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Communication

Estimation of Ammonium Ion Distribution between Cytoplasm and Vacuole Using Nuclear Magnetic Resonance Spectroscopy[1]

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ABSTRACT

Evidence is presented that intracellular ammonium is trapped in vacuoles of maize (*Zea mays* L.) root tips because of rapid movement of ammonia between cytoplasm and vacuoles. The concentration of cytoplasmic ammonium is estimated to be <15 μM at extracellular ammonium concentrations up to 1 mM. The implications for pathways of ammonium assimilation are discussed.

The concentration of NH_4^+ in the various compartments of plant cells has a direct bearing on the pathway of NH_4^+ assimilation, because the enzymes GDH[2] and GS have very different affinities for NH_4^+ (25). Published estimates of the concentration of NH_4^+ in maize leaf mitochondria (31), root cytoplasm (10), and soybean nodule cytosol (7, 29) have been in the millimolar range, similar to the K_m of GDH for NH_4^+. These results suggest that GDH could catalyze significant assimilation of NH_4^+ in plants. However, Streeter (27) estimated the NH_4^+ concentration in soybean nodule cytosol to be "essentially nil."

The cytoplasmic NH_4^+ concentration may also be important with respect to pH gradients between intracellular compartments; millimolar concentrations of NH_4^+ can collapse transmembrane pH gradients in vitro (3). Solutions of NH_3 have been shown to rapidly and dramatically increase intracellular pH (15, 21). Biomembranes are highly permeable to NH_3 (6). However, the significance of transmembrane fluxes of NH_3 in plant cells exposed to solutions in which NH_4^+ predominates over NH_3, as occurs under normal physiological conditions, is unclear (cf. refs. 6, 10, 12, 13, 16). Here, we describe the effects of extracellular NH_4^+ on cytoplasmic and vacuolar pHs using ^{31}P and ^{13}C NMR, respectively. We present evidence for rapid equilibration of NH_3 between cytoplasm and vacuole and estimate the concentrations of NH_4^+ in the different intracellular compartments.

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[2] Abbreviations: GDH, glutamate dehydrogenase; GS, glutamine synthetase.

MATERIALS AND METHODS

Maize (*Zea mays* L.; Funk hybrid 4323 from Germain's Seeds, Los Angeles, CA) root tips were harvested and treated as described previously (19). Tissue samples were initially perfused with 100 mL of oxygenated medium that was recirculated for 3 h at 10 mL/min ("pretreatment"). The pretreatment perfusion medium contained 50 mM Glc (^{31}P NMR experiments) or [1-^{13}C]Glc (Isotech, Miamisburg, OH; ^{13}C NMR experiments) in 0.1 mM $CaSO_4$, 10 mM Mes (brought to pH 6.5 with Tris), plus the antibiotics gentamycin and amphotericin B at 50 and 2.5 mg/L, respectively. The extracellular pH increased to approximately 7 after 3 h. Ammonium treatment consisted of supplementing the oxygenated medium with various concentrations of ammonium sulfate; at the highest NH_4^+ concentration (10 mM), extracellular pH decreased less than 0.1 pH unit. Experiments were carried out at room temperature.

^{31}P and ^{13}C NMR spectra were obtained using a General Electric GN500 spectrometer as described previously (1). Cytoplasmic and vacuolar pH were measured using ^{31}P NMR, which is most accurate for cytoplasmic pH measurements (17); only the large vacuolar pH changes that occur at high extracellular NH_4^+ were detectable by ^{31}P NMR (data not shown; cf. ref. 13), because of the insensitivity of ^{31}Pi chemical shifts at pH values below 6 (20). ^{13}C NMR spectra of ^{13}C-labeled malate were used to obtain sensitive measurements of vacuolar pH (1, 18, 26).

At the end of NMR experiments, samples were frozen in liquid nitrogen. Low mol wt metabolites were extracted with 5% $HClO_4$ and then centrifuged and neutralized with KOH. ^{13}C NMR analysis in vivo (cf. ref. 19) and of corresponding tissue extracts indicated no significant breakdown of amino acids (e.g. Gln) during or subsequent to extraction (data not shown). Ammonium in extracts was measured using an NH_3 electrode (Orion Research, Boston, MA) according to the manufacturer's directions.

RESULTS AND DISCUSSION

Exposure of oxygenated maize root tips to millimolar concentrations of NH_4^+ (external pH 7) leads to an increase in vacuolar pH, as determined using ^{13}C NMR spectroscopy (Fig. 1, Table I). Malate ^{13}C NMR signals reveal heterogeneity in vacuolar pH (1, 8); the vacuolar pH values given in Table

