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OPINION

Nitrogen acquisition, PEP carboxylase, and cellular pH homeostasis: new views on old paradigms

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ABSTRACT

The classic biochemical pH-stat model of cytosolic pH regulation in plant cells presupposes a pH-dependent biosynthesis and degradation of organic acids, specifically malic acid, in the cytosol. This model has been used to explain the higher tissue accumulation of organic acids in nitrate (NO_3^-)-grown, relative to ammonium (NH_4^+)-grown, plants, the result of proposed cytosolic alkalinization by NO_3^- metabolism, and acidification by NH_4^+ metabolism. Here, a critical examination of the model shows that its key assumptions are fundamentally problematic, particularly in the context of the effects on cellular pH of nitrogen source differences. Specifically, the model fails to account for proton transport accompanying inorganic nitrogen transport, which, if considered, renders the H^+ production of combined transport and assimilation (although not the accumulation) to be equal for NO_3^- and NH_4^+ as externally provided N sources. We show that the model's evidentiary basis in total-tissue mineral ion and organic acid analysis is not directly relevant to subcellular (cytosolic) pH homeostasis, while the analysis of the ionic components of the cytosol is relevant to this process. A literature analysis further shows that the assumed greater activity of the enzyme phosphoenolpyruvate (PEP) carboxylase under nitrate nutrition, which is a key characteristic of the biochemical pH-stat model as it applies to nitrogen source, is not borne out in numerous instances. We conclude that this model is not tenable in its current state, and propose an alternative model that reaffirms the anaplerotic role of PEP carboxylase within the context of N nutrition, in the production of carbon skeletons for amino acid synthesis.

Key-words: ammonium; biochemical pH-stat; biophysical pH-stat; cytosolic pH; ion transport; malate; nitrate; nitrogen assimilation; PEP carboxylase.

INTRODUCTION

> "Model pH-stats are real enough – at least in the mind of their originators . . . The extent to which they represent reality in the fine control of cytosolic pH remains to be established." – Davies DD (1986), 'The fine control of cytosolic pH'

A remarkable feature of plant cells is that they are able to homeostatically maintain the pH of the cytosolic compartment (pH_{cyt}), which in an unstressed cell is about 7.2–7.5 (Felle 2001), corresponding to a $[H^+]_{cyt}$ of 32–63 nM. These values are even lower than that of the cytosolic calcium pool (~200 nM), and, like calcium homeostasis, proton homeostasis in the cytosol is sustained despite the adjacency of apoplastic and vacuolar spaces, which contain proton (and calcium) pools several orders of magnitude more concentrated than the cytosolic pool. Cytosolic pH homeostasis is a result of several processes, including the activities of intracellular open- and closed-system buffering components such as bicarbonate, phosphates, and protein buffers, and the active pumping, and channel-mediated transport, of protons between cytosol and apoplast or vacuole, in concert with the charge-compensating movement of other ions (Kurkdjian & Guern 1989; Felle 2001). The closed-system buffering capacity of the cytosol, however, appears to be limited relative to the scale of changes in cytosolic $[H^+]$ that are expected from normal metabolic reactions pertaining to growth and maintenance (Pfanz & Heber 1986; Kurkdjian & Guern 1989; Sakano 2001), while pH maintenance by the electrogenic pumping of protons across membranes bounding the cytosol may be limited by the availability of charge-compensating ion transport, which is required for maintenance of electrical homeostasis in the cell (Gerendás & Schurr 1999). Several biochemical or metabolic 'pH stats' have been proposed as additional mechanisms responsible for the control of pH homeostasis in the cytosol, the most widely recognized of which functions via the cytosolically localized, pH-dependent formation or destruction of carboxylic acid groups, in particular the 4-C carboxyl group of malic acid (Davies 1986; Raven 1986; Gerendás & Ratcliffe 2002). Its mechanism is thought to be regulated by the complementary pH-optima of the implicated enzymes (Fig. 1): specifically, the high-pH optimum of the carboxylation enzyme, phosphoenolpyruvate (PEP) carboxylase (in concert with the non-limiting activity, or co-regulation, of malate dehydrogenase), and the low-pH optimum of the decarboxylation enzyme, malic enzyme (Davies 1973; Mathieu 1982).

