Regulation of Vacuolar pH of Plant Cells

I. Isolation and Properties of Vacuoles Suitable for ^{31}P NMR Studies

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ABSTRACT

For the first time, the ^{31}P nuclear magnetic resonance technique has been used to study the properties of isolated vacuoles of plant cells, namely the vacuolar pH and the inorganic phosphate content. *Catharanthus roseus* cells incubated for 15 hours on a culture medium enriched with 10 millimolar inorganic phosphate accumulated large amounts of inorganic phosphate in their vacuoles. Vacuolar phosphate ions were largely retained in the vacuoles when protoplasts were prepared from the cells and vacuoles isolated from the protoplasts. Vacuolar inorganic phosphate concentrations up to 150 millimolar were routinely obtained. Suspensions prepared with 2 to 3 × 10⁶ vacuoles per milliliter from the enriched *C. roseus* cells have an internal pH value of 5.50 ± 0.06 and a mean *trans*-tonoplast ΔpH of 1.56 ± 0.07. Reliable determinations of vacuolar and external pH could be made by using accumulation times as low as 2 minutes. These conditions are suitable to follow the kinetics of H⁺ exchanges at the tonoplast. The ^{31}P nuclear magnetic resonance technique also offered the possibility of monitoring simultaneously the stability of the *trans*-tonoplast pH and phosphate gradients. Both appeared to be reasonably stable over several hours. The buffering capacity of the vacuolar sap around pH 5.5 has been estimated by several procedures to be 36 ± 2 microequivalents per milliliter per pH unit. The increase of the buffering capacity due to the accumulation of phosphate in the vacuoles is, in large part, compensated by a decrease of the intravacuolar malate content.

The vacuolar membrane is the site of transport systems leading to the accumulation of various solutes. It is widely accepted that in most cases these accumulations are energized by the proton electrochemical gradient ($\Delta\mu H^+$) across the tonoplast (17, 32).

The vectorial electrogenic H⁺-pump ATPase, thought to be responsible for building $\Delta\mu H^+$ across the tonoplast has been the most intensively studied of these systems (30). Other ionic exchanges of potential significance for the regulation of vacuolar pH, namely H⁺-pumping pyrophosphatase (34), H⁺/Na⁺ (4) and H⁺/Ca²⁺ (6, 28) antiports and NO₃⁻/H⁺ symport (5) have been described. Most of these studies have been made using tonoplast vesicles. The pH variations inside the vesicles have been monitored by measuring the uptake of radiolabeled lipophilic bases or by the fluorescence quenching of acridine derivatives. These techniques are of a semiquantitative type,

allowing the measurement of intravesicular acidification or alkalinizations without measurement of the absolute pH values. Only a few studies have been devoted to isolated intact vacuoles (11, 12, 19, 31, 33) and, due to technical limitations, no systematic study has been devoted to the measurement of the internal pH of isolated vacuoles undergoing H⁺ or proton-equivalent exchanges with their surrounding medium. The aim of this study was to use the possibilities offered by ^{31}P NMR to investigate some of the characteristics of the trans-tonoplast pH gradient of isolated intact vacuoles.

Due to its low sensitivity, it has been difficult to use the ^{31}P NMR technique for measuring the proton gradient between isolated cellular organelles and their surrounding medium. This is most likely due to the fact that organelle suspensions usually offer only a limited internal volume which, added to inorganic phosphate pools of moderate size, leads to weak NMR signals. It is relevant to note that, in higher plants, there is only one report concerning the ^{31}P NMR spectrometry of isolated spinach chloroplasts (9). Due to the large size of vacuoles compared to other organelles, to the development of large scale isolation procedures, and to the possibility of loading the vacuolar compartment of various plant cells with phosphate (3, 7, 13, 25, 27), these organelles potentially offer the possibility of overcoming these limitations.

This paper describes the preparation of vacuoles with an internal Pi[1] content high enough to obtain a clear ^{31}P NMR vacuolar signal within reasonable accumulation times. From the intravacuolar and external Pi signals, the vacuolar pH, the external pH, and the proton gradient across the tonoplast of intact vacuoles can be measured. Some of the properties of such a system are described.

MATERIALS AND METHODS

Cell Strain and Culture Conditions

Protoplasts and vacuoles used in this study were isolated from the cell line C20 of *Catharanthus roseus* grown at 27°C under continuous light (2,5 W·m⁻²) in a B5 Gamborg medium containing 1 μM 2,4-D and 60 nM kinetin. Cells at the onset of the stationary phase (7 d-old suspension) were trans-

[1] Abbreviations: Pi, inorganic phosphate; FCCP, p-trifluoromethoxycarbonyl cyanide phenylhydrazone; pHe, external pH; pHᵢ, internal pH; pHᵥ, vacuolar pH; TPP⁺, cation of tetraphenyl phosphonium; Bis-Tris propane,

ferred into a fresh culture medium. Pi-loaded cells were obtained by adding potassium phosphate (10 mM final concentration, pH 5.0) to the culture medium either 60 h (for 4 d-old suspensions) or about 15 h (for 6 d-old suspensions) before the experiments.

