Plant Physiol. (1992) 99, 665–671
0032-0889/92/99/0665/07/$01.00/0

Received for publication October 8, 1991
Accepted January 20, 1992

Proton/L-Glutamate Symport and the Regulation of Intracellular pH in Isolated Mesophyll Cells[1]

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ABSTRACT

Addition of L-[U-^{14}C]glutamate to a suspension of mechanically isolated asparagus (*Asparagus sprengeri* Regel) mesophyll cells results in (a) alkalinization of the medium, (b) uptake of L-[U-^{14}C] glutamate, and (c) efflux of [^{14}C]4-aminobutyrate, a product of glutamate decarboxylation. All three phenomena were eliminated by treatment with 1 millimolar aminooxyacetate. *In vitro* glutamate decarboxylase (GAD) assays showed that (a) 2 millimolar aminooxyacetate eliminated enzyme activity, (b) activity was pyridoxal phosphate-dependent, and (c) activity exhibited a sharp pH optimum at 6.0 that decreased to 20% of optimal activity at pH 5.0 and 7.0. Addition of 1.5 millimolar sodium butyrate or sodium acetate to cell suspensions caused immediate alkalinization of the medium followed by a resumption of acidification of the medium at a rate approximately double the initial rate. The data indicate that (a) continued H$^+$/L-glutamate cotransport is dependent upon GAD activity, (b) the pH-dependent properties of GAD are consistent with a role in a metabolic pH-stat, and (c) the regulation of intracellular pH during H$^+$/L-Glu symport may involve both H$^+$ consumption during 4-aminobutyrate production and ATP-driven H$^+$ efflux.

An ATP-driven, outwardly directed H$^+$ pump establishes a H$^+$ electrochemical gradient across the plasma membrane of plant cells. The active accumulation of sugars and amino acids is coupled to and driven by a flux of H$^+$ down this gradient. Accumulation involves binding of H$^+$ and organic solutes at the external face of a carrier protein, in a simple molar stoichiometry, prior to their coupled entry into the cell. Without regenerating mechanisms, electrogenic proton symport processes will dissipate their driving forces. These are the concentration gradients of the sugar or amino acid, the proton gradient, and the electrical gradient (20). An influx of protons represents an acid load imposed on the cytoplasm that, depending on the efficiency of the pH-regulating processes, may or may not result in a significant decrease in pH. That there are processes compensating for imposed acid loads is indicated by a tight control of cytoplasmic pH values varying within a narrow pH range of 7.0 to 7.4 and by the rapid recovery to initial pH values (7, 8, 11). Regulation of an acid load could involve passive physiochemical buffering, ATP-driven transfer of H$^+$ to the vacuole, or external medium and H$^+$ consumption in metabolic reactions. In the metabolic pH-stat model, lower pH values activate enzymes that catalyze proton-consuming decarboxylation reactions (6, 12, 17). Similarly, symport-induced depolarization is often reversed during the continued alkalinization of the external medium (10, 11, 20). Charge compensation may involve K$^+$ efflux (11, 20, 28) or stimulation of electrogenic, ATP-driven H$^+$ efflux (11, 20, 21).

In isolated *Asparagus* mesophyll cells, a H$^+$/L-Glu symport process causes rapid alkalinization of the medium for 30 to 40 min (14, 15). Calculations indicate that, without active compensating mechanisms, the cytoplasmic pH would decline by 1.9 pH units/h. Measured stoichiometries, however, ranged from 3.0 to 7.7:1. (15), far higher than theoretical or measured stoichiometries found in other systems (20, 21, 28). The elevated stoichiometry resulted from an underestimation of labeled L-Glu uptake due to its decarboxylation and the rapid efflux of the labeled product GABA[5] (1, 15).

The present report is concerned with the mechanisms by which *Asparagus* mesophyll cells maintain the driving forces for H$^+$/L-Glu symport. In particular, (a) AOA, an inhibitor of GAD, was employed to test the importance of L-Glu decarboxylation in continued H$^+$/L-Glu symport, and (b) the pH-dependent properties of GAD were investigated to evaluate the potential role of GABA production in reducing cytoplasmic acidification.

MATERIALS AND METHODS

Mesophyll cells were isolated from mature asparagus (*Asparagus sprengeri* Regel) cladophylls (4), suspended in 1 mM $CaSO_4$ unless noted otherwise, and counted. All concentrations quoted are final after dilution with the suspension medium.