Although the biochemical pH-stat concept has received some degree of criticism & revision (Smith & Bown 1981;

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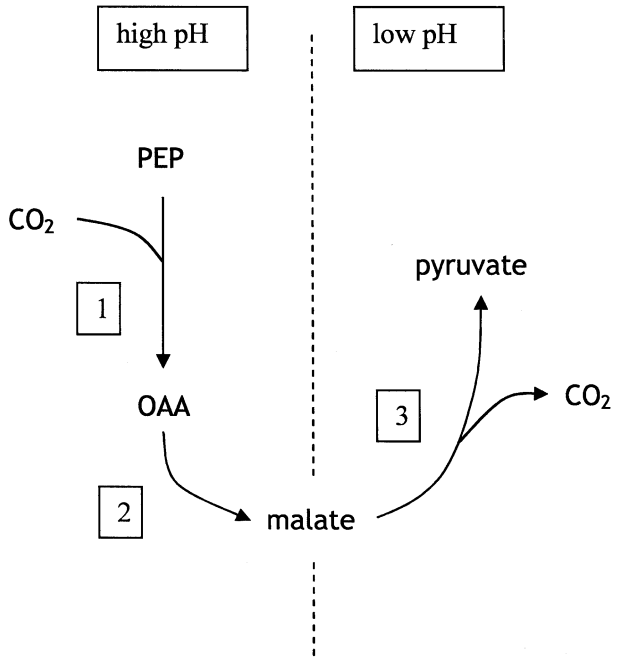

Figure 1. Summary of traditional concept of biochemical pH-stat. Enzymes 1 (PEP carboxylase) and 2 (malate dehydrogenase) operate when cytosolic pH is high, while enzyme 3 (malic enzyme) operates when pH is low. Note that the PEP carboxylase/malate dehydrogenase sequence does not actually release protons. The protons associated with the two carboxylic acid groups of malate were previously present; one was released in glycolysis (prior to the formation of PEP), the other produced and released in the solubilization of CO_2 via carbonic anhydrase. PEP, phosphoenol pyruvate; OAA, oxaloacetate.

Roberts *et al*. 1984; Davies 1986; Leport *et al*. 1996; Savchenko *et al*. 2000; Sakano 2001), it has become so widely accepted over the last few decades that it can be considered 'textbook knowledge' (Dennis & Turpin 1990; Salisbury & Ross 1992; Marschner 1995; Mengel & Kirkby 2001). In particular, cytosolic pH regulation by this mechanism is routinely implicated as a major response to the differential pH stresses proposed to result from variations in plant nitrogen nutrition (Kirkby & Mengel 1967; Raven & Smith 1974; Raven 1985; Schweizer & Erismann 1985; Allen & Smith 1986; van Beusichem, Kirkby & Bass 1988; Müller *et al*. 1990; Marschner 1995; Sagi *et al*. 1998; Pasqualini *et al*. 2001). In this view, nitrate (NO_3^-) assimilation is considered to be a proton-consuming process, as the summary reaction through nitrate and nitrite reductases suggests: $NO_3^- + 4[NAD(P)H + H^+] + 2H^+ \rightarrow NH_4^+ + 4[NAD(P)^+] + 3H_2O$. Ammonium ($NH_4^+$) assimilation, by contrast, is generally viewed as a proton-producing process, because NH_3, the inorganic substrate for glutamine synthetase, is produced from the deprotonation of ammonium: $NH_4^+ \rightarrow NH_3 + H^+$ (Kirkby & Mengel 1966; van Beusichem *et al*. 1988). [See note (1) in the Appendix.]