Protoplast and Vacuole Isolation

Vacuoles were isolated by disruption of protoplasts by an osmotic shock and the vacuoles were purified by flotation on a single-step gradient of Nycodenz according to Renaudin *et al.* (26). In some experiments (with the 6 d-old suspensions), the EDTA concentration in the lower phase of the gradient used for the isolation of the vacuoles was reduced to 1 mM and a Bis-Tris propane buffer (25 mM) adjusted to pH 7.3 with Mes was used instead of the Hepes-KOH buffer for the harvest of vacuoles. Centrifugation time to harvest the vacuoles was increased to 8 min.

pH Measurements

^{31}P NMR spectra were obtained with a Brucker Aspect 3000 spectrometer operating at 161.932 MHz. Unless otherwise indicated, a 20 mm diameter probe was used. Typically, 10 ml of the vacuole suspension containing 1 to 2 × 10^6 vacuoles·ml^{-1} were used. D$_2$O was added to the sample (2 ml of a 0.55 M sorbitol solution) to lock the field frequency. Chemical shifts were measured relative to the signal of 100 mM methylene diphosphonate (MDP) contained in a capillary tube included in the suspension. The pulse repetition time was 1.2 s and the total acquisition time was usually 10 min. Calibration curves relating pH to Pi chemical shifts were established as described in Guern *et al.* (10). We checked that the presence of sorbitol (0.7 M) had no significant effect on the Pi chemical shift.

The internal pH of individual vacuoles was measured by using 9-aminoacridine fluorimetry according to Manigault *et al.* (20) and Kurkdjian *et al.* (15).

Pi Measurements

Samples of the filtered and washed cell, protoplast and vacuole suspensions were acidified at 0 to 4°C with 6% HClO$_4$ for 20 min. The acidified extracts were then neutralized with K$_2$CO$_3$ and the Pi content of the different samples was determined spectrophotometrically according to Fiske and Subbarow (8). The volumes of the samples were adjusted so that the sorbitol concentration was maintained at a low level in order to avoid interference with the Pi reagent.

RESULTS AND DISCUSSION

Enrichment of Cells with Phosphate and Isolation of Vacuoles from Pi-enriched Cells

