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Cytoplasmic pH Regulation in *Acer pseudoplatanus* Cells

I. A ^{31}P NMR DESCRIPTION OF ACID-LOAD EFFECTS

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ABSTRACT

Modifications of cytoplasmic pH in *Acer pseudoplatanus* L. cells cultivated in suspension have been induced by acid-loads and studied by using ^{31}P nuclear magnetic resonance spectroscopy. The initial drop of cytoplasmic pH, observed in the first minutes of exposure to weak lipophilic acids, was followed by a slow recovery to reach a plateau phase with a pH value lower than the initial one. Conversely, removal of the acid led to a sharp increase of cytoplasmic pH with in most cases an overshoot toward more alkaline values than the initial one and a subsequent decrease to more acidic values. This shows that *A. pseudoplatanus* cells powerfully regulate their cytoplasmic pH both on the acid side of their normal pH, during the acid-load, and on the alkaline side, after removal of acid. Similar results were obtained with different types of acid-loads, *i.e.* treatments with propionic or benzoic acid or bubbling with CO_2-enriched air. This indicates that the occurrence of pH regulation does not depend upon the method used to acid-load the cells. The time courses of cytoplasmic pH observed for *A. pseudoplatanus* and also *Catharanthus roseus* cells are similar to those recorded for animal cells but different from those described for other plant materials for which no recovery phase was observed. This can be explained by different balances between the initial rate of proton influx brought in by the acids, and the capacity of proton consumption by the regulatory mechanisms. The existence of the recovery phase offers a unique possibility to study the regulation of the cytoplasmic pH of plant cells, as it has been done in animal systems.

The study of transient changes in pH_i[2] has brought considerable progress in the understanding of pH_i regulation in animal cells (27). A classical approach has been to load the cells with weak organic acids. A number of workers have observed a fall in pH_i after exposing animal cells to propionic, butyric, or benzoic acid (see Ref. 27 for a review) and studied the regulations involved in the recovery from cytoplasmic acidification.

This approach has been less used with plant materials due to the only recent adaptation to plant cells of the main techniques for pH_i measurement. pH_c modifications under the influence of acid-loads have been directly studied using a variety of techniques. The [^{14}C]DMO uptake technique was used to show that acid-loads reduce pH_c and modify CAM in *Kalanchoë tubiflora* (17). Specific H^+-microelectrodes allowed the continuous monitoring of the evolution of the pH_c during the penetration of weak acids in *Neurospora* cells (28), maize coleoptiles (7), and root hair cells from *Sinapis alba* (2). During the last few years, the ^{31}P NMR technique has been adapted to plant material, allowing simultaneous determinations of vacuolar and cytoplasmic pH values (24). The hypoxic acidification of the cytoplasm of maize root tips under hypoxia has been very elegantly studied by Roberts *et al.* (22, 23). These authors used acetic acid (22) and CO_2 (23) as acidifying agents and demonstrated large cytoplasmic acidifications.

The effects of acid-loads have been also indirectly studied (*i.e.* without measuring the pH_i by their effect on the electrogenic proton pump at the plasmalemma and the subsequent modifications of ionic exchanges. Weak acids like butyric acid induce a hyperpolarization associated with a K^+ influx in maize roots (18). Butyric and benzoic acids hyperpolarize oat coleoptiles (1). These results are in good agreement with more recent ones associating pH and potential measurements with microelectrodes in maize (7) and *S. alba* roots (2) which demonstrate unambiguously that the hyperpolarization is a consequence of cytoplasmic acidification.

Acid-loading of plant cells now appears to be as a useful tool to stimulate the plasmalemma proton pump (1, 7, 18, 28) and to study the relationship between pH_c and growth (7).

We have shown with *Acer pseudoplatanus* cells cultivated in liquid medium that the effects of acid-loads are more complex than those generally described for plant systems. Acid-loading induces a strong initial acidification of the cytoplasm after which the pH_c increases to stabilize, after 0.5 h, at a value lower than the initial one. The pH_c recovery indicates the operation of powerful pH regulatory mechanisms in plant cells.

The presentation of the results of this study has been divided into two parts. The present paper concentrates on the description of the various effects of different acid-loads on cytoplasmic and vacuolar pH monitored with the ^{31}P NMR technique adapted to our cell cultures. The second paper is devoted to the study of the mechanisms involved in the pH_c regulation during acid-load.