Concurrent Measurements of L-Glu Uptake and GABA Efflux

A 4.2-mL cell suspension containing 8×10^6 cells was adjusted to pH 6.0, illuminated (4.6×10^{-4} mol·m^{-2}·s^{-1}), and maintained at 30°C in a glass, water-jacketed incubation

[1] Supported by grant No. A4453 from the Natural Sciences and Engineering Research Council of Canada to A.W.B.

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[5] Abbreviations: GABA, 4-aminobutyrate; GAD, glutamate decarboxylase; AOA, aminooxyacetate; PLP, pyridoxal phosphate.

vessel (15). Prior to addition of [U-^{14}C]L-Glu, 200 μL of cell suspension was removed to serve as a scintillation counter blank. The cells were washed with 5 mL of 1 mM $CaSO_4$, and then twice with 10 mL of 1 mM $CaSO_4$, and were collected on Millipore 0.45-μm filters. L-[U-^{14}C]Glu (2.50 nCi/nmol) was then added to the remaining cells to give a final concentration of 0.82 mM. Aliquots of 200 μL were removed immediately and at 5-min intervals for 30 min. The cells were washed and collected on Millipore filters as described above. The filters were transferred to scintillation vials containing 1.0 mL of methanol. Fourteen milliliters of ACS (Amersham) scintillation fluid was then added to the vials. Concurrent with the L-Glu uptake assay, 200-μL aliquots of cell suspension were removed at 5-min intervals to determine the amount of radioactive GABA in the medium. These aliquots were centrifuged at 2400*g* for 2 min to sediment the mesophyll cells, and the medium was stored at −20°C prior to assays for [^{14}C]GABA. Aliquots of the supernatant fluids together with unlabeled GABA were applied to TLC plates (MN Kiesel gel G). Plates were developed with a phenol:water (75:25, w/w) solvent, dried, and treated with ninhydrin. Areas containing GABA were transferred into scintillation vials for counting. The moles of GABA in each sample and the corresponding L-Glu uptake measurements were used to calculate the rates of L-Glu uptake and GABA release. Rates of total L-Glu uptake were obtained from the sum of these two values.

Measurement of H^+ Fluxes

Cells (8×10^6) were suspended in 4 mL of medium, adjusted to pH 6.0, and maintained as described above. Rates of alkalinization or acidification were measured with a Radiometer (Copenhagen) recording pH apparatus (15). After a constant rate of extracellular acidification had been established, 2.5 mM L-Glu was added and the rate of alkalinization recorded. The buffering capacity of the medium was determined before and after L-Glu addition. Net rates of proton efflux or influx were measured in nmol $H^+ \cdot 10^6$ cells$^{-1} \cdot$ min^{-1} using the rate of pH change and the corresponding buffering capacity of the medium. The influence of weak acids on net H^+ efflux was determined by the same procedures. Butyrate or acetate (adjusted to pH 6.0 with NaOH) was added to give a concentration of 1.5 mM. Rates of proton efflux before and after addition were calculated from the acidification rates and the corresponding buffering capacity.

Measurement of *in Vitro* GAD Activity

About 10 g of mature cladophylls were collected and washed, and the mesophyll cells were isolated prior to their disruption with a French press (Aminco) at 8270 kPa. This extract was then centrifuged at 590*g* for 5 min. The supernatant fluid was then centrifuged at 190,000*g* for 1 h at 4°C in a B-60 Damon/IEC ultracentrifuge. The resulting clear supernatant fluid was subsequently desalted with a PD-10 Sephadex G-25 (Pharmacia) column. Eluates were frozen in liquid nitrogen and lyophilized overnight. Protein concentration in the lyopholized material was determined (2) with BSA (Sigma) as a standard. At −20°C, GAD activity was stable for several months.