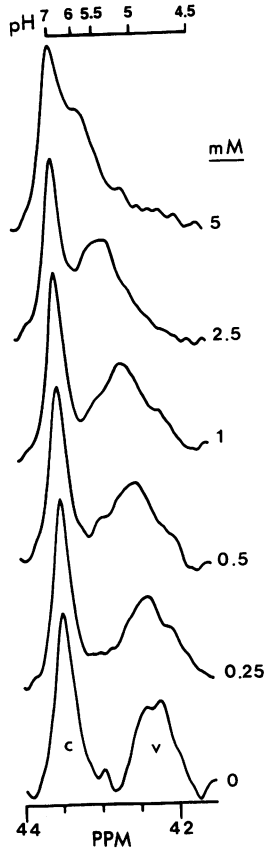

Figure 1. Effect of exogenous ammonium sulfate on vacuolar pH in oxygenated maize root tips. In vivo ^{13}C NMR partial spectra of cytoplasmic (c) and vacuolar (v) [3-^{13}C]malate resonances in root tips treated with $(NH_4)_2SO_4$ at concentrations indicated at right. The top scale of the spectra indicates the chemical shifts of the malate resonance at different pHs, determined by titration of ^{13}C-labeled root tip extracts (cf. ref. 1). Spectra were acquired 30 to 60 min after addition of NH_4^+, 3.5 to 4 h after perfusion in oxygenated 50 mM [1-^{13}C]Glc began.

I are average values. These results are similar to previous reports of increases in vacuolar pH in NH_4^+-fed plants using the 5,5-dimethyloxazolidine-2,4-dione method (16) and ^{31}P NMR (13). The value of the ^{13}C NMR method used here is that it provides greater time resolution than the 5,5-dimethyloxazolidine-2,4-dione method and greater sensitivity to vacuolar pH changes than the ^{31}P NMR method (see "Materials and Methods"). In vivo, ^{31}P NMR spectroscopy indicated no significant effects of NH_4^+ treatments on cytoplasmic pH (Table I).

The NH_4^+-induced vacuolar pH changes occur quickly, being greatest 30 to 60 min after addition of 5 mM NH_4^+; thereafter, partial recovery of vacuolar pH to more acidic values is apparent (Fig. 2). This partial recovery is not due to depletion of exogenous NH_4^+, which was insignificant in the experiment in Figure 2 (data not shown). The recovery may be due to activation of proton pumps on the tonoplast membrane in response to the decreased proton electrochemical gradient between cytoplasm and vacuole, as has been described for tonoplast H^+-ATPase activity in vitro using ionophores (28).

The above results indicate rapid net movement of NH_3 from the external medium into vacuoles and its accumulation there as NH_4^+ through combination with vacuolar protons. The magnitude of the NH_4^+-induced increases in vacuolar pH is consistent with the accumulation of most intracellular NH_4^+ in vacuoles (Table I), based on entry of this NH_4^+ into vacuoles as NH_3, and a buffering capacity of vacuoles in maize root tips of approximately 7 μeq H^+/g tissue (19). Trapping of NH_4^+ in an acidic compartment is predicted in systems in which membrane permeability to NH_3 greatly exceeds that for NH_4^+ (6). High membrane permeability to NH_3, in solutions in which NH_4^+ predominates, is indicated by the ability of millimolar concentrations of ammonium chloride to collapse pH gradients across tonoplast vesicles (3) and to alkalinize chloroplast compartments (2, 4) in vitro. Illustrated descriptions of this process are given in refs. 2 and 6. In such systems, the ratio of NH_4^+ concentrations in different compartments equals the ratio of H^+ concentrations at equilibrium (6). From this relationship, we can estimate the concentration of cytoplasmic NH_4^+ at different extracellular NH_4^+ concentrations (Table I). Because mitochondrial pH is expected to be similar to or slightly higher than cytoplasmic pH, mitochondrial NH_4^+ concentrations can be estimated to be similar to or slightly lower than cytoplasmic levels. Cytoplasmic NH_4^+ remains $\leq$15 μM until extracellular ammonium levels reach 5 mM or higher. These higher extracellular NH_4^+ concentrations may be toxic (cf. refs. 12, 23).

Several factors could cause deviation of cytoplasmic NH_4^+ levels from the estimated values in Table I. First, vacuolar pH heterogeneity (8), noted above, will lead to different

Table I. *Effect of Extracellular NH_4^+ on Intracellular pH Values and Intracellular NH_4^+ in Maize Root Tips*

Root tips were perfused for 3 h with 100 mL of oxygenated 50 mM glucose for 3 h, then ammonium sulfate was added to give the indicated concentration, and perfusion was continued for 3 h, at which time the tissue was frozen and extracted. Intracellular pH measurements were made during the last 30 min of perfusion. Intracellular ammonium was measured in cell extracts.

Treatment $[NH_4^+]$	Cytoplasmic pH	Vacuolar pH	Intracellular NH_4^+ Content	Estimated Cytoplasmic $[NH_4^+]$
mM			μmol/g[a]	μM[b]
0	7.6	4.9	0.48 ± 0.06 (9)	3
0.5	7.6	5.0	0.73 ± 0.14 (6)	5
1	7.6	5.1	1.14 ± 0.64 (4)	10
2	7.6	5.1	1.74 ± 0.34 (7)	15
5	7.6	5.3	7.24 ± 1.11 (7)	103
10	7.6	5.9	7.96 ± 2.06 (4)	438

[a] Tissue fresh weight; mean ± SE (*n*). [b] Calculated from:

$$\frac{1000 \times [H^+]_c}{0.65[H^+]_c + 0.35[H^+]_v} \times NH_4^+ \text{ content}$$

where the subscripts c and v refer to cytoplasm and vacuole, respectively, and the values 0.65 and 0.35 represent the estimated proportions of root tips occupied by cytoplasm and vacuole, respectively (cf. ref. 24).

