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Cytoplasmic pH Regulation in *Acer pseudoplatanus* Cells

II. POSSIBLE MECHANISMS INVOLVED IN pH REGULATION DURING ACID-LOAD

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ABSTRACT

Qualitative and quantitative aspects of the mechanisms involved in the regulation of cytoplasmic pH during an acid-load have been studied in *Acer pseudoplatanus* cells. Two main processes, with about the same relative importance, account for the removal of H^+ from the cytoplasm, namely a 'metabolic consumption' of protons and the excretion of protons or proton-equivalents out of the cells. The metabolic component corresponds to a change in the equilibrium between malate synthesis and degradation leading to a 30% decrease of the malate content of the cells during the period of cytoplasmic pH regulation. Various conditions which severely inhibit the activity of the plasmalemma proton pump ATPase reduce, at most by 50%, the excretion of H^+. This suggests that, besides the plasmalemma proton-pump, other systems are involved in the excretion of proton-equivalents. Indirect information on qualitative and quantitative features of these systems is described, which suggests the involvement of Na^+ and HCO_3^- exchanges in the regulation of cytoplasmic pH of acid-loaded cells.

In the accompanying paper (10) we have shown, using the ^{31}P NMR technique, that *Acer pseudoplatanus* cells are able to regulate their pH_c[2] during acid-load. The presence of a recovery phase following the initial acidification is a direct expression of the operation of the regulatory mechanisms. As such, it offers the possibility of studying these mechanisms by using the general strategy used for animal systems (for reviews, see Refs. 4, 18, 22).

In animal cells, three mechanisms minimize the extent of pH_c decrease during an acid-load: (a) the intracellular buffering capacity, (b) the excretion of acid equivalents, and (c) the metabolic consumption of intracellular acids. Roos and Boron (22) and Boron (4) have suggested that metabolic consumption offers only short-term solutions for resisting acid-loads. The central role in maintaining pH_c at a steady value in animal cells seems to be played by mechanisms which induce the excretion of acid-equivalents from the cells. Bicarbonate-chloride exchange and/or sodium-proton exchange, are generally involved in these cases.

It has now been clearly demonstrated that in plant cells, the plasmalemma proton-pumping ATPase is activated by acid-loading the cells. The hyperpolarization observed by Lyalin *et al.* (14), Marrè *et al.* (16) and Bates and Goldsmith (1) after acid-loading diverse plant materials suggested that the pump was activated by the cytoplasmic acidification and most likely contributed to the pH recovery. More recently, Brummer *et al.* (5) and Bertl and Felle (2) have given a very elegant demonstration that the hyperpolarization of the cell is the consequence of the pump activation by the cytoplasmic acidification. As a consequence, it is rather widely accepted that, in plant cells, the plasmalemma proton-pumping ATPase could be a major mechanism for regulating cytoplasmic pH by excreting acid-equivalents outside the cells. However, there is no quantitative evidence, relating the amount of protons excreted to the associated cytoplasmic pH increase, to support this assumption. Furthermore, in the fungus *Neurospora*, it has been demonstrated that the metabolism alone is able to control the cytoplasmic pH even when the pump is inhibited (24).

Quantitative data are lacking on the actual parts taken by the different mechanisms likely involved in the resistance to acid-loads: H^+ excretion from the cytoplasm to the extracellular medium and/or to the vacuole and metabolic consumption of acids. The aim of this work was to identify the mechanisms involved in the regulation of cytoplasmic pH in *A. pseudoplatanus* cells under acid-load and to get qualitative and quantitative information on their relative importance and their cooperation.

MATERIALS AND METHODS

Cell Culture Conditions and NMR Experimental Conditions. *Acer pseudoplatanus* L. cells were cultivated as described previously (13) in the medium modified by Bligny (3). Conditions used to run the NMR measurements, to acid-load the cells and to measure PA uptake have been described in the first paper of this series (10).

CO_2 Incorporation Conditions. Fifteen ml of cell suspension (about 2.2 g fresh weight), buffered to pH 6.5 with Mops-NaOH (100 mM), were incubated in a beaker shaken at 150 rpm at 26°C. $NaH^{14}CO_3$ (2.1 MBq·μmol^{-1} CEA Saclay, France) was added at a final concentration of 22 μM in the suspension culture. Aliquots of 500 μl of cell suspension were then fixed at intervals during 10 min, with 1 ml of a mixture HCOOH: ethanol: water (3:6:1, v/v/v). The residual $^{14}CO_2$ was removed by bubbling air during 1 h. Radioactivity of 50 μl of the extract was then determined by liquid scintillation counting using an Intertechnique SL 4000 spectrometer.