MATERIALS AND METHODS

Cell Culture Conditions for ^{31}P NMR Experiments. *A. pseudoplatanus* L. cells were cultivated as described previously (16) in the medium modified by Bligny (3). They were maintained in exponential growth by subculturing every 7 d. About 10 d before the NMR experiments, cells were filtered on a 300 μm nylon sieve to remove, if necessary, the biggest cell clusters. Cells and

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[2] Abbreviations: pH_i, intracellular pH; pH_c, cytoplasmic pH; DMO, 5,5-dimethyl-2,4-oxazolidine-dione; G6P, glucose-6-phosphate· ^{31}P NMR, nuclear magnetic resonance of ^{31}P.

small clusters were collected after sedimentation, transferred in Mn^{2+}-depleted Bligny medium and subcultured every 3 d in this medium. *Catharanthus roseus* cells were cultivated in a B5 Gamborg medium supplemented with 1 μM 2,4-D and 60 nM kinetin. Before NMR measurements, about 1.6 g fresh weight of cells were collected by filtration on a Whatman glass fiber filter (GF/A). Cells were washed five times with 10 ml of Mn^{2+} and Pi-depleted culture media buffered to pH 6.5 with 50 mM Mops-NaOH or to pH 5.0 with 50 mM Mes-NaOH.

NMR Experimental Conditions and pH Calibration Curves. Basically, the ^{31}P NMR technique was adapted to the study of our cell suspensions as described previously (19). Spectra were recorded without spinning at 161.93 MHz with a WM 400 Brucker spectrometer without relaxation delay and with a 25 μs pulse width. Typically, 8K data points were collected over a width of 10,000 Hz. Methylene diphosphonic acid (50 mM Tris buffer solution [pH 8.9]) located in a capillary tube was used as internal reference (chemical shift: 16.2 ppm). Chemical shifts were expressed with reference to H_3PO_4 in 15% $HClO_4$ (v/v).

Cell suspensions were bubbled with air (about 15–20 ml per min) with three capillary tubes abutting to the bottom of the NMR tube and connected to a Gilson HP4 peristaltic pump. Gases were mixed in an Erlenmeyer flask containing a magnetic stirrer. Gas flows were controlled with flow meters and the gas mixture was collected from the Erlenmeyer flask by the pump. The CO_2 concentration of the mixtures was measured with a Multi-Gas-Detector 21-31 Dräger.

Calibration curves relating pH to G6P and Pi chemical shifts were established with a solution containing 100 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1mM Pi, and 1 mM G6P, according to Roberts *et al.* (25). Successive spectra were recorded with this solution adjusted to various pH values.

Propionic Acid Uptake. Cells were incubated in their culture medium in a beaker, shaken at 180 rpm (26°C) at a population density close to the one used in the NMR tube (about 1.8 g fresh weight for 10 ml of cell suspension). External pH was buffered with Mops-NaOH (50 mM; pH 6.50). Radioactive [2-^{14}C]propionic acid (37 kBq·μmol^{-1}, 50 mM final concentration in the suspension culture), adjusted to pH 6.50 with NaOH, was added. At various times, an aliquot of the suspension was rapidly filtered and washed for a few seconds with 3 ml of the buffered culture medium containing unlabeled propionic acid, on Whatman glass fiber filter (GF/A). The radioactivity of cells was measured by liquid scintillation counting using an Intertechnique SL 4000 spectrometer.

RESULTS

Propionic Acid Uptake and Cytoplasmic Acidification. Cells (about 1.6 g fresh weight) were suspended in a total volume of 8 ml as described in "Materials and Methods" and spectra accumulating 256 scans of 1.2 s each were collected. At zero time, propionic acid (50 mM final concentration, pH 6.5) was injected in the suspension. Figure 1A shows the evolution of the pH_c of *A. pseudoplatanus* cells during propionic acid penetration into the cells.

The uptake of propionic acid was measured in similar conditions of extracellular pH, propionic acid concentration, and cell population density as described in "Materials and Methods." Figure 1B shows the time course of propionic acid penetration into the cells.

pH_c determined from the G6P chemical shifts (Fig. 1A) rapidly and dramatically decreased after acid injection (0.7 pH unit in less than 5 min). This is in good agreement with a rapid influx of the neutral form of propionic acid and its dissociation within the cells according to the pH_c. One striking fact was that, soon after the initial acidification, pH_c gradually increased (0.4 pH unit during 0.5 h) to a steady value (about pH 7.15) in spite of the continued penetration of propionic acid into the cells (Fig. 1B). This indicated the operation of powerful pH regulating system.