As already observed by Brodelius and Vogel (7) and by Ashihara and Usaki (1) *Catharanthus roseus* cells, when incubated on a culture medium enriched with phosphate (10 mM), were able to take up Pi very rapidly during the first 10 h (Fig. 1). The uptake proceeded afterward at a slower rate. The phosphate content reached after 12 to 15 h was about 30 to 50 times that of control cells.
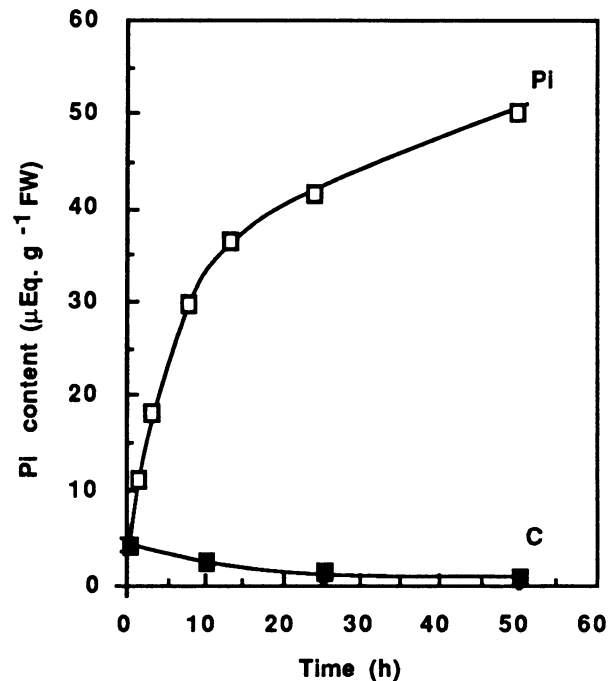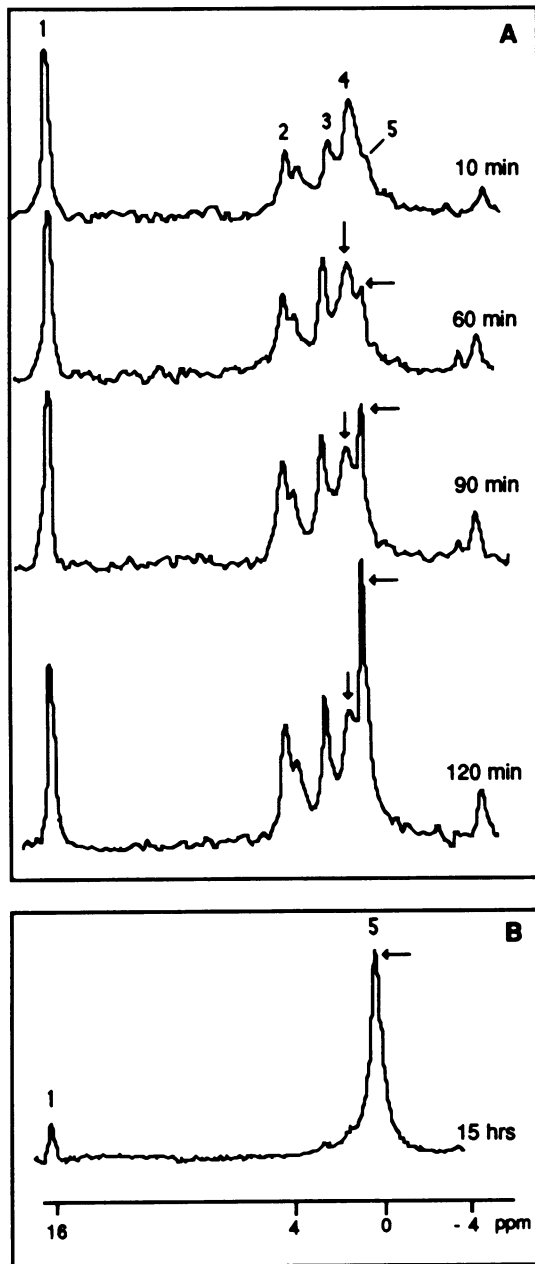

Figure 1. Time course of Pi uptake by *C. roseus* cell suspensions cultivated on a Pi-enriched medium compared to cells grown on their normal culture medium. Cells (about 2 × 10^5 cells·mL^{-1}) were cultivated for 36 h in the standard medium. Potassium phosphate (10 mM final concentration, pH 5.0) was then injected into the flasks (time 0). The inorganic phosphate contents of cells grown either on normal (C) or Pi-enriched medium (+Pi) were determined on aliquots of cell suspensions as described in "Materials and Methods."

Figure 2A shows that the fast uptake of Pi by *C. roseus* cells could be monitored directly in the NMR tube. Feeding Pi-starved cells (at the end of the log phase) with Pi, induced, after a limited increase of the cytoplasmic Pi during the first hour, a strong accumulation of Pi into the vacuole. The inorganic phosphate accumulated after 15 to 60 h was essentially located in the vacuolar compartment; cells displayed a large vacuolar peak with hardly recognizable cytoplasmic peaks (Fig. 2B). When protoplasts and vacuoles were prepared from Pi-enriched cells, the ^{31}P NMR spectra (Fig. 3) revealed that the vacuoles retained a large amount of Pi in agreement with the results obtained by Martinoia *et al.* (22) showing the low permeability of the tonoplast for Pi.

The yields of protoplasts on a cell basis were 78% ± 6 (10 experiments mean ± SE) and 62% ± 2.7 (15 experiments) for normal and Pi-loaded cells, respectively. The yields of purified vacuoles on a protoplast basis, were 29% ± 2.6 (9 experiments) and 29.8% ± 1.6 (27 experiments) for protoplasts from normal and Pi-loaded cells, respectively. This demonstrates that the efficiency of the procedure used to prepare protoplasts and vacuoles was not affected by loading the cells with large amounts of Pi. Vacuoles were heterogeneous in size, with diameters distributed over about one order of magnitude. The mean vacuolar diameter was 25.3 ± 2.8 μm and the mean vacuolar volume calculated from the mean volume of 900 vacuoles distributed in classes according to their diameter was 11.6 ± 1 μL/10^6 vacuoles (9 experiments).