Malate Contents and Metatoblism. Thirty ml of cell suspension were incubated in a beaker in the same conditions of population density, aeration and culture medium as those used

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[2] Abbreviations: pH_c, cytoplasmic pH; DCCD, dicyclohexylcarbodiimide; EB, erythrosin B; FC, fusicoccin; G6P, glucose-6-phosphate; PA, propionic acid.

in the NMR tube. Aliquots of cell suspension (about 100 mg fresh weight) were filtered under vacuum and fixed with 2 ml of 10% (v/v) $HClO_4$. The extracts were neutralized with saturated sodium carbonate and malate was determined by enzymic analysis as described previously (17). The radioactivity present in the C_4 of malate was determined as the $^{14}CO_2$ evolved after incubation for 3 h of 400 µl of decanted extracts in the presence of 0.04 unit of malic enzyme (EC 1.1.1.40) in a 1.6 ml reaction mixture containing 50 mM Tris-Mops (pH 7.4), 20% glycerol, 2.5 mM $MgCl_2$, and 1.4 mM NADP. Radioactivity present in the pyruvate formed was determined as the corresponding 2,4-dinitrophenylhydrazone upon addition of 400 µl of saturated 2,4-dinitrophenyl hydrazine in a 4 N HCl. The hydrazone was extracted by three washes with 1 ml ethyl acetate. Ethyl acetate extracts were pooled and washed twice with 2 ml 0.1 N HCl. Pyruvate recovery was 80 to 90% with less than 5% contamination by malate. The radioactivity of the pyruvate formed in the presence of malic enzyme mainly corresponding to the radioactivity present in the C_1 of malate, was calculated after substracting the radioactivity recovered from the sample without malic enzyme.

H^+, K^+, and Na^+ Uptake. Kinetics of the disappearance of H^+ from the culture medium during acid uptake were determined by continuously recording changes of external pH. Calculation of the H^+ uptake associated with propionic acid uptake was made from the pH changes and the buffering capacity of the cell suspension estimated by back titration with 0.1 N HCl. K^+ and Na^+ uptake during the acid-load were followed by using ^{86}Rb and ^{22}Na. At the end of the desired incubation time, the cells were rapidly collected by filtration under vacuum. The radiolabel associated with the cell walls was removed by two washes with 5 ml ice-cold buffered solution used for resuspending the cells. In the experiments with ^{22}Na, $CaCl_2$ (10 mM) was added to the washing medium.

^{86}Rb content of the cells was determined by Cerenkov counting and ^{22}Na content by liquid scintillation counting using an Intertechnique SL4000 spectrometer.

For the Na^+ content analysis, the cells were collected and washed with 5 ml of Tris-Mes (25 mM, pH 6.5) solution containing 20 g/L saccharose. Na^+ contents were determined by flame photometry after extraction with 0.1 N HCl.

Unless otherwise indicated, all the experiments were run in fresh culture medium buffered to pH 6.5 with 50 mM Mops-NaOH.

RESULTS AND DISCUSSION

Importance of Proton Consumption during the Recovery from Acid-Load. A range of values for the intensity of proton consumption was calculated taking into account the uncertainties of the techniques used and the assumptions made for the calculations. As shown in Figure 1, the amount of protons consumed during the period of recovery from the acid-load (from 2 to 20 min) can be roughly estimated as the sum of two components: H_1^+ corresponding to the pH_c increase (0.4–0.6 pH unit) and H_2^+ corresponding to the continuous entry of PA. The amount of protons corresponding to the pH_c increase (H_1^+) was roughly estimated as 10 to 20 $\mu Eq \cdot ml^{-1}$ of cytoplasm, assuming a mean buffer capacity of 30 $\mu Eq\ H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$ (10). During the same time, the amount of PA absorbed by the cells was 13 ± 1.1 $\mu mol \cdot g^{-1}$ fresh weight. Calculations taking into account the relative volume of the cytoplasm (assumed to be 10%) and the cytoplasmic and vacuolar pH values (10) show that this uptake corresponds for the major part to an increase of the cytoplasmic acid content giving a rough estimate of 130 ± 11 $\mu Eq\ H^+ \cdot ml^{-1}$ cytoplasm for H_2^+. Thus a reasonable range for the amount of protons consumed during the recovery period of 20 min is 130 to 160 $\mu Eq\ H^+ \cdot ml^{-1}$. This amount, quite large compared to the buffering power of the cytoplasm (30 $\mu Eq\ H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$),

