Cytosolic Concentrations and Transmembrane Fluxes of NH_4^+/NH_3. An Evaluation of Recent Proposals

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Ammonium (NH_4^+) is an important, perhaps underestimated, nitrogen source for plant growth (Bloom, 1988; Glass, 1988; Kronzucker et al., 1997). In many agricultural soils it is present in millimolar concentrations in soil solution (Wolt, 1994), whereas in mature forest soils NH_4^+:NO_3^- ratios as high as 56:1 have been reported (Stark and Hart, 1997). In many such soils, NH_4^+ may constitute the only inorganic nitrogen source available to plants (Van Cleve et al., 1993). Physiological studies have demonstrated that NH_4^+ fluxes commonly exceed those of NO_3^- from equimolar solutions, and when both ions are present, NO_3^- influx is reduced within minutes of exposure to NH_4^+ (Lee and Drew, 1989; Gazzarini et al., 1999; Kronzucker et al., 1999). As a byproduct of photorespiration and the shikimic acid pathway, as well as through the turnover of various nitrogen pools, large quantities of NH_4^+ are also generated internally (Joy, 1988; Feng et al., 1998). Although much of this NH_4^+ is re-assimilated through the activity of Gln synthetase (GS), many plants show high values of NH_4^+ efflux, and some develop symptoms of toxicity when exposed to millimolar concentrations of NH_4^+ in nutrient media, particularly when NH_4^+ is the sole source of nitrogen. The actual mechanism of NH_4^+ toxicity is still unclear, despite extensive studies (Gerendas et al., 1997). For these reasons it is important to characterize subcellular compartmentation and fluxes of NH_4^+.

Because of the potential toxicity of NH_4^+/NH_3, it is often assumed that plant cytosolic NH_4^+ concentrations ($[NH_4^+]_c$) are maintained at very low (submillimolar) levels, through the high activity and high affinity of GS for NH_4^+ (Pearson and Stewart, 1993; Gerendas et al., 1997). This is despite the fact that millimolar values for $[NH_4^+]_c$ have been measured using NMR, efflux analysis, ion-specific microelectrodes, and tissue fractionation (Fentem et al., 1983; Lee and Ratcliffe, 1991; Wang et al., 1993a; Wells and Miller, 2000), and that large plasma membrane fluxes of NH_4^+ have been reported (Lee and Ayling, 1993; Wang et al., 1993b; Min et al., 1999). Nevertheless, in a recent review of NH_4^+ transport, Howitt and Udvardi (2000) conclude that some estimates of $[NH_4^+]_c$ are probably orders of magnitude too high. First, they argue that "it is difficult to reconcile the estimates of cytosolic NH_4^+ concentrations made by this group (referring to the data of Wang et al., 1993a) with the high affinity of cytosolic GS for NH_4^+ (K_m = 10–20 μM)." Second, they cite the findings of a single NMR study by Roberts and Pang (1992) who estimated $[NH_4^+]_c$ to be 3 to 10 μM for external $[NH_4^+]$ ($[NH_4^+]_o$) up to 1 mM. Howitt and Udvardi (2000) go on to suggest that both influx and efflux of NH_3 are mediated by passive diffusion through a reversible low-affinity transport system (LATS), previously considered to be an NH_4^+ transporter. Finally, they suggest that NH_4^+ entry to the vacuole is via passive permeation of NH_3 and acid trapping of NH_4^+. These speculations are at variance with a large body of evidence in the literature and therefore need to be critically examined.

CYTOSOLIC NH_4^+ CONCENTRATIONS

Howitt and Udvardi (2000) suggest that $[NH_4^+]_c$ must be considerably lower than the 3.6 mM estimated by Wang et al. (1993a) for plants grown at low external $[NH_4^+]$ because of the reported low K_m values for NH_4^+ (10–20 μM) of GS. However, it must first be emphasized that K_m values of enzymes determined in vitro might be very different from those operating in vivo because of regulation by allosteric effectors. Furthermore, the extensively studied glycolytic enzymes have $K_m/[substrate]_c$ ratios that vary from 0.02 to 333 (Fersht, 1985), and similar deviations of $K_m/[substrate]_c$ from unity can be found for the enzymes nitrate reductase and pyruvate kinase. In the first case, $K_m/[NO_3^-]_c$ can be as low as 0.01 (Lee and Clarkson, 1986; Siddiqi et al., 1991; Miller and Smith, 1992; Kanayama et al., 1999), whereas in the second, the ratio can be as low as 0.02 (Memon et al., 1985a, 1985b; Walker et al., 1996).

