate.

ATPase Assay. Assays were performed in a final volume of 1 ml. Inhibitors were present at the concentrations indicated. When they were added as alcoholic solutions, the controls also contained an equal aliquot of alcohol without inhibitor. The final alcohol concentration in the assay mixtures was 1% w/v. Reactions were started by the addition of an aliquot of tonoplast membranes containing approximately 100 μg of protein and an ATPase activity varying between 0.2 to 0.3 μmol/min·mg protein at pH 7.0. Mixtures were incubated for 30 min. Enzymic hydrolysis of ATP was stopped with 250 μl of ice-cooled 20% (w/v) TCA. The mixtures were centrifuged at 7,500*g* for 5 min at ambient temperature. The liberated phosphate present in the supernatant was assayed according to the method of Taussky and Shorr (24). In each case, the results are expressed as per cent of control activity in the absence of inhibitor. As the same amount of protein is used in each experiment, *e.g.* 0.1 mg/ml, reported inhibitions are expressed on a molar basis. Consequently, this data may be compared with those published elsewhere (8).

Inhibition as a Function of Assay pH. ATPase activity of *Hevea* tonoplast was measured in the presence of inhibitor at different pH values. The desired pH value was obtained by the addition of Tris-base.

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[2] Supported by a Fellowship from Alexander von Humboldt-Stiftung (Bonn, Federal Republic of Germany). Present address: Universität Bayreuth, Lehrstuhl für Pflanzenphysiologie, Postfach 3008, Universitätsstrasse 30, D-8580 Bayreuth, Federal Republic of Germany.

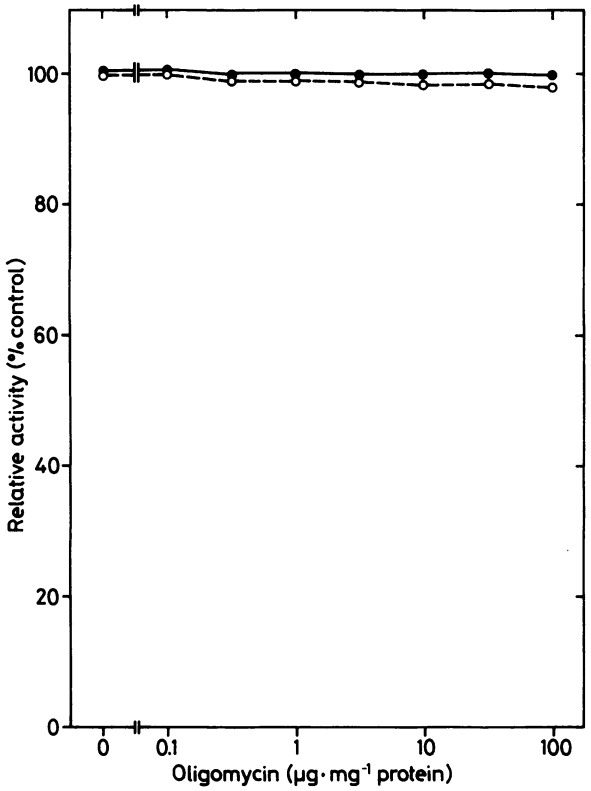

FIG. 1. Effect of oligomycin on *Hevea* tonoplast ATPase: assayed at pH 7.0 (●——●) and at pH 8.5(O- - -O), as described in "Materials and Methods." Control rates were, respectively, 0.21 μmol min^{-1} mg^{-1} protein at pH 7.0 and 0.08 μmol min^{-1} mg^{-1} protein at pH 8.5.