Figure 3C shows a typical ^{31}P NMR spectrum of a vacuolar

Figure 2. Time course of Pi accumulation in *C. roseus* cells. A, Uptake of Pi was followed by perfusing cells (about 4.5 g fresh weight) in the NMR tube with a Mn^{2+} and Pi depleted Gamborg medium buffered to pH 6.5 with 1 mM BTP-Mes. At time 0, 2 mM Pi was injected in the perfusing medium. ³¹P NMR spectra were taken by accumulating 512 scans (1.2 s each), at time intervals during 2 h. Peak assignments were: 1, MDP (capillary tube with a 100 mM solution); 2, glucose-6-phosphate; 3, cytoplasmic Pi; 4, external Pi (↓); 5, vacuolar Pi (←). Spectra were digitalized at 300 dpi by using a Thunderscan scanner coupled to a Macintosh computer. B, ³¹P NMR spectrum of cells incubated on a Pi-enriched medium (10 mM) for 15 h. About 1.6 g fresh weight of the Pi-loaded cells were washed and resuspended on a Mn^{2+} Pi-depleted medium (pH 5.5). Oxygenation of the suspension was obtained by bubbling air, as described in (10). The scale is about one-fourth the one of A.
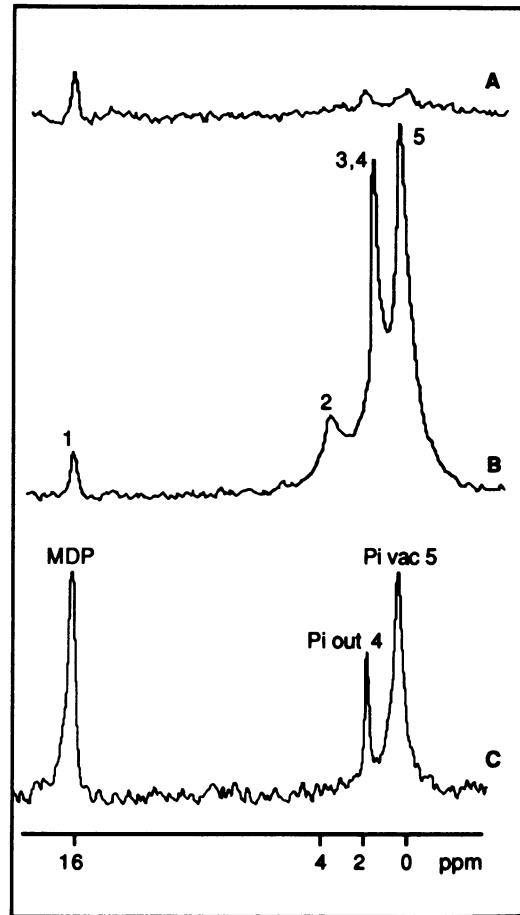
Figure 3. Typical spectra of protoplast populations isolated from control (A) and Pi-loaded (B) cells and of a vacuole population isolated from Pi-loaded cells (C). In each case, the volume of the suspension was 12 ml. A, 6 × 10⁷ protoplasts prepared from normal cells; B, 5 × 10⁷ protoplasts from Pi-loaded cells; C, 1.6 × 10⁷ vacuoles isolated from Pi-enriched cells. Peak assignments were the same as in Fig. 2. External and vacuolar pH values determined by the position of the respective Pi resonances were 7.01 and 5.28.

preparation obtained from Pi-enriched cells. Two Pi peaks were revealed corresponding to the vacuolar acidic pool and to the extravacuolar phosphate. The Pi contents of the vacuolar preparations were estimated from the cumulated areas of the intravacuolar and external Pi peaks relative to the area of the peaks of calibrated Pi solutions and compared to the colorimetric measurements of the Pi contents of the vacuolar preparations. A good agreement was observed between the results of these two determinations, indicating that, in contrast to the results obtained by Brodelius and Vogel (7), prolonged culture in the presence of elevated Pi did not result in the accumulation of Pi as NMR invisible compounds such as metal complexes of phosphate with a reduced mobility. Furthermore, determination of the relative area of the peaks under the resonance of the external and vacuolar Pi revealed that about 60 to 80% of the Pi present in the preparation was located in the vacuoles.

The mean Pi contents of cells, protoplasts, and isolated vacuoles were compared (Table I) for the two procedures used to load the cells with inorganic phosphate. Pi content of the

Table I. *Inorganic Phosphate Contents of Cells, Protoplasts, and Vacuoles Isolated From C. Roseus Cells Grown on a Pi-Enriched Medium*

C. roseus cells were grown on a Pi-enriched medium (10 mM) either for 15 h (6-d-old suspension, A) or 60 h (4-d-old suspension, B). Numbers represent the mean Pi content ± SE. Values in parentheses represent the number of independent experiments. Total Pi contents present in the vacuolar preparations were determined by colorimetric measurements. The distribution of Pi between the vacuoles and the external medium was calculated from the relative area of the peaks corresponding to vacuolar and external Pi resonances on the ^{31}P NMR spectra.