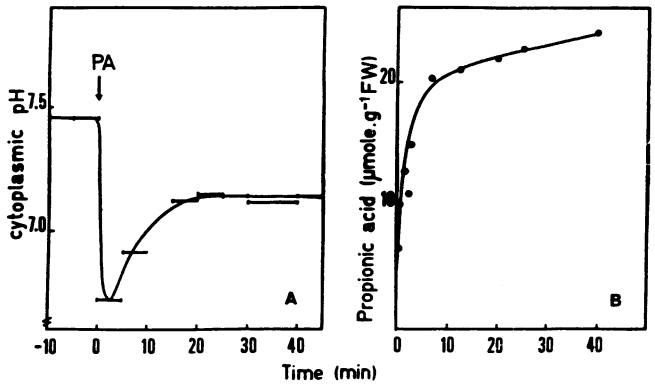

FIG. 1. Propionic acid (PA) penetration into *A. pseudoplatanus* cells and its effects on the cytoplasmic pH. pH_c values (A) were determined from the G6P chemical shifts. Each pH value is plotted as an horizontal bar. The length of a short bar is the duration (about 5 min) during which scans were accumulated (256 scans, 1.2 s each). The large bars represent pH values determined from the cumulation of scans corresponding to several elementary spectra. The cell suspension was prepared and bubbled with air as described in "Materials and Methods." Propionic acid (50 mM final concentration; adjusted to pH 6.5 with NaOH) was added at zero time (PA). Propionic acid uptake (B) was measured separately as described in "Materials and Methods." FW, fresh weight.

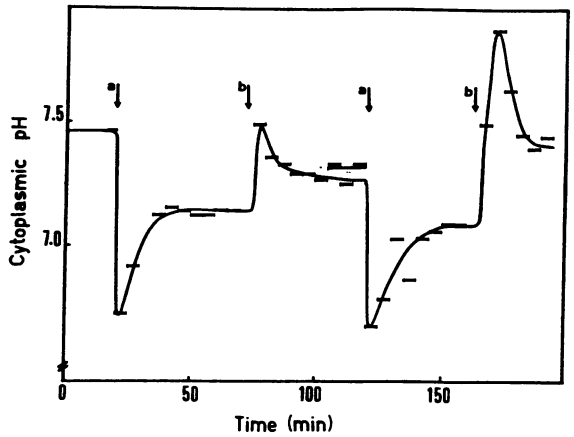

FIG. 2. Effect of propionic acid on the pH_c of *A. pseudoplatanus* cells. (a) injection of 50 mM propionic acid (adjusted to pH 6.5 with NaOH); (b) removal of propionic acid (cells were rapidly filtered and washed with acid free medium). Other conditions were the same as in Figure 1.

Reversibility of the Acid-Load Induced by Propionic Acid. When the acid-load was stopped by washing the cells (Fig. 2), the pH_c rapidly increased to a pH value higher than or equal to the initial value. The overshoot is a well known phenomenon in animal cells (4) for which the classical interpretation is that, since protons have been neutralized during the recovery phase, pH_c upon acid removal is increased to a value higher than the initial one. Afterward, pH_c gradually decreased to a steady value (about pH 7.25) indicating the operation of a regulating system competing with the overalkalinization. The new steady state level, lower than the initial one (pH 7.5), was more unexpected. The same behavior was observed when measuring pH_c from the Pi chemical shifts (results not shown).