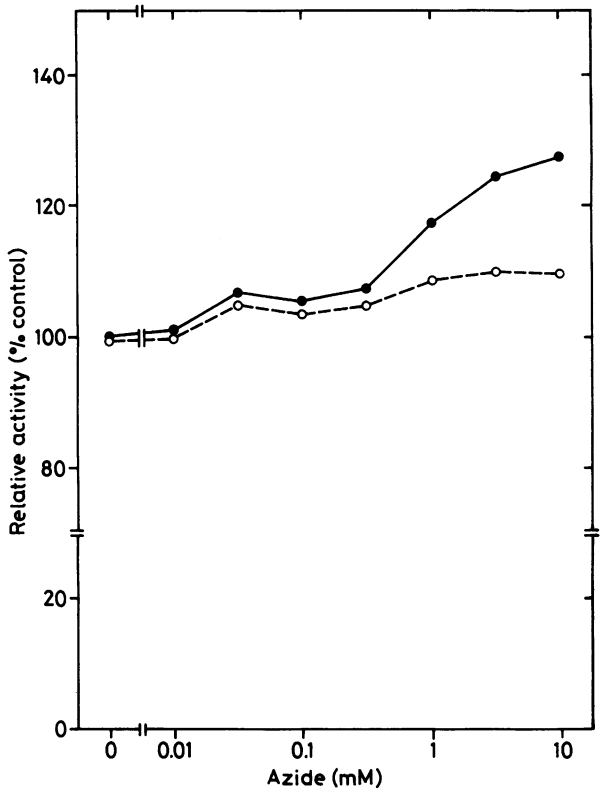

FIG. 2. Effect of azide on *Hevea* tonoplast ATPase. Symbols and reaction conditions are described in the legend to Figure 1 and in "Materials and Methods." Control activities were, respectively, 0.25 μmol min^{-1} mg^{-1} protein at pH 7.0 and 0.08 μmol min^{-1} mg^{-1} protein at pH 8.5.

Protein Assay. Protein was estimated by the method of Lowry *et al.* (14) with BSA as standard. Samples were precipitated and washed with ice-cold 10% (w/v) TCA. Then they were centrifuged at 7,500*g* for 5 min at ambient temperature. Protein was solubilized from the pellet with 0.1 N NaOH.

Reagents. Common laboratory reagents and chemicals not mentioned below were obtained from Labosi, Paris, France; Fluka Feinchemikalien GmbH, Federal Republic of Germany; Merck, Darmstadt, Federal Republic of Germany; and Sigma Chemical Co. Trimethyltin chloride and DIPCD[3] were obtained from ICN Pharmaceuticals, Inc., K and K Labs Division, New York; Dio-9 was purchased from Koninklijke Nederlandsche Gist and Spiritus Fabriek, Delft, The Netherlands. Oligomycin was a gift from Dr. E. R. Kashket, The Boston University School of Medicine. Octylguanidine was kindly donated to us by Dr. M. I. De Michelis and Prof. E. Marrè, Istituto di Scienze Botaniche dell' Università, Milano, Italy. In addition, phaseolin was a gift from Dr. Van Etten, Cornell University.

RESULTS

Many investigators have reported the activities of different inhibitors and their effectiveness against plasma membrane and mitochondrial ATPases. Table I summarizes the properties of many of these compounds that have been tested for their activity using the *Hevea* tonoplast ATPase.

Specific Inhibitors of Mitochondrial ATPase. In Table I group I includes classical inhibitors of mitochondrial ATPase (oligomycin, trimethyltin, azide) which have no effect on plasma membrane ATPase.

Oligomycin is a potent inhibitor of mitochondrial ATPase (23). As illustrated by Figure 1, even at 100 μg/mg protein, it does not inhibit *Hevea* tonoplast ATPase, at the two pH values tested, respectively 7.0 and 8.5.

Similarly, NaN_3, which inhibits mitochondrial ATPase from plant and animal tissues (8, 9, 22) does not inhibit the *Hevea* tonoplast ATPase at either pH 7.0 or pH 8.5 (Fig. 2). Azide even stimulated the ATPase by 20 to 30% at concentrations up to 1.0 mM.

Alkyltin compounds, *e.g.* trimethyltin chloride, which have been shown to interfere with the functioning of other H^+-translocating ATPases (11) cause 50% inhibition of tonoplast-bound ATPase from *Hevea* at 0.32 μmol/mg protein and a nearly complete inhibition is obtained at 1 μmol/mg protein (Fig. 3). Consequently, in terms of concentration, I_{50} was obtained at 32 μM, similar to the value reported for the mitochondrial ATPase from *Schizosaccharomyces pombe* (8).

Specific Inhibitors of Electron Transport. Rotenone and antimycin A, two typical electron transport inhibitors, listed in group 2 (Table I), had no significant effect (Fig. 3), even at high concentrations.