GAD activity was assayed by a radiometric method based on the formation of $^{14}CO_2$ from L-[U-^{14}C]Glu (27). A typical 200-μL system contained 2.5 mM L-[U-^{14}C]Glu (18.4 nCi/nmol), 0.5 mM PLP, and 160 μg protein in 0.2 M K_2HPO_4: 0.1 M citric acid buffer, pH 5.8. Deviations from these conditions are noted in "Results." Enzyme-free controls were included in each experiment. The incubation vessel was a 14 × 85 mm glass test tube. After L-[U-^{14}C]Glu was introduced, the tubes were immediately sealed with a serum rubber stopper (Kontes, Vineland, NJ). Inserted through the stopper was a plastic center well (Kontes) containing 300 μL of methylbenzethonium hydroxide (10% [w/v] in ethanol) and filter paper to increase the absorptive surface area. Vessels were then incubated at 37°C for 30 min in a shaking water bath. The reaction was terminated by introducing 20 μL of 0.5 N sulfuric acid. After another 40 min of incubation to capture released $^{14}CO_2$, the center wells were transferred to vials for liquid scintillation counting.

The other product of the GAD-catalyzed reaction, [^{14}C]GABA, was identified by TLC and autoradiography. TLC plates were developed as described above, sprayed with beta-decay enhancer (Dupont), and exposed to x-ray film (Kodak) at −70°C for 2 to 4 weeks.

Measurement of Cell Volume

Volume estimates were based on the cross-sectional areas of 1-mm thick serial sections of mesophyll cells from Epon-embedded, intact cladophylls. The sections were heat-fixed on glass slides and stained with toluidine blue solution. They were viewed with a light microscope at 400× magnification, and the cross-sectional areas were determined with a microcomputer imaging device software package (Imaging Research Inc., Brock University, St. Catharines, Ontario). Each section was converted to volume by the formula $V = Sh$, where V is the sectional volume, S is the sectional area, and h is the sectional thickness. The volumes of all sections were added to give the volume for one cell and repeated for 10 different cells.

A second method involved measuring cell dimensions at 400× magnification with an eyepiece calibrated with a micrometer scale. Measurements of length and radius midway along the length were made on more than 100 isolated cells. Calculations of volume assumed that the cells were cylindrical in shape.

Measurement of Cytoplasmic Buffering Capacity

Twenty × 10^6 cells suspended in 10 mL of 1 mM $CaSO_4$ were stirred without aeration in ambient light. The extracellular buffering capacity was determined by adding aliquots of 1 mN HCl. The buffering capacity between 6.5 and 7.3 was calculated and expressed in units of μmol $H^+ \cdot 10^6$ cells$^{-1} \cdot$ pH unit^{-1}. The cells were then disrupted by passage through a French press (Aminco) at 8270 kPa. Light microscope observations indicated that the resulting homogenate was cell free. The buffering capacity of the homogenate was determined between pH 6.5 and 7.3 and calculated in the same units. The cytoplasmic buffering capacity was then calculated by subtracting the extracellular buffering capacity from the buff-

ering capacity of the homogenate (11). Cell volume measurements expressed in $mL \cdot 10^6$ $cells^{-1}$ were used to calculate buffering capacity in units of $\mu mol\ H^+ \cdot mL^{-1}$ cytoplasm·pH $unit^{-1}$.

RESULTS

Measurements of cell volume indicated a value of 4.7 (±0.09 SD) $pL \cdot cell^{-1}$ by the serial section technique and 5.8 (±1.6 SD) $pL \cdot cell^{-1}$ by measurements of length and width. The former value corresponds to 4.7×10^{-3} $mL \cdot 10^6$ $cells^{-1}$. Employing this value, the extracellular buffering capacity was calculated to be 7.4 $\mu mol\ H^+ \cdot mL^{-1}$ cytoplasm·pH $unit^{-1}$, and the corresponding value for the homogenate was 22.3 $\mu mol\ H^+ \cdot mL^{-1}$ cytoplasm·pH $unit^{-1}$. Subtraction of these values resulted in a cytoplasmic buffering capacity of 14.9 $\mu mol\ H^+ \cdot mL^{-1}$ cytoplasm·pH $unit^{-1}$.