Comparison of Different Acid-Loads. When using other effectors like benzoic acid (Fig. 3) to acid-load *A. pseudoplatanus* cells, the time course of pH_c was qualitatively similar to the one described for propionic acid-load *i.e.* a rapid and large decrease

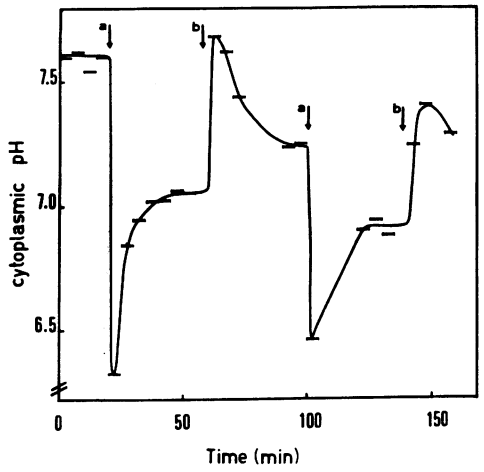

FIG. 3. Effect of benzoic acid on pH_c of *A. pseudoplatanus* cells. pH_c values were determined from Pi chemical shift. (a) benzoic acid injection (50 mM final concentration; adjusted to pH 6.5 with NaOH); (b) benzoic acid removal. Other conditions were the same as in Figure 1.

after acid injection followed by a progressive recovery and the opposite behavior after removing the acidifying agent.

When *A. pseudoplatanus* cells were acid-loaded by bubbling CO_2 enriched air (25%) modifications of the Pi and G6P chemical shifts indicated a pH_c decrease (data not shown). This acidification showed the same pattern as those described for propionic and benzoic acid with a recovery following the initial drop of pH_c and a large alkalinization after CO_2 removal. Such results are in good agreement with those known for some time for animal cells (8) or demonstrated recently for maize roots submitted to hypoxia (23).

The similar patterns of cytoplasmic acidification for three different acidifying agents indicate that the reaction of plant cells to the acidification of their cytoplasm is not specific to the acid applied. This also renders unlikely significant effects of the metabolism of the acids in the regulation of pH_c.

As a matter of fact, no $^{14}CO_2$ efflux from the cell suspensions incubated with [2-^{14}C]propionate was observed for a period of 40 min following the acid-load, strongly suggesting that propionate was not extensively metabolized and its carbon atoms recycled to CO_2.

At first sight, the concentration of acid used to load the cells (50 mM) appears rather high compared to those used by various authors (Table I). In fact, according to the basic principle of the acid-load procedure (*i.e.* only the protonated form of the acid diffuses through the membranes), the intensity of the cytoplasmic acidification must be dependent on the external concentration of the protonated acid. Figure 4 shows that the behavior of the cells facing acid-load with propionic acid at different concentrations and external pH values is qualitatively the same, with an intensity of cytoplasmic acidification related to the respective concentrations of the protonated acid (1.2 mM, 1.9 mM, and 6.4 mM for A, B, and C, respectively). This explains why extensive acidification can be obtained with millimolar concentrations of lipophilic acids (acid + conjugated base) provided that external pH is low.

No detailed study of the influence of external pH on the initial rate of uptake of propionic acid was made in order to check that the permeability of the anionic form was low compared to that of the neutral form. Precise measurements of the initial rates of uptake are rendered difficult by the high rate of uptake and the rapid acidification of the cytoplasm which in turns modifies the net entry of acid. However, with this limitation in mind, rough calculations from the experiments depicted in Figure 4 show that the extent of the initial cytoplasmic acidification is related to the external concentration of neutral propionic acid. Furthermore, simple measurements of [^{14}C]propionic acid uptake (0.5 mM) at two pH values reveal a 7 to 8 times stimulation of the uptake at pH 5.2 compared to pH 6.5 (respectively 2.3 and 0.3 $\mu mol \cdot g^{-1}$ fresh weight absorbed after 2 min), in rather good agreement with what would be expected from a preferential entry of the neutral form.

Comparison of Different Cell Strains. To check that the time course of pH_c changes in plant cells facing a strong acid-load was not specific of a particular cell strain, we studied the response of *C. roseus* cells to a treatment with propionic acid. Results depicted in Figure 5 show that these cells exhibit a cytoplasmic pH evolution quite similar to that observed for *A. pseudoplatanus* cells, *i.e.* a typical recovery phase following the initial acidification.

Vacuolar pH Modifications. Broad vacuolar Pi peaks due to a large vacuolar pH variability in cell populations (14, 15) and low sensitivity of pH measurement due to the shape of the Pi calibration curve in the vacuolar pH range 5.0 to 6.0 are responsible for a rather low precision for vacuolar pH determination. In spite of these limitations, simultaneous variations of cytoplasmic and vacuolar pH in acid-loaded cells were monitored.