The proposed rectification of these opposite pH_{cyt} stresses by the biochemical pH-stat is commonly used to explain the well-documented increases in malate and other organic acid anions that are observed in nitrate-grown relative to ammonium-grown plants (Kirkby & Mengel 1966; van Beusichem *et al*. 1988; Lüttge *et al*. 2000; Pasqualini *et al*. 2001; however, diurnal oscillations in tissue malate contents complicate this picture – see Lang & Kaiser 1994). This result is somewhat counter-intuitive, in that malate accumulation is usually associated with plant cells absorbing more cations than anions (Ulrich 1941; Kirkby & Mengel 1967; Hiatt & Hendricks 1967; van Beusichem *et al*. 1988). Despite taking up more anions than cations, however, NO_3^--grown plants *accumulate* substantially fewer anions, due to the reduction of nitrate to ammonium; this 'anion deficit' (and the associated malate buildup) is even greater than that found with NH_4^+-grown plants (see van Beusichem *et al*. 1988).

In the present paper, we raise several key questions regarding fundamental principles and assumptions of the biochemical pH-stat model in the context of nitrogen assimilation. We argue that the prevailing view is incorrect, and propose alternative explanations for the physiological differences, as a function of N source, that proponents of the existing model have sought to explain.

THE PROTON ECONOMY AND NITROGEN ACQUISITION

Ion transport in plant cells is intimately tied to cellular $[H^+]$ regulation, since the energy sources driving most transport events are the electrical and $[H^+]$ potential differences across cell membranes, energized by H^+-pumping ATPases and pyrophosphatases (Serrano 1990; Barkla & Pantoja 1996; Sze, Li & Palmgren 1999; Martinoia, Massonneau & Frangne 2000; Palmgren 2001; Sakano 2001). The coupling of ion fluxes with co- or counter-fluxes of protons has emerged as such a ubiquitous theme in plant mineral nutrition that both active and passive ion transport can be regarded as based upon a 'proton economy', while the plasma membrane proton ATPase has been designated a 'master enzyme' (Serrano 1990; Marschner 1995), since it is the mechanism responsible for the primary establishment, and steady-state maintenance, of the plasma membrane electrical potential (Fig. 2). The quantity and direction of proton-coupled transport, however, is constrained by cellular requirements to maintain electrical homeostasis, as even a small imbalance between transported positive and negative charges across a membrane can have a substantial impact on the degree of membrane polarization (Clarkson 1974; Gerendás & Schurr 1999; Nobel 1999). For this reason, electroneutral ion fluxes must be achieved, except in the generation of the minute charge differential that brings about the membrane electrical potential. Studies of the mechanisms of isolated (unidirectional) ion fluxes indicate that ions typically cross the plasma membrane with an initial, electrogenic overshoot of positive charge, as membrane depolarization measurements upon sudden (re)supply of the ion show. In the case of nitrate, the anion assumes a cationic behaviour because the mechanism of its transport involves the cotransport of more than one proton (typically, two), and hence causes a depolarization (Meharg & Blatt

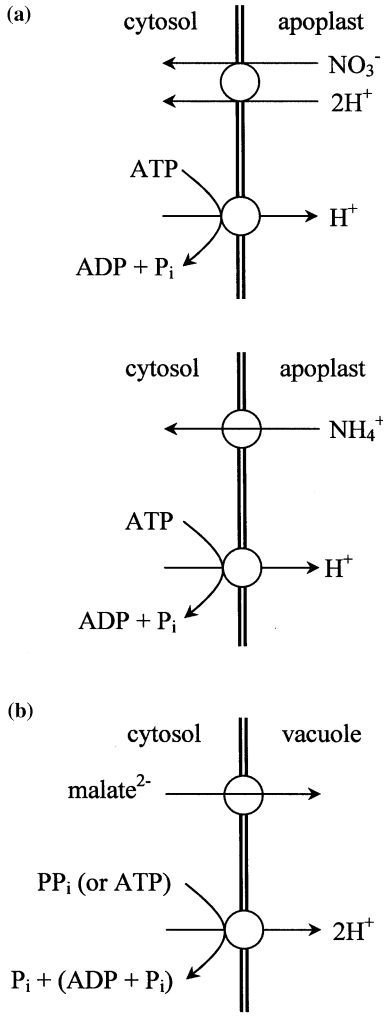

Figure 2. The role of proton transport as charge-balancer in inorganic nitrogen transport. (a) Transport of NO_3^- and NH_4^+ across the plasma membrane, rectified by proton pumping by membrane ATPase (Ullrich *et al*. 1984; Ullrich & Novacky 1990; Glass *et al*. 1992; Wang *et al*. 1994; Mistrik & Ullrich 1996); (b) Transport of malate ion across the tonoplast, from cytosol to vacuole, rectified by proton pumping by ATPase and/or pyrophosphatase (Gout *et al*. 1993; Cheffings *et al*. 1997).