The apparent rate of L-[U-^{14}C]Glu uptake and the release of [^{14}C]GABA were both linear over a 30-min incubation period. The total L-Glu uptake was calculated from the sum of the apparent L-Glu uptake and the observed GABA efflux. Approximately 82% of the L-Glu taken up by the cells was exported as GABA (Fig. 1). AOA at a concentration of 1 mM

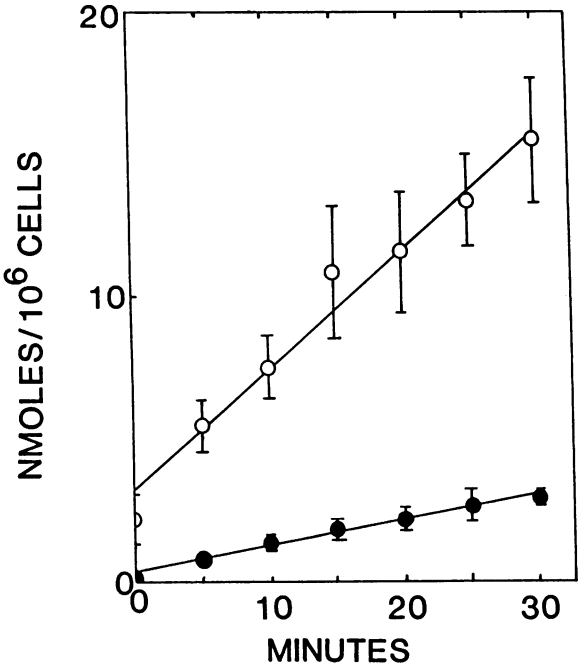

Figure 1. The apparent rate of L-[U-^{14}C]Glu uptake and release of [^{14}C]GABA over time. Cells were incubated and stirred in 4.2 mL of 1 mM $CaSO_4$ in the light at 30°C. Immediately after the addition of 0.82 mM L-[U-^{14}C]Glu and every 5 min thereafter for 30 min, 2 × 200-μL aliquots of cell suspension were removed. The cells from one aliquot were collected and washed on a Millipore filter apparatus, and the ^{14}C content of the cells was determined to give a measure of L-Glu uptake. The other aliquot was centrifuged to separate the cells from the medium. [^{14}C]GABA in the medium was isolated by TLC and radioactivity determined to give a measure of GABA efflux. Each point indicates the mean (± SD) of five trials. GABA efflux (○); L-Glu uptake (●).

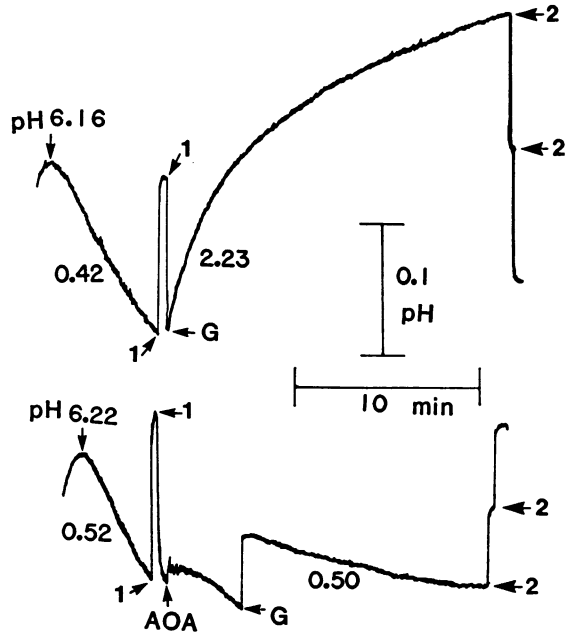

Figure 2. Representative tracings showing L-Glu-dependent alkalinization and inhibition by AOA. Upper trace, Cells were incubated, stirred, and aerated in 10 mL of 1 mM $CaSO_4$ in the light at 30°C. Once a constant rate of acidification was reached, the cell suspension was titrated with 20-nmol additions of NaOH and HCl (1); 2.5 mM L-Glu was then immediately added (G). After 20 min, the cell suspension medium was titrated with 100-nmol additions of HCl (2). Lower trace, Same as upper trace but (a) 2 mM AOA was added to the cells after the initial titration, and (b) the cell suspension medium was titrated with 100-nmol additions of NaOH at the end of the experiment (2). Rates of acidification and alkalinization are expressed in nmol $H^+ \cdot 10^6$ $cells^{-1} \cdot min^{-1}$.

essentially eliminated L-Glu-dependent alkalinization, L-Glu uptake, and GABA efflux (Figs. 2–4). The data indicate that at time zero, some L-Glu is converted to GABA (Fig. 1). This may result from the time required to separate the suspension medium from the cells immediately after adding L-Glu. Another possible explanation is that a minor radioactive contaminant, which was present in the commercial preparation of L-[U-^{14}C]Glu, had a chromatographic mobility slightly less than that of GABA, and some portion may have been eluted with GABA prior to counting of radioactivity.