As shown by Figure 6, vacuolar pH is rapidly decreased by about 0.3 pH unit after propionic acid injection, but without any

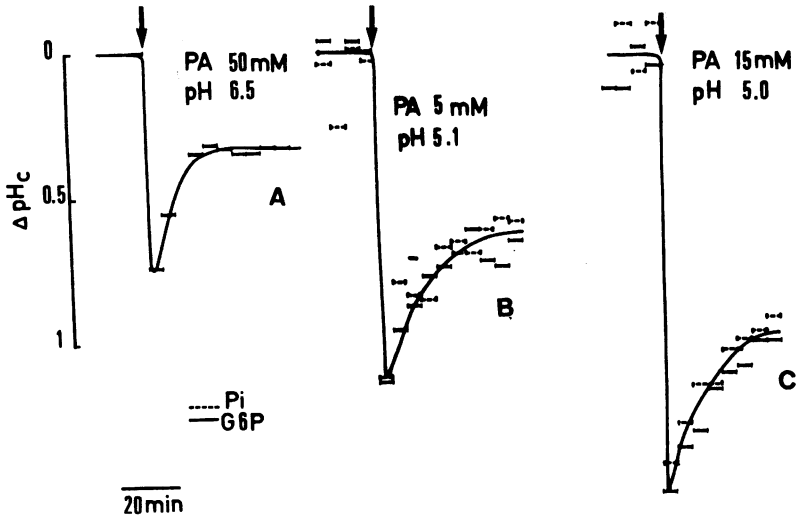

FIG. 4. Cytoplasmic acidification of *A. pseudoplatanus* cells as a function of the extracellular concentration of propionic acid (PA) and pH of the suspension medium. Cells were treated as described for Figure 1 and 2. pH_c variations were followed from the G6P (——) and Pi (----) chemical shifts.

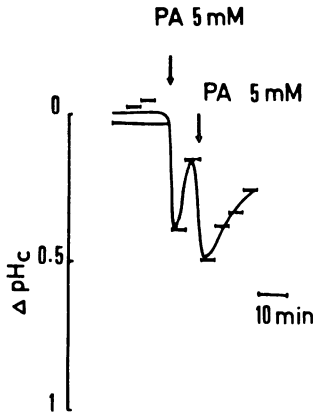

FIG. 5. Acid-loading of *C. roseus* cells. pH_c values were determined from the G6P chemical shifts. *C. roseus* cells were grown and processed as described in "Materials and Methods." The cells were washed with a Mn^{2+} and Pi depleted culture medium buffered to pH 5.0 with 50 mM Mes-NaOH, filtered and resuspended with 6 ml of washing medium in a 15 mm diameter NMR tube. About 2 ml of D_2O were added to lock the field frequency. The initial pH_c was 7.51. Cells were treated first with 5 mM propionic acid (PA) adjusted to pH 5.0 with NaOH and 10 min later they received a second injection of 5 mM acid (pH 5.0).

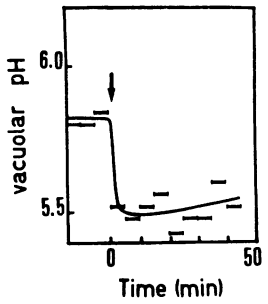

FIG. 6. Effect of propionic acid on the vacuolar pH of *A. pseudoplatanus* cells. Vacuolar pH values were determined from the Pi chemical shifts. Cells were treated at time zero with 50 mM propionic acid (pH 6.5).

important recovery. When the rather crude technique of cell sap extraction and pH measurement (13) was used to estimate vacuolar pH, a similar decrease (about 0.5 pH unit) was observed.

DISCUSSION

Measurement of Cytoplasmic Acidification. In spite of the rather low time resolution of our measurements, the time courses for cytoplasmic and vacuolar pH changes in acid-load cells could be followed with rather good reproducibility. However, as in most cases pH values were estimated from spectra accumulating 256 scans of 1.2 s each in order to ensure a proper signal to noise ratio, the time resolution of the kinetics was only 5 min. Figures 1 to 5 show that this time resolution was too low to give a suitable qualitative and quantitative description of the initial acidification during the very first minutes of acid-loading, but good enough to follow pH_c recovery. Experiments in which the time resolution of the technique was improved by reducing the number of scans cumulated for each spectrum gave basically the same results (Fig. 7), showing that the maximum cytoplasmic acidification was attained within the first 2 min of exposure to the external acid.