1995; Mistrik & Ullrich 1996; Fig. 2a). Similar events occur with other transported anions (Mistrik & Ullrich 1996). These depolarization events are quickly stabilized, or fully rectified, by the plasma membrane ATPase (Ullrich *et al*. 1984; Glass *et al*. 1992; Wang *et al*. 1994; Mistrik & Ullrich 1996; White & Broadley 2001; Sakano 2001), indicating the attainment of electroneutrality. Thus, NH_4^+, K^+, and Ca^{2+} ions, which account for the great majority of cations normally taken into plant cells, must individually cross the plasma membrane in a counter-exchange with a resultant quantity of protons corresponding to the charge of the ion, while, similarly, the major anions NO_3^-, SO_4^{2-}, and $H_2PO_4^-$ are transported into the cell with cotransported protons, the quantity again corresponding to the charge on the transported nutrient ion (Ullrich & Novacky 1981, 1990; Serrano 1990; Mistrik & Ullrich 1996; Sze *et al*. 1999; Sakano 2001; also see Fig. 2a).

The proton-coupled transport processes for NO_3^- and for NH_4^+ are summarized in Fig. 2a, and the net result of proton coupling is that the chemical equivalents of nitric acid (NO_3^-, cotranported with one H^+) and ammonia (NH_4^+, counter-transported with one H^+) in effect appear inside the cytosol following the transport and subsequent membrane repolarization events (see Fig. 2a). For this reason, their accumulation poses a challenge to mechanisms maintaining cytosolic pH, since one is a strong acid, and the other a weak base that is nevertheless expected to be approximately 99% protonated at cytosolic pH (pK_a of $NH_4^+ = 9.25$). In other words, the uptake of nitrate is a cytosol-acidifying process whereas that of ammonium is a cytosol-alkalinizing one. These tendencies, rarely considered, work against the pH changes classically proposed to be involved in the subsequent metabolism of the ions taken up (see Introduction), although they agree with, if not fully explaining, the widely documented tendency for roots of nitrate-grown plants to alkalinize the external medium, and for roots of ammonium-grown plants to acidify it (Kirkby & Mengel 1966; van Beusichem *et al*. 1988; Magalhaes & Huber 1989; Marschner 1995; Mistrik & Ullrich 1996). Moreover, this agrees with a growing number of studies showing that a transient rise in cytosolic pH is stimulated by provision of ammonium (Kurkdjian, Leguay & Guern 1978; Roberts *et al*. 1982; Herrmann & Felle 1995; Giglioli-Guivarc'h *et al*. 1996; Yin *et al*. 1996; Kosegarten *et al*. 1997; Plieth, Sattelmacher & Knight 2000; Outlaw *et al*. 2002). [See note (2) in the Appendix.] NO_3^- transport has recently been shown to stimulate a transient lowering of pH_{cyt}, which is enhanced by provision of the nitrate reductase inhibitor, tungstate (Espen, Nocito & Cocucci 2004). These pH changes are expected to be only transient because subsequent assimilation (see below), in addition to flux regulation processes such as efflux, allostery of transport proteins, and differential expression of genes encoding transporters, rapidly alters the sizes of the 'HNO_3' and 'NH_3' pools. However, such pH stresses are potentially significant when the concentration of the pool is increasing, given that unassimilated NO_3^- and NH_4^+ often accumulate to substantial concentrations (approximately 4–30 mM or higher) within the cytosol (Lee & Clarkson 1986; Devienne, Mary & Lamaze 1994; Wells & Miller 2000; Britto *et al*. 2001b; Britto & Kronzucker 2003).