Specific Inhibitors of Plasma Membrane ATPase. Group 3 from Table I includes two compounds, orthovanadate and diethylstilbestrol, which come closest to being specific inhibitors of plasma membrane ATPase, as described in detail elsewhere (8).

Orthovanadate (5–10 μM) is a very effective inhibitor of the plasma membrane ATPases in animal and plant cells (1, 3, 8).

[3] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CMCD, 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate; EDAC, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, HCl; DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid; DIPCD, *N,N'*-diisopropylcarbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; NEM, *n*-ethylmaleimide; SITS, 4-acetamido-4'-isothiocyano-2,2'-stilbene disulfonic acid; I_{50}, 50% inhibition.

Table I. *Effect of Different Inhibitors or ATPases on the Activity of Hevea Tonoplast ATPase*

Inhibitor	Range of Concn. Tested	I_{50}[a]	Effect on Tonoplast ATPase	
			Concentration used	Activity[b] (in % of control[c])
Group 1: Specific mitochondrial inhibitors				
Oligomycin	0–1 mg mg^{-1} protein (0–100 $\mu g\ ml^{-1}$)	Not inhibited	100 $\mu g\ mg^{-1}$ protein (10 $\mu g\ ml^{-1}$)	98–101 (6)[d]
Trimethyltin chloride	0–1 $\mu mol\ mg^{-1}$ protein (0–0.1 mM)	32 μM	1 $\mu mol\ mg^{-1}$ protein (0.1 mM)	14–15 (5)
Sodium azide	0–10 mM	Not inhibited	10 mM	125–130 (5)
Group 2: Specific inhibitors of electron transport				
Rotenone	0–1.6 $\mu mol\ mg^{-1}$ protein (0–0.16 mM)	Not inhibited	1.6 $\mu mol\ mg^{-1}$ protein (0.16 mM)	103–105 (5)
Antimycin A	0–2 $nmol\ mg^{-1}$ protein (0–0.2 μM)	Not inhibited	2 $nmol\ mg^{-1}$ protein (0.2 μM)	101–105 (5)
Group 3: Specific plasma membrane inhibitors				
Orthovanadate	0–1 mM	Not inhibited	1 mM	95–98 (10)
Diethylstilbestrol	0–1 mM	Not inhibited	300 μM	100–102 (10)
Group 4: Mercurials and thiol reagents				
p-Hydroxymercuribenzoate	0–0.25 mM	50–100 μM	0.2 mM	25–30 (12)
p-Chloromercurisulfonate	0–0.25 mM	50–100 μM	0.2 mM	25–30 (12)
NEM	0–1 mM	35–40 μM	1 mM	4–5 (12)
Group 5: Other inhibitors different carbodimides				
CMCD	0–6 $\mu mol\ mg^{-1}$ protein (0–0.5 mM)	Not inhibited	6 $\mu mol\ mg^{-1}$ protein (0.6 mM)	96–97 (5)
DCCD	0–8 $\mu mol\ mg^{-1}$ protein (0–0.8 mM)	126 μM	8 $\mu mol\ mg^{-1}$ protein (0.8 mM)	8–9 (5)
DIPCD	0–6 $\mu mol\ mg^{-1}$ protein (0–0.6 mM)	Not obtained	6 $\mu mol\ mg^{-1}$ protein (0.6 mM)	63–65 (5)
EDAC	0–6 $\mu mol\ mg^{-1}$ protein (0–0.6 mM)	Not obtained	6 $\mu mol\ mg^{-1}$ protein (0.6 mM)	68–70 (5)
Chlorpromazine	0–3 mM	Not inhibited	2.5 mM	95–98 (3)
DIDS	0–80 μM	42–45 μM	60 μM	20–30 (6)
Dio-9	0–80 $\mu g\ ml^{-1}$	Not inhibited	80 $\mu g\ ml^{-1}$	54–58 (3)
EEDQ	0–300 $\mu M\ mg^{-1}$ protein (0–30 mM)	11.2 mM	300 $\mu M\ mg^{-1}$ protein (30 mM)	21–25 (8)
Octylguanidine	0–1 mM	Not inhibited	1 mM	102–108 (5)
Ouabain	0–0.15 mM	Not inhibited	0.15 mM	105–108 (5)
Phaseolin	0–0.12 mM	Not inhibited	0.12 mM	101–105 (5)
SITS	0–80 μM	40–50 μM	60 μM	20–30 (6)
Theobromine	0–1.0 mM	Not inhibited	1 mM	105–110 (5)
Theophylline	0–1.0 mM	Not inhibited	1 mM	108–110 (4)
Triphenylsulfonium chloride	0–0.5 mM	Not inhibited	0.3 mM	103–105 (5)