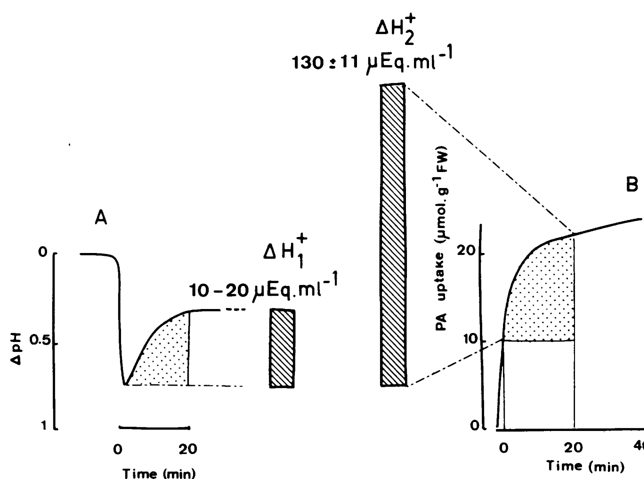

FIG. 1. Estimation of the intensity of proton consumption during the recovery phase of acid-loaded *A. pseudoplatanus* cells. The 'consumption' of H^+ in the cytoplasm was calculated as the sum of one component corresponding to the increase of pH_c (A) and a second component corresponding to the H^+ entry linked to PA uptake during the recovery phase (B). The buffering power of the cytoplasm was taken as 30 $\mu Eq\ H^+ \cdot ml^{-1} \cdot pH\ unit^{-1}$.

suggests the operation of strong pH regulatory mechanisms.

Relative Importance of Proton Excretion Outside the Cells in the pH_c Regulation. Evidence for proton excretion induced by the acid-load was obtained by comparing the theoretical uptake of protons linked to PA absorption to the real variation of H^+ concentration in the culture medium, calculated from extracellular pH measurements and the buffering capacity of the suspension medium. Figure 2 shows that a rather large discrepancy was revealed between the disappearance of protons measured experimentally (Fig. 2B, curve 2) and that which could be expected from PA uptake (Fig. 2A, curve 1). The difference between the two curves was assumed to correspond to the excretion of protons or proton-equivalents in the extracellular medium. According to this assumption, the amount of protons excreted during the recovery period (2–20 min) was about 9.1 ± 1.4 $\mu Eq\ H^+ \cdot g^{-1}$ fresh weight (Fig. 2B), *i.e.* about 90 $\mu Eq \cdot H^+ \cdot ml^{-1}$ cytoplasm (for a cytoplasmic relative volume of 10%). Thus, the overall proton excretion (*i.e.* the excretion of H^+ and proton-equivalents) could account for the disappearance of 60% of the protons 'removed' from the cytoplasm during the recovery period.

Metabolic Consumption of Organic Acids and pH_c Regulation during Acid-Load. As postulated by Davies (7), the 'biochemical pH-stat,' *i.e.* a pH control of the balance production/consumption of malate, is thought to be the major component of the cytoplasmic pH regulation in plant cells. As a matter of fact, $^{14}CO_2$ fixation by *A. pseudoplatanus* cells was strongly inhibited during acid-load in agreement with the pH sensitivity of P-enolpyruvatecarboxylase of these cells (17). Figure 3 shows that after a few minutes of acid-load the CO_2 fixation rate was inhibited by 90%. Then the fixation rate recovered slowly in parallel with the pH_c increase. The inhibition of CO_2 fixation was associated with a decrease of the malate content of the cells (Fig. 4) for the first 20 min after acid addition. The consumption of 3.7 ± 0.4 µmol of malate·g^{-1} fresh weight corresponded to about 30% of the initial content.

When cell suspensions were incubated with [^{14}C]bicarbonate for 20 min and then quickly washed out from external bicarbonate, the PA treatment increased by 1.5 to 2 times the rate of decrease of soluble radioactivity. The largest part (80–85%) of the radioactivity disappearing from the cells was recovered as $^{14}CO_2$. Results of Table I show that the radioactivity disappearing

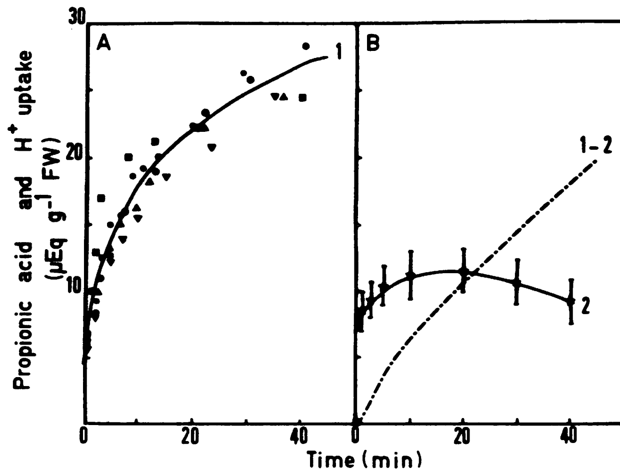

FIG. 2. Time course of PA uptake (A) and disappearance of protons from the extracellular medium (B). A, The uptake of [2-^{14}C]PA (50 mM, pH 6.5) was measured as described in the first paper of this series (10). The sets of different symbols correspond to independent experiments. B, The uptake of protons or proton-equivalents was measured as indicated in "Materials and Methods." Vertical bars represent two times the standard error of the means. The excretion of protons from the cells was calculated as the difference between curves 1 and 2.