Furthermore, catalytic rates of enzymes that are subject to allosteric regulation are typically determined by the concentration of regulatory molecules rather than by substrate concentration. For example, the hexokinase of erythrocytes has a K_m value of 0.1 mM for Glc, despite a cellular Glc concentration around 5 mM. Hexokinase activity is not regulated by the available Glc but as a result of allosteric inhibition

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from its product Glc-6-phosphate, with the result that large changes of Glc concentration hardly alter the rate of glycolysis (Fersht, 1985). GS is also known to be a highly regulated enzyme, located at a pivotal biochemical position to regulate plant nitrogen assimilation (Eisenberg et al., 2000), and available data indicate that $[NH_4^+]_c$ can vary considerably even in the same system under different conditions of $[NH_4^+]_o$ supply (Lee and Ratcliffe, 1991; Roberts and Pang, 1992; Wang et al., 1993a; Kronzucker et al., 1995). The rationale that an enzyme's K_m should necessarily track ambient substrate concentration to optimize reaction rate, therefore, fails to apply to such enzymes.

Roberts and Pang (1992), cited by Howitt and Udvardi (2000) in support of micromolar values of $[NH_4^+]_c$, incubated excised maize root tips in solutions containing from 0 to 10 mM NH_4^+, and used NMR signals from ^{31}P and ^{13}C to estimate cytosolic and vacuolar pH. $[NH_4^+]_c$ was then calculated from total root NH_4^+ to be from 2 to 438 μM (the upper limit being already $\geq$20 times higher than the reported GS K_m for NH_4^+), on the presumption that NH_3 rapidly equilibrated across the tonoplast and was trapped as NH_4^+ according to cytosolic and vacuolar pH values. In contrast, Lee and Ratcliffe (1991) used a more direct ^{14}N-NMR method to obtain $[NH_4^+]_c$ of 3 to 8 mM, almost 3 orders of magnitude higher than the Roberts and Pang results, when comparing values obtained at the same $[NH_4^+]_o$. All other methods (tissue fractionation, efflux analysis, and NH_4^+-specific microelectrodes) have yielded $[NH_4^+]_c$ values in the millimolar range (Kronzucker et al., 1995, and references therein; Wells and Miller, 2000).

PASSIVE DIFFUSION OF NH_3 THROUGH AN LATS?

Transport of NH_4^+ across the plasma membrane is biphasic (Ullrich et al., 1984; Wang et al., 1993b), corresponding to an active, saturable, high-affinity transport system and a passive, non-saturable LATS. Howitt and Udvardi (2000), however, propose that LATS transport occurs via a reversible NH_3 transporter. In support of this proposal, the authors cite a study of ^{14}C-methylamine ($CH_3NH_2/CH_3NH_3^+$) uptake by *Phaseolus vulgaris* leaves (Raven and Farquhar, 1981), but in fact this study showed that the relationship of influx to external pH and the equilibrium concentration of methylamine were "far higher than could be explained by the transport of CH_3NH_2 alone." Raven and Farquhar concluded that methylamine uptake, at least at pH values below 7, was predominantly as $CH_3NH_3^+$, and driven by the membrane electrical potential difference ($\Delta\psi$).

Howitt and Udvardi go on to claim that both NH_3 influx and efflux in roots, as well as leaves, probably occurs by diffusion through a reversible LATS, citing studies by Wang et al. (1993a) and Kronzucker at al. (1995). In fact, neither of these studies claimed that the efflux of ^{13}N was in the form of $^{13}NH_3$ because it is not possible to distinguish between the efflux of NH_3 and NH_4^+. Any NH_3 effluxing from cells into the external solution would immediately be protonated in the relatively acidic cell wall compartment (see Fig. 1, A and B), and thus all NH_4^+/NH_3 leaving the cytosol will "appear" as NH_4^+.

A further prediction arising from the suggestion that NH_3 efflux occurs passively via LATS is that NH_3 efflux should diminish as $[NH_4^+]_o$ (and thus $[NH_3]_o$) increases. However, measurements of ^{13}N tracer efflux have demonstrated that this flux increased from approximately 10% of influx to as high as 86% of influx as $[NH_4^+]_o$ is increased from 10 or 100 μM to 1 or 1.5 mM (Wang et al., 1993a; Kronzucker et al., 1995; Min et al., 1999). Such findings are inconsistent with passive efflux of NH_3.