	A		B	
	μmol Pi/10^6 units	mM	μmol Pi/10^6 units	mM
Cells	3.0 ± 0.26 (8)	57 ± 7.6	3.3 ± 0.3 (4)	49 ± 4
Protoplasts	3.0 ± 0.16 (19)	230 ± 12	2.6 ± 0.4 (6)	194 ± 26
Vacuoles	2.0 ± 0.12 (19)	156 ± 9	1.5 ± 0.16 (7)	118 ± 12
External		1.7 ± 0.12 (19)		1.1 ± 0.16 (7)

cells cultivated either 60 or 15 h on a Pi-enriched medium were similar and much higher (about 50 μmol·g^{-1} fresh weight) than the content (0.7 ± 0.12 μmol g^{-1} fresh weight) of cells grown at the normal Pi concentration of the culture medium (1.1 mM). We observed previously (26) that loss of Pi by isolated protoplasts (less than 25% of the cell content) was lower than the one observed for other anions and that phosphate ions appeared strongly concentrated in the vacuolar compartment during plasmolysis of the cells. The mean Pi content of the vacuoles was about 1.5 to 2 μmol/10^6 vacuoles, *i.e.* a concentration of about 150 mM, 100-fold higher than the external medium, demonstrating a large phosphate gradient between the vacuoles and their suspension medium.

Vacuolar pH Measurement on Isolated Vacuoles

Figure 3C reveals that the chemical shifts corresponding to the vacuolar and external Pi peaks were clearly distinct (about 0.04 and 1.61 ppm, respectively) demonstrating that a large proton gradient was maintained between the vacuole interior and the suspension medium (pH 5.28 and 7.01, respectively). The heterogeneity of the vacuolar populations in terms of the pH value of individual vacuoles was revealed by the broadness of the vacuolar Pi peak compared to that of external Pi, in good agreement with the distribution of vacuolar pH values over a 1.5 pH unit range we observed in cell, protoplast, and vacuole populations by using 9-aminoacridine microfluorimetry (15, 20).

Results of Table II show that the pH values of vacuoles of Pi-enriched cells, either *in situ* or isolated were very similar; the values found for the youngest cell suspensions (B) being slightly more acidic. Thus, the pH of the vacuolar content was not significantly modified during the isolation. Furthermore, the vacuolar pH values of the 6-d-old suspensions appeared very close to those found for *C. roseus* cells grown at normal Pi as demonstrated by the vacuolar pH values of 5.7 to 5.8 measured on normal cells by the ^{31}P NMR technique (data not shown). This last result was reinforced by measuring the internal pH of vacuoles isolated from normal and Pi-loaded cells by 9-aminoacridine microfluorimetry (Fig. 4). The mean vacuolar pH values were 5.58 ± 0.03 (8 independent experiments) and 5.58 ± 0.04 (5 independent experiments), respectively. Furthermore, the distribution of pH values in the vacuolar population was not modified by the

Table II. *Vacuolar pH Values of Cells and Isolated Vacuoles of C. roseus Cells Grown on a Pi-Enriched Medium*

C. roseus cells were grown on a Pi-enriched medium (10 mM) either for 15 h (A) or 60 h (B). pH$_v$ values were determined from the chemical shifts corresponding to the vacuolar and external Pi peaks of the ^{31}P NMR spectra. Values in parentheses represent the number of independent experiments. Mean ΔpH were calculated as the mean of difference between the vacuolar pH and that of the external medium for each independent experiment concerning isolated vacuoles.

	Vacuolar pH	
	A	B
Vacuoles *in situ*	5.60 ± 0.1 (2)	5.39 ± 0.17 (4)
Isolated vacuoles	5.50 ± 0.06 (19)	5.30 ± 0.07 (8)
Transtonoplast ΔpH	1.56 ± 0.07 (19)	1.64 ± 0.06 (8)

accumulation of Pi (Fig. 4). The mean *trans*-tonoplast ΔpH (about 1.6 pH units) measured for vacuoles isolated from Pi-enriched cells was similar to the one found for vacuoles isolated from cells grown in normal Pi (2).

These results demonstrate that, despite the large accumulation of Pi ions inside the vacuoles, the loading procedure did not induce significant modifications of the vacuolar pH, in agreement with the results obtained by Brodelius and Vogel (7) and Benhayyim and Navon (3).

Spectra, such as the one illustrated by Figure 3C, were routinely obtained by accumulating scans over 10 min, but reliable determinations of external and vacuolar pH could be made by using accumulation times as low as 2 min provided that the density of the vacuolar population is high enough (2–3 × 10^6 vacuoles·mL^{-1}). The surface to volume ratio of intact vacuoles being much lower than that of tonoplast vesicles, successive measurements every 5 to 10 min are frequent enough to follow the kinetics of most of the H$^+$ exchanges at the tonoplast.