Other than CO_2, labeled GABA was the only enzyme-dependent product of the *in vitro* GAD assays, and its level increased with addition of 0.5 mM PLP. The rate of labeled CO_2 release was linear for at least 30 min, and a twofold increase in CO_2 production was observed on the addition of saturating 0.5 mM PLP. Activity observed in the absence or presence of 0.5 mM exogenous PLP was completely eliminated by the addition of 2 mM AOA. The sulfhydryl reagents *p*-chloromercuriphenyl-sulfonate or Hg^{2+} inhibited decarboxylation, whereas GABA and succinate, metabolites of the GABA-shunt pathway, were not inhibitory (Table I).

A sharp pH optimum for GAD activity was observed at 6.0. This pattern was obtained with either a phosphate/citrate buffer or a potassium phosphate buffer (Fig. 5). GAD activity

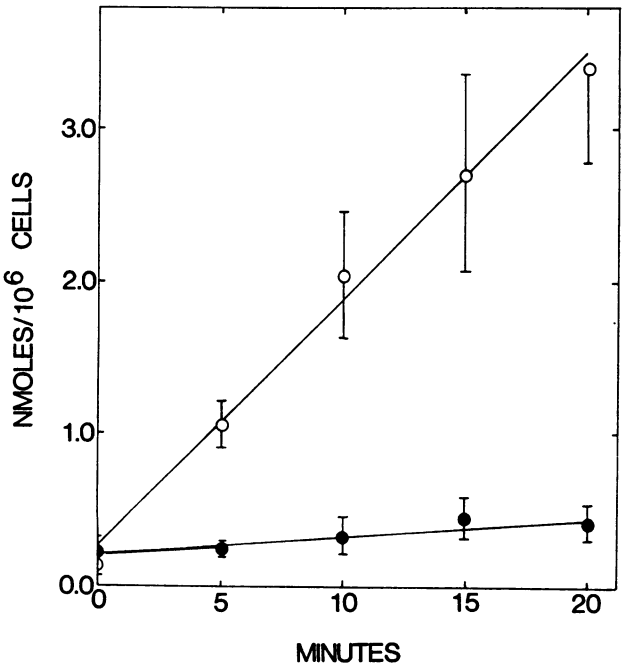

Figure 3. Rate of L-[U-^{14}C]Glu uptake in the presence and absence of 1 mM AOA. Cells were suspended in 1 mM $CaSO_4$ at pH 6.0 and 30°C. Immediately after the addition of L-[U-^{14}C]Glu (0.82 mM final concentration) and every 5 min thereafter for 20 min, a 200-μL aliquot was removed. The cells were washed and collected on a Millipore filter apparatus prior to scintillation counting and calculation of the apparent rate of L-Glu uptake. Cells were preincubated for 5 min with (●) or without (○) 1 mM AOA. Each point indicates the mean (±SD) of three trials.

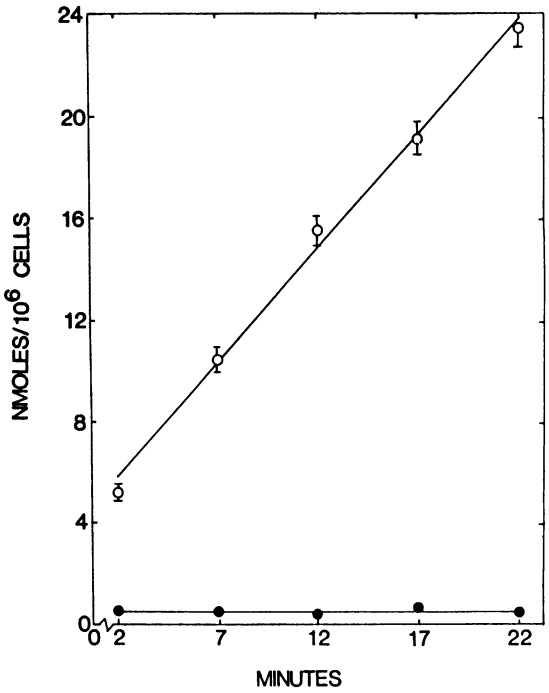

Figure 4. Rate of [^{14}C]GABA efflux in the presence or absence of 1 mM AOA. These results were obtained with the same cell suspension used to generate the data in Figure 3. Two minutes after the addition of L-[U-^{14}C]Glu and every 5 min thereafter for 20 min, a 200-μL aliquot of cell suspension was removed and centrifuged. Aliquots of the supernatant fluid together with unlabeled GABA were applied to TLC plates for chromatographic analysis. GABA spots detected with ninhydrin were transferred to scintillation vials for counting and calculation of the rate of GABA efflux. Cells were preincubated for 5 min with (●) or without (○) 1 mM AOA. Each point is the mean (±SD) of three trials.