[a] I_{50} was the value of inhibitor concentration giving 50% inhibition of ATPase activity.

[b] ATPase activity measured as the appearance of inorganic phosphate as described in "Materials and Methods," with a correction when inhibitor interfered with the normal development of the color.

[c] Control activity was 0.23 $\mu mol\ min^{-1}\ mg^{-1}$ protein at pH 7.0.

[d] The extent of variation with the number of replicas (parens).

As shown in Table I, orthovanadate produces no significant inhibition of *Hevea* tonoplast ATPase, even at a concentration as high as 1 mM.

Diethylstilbestrol (0.1–1 mM), considered to be a potent inhibitor of plasma membrane ATPase (2, 8) also produced no inhibition of *Hevea* tonoplast ATPase even at concentration as high as 1 mM (Table I). In some cases, at these higher concentrations, some inhibition could be observed. But, disruption and some disintegration of tonoplast membranes were noted. Consequently, this effect could be regarded as artefactual.

Mercurial and Thiol Reagents. Among the several thiol reagents which reacted with the *Hevea* tonoplast ATPase, NEM was the most effective (Table I). ATPase activity fell to less than 4 to 5% of the control value at 1.0 mM. I_{50} was estimated to be 35 to 40 μM. The other reagents could be regarded as less effective inhibitors.

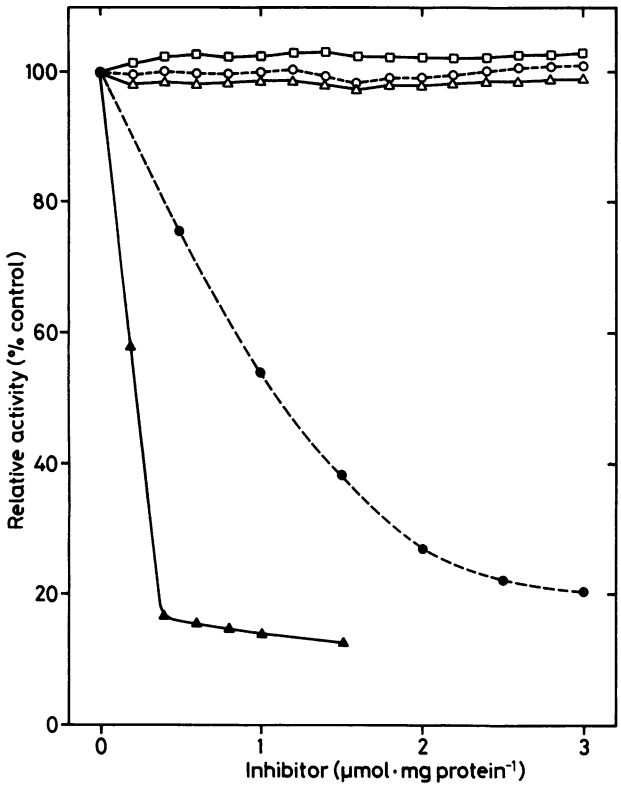

FIG. 3. Effect of different inhibitors on *Hevea* tonoplast ATPase: rotenone (□——□), antimycin A (○- - -○), octylguanidine (△——△), EEDQ (●- - -●), and trimethyltin chloride (▲——▲). Inhibitors are used as alcoholic solution in μmol range (rotenone, octylguanidine, EEDQ, trimethyltin chloride) or nmol range (antimycin A). For octylguanidine, and for EEDQ, the value must be magnified, respectively, by a factor of 10 and 100. In addition, except for octylguanidine where the inhibitor was expressed in terms of concentration, all the others were expressed per mg protein. Control activity was 0.24 μmol min^{-1} mg^{-1} protein at pH 7.0.