Buffering Capacity of the Vacuolar Sap

The buffering capacity of the vacuolar sap is an important parameter to determine in order to correlate vacuolar pH modifications to the intensity of exchange of protons or proton-equivalents. Only a few values are available from the literature (16) and most of them have been obtained by titrating cell saps or tissue juices, assuming that the expressed saps were mostly representative of the vacuolar contents.
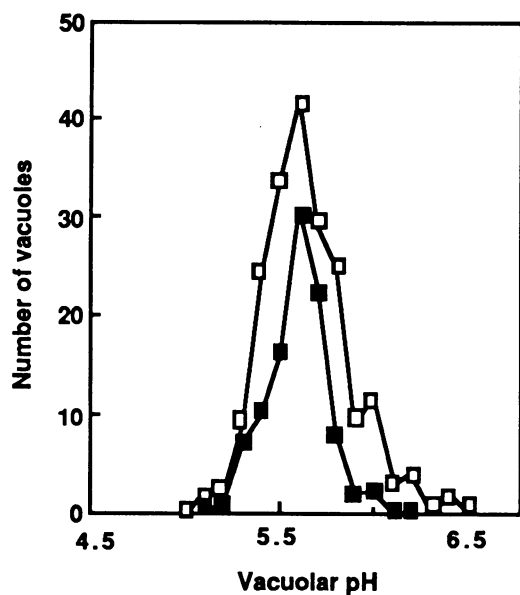
Figure 4. Distribution of vacuolar pH values in populations of vacuoles isolated from normal (□) and Pi-loaded cells (■). Vacuoles were incubated in the presence of 5 μM 9-aminoacridine (9AA). The vacuolar pH values were calculated from the intravacuolar accumulation ratio of 9AA determined by microfluorimetric measurement of the external and intravacuolar concentrations of 9AA as described previously (15, 20). The pH values corresponding to individual vacuoles were grouped by classes and the number of vacuoles corresponding to each class plotted as ordinates.

We used several procedures to estimate the buffering capacity of the vacuolar contents, namely loading vacuoles with weak lipophilic bases and measuring the corresponding alkalinization either by ^{31}P NMR spectrometry or 9-aminoacridine fluorimetry or calculating the buffering power associated with the vacuolar malate, citrate, and phosphate (Table III). The major uncertainties in using the weak-base loading procedure concerned the estimation of the cumulative vacuolar volume and the assumption that nicotine and benzylamine only diffused as their neutral form across the tonoplast (14). Direct calculation of the buffering capacity of a solution equivalent in its phosphate, malate, and citrate contents suffered from the uncertainties of the real values of the respective acid dissociation constants in the vacuolar environment. Nevertheless, a reasonable agreement was observed between the results obtained using the different procedures, with a mean buffering power of 36 ± 2 $\mu EqH^+mL^{-1} \cdot pH$ unit^{-1} (Table III). Interestingly, vacuoles from Pi-loaded cells have about the same buffering capacity at pH around 5.5 as vacuoles from normal cells, despite a 10-fold enrichment of their phosphate content. This was due to a reduced content of malate, the major buffering species in this pH region. However, around pH 6.5, Pi-enriched vacuoles have a buffering capacity about 5-fold higher than vacuoles from normal cells (results not shown).

Stability and Dissipation of the *trans*-Tonoplast Proton Gradient of Isolated Vacuoles