increased 12-fold when the pH was reduced from 7.5 to 6.0. Plots of enzymic activity resulted in K_m estimates of 5.8 and 10.3 mM for L-Glu at pH 6.0 and 7.0, respectively. V_{max} values decreased from 9.1×10^{-4} to 4.5×10^{-4} nmol CO_2 min^{-1} mg^{-1} protein with an increase in pH from 6.0 to 7.0.

Both sodium butyrate and sodium acetate caused a rapid alkalinization followed by a roughly twofold stimulation in the rate of proton efflux. Acidification rates were stimulated from 0.13 (±0.04 SD) to 0.26 (±0.10 SD) nmol $H^+ \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ by 1.5 mM butyrate, and from 0.14 (±0.03 SD) to 0.30 (±0.06 SD) nmol $H^+ \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ by the same concentration of acetate (Fig. 6). Controls indicated that neither alkalinization nor acidification were due to Na^+; cell-free controls demonstrated that these phenomena were cell-dependent.

DISCUSSION

The cytoplasmic buffering capacity of 14.9 μmol $H^+ \cdot mL^{-1}$ cytoplasm·pH $unit^{-1}$ is not substantially different from a value of 20 to 40 μmol $H^+ \cdot mL^{-1}$cytoplasm·pH $unit^{-1}$ found in cultured *Acer* cells (8). Calculations based on this value, cell volume (4.7×10^{-3} $mL \cdot 10^6$ $cells^{-1}$), and the rate of 2.5 mM L-Glu-dependent H^+ influx (2.23 nmol $H^+ \cdot 10^6$ $cells^{-1} \cdot min^{-1}$; Fig. 2) indicate that, in the absence of regulatory

Table 1. *In Vitro Inhibition of GAD*

Assy conditions were as in Figure 5. Values are the means of two separate experiments each performed in duplicate. Abbreviations: MSO, L-methionine sulfoximine; PCMS, *p*-chloromercuriphenyl sulfonic acid.

Compound	Concentrations	Inhibition
	mM	%
Amino oxyacetate[a]	2 and 4	100
GABA	2 and 5	0
Succinate	2 and 5	0
PCMS	2	88
Hg^{2+}	2	50
MSO	10	9

[a] Experiments were performed in the presence or absence of added 0.5 mM PLP.

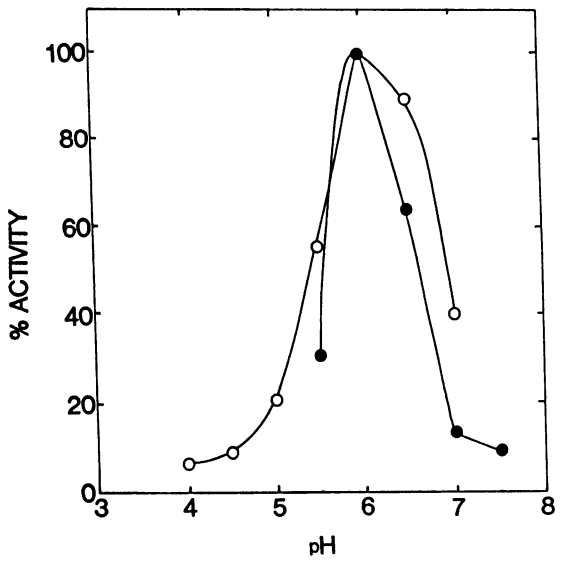

Figure 5. Effect of pH on GAD activity. The experimental conditions are described in "Materials and Methods." The assay system contained McIlvain's buffer (○) or potassium phosphate buffer (●). Values are the means of three experiments each involving duplicate assays.

mechanisms, the internal pH should decrease at a rate of 1.9 pH units·h^{-1}. Assuming that the cytosol occupies no more than 20% of cytoplasmic volume, H^+ influx into this compartment might be expected to generate a much more rapid change in pH. However, calculations based on cell volume and the redistribution of [^{14}C]5,5-dimethyloxazolidine-2,4-dione after the addition of 5 mM L-Glu (15) indicate a rate of 0.5 pH units·h^{-1}. Thus, active regulation of cytoplasmic pH is indicated. The possible role of GAD-catalyzed L-Glu decarboxylation in pH regulation has been investigated.