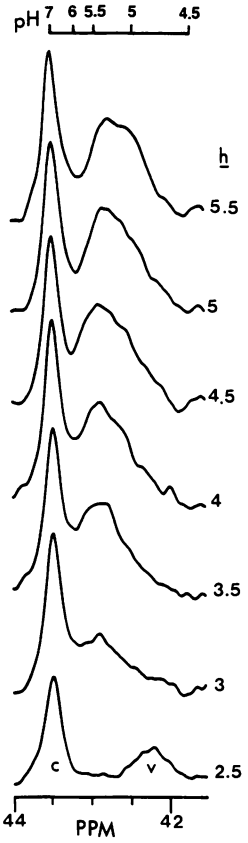

Figure 2. Time course for NH_4^+-induced vacuolar pH changes in oxygenated maize root tips. In vivo ^{13}C NMR partial spectra of cytoplasmic (c) and vacuolar (v) [3-^{13}C]malate resonances in root tips treated with 2.5 mM $(NH_4)_2SO_4$. Spectra were obtained consecutively from bottom to top during 30-min intervals, as indicated; NH_4^+ was added at 3 h. The pH scale relates malate chemical shifts to pH (see legend to Fig. 1). The increase in malate signal intensity over time reflects the stimulation of phospho*enol*pyruvate carboxylase by the NH_4^+ treatment (cf. ref. 30).

degrees of NH_4^+ trapping in different vacuoles/cells. Second, the outermost root tip cells will experience greater extracellular NH_4^+ levels than inner cells, this gradient in extracellular NH_4^+ being greatest at high treatment concentrations and early after NH_4^+ treatment begins. Both of these factors require that the estimated cytoplasmic NH_4^+ concentrations in Table I be taken as average values. Third, flux of NH_4^+ into the cytoplasm, from vacuoles or the extracellular space, would cause higher values of cytoplasmic NH_4^+ than those given in Table I. However, such NH_4^+ fluxes are likely to be much smaller than fluxes of NH_3, given the high intrinsic permeability of biomembranes to NH_3 (6). Furthermore, if NH_4^+ transport occurred via NH_4^+-H^+ exchange (reviewed in ref. 11), transport of NH_4^+ from cytoplasm to vacuoles would be favored, because of the pH gradient between these compartments, until cytoplasmic NH_4^+ reached the values reported in Table I. Fourth, incorporation of cytoplasmic NH_4^+ into amino acids will tend to lower the concentration of cytoplasmic NH_4^+ below the estimates given in Table I.

The results and analysis presented here may be useful in accounting for uncertainties about the pathway for NH_4^+ assimilation in higher plants (9, 14). Results of in vivo experiments have tended to support the view that GS is primarily responsible for NH_4^+ assimilation (9, 11, 22), whereas in vitro mitochondrial GDH catalyzes NH_4^+ assimilation in the presence of millimolar amounts of NH_4^+ (31, 32). The estimated cytoplasmic NH_4^+ concentrations of approximately 10 μM in Table I are of similar magnitude to measured K_m values of higher plant GS for NH_4^+ (10–20 μM), and much lower than the corresponding K_m values for higher plant GDH (10–80 mM) (25). Setting aside the complications of extrapolating in vitro K_m estimates to conditions in vivo (cf. ref. 12), micromolar concentrations of cytosolic and mitochondrial NH_4^+ would kinetically preclude significant catalysis of NH_4^+ assimilation by GDH and can explain why NH_4^+ assimilation in higher plants in vivo is virtually completely blocked by inhibitors of GS (9, 11, 22). Millimolar concentrations of NH_4^+ have been found in maize leaf mitochondria (31), in contrast to the much lower concentrations estimated here. This discrepancy may be due to mixing of the mitochondria with large pools of vacuolar NH_4^+ during extraction in the earlier study (31).

Our results also suggest a mechanism for NH_4^+ toxicity in plants, viz. vacuolar alkalinization. The increase in vacuolar pH at high extracellular NH_4^+ could be deleterious in two ways. First, accumulation of many metabolites and inorganic ions is driven by the proton motive force across the tonoplast (5); therefore, a collapse in the pH gradient between cytoplasm and vacuole will inhibit accumulation of metabolites in vacuoles and perturb cytoplasmic levels of these species. Second, movement of NH_3 into vacuoles will undermine the action of tonoplast proton pumps (28). This could increase intracellular ATP consumption by stimulating H^+-ATPase activity, as has been observed in vitro using tonoplast vesicles and ionophores (28).

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