Comparison of the above results to those described in the literature as to the *trans*-tonoplast pH gradient of isolated
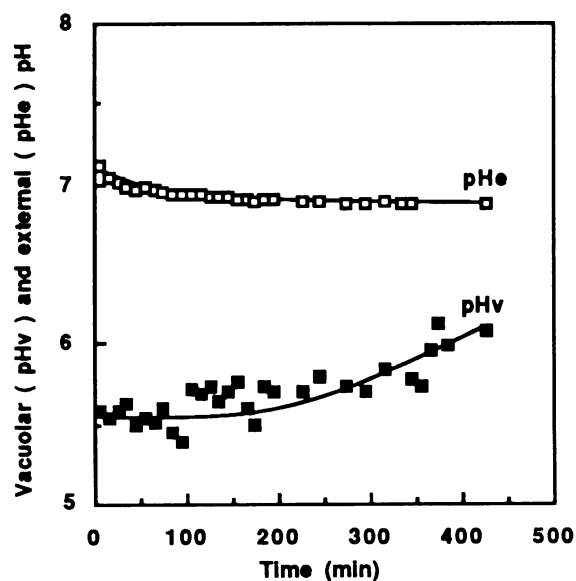
Figure 5. Time course of the evolution of the pH gradient between vacuoles and their suspension medium during aging of a vacuolar preparation. The vacuolar suspension was obtained from cells cultivated for about 15 h on a Pi-enriched medium. The number of vacuoles in the NMR tube was 3×10^7 at time 0 and 1.8×10^7 after 450 min. The vacuolar pH (pHv) and external pH (pHe) were determined from the corresponding Pi peaks of successive 10 min spectra.
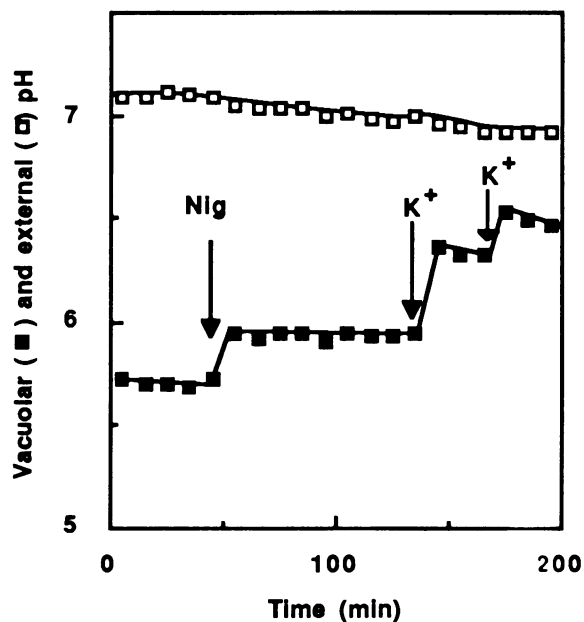
Figure 6. Effects of nigericin and K$^+$ on the pH gradient between vacuoles and their suspension medium. Vacuoles (3×10^7 vacuoles in 12 mL suspension) were isolated from cells enriched with Pi for 15 h. At the time indicated by arrow, nigericin (10 μM) or K$^+$ (20 mM) were added to the vacuolar suspension.

vacuoles reveals large variations (Table IV) from 0.2 pH unit (24) to a maximum of 2.7 pH unit (35). The simplest interpretation of such variations would be to admit that they were primarily related to the different biological systems investigated. In fact, we already demonstrated that (a) the procedure used to isolate vacuoles (specially using saline osmoticum)

Table III. *Estimation of the Buffering Capacity of the Internal Solution of C. roseus Vacuoles*

Pi-loaded vacuoles were incubated with 2 mM nicotine or benzylamine and the intravacuolar alkalinization (ΔpH) was measured at equilibrium either by ^{31}P NMR spectrometry (NMR) or by 9-aminoacridine fluorimetry (9AA). From the intra- and extravacuolar pH values at equilibrium and from the relative cumulative volume of the vacuoles (usually 2%), the internal concentration of base (Ci) was estimated according to the relation $Ci = Ce \cdot 10^{(pHe-pHi)}$ and the buffering power was calculated as $\beta = Ci/\Delta pH$. The second procedure used was to calculate from a simple computer program the buffering capacity, around pH 5.5, of a solution composed of the major buffering species of the vacuolar sap whose concentrations have been measured (pKa phosphate: 2.16, 6.8, 12.3; pKa malate: 3.4, 5.11; pKa citrate: 3.08, 4.74, 6.4).

Experiment	ΔpH	Technique	Estimated Ci	β
			mM	$\mu Eq \cdot ml^{-1} \cdot pH\ unit^{-1}$
Weak base-loading procedure				
Nicotine				
A	0.47	NMR	14.1	30
B	0.60	NMR	19.2	32
C	0.47	9AA	19.0	38
Benzylamine	0.38	9AA	7.2	37

	Phosphate	Malate	Citrate	β
	mM	mM	mM	$\mu Eq \cdot ml^{-1} \cdot pH\ unit^{-1}$
Calculations from the vacuolar concentrations of the major buffering species				
Normal vacuoles	15 ± 2 (6)*	71 ± 4 (6)	9 ± 1.2 (6)*	42
Pi-loaded vacuoles	156 ± 9 (19)	29 ± 7.6 (6)	20 ± 4 (6)	43

* Data from Renaudin et al. (26).