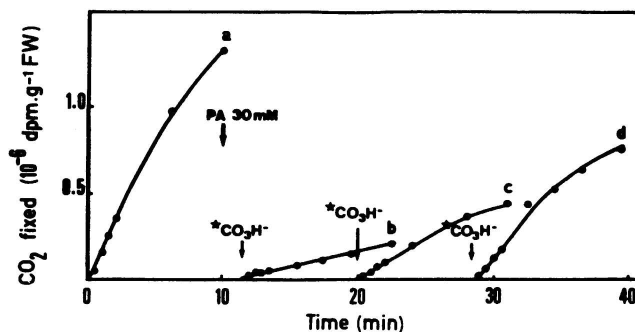

FIG. 3. Effect of PA on the rate of CO_2 fixation. At time 0, $H^{14}CO_3^-$ was injected in the control flask (a) and the amount of $^{14}CO_2$ fixed was measured during 10 min, as described in "Materials and Methods." At time t = 10 min, 30 mM PA (buffered to pH 6.5 with NaOH) was injected in three other flasks (b, c, d) and $H^{14}CO_3^-$ was added 2, 8, or 16 min after the acid injection and the $^{14}CO_2$ fixation was again measured during 10 min. FW, fresh weight.

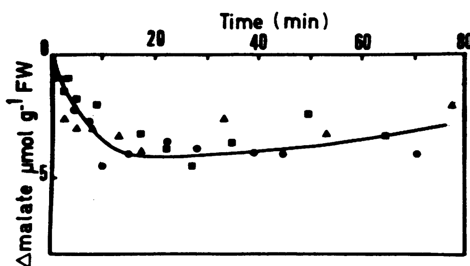

FIG. 4. Changes in the malate content of acid-loaded cells. At time 0, 50 mM PA (buffered to pH 6.5 with NaOH) was injected in the cell suspensions and changes in the malate content were followed during about 75 min from 600 µl aliquots processed as described in "Materials and Methods." Different symbols correspond to separate experiments. FW, fresh weight.

Table I. *Malate Metabolism after Treatment of A. pseudoplatanus Cells with PA*

A. pseudoplatanus cells (1 g fresh weight) were incubated in Erlenmeyer flasks in 10 ml of Bligny medium supplemented with 20 mM KCl and buffered to pH 6.5 with 50 mM Mops-NaOH. At time 0, the flasks were closed with a septum and 22 µM of NaH $^{14}CO_3$ (2.1 MBq µmol^{-1}) was injected. Ten min later, $HClO_4$ 5% (final concentration) was injected in the control flask and 50 mM Na propionate (pH 6.5) was injected into another flask. After a further 10 min incubation, the second suspension was fixed with $HClO_4$. After removal of the residual $^{14}CO_2$ by bubbling air through the fixed suspension, total radioactivity present in the soluble fraction and that present in malate were determined as described in "Materials and Methods."

	Soluble Fraction (a)	Malate			(a − d)
		C_4 (b)	C_1 (c)	Total (d)	
	dpm·mg⁻¹ fresh wt		*dpm·mg⁻¹ fresh wt*		
Control cells	1,404	560	438	998	406
PA treated-cells	806	200	143	343	463

from the cells (about 60%) corresponded specifically to a decrease of the ^{14}C present in malate, without any significant increase in the radioactivity of other soluble compounds. Furthermore, the two carboxyl groups of malate were decarboxylated to the same extent. As CO_2 was continuously removed, we considered that malate metabolization in the cytoplasm corresponded to the disappearance of two anionic charges. The metabolism of malate to CO_2 diffusing out of the suspension culture modifies the anion/cation balance in the cytoplasm and contributes to the increase of pH_c. The decrease of cytoplasmic malate probably induces an efflux from the vacuolar pool. If it is assumed that the transfer of vacuolar molecules back to the cytoplasm is an electroneutral process (*i.e.* cotransport $mal^{2-}/2K^+$ or countertransport $mal^{2-}/2Cl^-$) their consumption in the cytoplasm would also induce a decrease of the anion/cation ratio in the cytoplasm. Thus, whatever the initial location of the metabolized molecules, the consumption of about 3.7 ± 0.4 µmol of malate·g^{-1} fresh weight would account for the disappearance of about 74 ± 8 µEq H^+·ml^{-1} cytoplasm. This 'metabolic regulation' of the cytoplasmic pH appears quantitatively important as it could account for 45 to 55% of the total proton consumption during the recovery period.

Proton Transfer to the Vacuole as a Possible Mechanism of pH_c Regulation. Among the various possibilities for sequestrating intracellular acid, the vacuole appears a good candidate because of (a) its large volume compared to the cytoplasm and (b) the existence of a tonoplast proton-pump removing H^+ from the cytoplasm. As shown in the first paper of this series (10), vacuolar pH was decreased during acid load by about 0.3 pH unit. The buffering capacity of the vacuolar sap has been estimated to be 15 µEq H^+·ml^{-1}·pH unit^{-1} around pH 6 (11). Thus the decrease of vacuolar pH induced by acid-loading the cells corresponds roughly to 5 µEq H^+·g^{-1} fresh weight cells. Calculations show that most of this decrease could be accounted for by PA entry into the vacuole. For an extracellular concentration of PA of 50 mM in the overall cell suspension (the real external concentration is about 60 mM due to the high cell population density used in NMR conditions), an extracellular pH of 6.5 and a vacuolar pH in acid-loaded cells of 5.5 (10), the vacuolar concentration of PA at equilibrium should be about 6 mM. It appears that the vacuolar acidification results mostly from the entry of PA into the vacuole and does not correspond to a significant process in the regulation of pH_c.

