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Effect of nigericin on the H^+-translocating adenosine triphosphatase from tonoplast of *Hevea latex*

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Nigericin stimulated the ATPase activity of tightly-sealed membrane vesicles prepared from *Hevea brasiliensis* Müll.-Arg. lutoïds in the presence of K^+. This stimulation required a functioning membrane since it was membrane-bound and since it was not observed for the ATPase activity solubilized from the tonoplast by dichloromethane. The extent of nigericin-induced stimulation of tonoplast ATPase was proportional to the ΔpH collapsed by the ionophore in the presence of K^+.

Additional key words – Membrane vesicles, proton-motive force, tonoplast ATPase.

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Introduction

Vacuoles from *Hevea* latex are capable of hydrolyzing ATP (D'Auzac 1975, 1977, references in Marin 1985). This reaction is catalyzed by an enzyme bound to the tonoplast which also carries out an electrogenic influx of protons (Crétin 1982, Marin 1983, 1985, Marin et al. 1981a, b). The properties of this ATPase are well described (D'Auzac 1977; X. Gidrol 1984. Thesis, Univ. Aix-Marseille, France; Marin 1985). One important characteristic is its driving of the uptake of citrate (Marin 1982, cf. Marin 1985). In other less well characterized systems obtained from disrupted plant tissues the ionophore nigericin stimulates a microsomal anion-sensitive ATPase found to be a tonoplast-bound enzyme (Sze 1980, Churchill et al. 1983). It is not clear why this enzyme is sensitive to nigericin. The sensitivity could be related directly to the magnitude of pH gradient across the membrane of a tightly-sealed system, nigericin facilitating an electroneutral H^+/K^+ exchange across this membrane (Pressmann 1976). However, this nigericin stimulation of the membrane-bound enzyme could also be due to changes in the internal pH of such a vesicular system. Conditions required for maximal stimulation of the tonoplast H^+-translocating ATPase from *Hevea* latex are determined in the present paper. The results indicate that nigericin acts by dissipating the initial ΔpH rather than by a change in the internal pH.

Abbreviation – ΔpH, transmembrane pH gradient; Δψ, electrical transmembrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MES, 2-(N-morpholino)-ethanesulphonic acid; $TPMP^+$, triphenylmethylphosphonium iodide; Trizma, Tris-base.

Materials and methods

Plant material. Latex was obtained from trees of *Hevea brasiliensis* Müll.-Arg. (clone Prang Besar 86) growing on the I.R.C.A. (Inst. de Recherches sur le Caoutchouc en Afrique) experimental plantation at Bimbresso, Abidjan, Ivory Coast. The fluid cytoplasm was harvested in ice-cold flasks as described previously (Marin 1982, Marin et al. 1981b). This latex is very rich in orga-

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nelles called lutoïds which are equivalent to the vacuolar compartment of higher plants (D'Auzac et al. 1982).

Vacuole isolation and purification. Vacuoles were isolated and purified as described previously (Marin et al. 1981b). They were devoid of any contamination by non-vacuolar membranes (Marin 1982, D'Auzac et al. 1982). The vacuoles were lyophilized in the laboratory where the isolation and the purification processes were conducted (I.R.C.A., Bimbresso) and sent to France where the samples were preserved at −40°C for several months without appreciable loss of ATPase activity (Gidrol et al. 1984, Marin 1982).

Preparation of tonoplast. Tonoplast was resuspended as described elsewhere (Marin et al. 1981b) in the following medium: 25 m*M* MES, 25 m*M* HEPES and 5 m*M* β-mercaptoethanol adjusted to pH 6.0 with Trizma. One g of lyophilized material was added per 100 ml of medium. The membranes were collected by centrifugation at 15 000 *g* for 3 min at 4°C. The pellet was then washed twice with the same medium and adjusted to pH 7.5 with Trizma under the same conditions. The pellet corresponds to a highly purified tonoplast membrane fraction consisting of tightly-sealed vesicles (D'Auzac et al. 1982, Marin 1982, 1985).

Standard incubation conditions. Experiments were conducted at 30°C in the following medium: 50 m*M* MES, 50 m*M* HEPES and 5 m*M* β-mercaptoethanol, adjusted to pH 7.0 with Trizma. Ammonium molybdate was used at a final concentration of 0.1 m*M* to inhibit any acid phosphate activity (D'Auzac 1975, 1977). ATP (as Tris-salt) and magnesium (as sulfate salt) were added to a final concentration of 5 m*M*. Further details are given in the text.