PLASMA-MEMBRANE FLUXES OF NH_4^+/NH_3: A THERMODYNAMIC EVALUATION

To evaluate the hypotheses of Howitt and Udvardi, we have constructed a model for the directions of passive fluxes of NH_3 and NH_4^+ (Fig. 1, A and B), assuming $[NH_4^+]_c$ of 10 μM (Howitt and Udvardi, 2000) or 10,000 μM (Wang et al., 1993a). In this model, $[NH_4^+]_o$ was varied from 10 to 10,000 μM, pH values were set at 5, 7, and 5, respectively, for external solution, cytosol, and vacuole, $\Delta\psi$ values of -100 and $+10$ mV were selected for plasma membrane and tonoplast, respectively, (Wang et al., 1994), and $[NH_4^+]_v$ was set at 20,000 μM. This value was selected to represent a midpoint of a quite wide range of literature values (Lee and Ratcliffe, 1991; Roberts and Pang, 1992; Wang et al., 1993a; Wells and Miller, 2000). The quantitative analyses of the distribution of NH_4^+/NH_3, and directions of passive fluxes of these molecules that follow, are therefore based upon realistic values of pH, $[NH_4^+]_o$, $\Delta\Psi$, and $[NH_4^+]_v$. These values may vary somewhat under natural conditions but differences corresponding with orders of magnitude are unlikely.

The model predicts that when $[NH_4^+]_c$ is 10 μM (Fig. 1A), influx of NH_4^+ is passive until $[NH_4^+]_o$ falls below approximately 100 nM, a rare situation in most soils. Active influx of NH_4^+ would therefore be virtually unnecessary. This is a surprising conclusion given the abundant physiological and molecular evidence suggesting that the high-affinity transport system actively transports NH_4^+ at $[NH_4^+]_o$ of up to 655 μM (Ullrich et al., 1984; Wang et al., 1994; Gazzarini et al., 1999). In contrast, when $[NH_4^+]_c$ is 10,000 μM (Fig. 1B), active influx of NH_4^+ is required in the range of $[NH_4^+]_o \leq 500$ μM. This corresponds with the $[NH_4^+]_o$ at which a break between high- and low-affinity influx systems (Ullrich et al., 1984; Wang et al., 1993b; Kronzucker et al., 1996) and changes in membrane depolarization are observed (Wang et al., 1994). Figure 1A also indicates that if $[NH_4^+]_c$ is 10