Evidence for the Existence of Different Processes Responsible for the Excretion of Protons. The excretion of H^+ or proton-

equivalents appears to be largely involved in the recovery from the initial cytoplasmic acidification (60% of the overall 'removal' of protons). The hypothesis that the plasmalemma proton-pump ATPase could be responsible for this H^+ excretion was studied.

We observed that Rb^+ uptake was strongly stimulated during the acid-load (Fig. 5). Such a result is in good agreement with the well-described hyperpolarization induced by cytoplasmic acidification (2, 5, 16). The real problem was to obtain a reasonable estimation of the quantitative importance of the H^+-efflux catalyzed by the pump compared to the overall proton excretion. Several arguments showed that the H^+-pump is not the sole mechanism involved in proton excretion.

As the energy consumption by the pump is quite large in conditions where H^+ excretion is activated (9), we studied the acid-load effects under hypoxic conditions to reduce the energy supply of the pump. Figure 6 shows that under hypoxic conditions the pH_c was lowered by 0.5 pH unit as already described by Roberts *et al.* (21). When PA was injected into the cell suspension culture, the pH_c recovery, measured either from the Pi or the G6P chemical shifts, occurred as in aerated cells. This suggested that the H^+-pumping ATPase was not a major component of pH_c recovery; at least some of the mechanisms required to control pH_c operated at low energy levels in the cells.

A further support to the idea that the H^+ pump was not a major process of pH_c regulation arose from the observation (data not shown) that cells, thoroughly washed and resuspended 2 to 3 times in a Tris-Mes buffer to remove extracellular K^+, were still able to regulate their cytoplasmic pH during the acid-load given as Tris-propionate. Such a lowering of external K^+ probably reduces the rate of proton pumping which depends strongly on the presence of extracellular diffusible cations to counteract the hyperpolarization created by H^+-pumping (15).

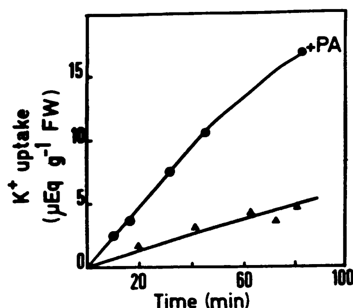

FIG. 5. Effect of propionic acid on K^+ uptake. K^+ uptake was determined as described in "Materials and Methods" in a control suspension or a cell culture receiving at zero time PA (50 mM, pH 6.5) given as Na^+ salt. FW, fresh weight.

As another approach to the study of the H^+-pump involvement, we used the inhibitor EB. This inhibitor, at 80 μM, strongly inhibited the activation by FC of net H^+ excretion by *A. pseudoplatanus* cells (Fig. 7A) without important effect on the pH_c recovery (Fig. 7B). The evidence brought by such a result was, however, rather weak as a study of the dose-activity curve of EB demonstrated (results not shown) that the inhibition by EB of FC- or PA-induced $^{86}Rb^+$ uptake was only 40% at 80 μM. Much higher EB concentrations (500–600 μM) were necessary to reach 80 to 85% inhibition of the PA- or FC-induced $Rb^+(K^+)$ uptake. Figure 8 illustrates the effect of EB (500 μM) on the uptake of labeled PA and on the associated H^+ uptake. Surprisingly, the inhibition of the H^+-pump by about 80% had only a slight depressive effect on PA uptake and only partially reduced the discrepancy between PA uptake and H^+ uptake. By assessing that the difference between the amounts of protons excreted during the period of recovery in control and EB-treated cells (respectively 12 and 8 $\mu Eq\ H^+\cdot g^{-1}$ fresh weight), represented about 80% of the activity of the pump, the intensity of H^+-efflux catalyzed by the pump was estimated as 5 $\mu Eq\ H^+\cdot g^{-1}$ fresh weight, *i.e.* about 50% of the overall proton excretion.

This value is in good agreement with the one which could be estimated from the PA-stimulated ^{86}Rb uptake (Fig. 5). The rate of K^+ uptake was 4.6 ± 1.3 $\mu Eq\cdot g^{-1}$ fresh weight during the 20 min period of pH_c recovery and it was likely driven by the hyperpolarization created by the activated pump. If a stoichiometry H^+-efflux/$Rb^+(K^+)$ uptake close to 1 is assumed, it follows that a reasonable value for the intensity of H^+ excretion through the pump during the period of pH_c regulation is about 5 $\mu Eq\cdot g^{-1}$ fresh weight.