ATPase assay. Assays were performed in a final volume of 1 ml. Reactions were started by the addition of an aliquot of membrane containing tonoplast fragments of approximately 100 µg protein. After incubation for 30 min, enzymatic hydrolysis of ATP was stopped with 250 µl of ice-cold 20% (w/v) trichloroacetic acid. The mixtures were then centrifuged at 7 500 *g* for 5 min at ambient temperature (20–25°C). The liberated phosphate present in the supernatant was assayed according to the method of Taussky and Shorr (1953). One unit of ATPase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of ATP h^{-1} under the assay conditions.

Protein assay. Samples were precipitated and washed with ice-cold 10% (w/v) trichloroacetic acid. After centrifugation at 7 500 *g* for 5 min at ambient temperature (20–25°C), the pellets were solubilized with 0.1 *M* NaOH and the solubilized proteins determined by the method of Lowry et al. (1951) with the modifications described by Marin (1982, 1983). Bovine serum albumin was used as standard.

Measurement of the proton motive force. The proton motive force is given by the equation:

$$\Delta p = \Delta\psi - 2.303\ RT\ \frac{\Delta pH}{F} \qquad (1)$$

where R, T and F have their usual thermodynamic meanings.

The membrane potential ($\Delta\psi$), transmembrane pH difference (ΔpH) and the osmotic volume of the vesicles were calculated from the uptake of radioactively labelled molecules according to the procedure described previously (Marin 1982, 1983, Marin et al. 1981a,b). [^{14}C]-TPMP$^+$ was used for the determination of membrane potential. The distribution of [^{14}C]-methylamine was used to measure ΔpH across the tonoplast membrane. Vesicular volumes were determined using ^3H_2O and [^{14}C]-dextran.

The experiment was started after the preincubation period (10 min) by the addition of 5 m*M* ATP in the presence of 5 m*M* $MgSO_4$. Assays were performed so that each series of vials contained ^3H_2O and a [^{14}C]-labelled compound with a ^{14}C:^{3}H ratio of 0.2. After the appropriate period, the vesicles were sedimented by centrifugation in an Eppendorf model 3200 desk microcentrifuge for 3 min at full speed (approximately 15 000 *g*). Further experimental details were as described previously (Marin 1982, 1983, Marin et al. 1981a,b). The distribution of the labelled compounds was determined from their relative activities in the pellet and in the supernatant (Marin 1982, 1983, Marin et al. 1981a,b). From these values, $\Delta\psi$ and ΔpH were calculated according to the methods described by Komor and Tanner (1976) and Johnson and Scarpa (1976), respectively.

Solubilization of tonoplast ATPase. Tonoplast ATPase was solubilized from the membrane by shaking the lyophilized material with dichloromethane (Marin et al. 1985). Analyses on polyacrylamide gels and by electron microscopy, and also the sensitivity to inhibitors, show that this simple and rapid method results in a highly purified active enzyme (Marin et al. 1985). The experiments described in the present paper were conducted with this purified enzyme.

Reagents. ATP (as disodium salt) was from Boehringer Mannheim France SA, Meylan, France. Nigericin was a gift from Dr J. Berger and Dr J. W. Westley from Hoffman-La Roche, Basel, Switzerland. [^{14}C]-Dextran (1.3–5.2 TBq mol^{-1}) was obtained from New England Nuclear Chemicals, Dreieich, West Germany; ^3H_2O (3.3 GBq mol^{-1}) from Amersham Buchler, Braunschweig, West Germany; and [^{14}C]-triphenylmethylphosphonium iodide (TPMP$^+$; 1.8 TBq mol^{-1}) and [^{14}C]-methylamine

hydrochloride (1.5 TBq mol^{-1}) from Commissariat à l'Energie Atomique, Gif-sur-Yvette, France. All other reagents and chemicals were obtained from Labosi, France; Fluka Feinchemikalien GmbH, Ulm, West Germany; Merck, Darmstadt, West Germany; and Sigma Chemical Co., Saint Louis, MO, U.S.A.