A good parallelism was observed between the time courses of cytoplasmic pH values monitored either from the Pi peak or from the G6P peak (Fig. 4). Such behavior illustrates an advantage of the ^{31}P NMR technique which allows a double determination of pH_c. However, in spite of their general similarity, some

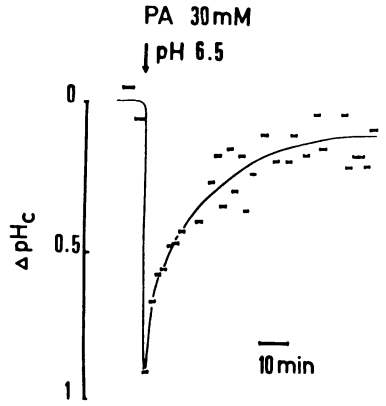

FIG. 7. Kinetics of pH_c modifications during acid-loading. pH_c values were determined from G6P chemical shifts of spectra cumulating 625 scans of 0.2 s, giving a time resolution of about 2 min (*i.e.* about 2.5 times better than the one corresponding to Figs. 1–6). The initial pH_c value was 7.61. PA, propionic acid.

differences between pH_c kinetics determined from G6P and Pi chemical shifts were observed. First, the pH values measured from the Pi peaks were often slightly higher than those measured from the G6P peaks. This discrepancy could be due to variations in ionic concentrations, especially of free Mg^{2+}, occurring during the acid treatment, with differential shifts of the Pi and G6P calibration curves (25). A second difference which occurred after acid removal remains unclear. The pH_c increase induced when the acidifying agent was removed, produced a drop in the intensity of the Pi peaks which reduced or abolished the accuracy of chemical shift determinations. In these cases, pH_c determination with Pi was impossible.

Estimation of the Buffering Power of the Cytoplasm. By accumulating scans during short periods (about 1 min) it was shown that the lowest value of pH_c was reached within 1.5 min after acid injection and corresponded to a pH drop of about 1 pH unit. Figure 1B shows that during this time, about 8 μmol of propionic acid were accumulated per g fresh weight. Calculations show that 1.5 min after acid injection, propionate should be distributed between the cytoplasmic and vacuolar compartments, with a large concentration difference (about 40 mM for the cytoplasm and 4 mM for the vacuole) linked to the respective pH (6.5 and 5.5) of these two compartments. Thus, a cytoplasmic buffering power of 40 μEq $H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$ was estimated for a cytoplasmic relative volume of 10%. However, if we assume that the pH_c regulatory mechanisms start without lag after the acid injection, extrapolating their contribution to the beginning of the acid-load period (to be detailed in the accompanying paper) reveals that during the first 1.5 min after acid injection 15 to 20 μEq $H^+ \cdot ml^{-1}$ cytoplasm were removed from the cells. Thus, a minimal buffering power of the cytoplasm appears to be 20 to 25 μEq $H^+ \cdot ml^{-1}$ cytoplasm $\cdot pH\ unit^{-1}$. These minimum and maximum values of the buffering power of the cytoplasm (20–40 μEq $H^+ \cdot ml^{-1}$ cytoplasm $\cdot pH\ unit^{-1}$) fall in the range of values published for animal cells (27) *i.e.* between 10 and 100 μEq $H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$ with a mean value of about 40. They appear in rather good agreement with the buffering capacity estimated by Sanders and Slayman (29) for *Neurospora* cells from butyric acid-loading experiments (30–35 μEq $H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$ at the normal pH_i of 7.2 to 7.4 and 60 μEq $H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$ when pH_i was acidified to 6.75) and that reported by Raven and Smith (21) for *Chlorella* (about 20 μEq $H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$). A mean value of 30 μEq $H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$ will be used in the remaining part of this study.

Evidence for Regulatory Mechanisms Responsible for the Recovery from Acid-Load. Table I shows the large variety of biological materials for which the cytoplasmic acidification of cells

Table I. *Comparison of Acid-Load Effects in Different Animal and Plant Systems*

The cytoplasmic pH modifications induced at maximal acidification and the rate of recovery, when observed, are compared for different cell types receiving various acid-loads.