Thus, all the experimental arguments show that the proton pump is only partly responsible for the excretion of protons out of the cells during the recovery period. Its activity accounts for only 40 to 50% of the apparent proton excretion and its relative importance in the overall pH_c regulation during the recovery period can be estimated as 30% of the total amount of H^+ disappearing from the cytoplasm. This conclusion differs from

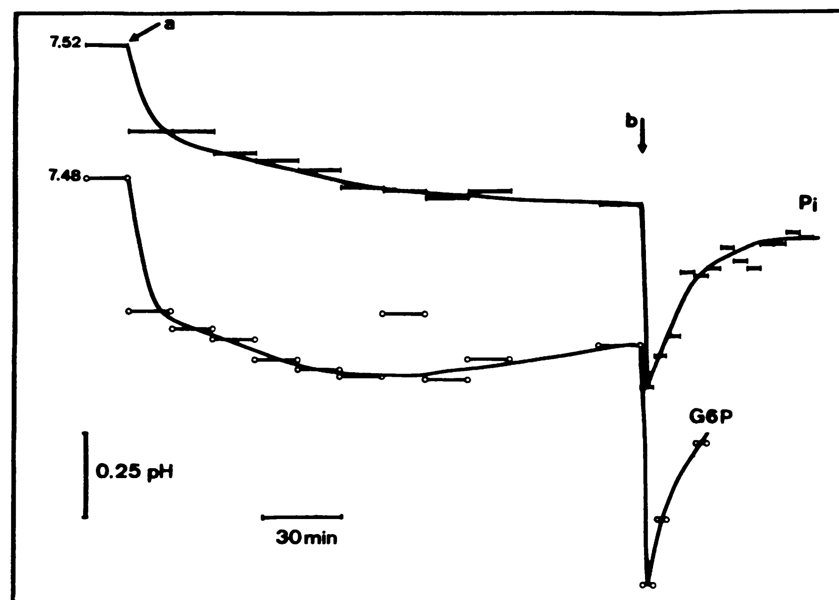

FIG. 6. Effect of PA on the cytoplasmic pH of *A. pseudoplatanus* cells bubbled with nitrogen. pH_c values were determined from G6P and Pi chemical shifts. Each pH value is plotted as an horizontal bar. The length of a bar is the duration during which scans were accumulated (0.5 s per scan). Usually, pH was determined from spectra cumulating 600 scans (about five minutes elementary spectra). The large bars represent the pH determined from the cumulation of scans corresponding to several elementary spectra. The initial pH values are indicated for each curve. Cells were first bubbled with air, then with N_2 (a), and 20 mM PA (buffered to pH 6.5 with NaOH) was injected (b).

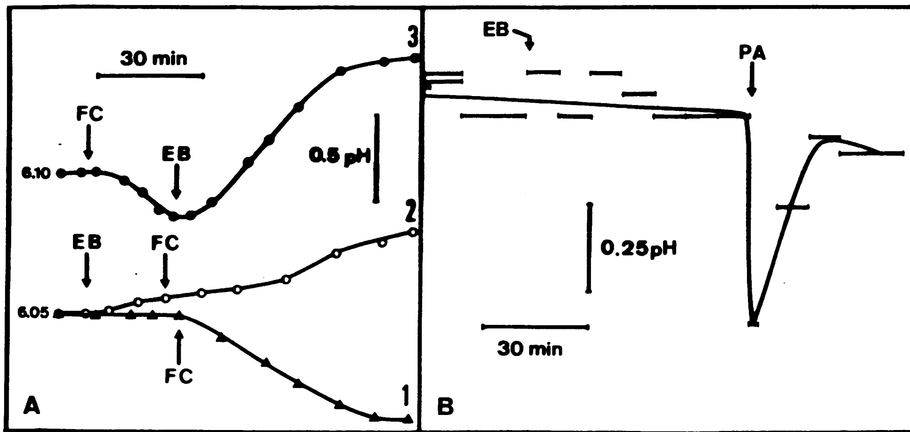

FIG. 7. Effect of EB on the FC-stimulated H^+ efflux and on the pH_c regulation during acid-load. A, Evolution with time of the extracellular pH of cell suspensions treated with FC (3 μM) and EB (80 μM). Curve 1, cells treated with FC. Curve 2, cells treated successively with EB and FC. Curve 3, cells treated successively with FC and EB. B, Evolution with time of the pH_c of cells treated with EB (80 μM) and acid-loaded 1 h after with PA (30 mM, pH 6.5).