Results

Dependence on the nigericin concentration

Tonoplast ATPase was stimulated significantly at uncoupler concentrations above 5 nM (Fig. 1). The nigericin concentration causing maximum stimulation varied from one lot of lyophilized material to another. It varied with the origin of the material (cultivar) and the tapping procedure which gave tonoplast vesicles which were more or less tightly sealed (B. Marin, unpublished data). Moreover, it was noted that the extent of stimulation by nigericin depended upon the age of the lyophilized material and the condition of preservation (if the material was not continuously kept at −40°C). When all these factors are taken into account, the extent of stimulation by nigericin varied between 25 and 120% above the control (in the absence of the ionophore). In addition, any treatment (physical and/or chemical) which modified the initial permeability of the tonoplast to protons decreased the effect of nigericin on the tonoplast ATPase activity. K^+ was required for stimulation of tonoplast ATPase by nigericin (Fig. 1). Stimulation of the ATPase was not observed when Triton X-100 at a final concentration of 0.05% (w/v; sufficient to disrupt the membrane without destroying the ATPase activity) was added to the medium. Moreover, no effect of nigericin was observed for the ATPase activity solubilized from tonoplasts by dichloromethane (Fig. 1). Consequently, the nigericin-stimulation of tonoplast ATPase appears to involve the integrity of the tonoplast vesicles, because it could be observed only with tightly sealed vesicles. Indeed, this effect has previously been observed for intact vacuoles freshly isolated from *Hevea* latex (Crétin 1982, Marin et al. 1981a) but never for vacuolar membranes made permeable by different treatments such as maceration with media used to prepare protoplasts from plant tissues (B. Marin, unpublished data).

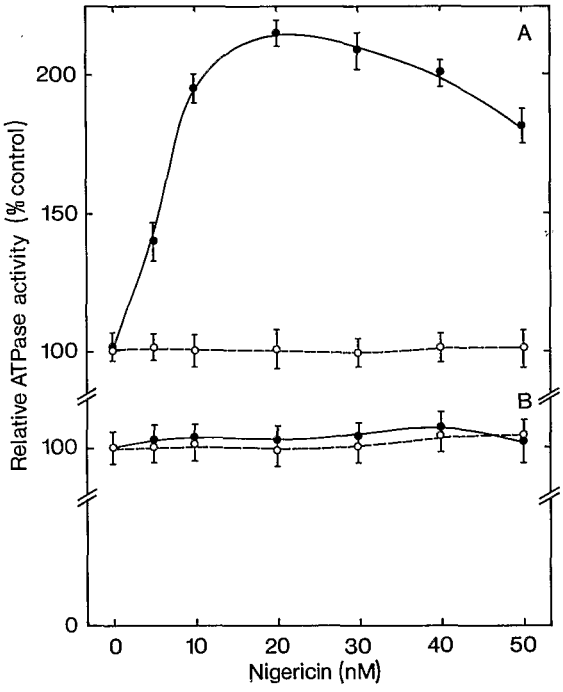

Fig. 1. Dependence of the tonoplast ATPase activity on nigericin concentration. The ATPase activity of tonoplast vesicles (A) and the solubilized tonoplast ATPase (B) were measured at pH 7.0 in the presence of 5 m*M* $MgSO_4$ and 5 m*M* ATP in an incubation medium containing nigericin ranging in concentration between 0 and 50 n*M*, in the presence (●——●) or absence (○- - -○) of K^+, added as sulfate (50 m*M*). The ATPase activity was expressed as percentage of the activity assayed in the absence of K^+-salt and nigericin. The results are from one experiment representative of four. Control values are as followed: native ATPase, 1.81 ± 0.12 units (mg protein)$^{-1}$; solubilized ATPase, 105.1 ± 0.2 units (mg protein)$^{-1}$.

Dependence on K^+ concentration

In the absence of added K_2SO_4, tonoplast vesicles do not contain sufficient endogeneous K^+ to permit any electroneutral H^+-K^+ exchange catalyzed by nigericin (Pressmann 1976). With the lyophilized material used in the present experiments, tonoplast vesicles contained no more than 3–5 μ*M* K^+. Since K^+ was not released by osmotically stressed vesicles, the K^+ present may be bound to the membranes.