The properties of GAD observed in this study are similar to those described in previous studies. GAD activity was dependent upon PLP as reported for GAD from leaf tissue (26), potato tuber (23), and squash fruit (13). AOA eliminated GAD activity measured in the presence or absence of PLP, suggesting that activity independent of exogenous PLP was due to PLP retained during enzyme extraction. Similar findings were obtained for potato tuber (23) and sunflower cotyledons (24). GAD was also inhibited by reagents known to react with sulfhydryl groups. A requirement for sulfhydryl groups has been reported previously (23, 26). GAD activity displayed a sharp pH optimum at about 6.0. This value agrees with values reported by other workers (23, 25, 26). The cytoplasmic pH of *Asparagus* mesophyll cells has been estimated at between pH 6.9 and 7.2 (5). Thus, any decrease in intracellular pH may well stimulate a large increase in GAD activity. The K_m value for L-Glu at pH 6.0 is 8.1 mM, within the range of 3.1 to 9.1 mM reported by other workers (9, 23, 24, 26). The large decrease in K_m and increase in V_{max} values with a pH decrease from 7.0 to 6.0 suggests an increased substrate affinity and increased catalytic rate at more acidic pH values.

The regulation of intracellular acidification through the stimulation of proton-consuming decarboxylating reactions at lower pH values has been proposed previously. Malate decarboxylation has been the primary focus of this metabolic pH-stat proposal (8, 12). However, the regulation of cellular pH through the decarboxylation of L-Glu has been suggested by recent studies concerning acidosis in response to anoxia (16, 18, 19). The present study is novel because the acid load is imposed by a specific H^+/L-Glu symport, and the molecular species involved in the symport system are also the substrates for decarboxylation and H^+ consumption. Given that 80% of the L-Glu entering the cells leaves as GABA (Fig. 1) and assuming a 2:1 stoichiometry for H^+/L-Glu cotransport (28), the data suggest that approximately 40% of the protons that enter with L-Glu are consumed during decarboxylation. This value may be an underestimation, because not all of the GABA produced is exported (3).

It is likely that the remainder of the protons that enter with L-Glu are accounted for through biophysical pH-stat processes and physicochemical buffering. It is difficult to demonstrate H^+/L-Glu-stimulated H^+ efflux while net H^+ influx is occur-