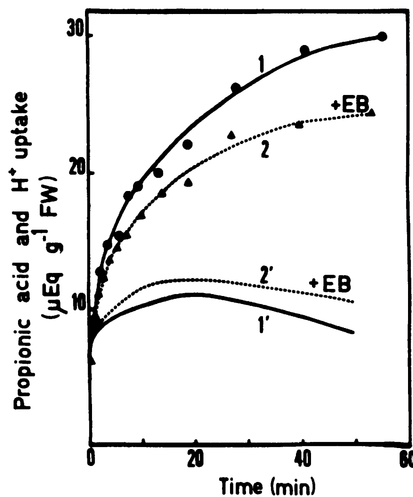

FIG. 8. Effect of EB (500 μM) on the uptake of PA and on the associated H^+ uptake. The uptake of [2-^{14}C] PA (50 mM, pH 6.5) was measured in control (curve 1) and EB-treated (500 μM) cells (curve 2). The corresponding extracellular alkalinization was measured and the uptake of protons was calculated (curves 1′ and 2′). FW, fresh weight.

the statement that H^+-ATPase has an important function in intracellular pH regulation in mung bean root-tip cells (25). However such an argument, based on the effects of DCCD, a general inhibitor of ATPases, whatever their type and location, is tenuous.

This analysis suggests the existence of other mechanisms responsible for the excretion of protons or proton-equivalents.

Evidence for the Involvement of External Bicarbonate and Na^+/H^+ Exchanges in the Regulation of pH_c of Cells under Acid-Load. In animal cells, the excretion of H^+ or proton equivalents is governed by an H^+/Na^+ antiport and/or a Cl^-/HCO_3^- antiport (see Ref. 22 for a review). Very few data are available in the literature on plants about H^+/Na^+ exchange except for barley root tips where an H^+/Na^+ antiport has been described (6, 20). The quantitative importance of such a system in the regulation of H^+ exchanges and the control of pH_c is unknown. In *Chlorella* cells, cytoplasmic acidification, by CO_2 or glucose uptake, has been shown to induce an efflux of Cl^- (8, 23), interpreted as an inhibition of a Cl^-/OH^- antiport. But in higher plants, nothing is known about the involvement of anion exchanges in the regulation of intracellular pH.

Indirect evidence suggesting that external bicarbonate could be involved in the regulation of pH_c of acid-loaded cells was obtained by testing the influence of lowering external HCO_3^- on the discrepancy between PA uptake and H^+ uptake (*i.e.* the rate of H^+ excretion). Table II shows that a 5 times reduction of the cell population density in a normal medium with a low Na^+ concentration (about 0.5 mM) and 500 μM EB to inhibit the proton-pump ATPase, induced a large reduction of proton excretion. Conversely, the rate of proton excretion was increased by bubbling CO_2 (0.2%) enriched air through the low density suspension.

The hypothesis that a Na^+/H^+ antiport could also be involved in the pH_c regulation of acid-stressed cells was supported by the following evidence. First, acid-loading with propionic acid (Tris salt) enhanced the uptake of ^{22}Na by *A. pseudoplatanus* cells (Fig. 9A). Their Na^+ content was increased by 2.7 ± 0.7 $\mu Eq \cdot g^{-1}$ fresh weight during 30 min of acid-loading with 50 mM propionic acid buffered to pH 6.5 with NaOH (*i.e.* in conditions used to run most of NMR experiments). EB (500 μM) which strongly inhibited the PA-induced $Rb^+(K^+)$ uptake (80%) was much less effective on the PA-induced Na^+ uptake (40%). These observations suggested that a large part of Na^+ uptake in acid-loaded cells was driven by a carrier distinct from the one involved in K^+ entry. Moreover, Na^+-loaded cells (40 mM, 24 h) resuspended in a weakly buffered acidic culture medium (pH 6.2) alkalinized the external medium at a rate significantly higher than that observed with K^+ loaded cells (Fig. 9B). Both results suggested that a H^+/Na^+ antiport was operating in *A. pseudoplatanus* cells, catalyzing an efflux of H^+ from acid-loaded cells, coupled to Na^+ entry and an uptake of H^+ from Na^+-loaded cells, coupled to the efflux of Na^+. This apparently reversible operation of an H^+/Na^+ antiport is quite comparable to the one described for animal cells (4, 12, 19).

The quantitative importance of such an exchange Na^+/H^+ in terms of intracellular pH regulation during acid-load was estimated from the assumption that one proton was excreted for each Na^+ ion absorbed. The increase of Na^+ content during the first 20 min following acid injection in the cell suspension would correspond to the excretion of 2 to 3 $\mu Eq\ H^+ \cdot g^{-1}$ fresh weight *i.e.* about 20 to 30% of the overall proton excretion during the period of pH_c recovery. Thus, the H^+/Na^+ antiport appears as one of the major components of H^+ excretion from acid-loaded cells.

The preliminary results showing the stimulating effect of external HCO_3^- on the excretion of protons are more difficult to interpret. Two different mechanisms operating in animal cells could be involved: either an exchange against strong anions (Cl^- or NO_3^-) or as in some invertebrates a more complex electroneutral system catalyzing coupled transports of Na^+, HCO_3^-, and Cl^- and possibly H^+ (4, 22). Further work is needed to get a more precise description of this component of the systems involved in proton excretion by acid-loaded cells.