When nitrate is assimilated, the net proton introduced to the cytosol in the nitrate transport step is effectively neutralized, as can be seen in a revised equation that integrates nitrate transport and reduction: $NO_3^-(ext) + H^+(ext) + 4[NAD(P)H + H^+](cyt) + H^+(cyt) \rightarrow NH_4^+(cyt) + 4[NAD(P)^+](cyt) + 3H_2O(cyt)$ (where 'ext' and 'cyt' refer to the extracellular or cytoplasmic location, respectively, of the substrates and products involved; also see Sakano 2001). In this equation, only a single proton is consumed in addition to that which entered in a symport step with nitrate. The present analysis differs from the tradi-

tional summary, which does not consider the transported proton, and therefore entails a net loss of two protons from the cell for every NO_3^- molecule taken up and assimilated. [See note (3) in the Appendix.] If this equation is added to that for ammonium deprotonation ($NH_4^+ \rightarrow NH_3 + H^+$) prior to its entry into the GS-GOGAT cycle (a necessary addition, considering that very little of the NH_4^+ produced in nitrate reduction accumulates in plant tissues!), then the summation of both processes is completely proton neutral: $NO_3^-(ext) + H^+(ext) + 4[NAD(P)H + H^+](cyt) \rightarrow NH_3(cyt) + 4[NAD(P)^+](cyt) + 3H_2O(cyt)$. In the case of ammonium nutrition, the proton lost from the cell in the transport step exactly counterbalances the proton released when NH_4^+ enters GS-GOGAT via NH_3. This also results in an integrated acquisition process that is proton-neutral, starting with uptake and continuing through to the amidation of 2-oxoglutarate, and the subsequent formation of glutamate (Fig. 3).

However, a more complete analysis of N uptake, reduction, and incorporation into amino acids should also take into account the proton balance associated with the provision of C substrates for reductant and amino acid skeletons. A step-by-step exposition then emerges as follows (also see Gerendás & Ratcliffe 2000). First, the uptake processes for NO_3^- and NH_4^+ are summarized in the following equations.

$$NO_3^-(ext) + H^+(ext) \rightarrow NO_3^-(cyt) + H^+(cyt) \quad (1)$$

$$NH_4^+(ext) \rightarrow NH_3(cyt) + H^+(ext) \quad (2)$$

Note that these equations indicate the net result of the N transport and ATPase activities at the membrane, as depicted in Fig. 2a. Starting from glucose as a source of reductant, NO_3^- reduction can then be written

$$3NO_3^-(cyt) + 6H^+(cyt) + 2C_6H_{12}O_6(cyt) + 6O_2(cyt) \rightarrow 3NH_4^+(cyt) + 12CO_2(cyt) + 9H_2O(cyt) \quad (3)$$

Adding Eqns 1 and 3 yields the following

$$3NO_3^-(ext) + 3H^+(ext) + 3H^+(cyt) + 2C_6H_{12}O_6(cyt) + 6O_2(cyt) \rightarrow 3NH_4^+(cyt) + 12CO_2(cyt) + 9H_2O(cyt) \quad (4)$$

Equation 4 reiterates the idea that only a single cytoplasmic proton is consumed in the combined transport and reduction of NO_3^-. The subsequent assimilation of NH_4^+ can also be expressed to include glucose as a source of reductant and carbon