The effectiveness of nigericin in enhancing the tonoplast ATPase rate increased with added K^+ up to 5 mM after which the effect of the ionophore was largely independent of the K^+ concentration (Fig. 2). This stimulation was not observed with the ATPase activity solubilized from tonoplast by dichloromethane in the absence or presence of 20 n*M* nigericin (Fig. 2).

Anion sensitivity

The nigericin-induced stimulation of native tonoplast ATPase varied according to the nature of the anion accompanying K^+ (Tab. 1). At pH 7.0, both in the absence and presence of 20 n*M* nigericin, the native tonoplast ATPase was stimulated by anions in the following order of effectiveness: Cl > Br. Sulfate, iminodiacetate and benzene sulphonate did not stimulate the tonoplast ATPase in the absence of nigericin. NO_3^- was inhibitory both in the presence and absence of nigericin. Whereas the solubilized tonoplast ATPase was differentially sensitive to the different anion tested, the nigericin-induced stimulation or inhibition was relatively independent of the nature of the anion tested when expressed on a percentage basis. This is true for all the anions tested ex-

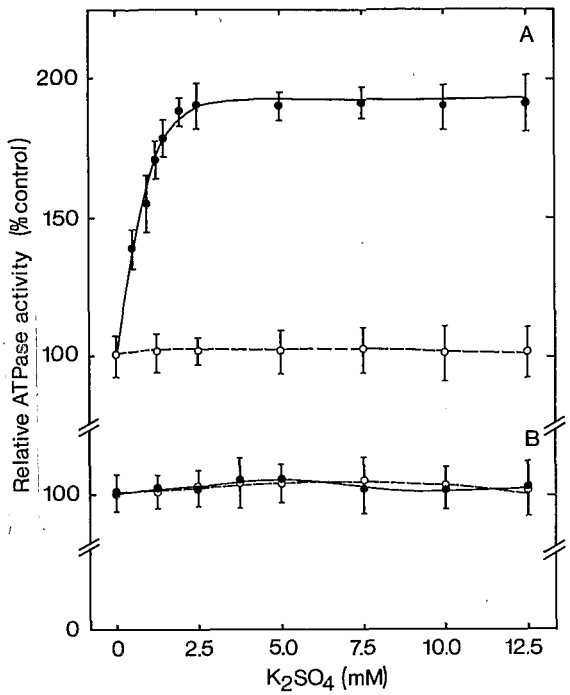

Fig. 2. Dependence of the nigericin stimulation of tonoplast ATPase on K^+ concentration. The ATPase activity of tonoplast ATPase activity (A) and the solubilized tonoplast ATPase (B) were measured in a medium containing 5 m*M* $MgSO_4$, 5 m*M* ATP and K_2SO_4 ranging between 0 and 12.5 m*M*, in the presence (●——●) or absence (○- - -○) of 20 n*M* nigericin. The ATPase activity was expressed as percentage of the activity assayed in the absence of K^+-salt and nigericin. The results are from one experiment representative of three. Control values are as followed: native ATPase, 1.75 ± 0.13 units (mg protein)$^{-1}$, solubilized ATPase, 109.3 ± 0.4 units (mg protein)$^{-1}$.

Tab. 1. Effect of different anions on the *Hevea* tonoplast ATPase. The ATPase activity of tonoplast vesicles (native) and the solubilized tonoplast ATPase (solubilized) was assayed at pH 7.0 as described in the experimental part. The final concentration of each salt was 50 m*M*. Nigericin concentration was 20 n*M*. The ATPase activity of tightly sealed tonoplast vesicles was 1.71 ± 0.11 units (mg protein)$^{-1}$. When the enzyme was solubilized from tonoplast membranes, the activity was 118.2 ± 0.4 units (mg protein)$^{-1}$. These values were set to 100%. All values represent the means of two to five experiments.

Treatment	ATPase activity			
	Native		Solubilized	
	− Nig.	+ Nig.	− Nig.	+ Nig.
No addition ($MgSO_4$ only)	100	103	100	102
KCl	171	340	143	141
KBr	161	280	131	134
KNO_3	42	55	39	40
K_2SO_4	103	210	102	98
K-imino diacetate	100	210	102	101
K-benzene sulphonate	98	200	95	96

cept NO_3^- which must be considered as a typical strong inhibitor of this ATPase (cf. Marin 1985). In contrast to the intact enzyme, nigericin had no effect on the activity of the solubilized ATPase (Tab. 1).