Type of Cells	Type of Acid-Load			Cytoplasmic Acidification		References
	Acid	Concentration	pH_e[a]	pH max	Rate of recovery	
					(pH units·min^{-1})	
Barnacle muscle	CO_2	5%		−0.25	ND[b]	(4)
Squid axon	CO_2	5%	7.7	−0.4	+0.0007	(6)
Snail neurons	CO_2	5%	7.0	−0.45	+0.005	(30)
Sheep Purkinge fibers	Acetic	20 mM	6.8	−0.30	+0.003	(10)
	Propionic	20 mM	6.8	−0.30	+0.002	(10)
Human fibroblasts	Isobutyric	15 mM	7.4	−0.20	+0.05	(20)
Mice thymocytes	Acetic	15 mM	7.3	−0.17	+0.04	(26)
Chlorella	DMO	15 mM	6.7	−0.25	0	(11)
	Propionic	15 mM	6.7	−0.29	0	(11)
	CO_2	1.5%	6.7	−0.34	0	(11)
Neurospora	Butyric	5 mM	5.8	−0.55	0	(29)
Z. mays roots	Acetic	1 mM	4.8	−0.43	0	(7)
	Acetic	2.5 mM	3.0	−0.4	0	(22)
S. alba roots	Acetic	1 mM	5.0	−0.21	0	(2)
A. pseudoplatanus cells	Propionic	50 mM	6.5	−0.9	+0.05	
	Benzoic	50 mM	6.5	−1.2	+0.07	
	CO_2	25%	6.5	−0.7	+0.03	

[a] pH_e = external pH. [b] ND = not determined.

under acid-load has been demonstrated. Most animal systems show a clear recovery phase after the strong initial acidification, during which pH_c increases to reach an equilibrium value lower than the initial one. Such an increase occurring when the cells are continuously submitted to the influence of the acid-load demonstrates the operation of pH regulatory mechanisms which have been identified and studied in a variety of materials (see Refs. 5 and 27 for reviews). The rate and extent of recovery were generally lower than the one we have observed for *A. pseudoplatanus* cells except for mice thymocytes (26) and human fibroblasts (20). In these two cases the curves of pH_c evolution during acid-load were strikingly similar to those of Figures 2 and 3. This strongly suggests that in *A. pseudoplatanus* cells, as observed in animal systems, mechanisms involved in the regulation of pH_c are activated by the acid-load and are responsible for the recovery from the strong initial acidification. One original point of our study was to compare the evolution with time of the uptake of propionic acid with the acidification induced by its accumulation. The most striking result of this comparison was that the recovery occurred well before the net uptake of acid stopped. Thus, the mechanisms responsible for the overall 'consumption' of protons in the cytoplasm exceed the entry of protons brought by propionic acid uptake during the period of recovery.

However, Table I shows that suspension cultured cells apparently behave peculiarly compared to the other plant materials already described for which the time courses of pH_c changes during an acid-load gave no evidence for a pH recovery (2, 7, 22, 29).

In fact, the time course of pH_c changes during an acid-load depends critically on the interplay between (a) the rate of uptake of the protonated acid, (b) the inception delay of the mechanisms regulating pH_c and activated by the cytoplasmic acidification, (c) the rate of proton consumption through the operation of these mechanisms, and (d) the magnitude of the passive efflux of the anionic form of the acid.

A first hypothesis to explain the different behaviors of our cells and the plant materials listed in Table I arises from the studies of Boron (5). This author has shown that the shape of the curve depicting the response of pH_i after the application of an acid-load is critically dependent on the permeability of the plasmalemma to the anionic form of the acid and consequently is largely determined by the intensity of the anion efflux from the cells. Typical recovery phases are only seen when the permeability to the anion is low enough to minimize the acidification linked to the passive efflux of anion. When the rate of proton consumption by the regulatory mechanisms is just balanced by the efflux of anions no recovery is observed after the initial acidification. It is difficult to discuss this hypothesis further as, unfortunately, most studies on plant systems failed to monitor the uptake of acids used to load the cells and no information is available on the relative permeabilities of the neutral and anionic forms of these acids. It might be relevant however to note that one of the forces driving the anion efflux, *i.e.* the transplasmalemma potential difference, is much lower in the *A. pseudoplatanus* cells we have used (about 50 mV) compared to the other plant systems listed in Table I (about 150–200 mV). Such a difference is probably due to the high K^+ concentration (about 20 mM) of the culture medium.