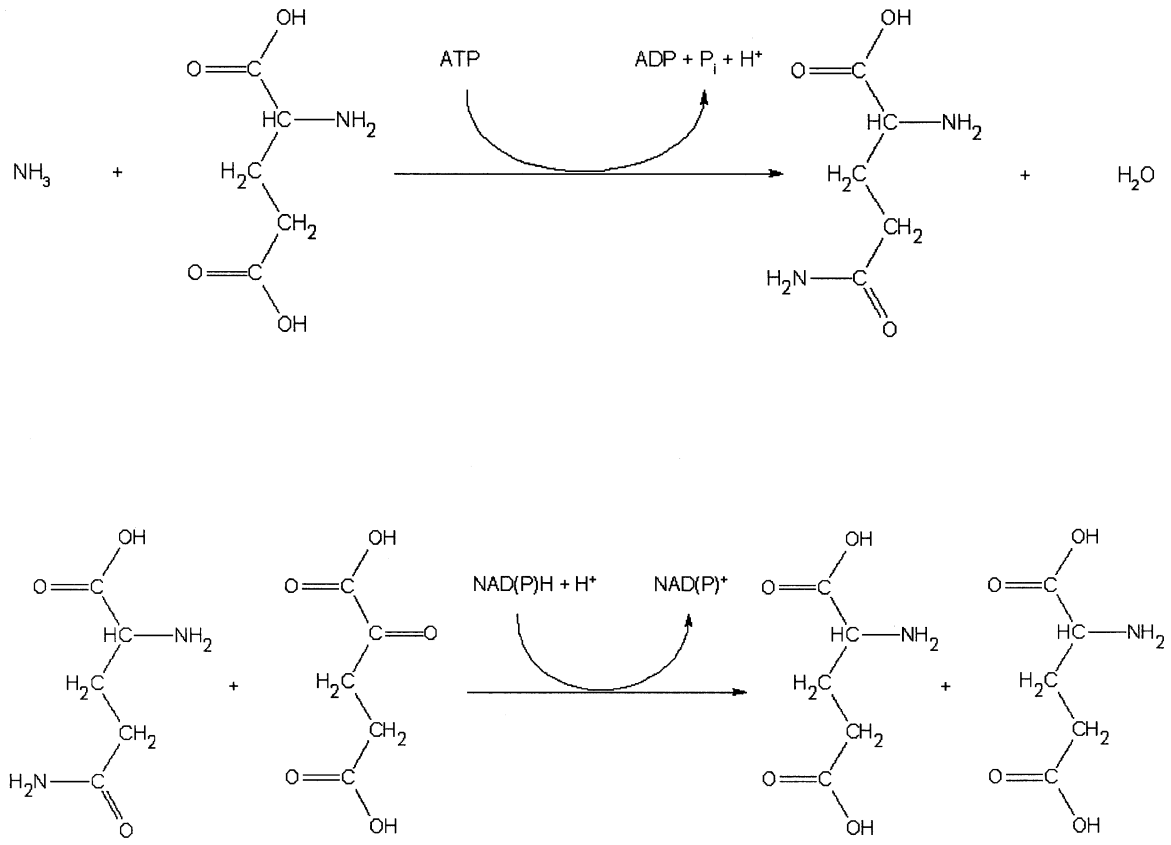

Figure 3. The proton-neutrality of ammonia assimilation. The first reaction is catalysed by glutamine synthetase (GS), and the second by glutamine 2-oxoglutarate aminotransferase (GOGAT). The protons associated with the hydrolysis of ATP and the oxidation of $NAD(P)H + H^+$ cancel each other out (Gerendás & Ratcliffe 2000; cf. Kosegarten *et al*. 1997), and are not relevant in the larger context because these compounds are regenerated in the steady state, under most conditions (Reid, Loughman & Ratcliffe 1985). For proton balance associated with the provision of C skeletons for this pathway, see text.

$$6NH_4^+(cyt) + 6C_6H_{12}O_6(cyt) + 9O_2(cyt) \rightarrow 6C_5O_4H_8N^-(cyt) + 6CO_2(cyt) + 12H^+(cyt) + 18H_2O(cyt) \quad (5)$$

When this equation is combined with Eqn 4, the net result of NO_3^- uptake, reduction, and assimilation is

$$6NO_3^-(ext) + 6H^+(ext) + 10C_6H_{12}O_6(cyt) + 21O_2(cyt) \rightarrow 6C_5O_4H_8N^-(cyt) + 30CO_2(cyt) + 6H^+(cyt) + 36H_2O(cyt) \quad (6)$$