Effect of nigericin on the proton-motive force in tonoplast vesicles

In the absence of MgATP, but in the presence of K_2SO_4, the transmembrane ΔpH was reduced when the nigericin concentration increased (Fig. 3). Thus, at a concentration which stimulates the ATPase activity (20 n*M*), the ionophore decreased the ΔpH component by 0.8–

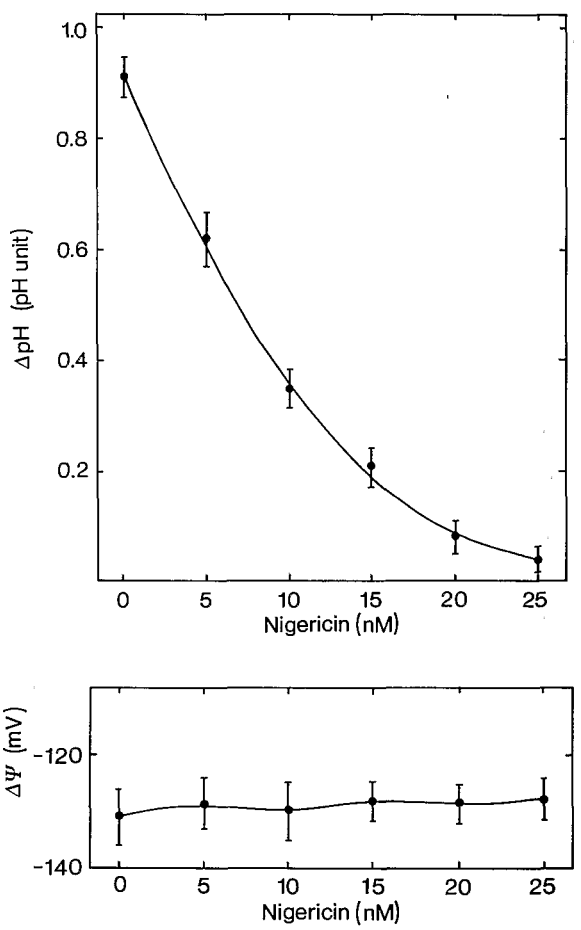

Fig. 3. Effect of nigericin on the magnitude of the electrochemical proton gradient in *Hevea* tonoplast vesicles. Tonoplast vesicles were added to an incubation medium adjusted at pH 7.0 by addition of Trizma, as described in Materials and methods. Data reported in this figure corresponds to a typical experiment where the magnitude of the electrochemical proton gradient was measured after the addition of the indicated nigericin concentration. The transmembrane pH gradient (ΔpH; top) and the electrical potential difference (Δψ; bottom) across the tonoplast were calculated from the transmembrane distribution at equilibrium of methylamine and $TPMP^+$, respectively. The results are from one experiment representative of three.

0.9 pH units. On the other hand, the membrane potential was not affected by the addition of nigericin, even at high concentrations (Fig. 3, bottom). Consequently, it can be postulated that nigericin stimulates the tonoplast ATPase activity because it decreases the pH gradient existing initially across the tonoplast membrane. Any constraint on the proton pump imposed is thus removed.

pH-dependence

The nigericin-induced stimulation was, on a percentage basis, relatively independent of the external pH value, with the greatest stimulation at the optimal value for the tonoplast ATPase (Fig. 4). In this experiment, as also demonstrated previously, the transmembrane pH gradient changes as the external pH varies (Marin 1982, 1983, Marin et al. 1982). However, this ΔpH also increases as the tonoplast-bound ATPase functions as a proton pump (Marin 1982, Marin et al. 1982). Consequently, when the tonoplast ATPase operates, the transmembrane ΔpH is maximal when external pH values are between 6.8–7.0 (Marin 1982, Marin et al. 1982). Under these conditions, when MgATP was added to the incubation mixture, the extent of the nigericin-stimulation of tonoplast ATPase was the greatest when the dissipated initial ΔpH was the greatest (Fig. 4). The collapsing effect induced by the ionophore was maximal under these conditions. The stimulation of the tonoplast ATPase was directly proportional to the magnitude of ΔpH across the membrane (Fig. 5).