A second quite simple hypothesis is based on two assumptions: the different plant materials studied (a) have about the same cytoplasmic buffering power and capacity to 'consume' protons through the regulatory mechanisms, but (b) they differ in their rate of uptake of the acid.

Two situations can arise according to the relative rate of proton consumption and the rate of acid uptake.

If the initial rate of acid uptake is in large excess compared to the rate of proton consumption, the influx of H^+ during the first minutes will exceed the capacity of regulation and a strong and rapid fall of pH_c should be observed. As the net uptake of acid decreases with time, the balance between proton influx and proton consumption is reversed and a recovery phase should occur. The plateau phase will correspond to a pH_c value for which the equilibrium between H^+ consumption and residual acid entry is reached.

If the initial rate of uptake of acid is not in so large an excess compared to the capacity to consume protons, the initial acidification should be smaller and a slow continuous decrease of pH_c should be observed until the equilibrium is reached.

According to this hypothesis, the two situations should lead to rather similar pH_c values at the plateau phase in spite of large

differences in rates of initial acidification and in shapes of the curves depicting pH changes. It is interesting to note that the extent of cytoplasmic acidification calculated as the difference between initial and plateau phase pH values are of the same magnitude as those reported for other plant systems (respectively 0.3, 0.5, and 0.3 pH unit for propionic, benzoic, and CO_2 loadings).

Two factors could explain why the rate of uptake of acids is higher in the cell suspensions we used compared to other materials. In organ fragments such as root segments, the rate of uptake by the overall cell population is probably limited by the rate of diffusion of the acid in the tissue. For example, the rate of uptake of butyric acid measured on maize roots by Marrè *et al.* (18) is about 12 times lower than the rate of uptake of propionic acid in our cells (0.55 against 6 $\mu mol \cdot min^{-1} \cdot g^{-1}$ fresh weight, for an external concentration of neutral acid of 1 mM at pH 5). Such a limitation in the rate of uptake of acid by root segments could account for the slow cytoplasmic acidification observed by Roberts *et al.* (22) in maize roots loaded with acetic acid. The second factor, operating at the very surface of the cell, is a limitation of uptake due to unstirred layers. The importance of this factor in the uptake of weak acids has been emphasized by Gutknecht and Tosteson (12) and reviewed by Roos and Boron (27). We have obtained some evidence that even in the case of our vigorously shaked cell suspensions, uptake of propionic acid could be limited by unstirred layers. The rate of uptake from a 50 mM acid solution in the culture medium (low buffer capacity corresponding to a phosphate concentration about 3.5 mM) at constant pH 6.5 maintained by continuous titration, was 30% lower than the rate of uptake from a 50 mM acid solution in a culture medium with a buffer concentration of 50 mM and a pH of 6.65 at equilibrium. This shows that a high buffer concentration in the incubation medium and an adequate renewal of the medium in the vicinity of the absorbing surfaces are necessary to depress the influence of unstirred layers.

CONCLUSIONS

The analysis of pH_c changes in *A. pseudoplatanus* cells submitted to acid-loads, compared to the reactions of animal and plant systems already studied, shows that plant cells, as other biological systems, are equipped with powerful mechanisms of regulation which resist large cytoplasmic acidification or alkalinization. Differences in the kinetics of reactions to acid-loads are observed and probably depend on different balances between rates of proton entry and proton consumption. The existence of a typical recovery phase following a strong initial acidification in two cell strains grown as cell suspensions offers a unique possibility to study the mechanisms involved in the control of pH_c.

The central role in maintaining pH_c in animal cells is played by transport mechanisms that remove protons from the cells, the biochemical mechanisms offering only short-term means to resist acid-load. The main role seems to be played by HCO_3^--Cl^- exchange and/or Na^+-H^+ exchange (see Refs. 5 and 27 for reviews).

In plant cells, little is known about the mechanisms responsible for the regulation of pH_c. The extrusion of H^+ by the proton pump at the plasmalemma activated by the cytoplasmic acidification (1, 7, 18), the metabolic consumption of protons by the 'biochemical pH-stat' described by Davies (9) and a transfer of protons from the cytoplasm to the vacuole as suggested by the observed vacuolar acidification are possible candidates.

The second paper of this series will be devoted to a study of the possible involvement of these mechanisms and their cooperative action in the resistance to acid-loads.

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