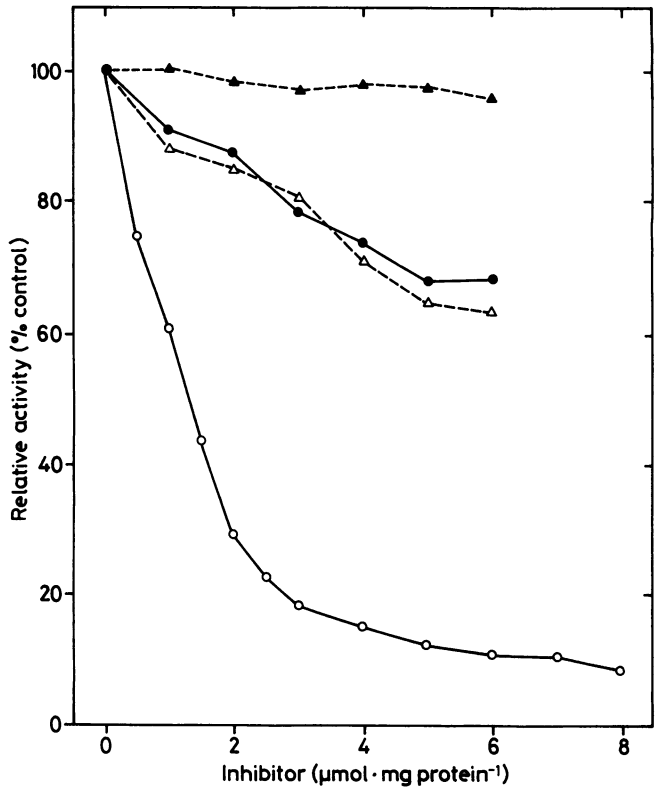

FIG. 4. Effect of different carbodiimides on *Hevea* tonoplast ATPase: CMCD (▲- - -▲), DCCD (○——○), DIPCP (△- - -△), EDAC (●——●). Control activity was 0.21 μmol min^{-1} mg^{-1} protein at pH 7.0.

Other Inhibitors. A number of other compounds (Group 5, Table I) were tested but they were found to have no significant effect on the *Hevea* tonoplast ATPase. These compounds included: ouabain, phaseolin, theobromine, theophylline, octylguanidine, and triphenylsulfonium chloride.

DCCD has been previously shown to be an effective inhibitor of *Hevea* tonoplast ATPase (6). Fifty % inhibition was observed at 1.26 μmol/mg protein (Fig. 4). Among the various carbodiimides tested, DCCD is the most effective inhibitor, whereas CMCD, EDAC, and DIPCD had only a slight inhibitory effect (Fig. 4). For instance, 22% inhibition was observed by 5 mM EDAC.

EEDQ was also an effective inhibitor (Fig. 3). *Hevea* tonoplast ATPase was inhibited by about 80% when EEDQ was used at 30 mM. I_{50} was 11.2 mM.

Dio-9 inhibited the tonoplast ATPase activity by 46% when used at 80 μg/ml (Table I).

Special attention has been paid for the effect of two anion channel blockers, DIDS and SITS. These two molecules inhibited by 70 to 80% the *Hevea* tonoplast activity when used at 60 μM (Table I).

DISCUSSION

The specific aim of the work presented in this paper is to examine the relationship between the *Hevea* tonoplast ATPase and other ATPases described in fungi and in higher plants, with respect to the sensitivity to known ATPase inhibitors.

It seems clear on the basis of inhibitor sensitivities that the *Hevea* tonoplast ATPase is distinct from other transport ATPases in the mitochondrion, chloroplast, and plasmalemma (8, 10, 15).

In practical terms, we have found no specific inhibitor to characterize the *Hevea* tonoplast ATPase, since DCCD and EEDQ are known to inhibit all H^+-translocating ATPases. In addition, trimethyltin is reported as an effective inhibitor of mitochondrial ATPase (8, 11).