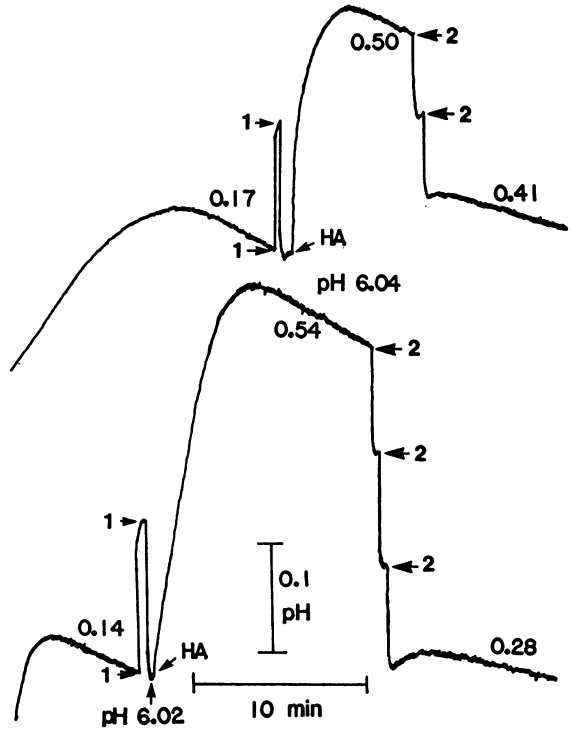

Figure 6. Representative tracings showing the effect of two weak acids on the rate of cell-dependent H^+ efflux. Cells were incubated, stirred, and aerated at 30°C in the light. When a steady rate of acidification was obtained, the medium was titrated with 20 nmol of NaOH and 20 nmol of HCl (1). This was followed by the addition of a weak acid (HA), 1.5 mM Na butyrate (upper trace), or 1.5 mM Na acetate (lower trace). After a new rate of acidification was obtained, the medium was titrated with 50-nmol additions of HCl (2) to lower the pH to its previous level and to measure the new buffering capacity at that point. Rates of H^+ efflux before and after addition of weak acid were then calculated using the appropriate buffering capacity. Rates are expressed in nmol $H^+ \cdot 10^6$ cells$^{-1} \cdot$ min^{-1}.

ring (Fig. 2). However, this possibility can be demonstrated with weak acids that cross the plasma membrane in their uncharged form and dissociate to acidify the cytosol (6, 8, 12). In the present study, weak acids stimulated H^+ efflux, suggesting that cytoplasmic acidification stimulates ATP-driven proton efflux (Fig. 6). The initial alkalinization observed after the addition of a weak acid is most probably due to the rapid influx of the protonated uncharged species, resulting in protonation of the external charged species to maintain equilibrium. Other workers have also observed a stimulation of proton efflux resulting from exposure to weak permeant acids (6, 12, 19, 22) or sucrose/H^+ cotransport (11). However, continued medium alkalinization (Fig. 2) in response to L-Glu demonstrates that ATP-driven H^+ efflux can not cope with the entire acid load imposed by H^+/L-Glu symport. Similarly, in other systems, rates of H^+ pumping alone were not enough to compensate for the imposed acid load (6, 11, 12, 19, 22). In *Acer* cells, estimates for the relative contribution of biophysical pH-stat and metabolic pH-stat processes in resisting acid load suggest an equal contribution from each (12).

The data in this report provide evidence that H^+/L-Glu cotransport and GABA efflux in *A. sprengeri* mesophyll cells are sustained by H^+-stimulated GAD activity. GAD may not only maintain the symport by removing H^+ and L-Glu, but may also act as a metabolic pH stat. Increased plasma membrane H^+ ATPase activity may also play a role in regulating cellular pH during H^+/L-Glu symport. A working hypothesis is indicated in Figure 7. This model is consistent with sustained medium alkalinization (Fig. 2), L-Glu uptake (Fig. 3), and GABA efflux (Fig. 4). In addition, it indicates approximately equivalent roles for GABA production and ATP-driven H^+ efflux in resisting cytoplasmic acidification.

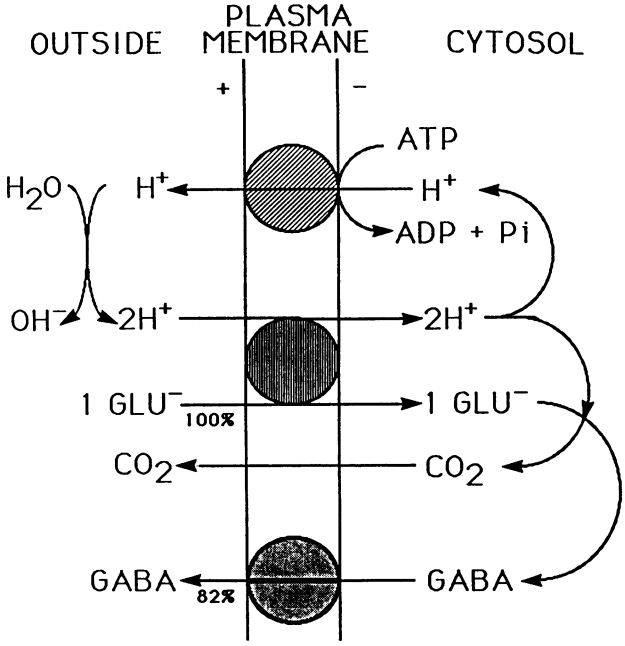

Figure 7. Two active mechanisms of pH regulation that may ameliorate an acid load during H^+/L-Glu cotransport. A metabolic pH-stat consumes H^+ when GAD converts L-Glu to GABA + CO_2. An ATP-driven biophysical pH stat pumps H^+ out of the cytosol into the extracellular medium.

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