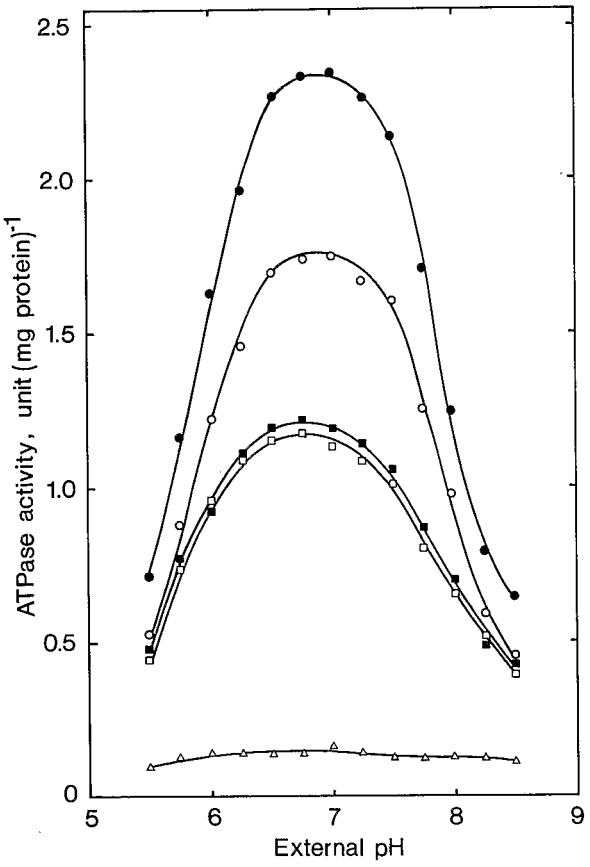

Fig. 4. Effect of external pH on the stimulation of *Hevea* tonoplast ATPase by nigericin. Tonoplast vesicles were incubated at different pH values from pH 5.0 to pH 8.0. Sufficient Trizma or H_2SO_4 was added to bring the pH to the desired value. In each case, 5 m*M* ATP and 5 m*M* $MgSO_4$, buffered at the tested pH value, were added. The activity was measured in the presence (●) or in the absence (○) of 20 n*M* nigericin in a medium containing 50 m*M* K_2SO_4. For control, K^+ was omitted, and 20 n*M* nigericin was either absent (□) or present (■). When ionophore was omitted, 0.5% ethanol was added. △, without Mg^{2+} in the medium. The results are from one experiment representative of three.

Discussion

The data reported in the present paper describe the conditions required for maximal stimulation of tonoplast ATPase from rubber tree (*Hevea brasiliensis*) by nigericin. This stimulation is due to the preexisting ΔpH across the tonoplast membrane and not to the value of the internal pH of the tonoplast vesicles. The tonoplast ATPase activity is directly related to the transmembrane

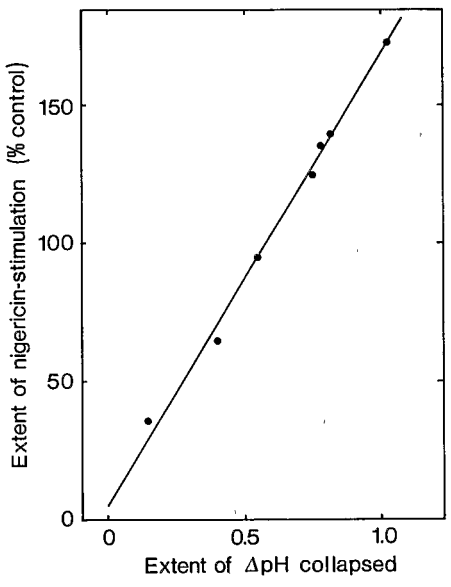

Fig. 5. Dependence of the extent of the nigericin-stimulation of tonoplast ATPase on the initial magnitude of ΔpH collapsed by the ionophore. These data come from different experiments conducted on tonoplast vesicles exhibiting different initial ΔpH gradient. The same amount of lyophilized tonoplast fractions was vesiculated at different pH values but the resulting tonoplast vesicles were incubated at the same pH value (7.0). The extent of nigericin-stimulation of tonoplast ATPase was measured after the addition of 20 n*M* nigericin. The results are presented as percentage of the rate of ATP hydrolysis and correspond to the means ± SE. The value of the control is 2.05 ± 0.15 units (mg protein)$^{-1}$.