This summary equation shows that the overall proton balance of NO_3^- utilization yields one proton generated in the cell, and one proton consumed from the external medium, for every NO_3^- ion incorporated into glutamate. In the case of NH_4^+, the NH_3 transported into the cell in step (2) above will be mostly in the protonated form

$$NH_3(cyt) + H^+(cyt) \rightarrow NH_4^+(cyt) \quad (7)$$

Combining Eqns 2, 5 and 7 yields the summary reaction for NH_4^+ uptake and assimilation

$$6NH_4^+(ext) + 6C_6H_{12}O_6(cyt) + 9O_2(cyt) \rightarrow 6C_5O_4H_8N^-(cyt) + 6CO_2(cyt) + 6H^+(cyt) + 6H^+(ext) + 18H_2O(cyt) \quad (8)$$

In this case, there is also one H^+ produced in the cell for every molecule of inorganic N incorporated into glutamate, but, unlike with NO_3^-, the assimilation of NH_4^+ also generates a proton in the external medium. [See note (4) in the Appendix.]

AN ANALYSIS OF INORGANIC CYTOCHEMISTRY AND ITS COMPARTMENTATION

It may appear that the acidification of the cytosol due to H^+/NO_3^- symport could be offset by the higher uptake of cations (especially K^+ and Ca^{2+}) that is seen with nitrate-grown relative to ammonium-grown plants (Kirkby & Mengel 1966; van Beusichem *et al*. 1988, by virtue of increased H^+/K^+ antiport at the plasma membrane. Indeed, Espen *et al*. (2004) showed that nitrate-dependent cytosolic acidification was reduced when K^+ was provided as the counterion. In the context of a nutritional steady state (which this cited study did not investigate), however, it is crucial that the increased net flux of K^+ does not result in an increase in the *cytosolic* K^+ pool; rather, this pool is homeostatically maintained at approximately 100 mM under most circumstances (Walker, Leigh & Miller 1996; Leigh 2001), and independently of N-source, except under conditions of extraordinarily high NH_4^+ supply (Kronzucker, Szczerba & Britto 2003). Therefore, the additional K^+ flux results in a greater accumulation of K^+ in the vacuole, and the associated H^+ efflux at the plasma membrane (in the initial transport step for K^+) is compensated for by equivalent proton fluxes at the tonoplast, directed in this instance from vacuole to cytosol.

The difference in pool sizes of strong cations and strong anions is considered an independent variable determining pH in living systems (Stewart 1983; Gerendás & Schurr 1999). If, as postulated in the biochemical pH-stat model, malate and other organic anions are effective agents for pH_{cyt} homeostasis, it must be argued that these compounds variably accumulate in the cytosol to counterbalance a variable excess of cations in that compartment. Crucially, however, no evidence exists to show that nitrate-grown plants accumulate more organic anions *in the cytosol* than ammonium-grown plants, nor is there evidence to indicate that there is a larger accumulation of inorganic cations in the cytosol, relative to anions, in nitrate-grown plants. On the contrary, an analysis of literature values of cytosolic ion content strongly suggests that an excess of inorganic cations over inorganic anions prevails to a greater extent in *ammonium-grown* plants. As indicated in Table 1, the cytosolic pools of most major inorganic ions are independent of nitrogen source. This applies in particular to K^+, Ca^{2+}, Mg^{2+}, and P_i, the cytosolic concentrations of which are held under strict homeostatic control over broad ranges of culture conditions, including nutrient supply (Lee & Ratcliffe 1993a, b; Felle 2001; Igamberdiev & Kleczkowski 2001; Leigh 2001; Kronzucker *et al*. 2003). Similarly, N-source independence has been shown for cytosolic Na^+ and Cl^- pool sizes under non-salinity conditions (Speer & Kaiser 1994; Ger-

Table 1. Nitrogen-source effects upon the distribution of the main inorganic ions within tissue and cytosol of nitrate- and ammonium-grown plants

	Total tissue		Cytosol		
	NO_3^-	NH_4^+	NO_3^-	NH_4^+	References
Anions					
NO_3^-	>		>		a
$H_2PO_4^-$ + HPO_4^{2-}	<		=		b
SO_4^{2-}	<		=		c
Cl^-	=		=		d
Cations					
NH_4^+	<		<		e
K^+	>		=		f
Na^+	=		=		g
Ca^{2+}	>		=		h
Mg^{2+}	>		=		i
Cations – anions	>		<		