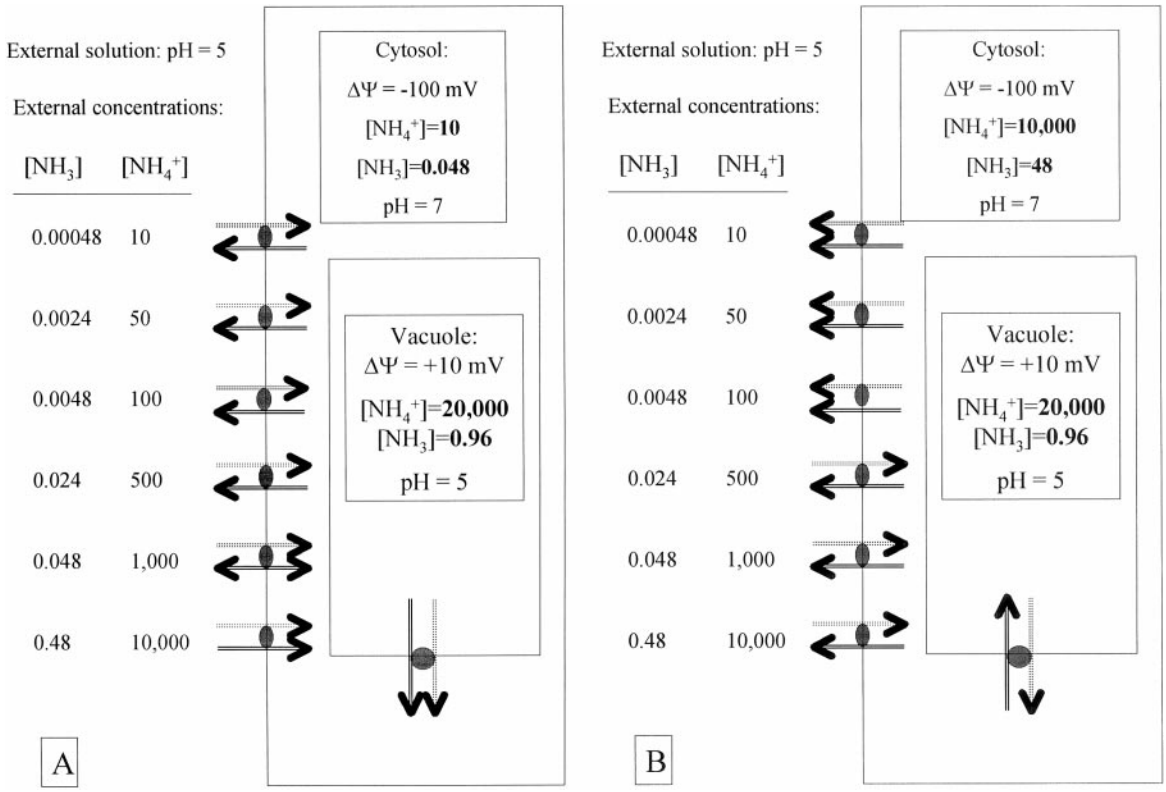

Figure 1. A, Directions of passive NH_4^+ (dotted arrows) and NH_3 (solid arrows) fluxes as functions of external $[NH_4^+]$, (all concentrations are in μmol L^{-1}), calculated from the Nernst equation, with cytosolic $[NH_4^+]$ set at 10 μM and vacuolar $[NH_4^+]$ at 20,000 μM. External $[NH_4^+]$ was set at values from 10 to 10,000 μM. The pK_a for dissociation of NH_4^+ was set at 9.32; external, cytosolic, and vacuolar pH values were set at 5, 7, and 5, respectively; plasma membrane and tonoplast $\Delta\Psi$ were set at −100 and +10 mV, respectively. B, As in Figure 1A, except that cytosolic $[NH_4^+]$ is set at 10,000 μM.

μM, passive NH_3 influx is not energetically feasible unless $[NH_4^+]_o$ is ≥1 mM. It is entirely impossible if $[NH_4^+]_c$ is 10 mM (Fig. 1B), because the gradient for NH_3 is always from cytosol to cell wall. In this regard, it is instructive to examine the effects of pH on $^{13}NH_3/^{13}NH_4^+$ influx. Published data reveal that low-affinity putative $^{13}NH_4^+$ fluxes declined from 18.63 to 11.44 μmol g^{-1} h^{-1} as external pH increased from 4.5 to 7.5 in rice roots (Wang et al., 1993b). Thus, despite a 1,000-fold increase of $[^{13}NH_3]_o$, tracer influx actually declined by 40%; a similar decline of $^{13}NH_4^+$ fluxes between pH 5 and 7, was observed by Kosegarten et al. (1997) using rice roots. The results argue against NH_3 influx at typical values of soil pH. This is not to suggest that NH_3 influx may not occur as the pK_a for NH_3/NH_4^+ (9.32) is approached or exceeded. However, this situation is rare in most soils.

VACUOLAR FLUXES OF NH_3/NH_4^+

With respect to vacuolar accumulation of NH_4^+, Howitt and Udvardi (2000) propose that the flux of NH_4^+ from cytosol to vacuole must be active. This is confirmed in our model for conditions where $[NH_4^+]_c$ is 10 μM, or when $[NH_4^+]_c$ is 10 mM and vacuolar $[NH_4^+]$ ($[NH_4^+]_v$) exceeds 5 mM. However, the authors consider it more likely that passive transport of NH_3 across the tonoplast, and acid trapping in the vacuole, provide the mechanisms for vacuolar NH_4^+ accumulation. This situation might apply when $[NH_4^+]_v$ is ≤1 mM, corresponding to an $[NH_3]_v \leq$ 0.048 μM. Above this value, however, as shown in Figure 1A, the gradient for NH_3 permeation across the tonoplast is in the opposite direction (from vacuole to cytosol), requiring active transport of NH_3 to the vacuole. The data reported by Lee and Ratcliffe (1991), Roberts and Pang (1992), and Wang et al. (1993a) provide $[NH_4^+]_v$ values that are >1 mM. This leads to the unlikely scenario whereby transport of NH_3 to the vacuole requires an active NH_3 flux, not a passive flux as they propose. Together with a passive leak of NH_3 in the opposite direction, this scenario would result in a futile cycling of NH_3 between cytosol and vacuole. In contrast, when $[NH_4^+]_c$ = 10,000 μM (Fig. 1B), the flux of NH_3 to the vacuole will always be passive, and remobilization of NH_4^+ to the cytosol is also passive.

CONCLUSIONS

$[NH_4^+]_c$ values in the millimolar range are confirmed by four different methodologies.

The single literature value for $[NH_4^+]_c$ that conforms to a micromolar value (Roberts and Pang, 1992) is predicted from measurements of cellular pH and total-tissue $[NH_4^+]$ in roots, rather than being based upon direct measurements of $[NH_4^+]_c$.

Conjectures regarding cytosolic [ion] based upon in vitro measurements of enzyme K_m values are untenable.

If $[NH_4^+]_c$ were 10 μM, then (a) Active transport of NH_4^+ would be unnecessary, unless $[NH_4^+]_o$ falls below 100 nM; (b) Passive influx of NH_3 is feasible at $[NH_4^+]_o \geq 1000$ μM, but the pH profile for ^{13}N tracer influx appears to contradict this possibility; (c) Passive efflux of NH_3 is thermodynamically feasible, but appears to be contradicted by the observation that ^{13}N efflux increases as $[NH_4^+]_o$ (and hence $[NH_3]_o$) is increased; and (d) If $[NH_4^+]_c$ is 10 μM, NH_3 can only traverse the tonoplast passively as long as $[NH_4^+]_v \leq 1$ mM, an unlikely situation when NH_4^+ is available in external media.

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