Table II. *Influence of the Density of Cell Population and of the Partial Pressure of CO_2 on the Intensity of Proton Excretion by Acid-Loaded Cells*

Cells were incubated in a Bligny culture medium buffered with 1 mM Mops adjusted to pH 6.5 with Tris. Cells were acid-loaded with 50 mM of Tris propionate (pH 6.5). Experiments were run in the presence of 500 μM EB. Propionate and H^+ uptakes were measured during 10 min. Proton excretion was calculated as the difference between PA uptake and H^+ uptake and its relative importance (%) compared to the theoretical proton uptake (PA uptake) indicated in parentheses.

Density of Cell Population	Gaseous Phase	H^+ Uptake (A)	PA Uptake (B)	Proton Excretion
mg fresh wt·ml^{-1}		*μEq·g^{-1} fresh wt*	*μmol·g^{-1} fresh wt*	*μEq·g^{-1} fresh wt*
110	Air	7.0	15.4	8.4 (55)
21	Air	13.1	14.7	1.6 (11)
21	Air + CO_2 0.2%	11.1	15.7	4.6 (29)

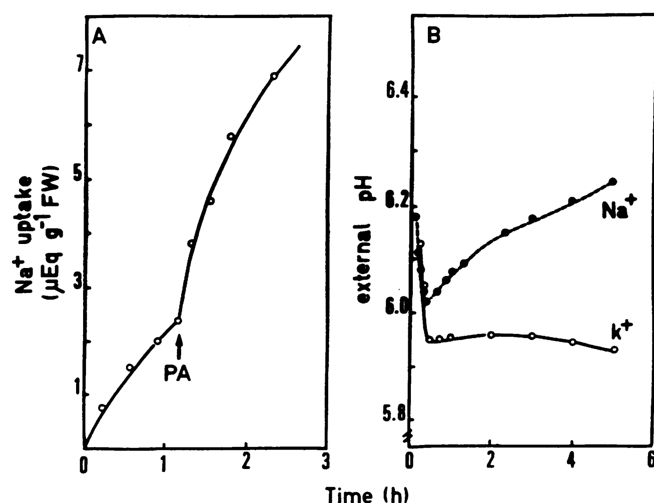

FIG. 9. Stimulating effect of acid-load on the uptake of Na^+ in *A. pseudoplatanus* cells and evidence for the existence of a Na^+/H^+ exchange. A, Cells were washed and resuspended in a Bligny medium buffered to pH 6.5 with 50 mM Mops-NaOH. Carrier free ^{22}Na was injected in the suspension at time 0 to give a final specific radioactivity of 25 kBq μEq^{-1} and 70 min later cells were loaded with 50 mM Tris-propionate. At times intervals, aliquots of cell suspension were taken up and analyzed for their ^{22}Na content as described in "Materials and Methods." B, Cells were first incubated for 24 h in the presence of KCl or NaCl (40 mM). At time 0, the cells were rapidly washed and resuspended in a Bligny medium supplemented with 20 mM KCl buffered to pH 6.2 with Tris, to give a final cell population density of 250 mg fresh weight (FW)·ml^{-1}. Evolution of the external pH was followed during 5 h for KCl loaded cells and NaCl loaded cells. The rate of net proton uptake after 3 h was 0.22 μEq $H^+ \cdot g^{-1}$ fresh weight and −0.06 μEq $H^+ \cdot g^{-1}$ fresh weight for the NaCl and KCl loaded cells, respectively.

CONCLUSION

Metabolic consumption of protons and proton excretion in the extracellular medium are the main mechanisms by which acid-loaded cells of *A. pseudoplatanus* cells regulate their pH_c. The most striking result obtained was the discovery that only one-half of the H^+-excretion from acid-stressed cells was driven by the proton-pump ATPase. A tentative proposal was made as to the identity of the systems responsible for the excretion of the remaining half. Preliminary results showed that an H^+/Na^+ antiport was apparently strongly activated by the cytoplasmic acidification. A stimulating effect of external HCO_3^- on proton excretion was also demonstrated but the mechanisms involved were not identified.

A rather complex picture of pH_c regulation in plant cells emerges from this study. Plant cells are equipped with different systems which remove protons from the cytoplasm under conditions of acid-load. Their relative contribution to the regulation of the pH_c is most likely dependent on the type of pH stress (intensity and duration) encountered by the cells. In this respect, the acid-load technique corresponds to a peculiar physiological situation where an intense acid-stress induces regulating responses in the short-term. For the long-term pH homeostasis, the relative importance of the different systems is most likely modified with a decrease of the role of malate consumption and an increase of the importance of the primary active H^+ efflux. The diversity of mechanisms available to plant cells is probably at the basis of their ability to resist acid-stresses under a variety of physiological situations.

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