Table IV. *Transtonoplast pH Gradient between Vacuoles Isolated from Different Biological Systems and Their Suspension Medium*

Biological System	ΔpH	pH$_e$	Technique of pH Measurement	References
Acer pseudoplatanus cells	1.44	7.0	9-Aminoacridine	(16)
	1.43	7.2	H$^+$-Microelectrodes	(16)
Beet roots	0.6–1.2	7.6	Methylamine	(23)
Beet roots	0.3	7.6	Valinomycin equilibrium	(12)
Castor bean endosperm	0.2	6.0	Methylamine	(24)
Catharanthus roseus cells	1.65	7.1	^{31}P-NMR	(2)
	1.50	7.4	9-Aminoacridine	(2)
	0.73ᵃ	7.1	^{31}P-NMR	(2)
	0.85ᵃ	7.4	9-Aminoacridine	(2)
Kalanchoë daigremontiana leaves	2.0	7.4	H$^+$-Microelectrodes	(11)
Sugarcane cells	1.41	6.5	Quinacrine	(31)
Tulipa petals	1.0	8.0	Endogenous pigments	(18)
Valerianella locusta leaves	2.2–2.7	7.6	9-Aminoacridine	(35)

ᵃ Vacuoles prepared in a NaCl osmoticum instead of sorbitol.

can drastically affect the vacuolar pH (2) and (b) the acidity of the vacuoles can be overestimated according to the measurement technique used (16). Furthermore, several reports (18, 21, 29) described large alkalinization of vacuoles during their isolation or their incubation as a suspension of purified isolated vacuoles. This called for a study of the stability of the proton and Pi gradients we measured between freshly isolated C. roseus vacuoles and their suspension medium.

The evolution of the *trans*-tonoplast ΔpH was monitored for several hours (Fig. 5), revealing a rather good stability with a decrease of less than 0.3 pH unit during the first 3 h. More than 50% of the vacuoles were still present in the NMR tube after 450 min. A progressive flotation of the vacuoles which slowly accumulated outside the volume read by the receiving coil of the spectrometer, induced a marked drop of the vacuolar peak without a correlative increase of the external peak. This flotation was strongly reduced when vacuoles were collected on a ficoll instead of a Nycodenz gradient (results not shown).

Unlike the stability of ΔpH under normal conditions, the proton gradient could be dissipated, at least partly, by a variety of procedures. First, as described above, the uptake and vacuolar accumulation of 2 mM nicotine produced a shift of the vacuolar pH toward more alkaline values (+0.47 pH unit) whereas the external medium was acidified (−0.18 pH unit). FCCP (5 μM) alone or in the presence of the diffusible cation

TPP$^+$ (3 mM) induced significant alkalinization of the vacuolar interior (0.3 and 0.8 pH unit, respectively). Figure 6 shows the effect of nigericin (10 μM) on the ΔpH of isolated vacuoles. Nigericin which catalyzes an electroneutral H$^+$/K$^+$ exchange had only a limited effect on the pH of vacuoles which exhibit a strong outwardly directed K$^+$ gradient. Indeed, increasing the external K$^+$ concentration stimulated further the H$^+$/K$^+$ exchange, giving a threefold reduction of the ΔpH with 40 mM K$^+$ in the external medium. Interestingly, the partial abolition of the ΔpH across the tonoplast was accompanied by a strong reduction of the vacuolar Pi peak (about 2.5-fold), much higher than the decrease expected from the breakage of the vacuoles during the gentle homogenization of the added effectors. This suggested that the inorganic phosphate gradient between the vacuoles and their suspension medium was sensitive to the size of the ΔpH. This hypothesis was reinforced by the observation that decreasing the external pH to 6.3 induced a large decrease of the vacuolar Pi peak which could not be accounted for by vacuolar bursting (data not shown).

CONCLUSION

The results reported in this paper demonstrate that it is possible to prepare vacuoles from Pi-loaded *Catharanthus roseus* cells which are suitable for measuring *trans*-tonoplast proton and phosphate gradients by using ^{31}P NMR. A large scale isolation procedure allowed us to monitor routinely 2 to 3 × 10^7 vacuoles in 12 mL suspension. Due to the large intravacuolar concentration of Pi (120–160 mM), the vacuolar Pi signal was large enough to allow the determination of the vacuolar pH and Pi content during accumulation times as short as 2 min, with a reasonable signal to noise ratio, opening the possibility of undertaking kinetic studies. The pH of isolated vacuoles was close to that measured for vacuoles *in situ*. Furthermore, the accumulation of Pi in the vacuoles did not modify their pH or their buffering power around pH 5.5. Thus, vacuoles prepared according to the procedure described represent a good model to study *trans*-tonoplast exchanges in order to complement the information obtained using tonoplast vesicles.

One major point of interest was that isolated vacuoles retained a large and stable ΔpH across the tonoplast for a rather long period of time, with only a slow decrease of the vacuolar Pi. The second paper of this series will illustrate the application of the technique described here to approach some of the major ionic exchange systems at the tonoplast and give a quantitative description of their involvement in the regulation of the internal pH of isolated, intact vacuoles.

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