Hevea tonoplast ATPase is known to be a proton-translocating pump (4, 17). It is tempting to correlate this observation with the inhibitory effect of DCCD and trimethyltin. The observation that the highest inhibitory effects were produced by the most lipophilic carbodiimides indicates either a lipid-embedded inhibition site at the ATPase, or that the inhibitors have first to cross the vesicle membrane to bind from inside. DCCD is known to inhibit the ATPase of mitochondria, chloroplast, and bacteria (15) where it is thought to bind the proteolipid in the F_0 unit and the β-subunit of the F_1 portion. The other inhibitory agent, trimethyltin, belongs to a group of structurally related agents which also act by binding to a proteolipid, probably near but not identical with the site of DCCD binding (11). The magnitude of inhibition caused by DCCD and trimethyltin chloride are not identical, as shown previously for the H^+-translocating ATPase of chromaffin granules (11). Trimethyltin chloride, on a molar basis, is a much better inhibitor than EEDQ or DCCD.

The orthovanadate insensitivity suggests that the *Hevea* tonoplast ATPase does not have a phosphorylated intermediate and thus resembles the ATPases of mitochondria and chloroplasts (15), although some differences in inhibitor sensitivity are evident. These differences indicate at least the nonidentity of these ATPases with the *Hevea* tonoplast ATPase. In addition, the absence of inhibition of ATPase activities by these agents further confirms that contamination by other cellular membranes is

negligible and that the measured activity is due to *Hevea* tonoplast ATPase. Consequently, the insensitivity of the tonoplast ATPase to orthovanadate and azide may be used to distinguish vacuolar membranes from plasmalemma, mitochondria, or chloroplasts.

The results reported in this study indicate certain similarities and differences with respect to other tonoplast ATPases.

Thus, EDAC has been reported as a potent inhibitor of tonoplast ATPase from *Hippeastrum* and *Tulipa* (13). In addition, diethylstilbestrol has been described as an effective inhibitor but at different concentration for vacuoles of *Saccharomyces carlsbergensis* (21) and of *Beta* roots (25). The reason for these differences in inhibitor sensitivity among the presently identified tonoplast-bound ATPases is not clear. However, several explanations are possible. First, the vacuoles used were prepared from different sources, and the preparation procedures used differ. The *Hevea* vacuoles are prepared by a procedure which is likely to involve the least damage to the tonoplast ATPase compared to other procedures, where for example there is a lengthy incubation in cell wall digesting enzymes (20) which might result in some modification and some denaturation of tonoplast membranes (20). Consequently, the loss of some inhibitor sensitivity could be explained by a modification of the native properties of tonoplasts by the procedures used to isolate them (20). The procedure used to isolate vacuoles from *Beta* roots (25) was also somewhat drastic for the tonoplast. Consequently, for all these reasons, as suggested elsewhere (7), the organelles from *Hevea* latex could be regarded as a vacuolar model having preserved their native properties. The lyophilization procedure did not modify the properties of tonoplast membranes from *Hevea* latex and especially those of the tonoplast-bound ATPase (7, 16). Second, even if much effort is spent to obtain a very highly purified vacuole preparation, this preparation could be still contaminated by some fragments of plasma membrane. This is sufficient to explain the observed differences among the presently identified tonoplast-bound ATPases. Third, these differences could be related to the different composition of tonoplast membranes. Tonoplasts from *Hevea* latex, for example, are extremely rich in phosphatidic acid (7). A modification of the hydrophobic environment near the site of ATP hydrolysis at the tonoplast level could cause such differences as those reported for the different carbodiimides, especially EDAC, and for diethylstilbestrol.

In addition, the effects of DIDS and SITS could be correlated with the activation of *Hevea* tonoplast ATPase by Cl^- (B. Marin, unpublished data). This activation by Cl^- is complex. First, in the presence of Cl^-, the electrogenicity of the proton pump decreases and the positive charge of the tonoplast was somewhat compensated by Cl^-. Second, a direct effect of Cl^- on some enzymic site involved in ATP hydrolysis could be also evidenced.

Furthermore, as NEM and sulfhydryl reagents severely inhibit *Hevea* tonoplast ATPases, the intervention of some sulfhydryl groups is evidenced. They should be implicated in some process which can modulate the overall proton conductivity of the tonoplast proton pump.

Further studies, including the solubilization and reconstruction of the active H^+-translocating ATPase from tonoplast systems, should clarify these points.

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