ΔpH as measured by methylamine uptake. The higher the ΔpH across the tonoplast, the higher is the effect of nigericin.

Since the tonoplast ATPase activity is increased as the ΔpH across the membrane is decreased, the tonoplast ATPase activity is under thermodynamic (or kinetic) regulation by the ΔpH component of the proton-motive force. Consequently, this ATPase cannot be considered as fundamentally different from the other H^+-translocating ATPases classified as F_0F_1-type enzymes (Maloney 1982). As for the other species belonging to this class, the rate of ATP hydrolysis by a H^+-translocating ATPase in well-coupled membranes is affected by the magnitude of the transmembrane proton gradient generated by the enzyme (Maloney 1982).

It would be interesting, but quite difficult, to compare the nigericin-stimulation of tonoplast ATPase from *Hevea* latex with the anion- and nigericin-sensitive, H^+-translocating ATPase found in microsomal membranes from tobacco callus (Sze 1980, 1982), oat seedlings (Churchill and Sze 1983, Churchill et al. 1983) and pumpkin hypocotyls (Scherer 1984).

The principal advantage of the *Hevea* vesicles over other vesicles that have been used in similar studies is that the former are uncontaminated by non-vacuolar membranes, whereas the latter may be contaminated. Another advantage is that the tonoplast can be either freshly prepared from *Hevea* latex or from lyophilized material (Gidrol et al. 1984). No differences were observed if the lyophilized material was stored at −40°C. The conditions of preservation affects the integrity of tonoplast and modifies the efficiency of the method used to form tightly-sealed vesicles. Thus, if improperly stored (at higher than −40°C), the vesicles could become too permeable to protons (an artefactual property of the tonoplast) and the stimulation by nigericin lost. Thus, from the present work, it can be concluded that (nigericin + K^+)-stimulation of ATPase is a characteristic of vacuolar ATPase per se in well-coupled tonoplast vesicles.

In spite of the abundant literature concerning the tonoplast system from fungi and higher plants (cf. Marin 1985), it would be difficult to define the typical features of this membrane. Certainly, the best-known example is the tonoplast-related structure found in *Hevea* latex (cf. Marin 1985). Initially, the lutoïds have been described as a vacuo-lysosomal system (cf. Pujarniscle 1968) but recently they have been classified as a vacuolar system (cf. D'Auzac et al. 1982). More important than the degree to which the lutoïds is a vacuolar system (cf. D'Auzac et al. 1982, Marin 1982), however, is the question of the stimulation of tonoplast ATPase by nigericin. This stimulation is due to the dissipation of ΔpH across the membrane in the presence of K^+ when the vesicles are tightly sealed, as shown in the first description of nigericin-stimulated ATPase in plants (Sze 1980). It has been claimed that in most systems nigericin-stimulated ATPase is associated with the tonoplast. However, this effect of nigericin is not at all specific for this enzyme. The lack of effect in other membranes reflects only their inability to form sealed membrane vesicles. The tonoplast appears to form sealed vesicles readily, especially when it is not damaged during the isolation. Therefore, the tonoplast is able to develop a pH gradient that can be dissipated by nigericin, as described here for tonoplast vesicles from *Hevea,* only when the assay medium contains K^+. Another way to dissipate this transmembrane ΔpH is the use of protonophores such as FCCP, especially at low concentrations. That lutoïds from *Hevea* can develop and maintain a pH gradient is well known (Marin et al. 1981a, b, Crétin 1982, Marin 1982, 1983). This material permits us to examine the degree of coupling between the tonoplast ATPase activity and the magnitude of the proton-motive force across the membrane. This tonoplast ATPase drives electrogenic H^+ transport into a tightly-sealed vesicle (Marin 1982, 1983, cf. Marin 1985). Consequently, any reduction of the proton-motive potential against which the enzyme must operate involves a stimulation of this enzyme. Such an effect is expected. The results reported in the present paper confirm this property which is typical for any electrogenic H^+-translocating ATPases (cf. Maloney 1982). The tonoplast-bound H^+-translocating ATPase from *Hevea* latex is also controlled by the magnitude of the proton-motive force (or by the protons).

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