Inequality or equal signs indicate comparisons of ionic pool sizes, between NO_3^- or NH_4^+ growth conditions. The bottom line of the table indicates the difference between major inorganic cations and anions for each nitrogen condition. Total tissue data is widely available and fairly consistent among plant species (see, for instance, Kirkby & Mengel 1966; van Beusichem *et al*. 1988). Cytosolic pool size comparisons for these ions were determined using data from multiple sources: (a) Miller & Smith (1996); Britto & Kronzucker (2003); (b) Lee & Ratcliffe (1993ab); (c) Bell *et al*. (1994); (d) Britto *et al*. (2004); (e) Wang *et al*. (1993); Wells & Miller (2000); (f) Leigh *et al*. 2001; Kronzucker *et al*. (2003); (g) Speer & Kaiser (1994); Flowers & Hajibagheri 2001; (h) Bush (1995); (i) Igamberdiev & Kleczkowski (2001); See text for further details.

endás & Schurr 1999; Britto *et al*. 2004). In addition, variations in the cytosolic SO_4^{2-} pool, while at present not well investigated, appear to be minimal, and the overall concentration of this pool, relative to potassium and inorganic nitrogen pools, is generally small (Bell, Cram & Clarkson 1994; Speer & Kaiser 1994; Buchner, Takahashi & Hawkesford 2004). Against this backdrop of relatively constant ion pools, the pools of inorganic N themselves can be superimposed, sizes of which have been shown by a variety of methods to typically fall into a 4–30 mM range, although some evidence suggests that they can be substantially higher (Britto *et al*. 2001b; Britto & Kronzucker 2003). The charge contribution from cytosolic N pools clearly establish a larger cytosolic excess of cations over anions in the case of NH_4^+ nutrition relative to NO_3^- nutrition. Taken together, the data in Table 1 therefore indicate that the cytosol of ammonium-grown plants is likely to accumulate a larger amount of organic anions (the identity and relative quantities of which have yet to be determined) than nitrate-grown plants, because the steady-state pH values for the cytosol of NH_4^+ or NO_3^--grown plants tend to be virtually identical (Bligny *et al*. 1997), within 0.2 pH units of one another (Gerendás, Ratcliffe & Sattelmacher 1990). In summary, the direction of this subcellularly localized strong ion difference directly contradicts the classical pH-stat paradigm as it applies to the cytosolic compartment.

If no differences in H^+ production or consumption occur as a result of differences in inorganic nitrogen source, then, the question remains as to why nitrate-grown plants have a pronouncedly higher accumulation of organic acid anions, particularly malate, relative to ammonium-grown plants. A straightforward explanation for this may be found in the greater *tissue* accumulation of cations by nitrate-grown plants (see above). For the achievement of electroneutrality by the plant, these additional positive charges require charge-balancing by strong anions, of which malate is a good candidate (Torii & Laties 1966). However, it must be emphasized that this differential, N-source-dependent accrual of inorganic ions and organic anions does not occur in the cytosol, but specifically in the vacuole. Evidence from organelle fractionation studies in spinach, barley, and potato, for instance, showed that malate concentrations in the cytosol (and the chloroplast stroma) were below detection limits, and at least an order of magnitude smaller than those in the vacuole (Winter, Robinson & Heldt 1993, 1994; Leidreiter *et al*. 1995; see also review by Martinoia & Rentsch 1994); by contrast, the concentrations of other metabolic intermediates, including amino acids, were almost always substantially larger in the cytosol than in the vacuole. Malate was an interesting exception in this regard because its relatively low cytosolic concentration is congruent with its role as a potent negative feedback agent upon PEP carboxylase (see below). Conversely, this pattern is inconsistent with malate's postulated role as a cytosolic proton source. This is because, mechanistically, the transport of organic anions to the vacuole is subject to the constraints of the 'proton economy' as described above; that is, their movement is accompanied by the flux of protons in the same direction (Fig. 2b), and therefore the cytosolic synthesis and subsequent transport to the vacuole of malic acid cannot contribute to alleviating cytosolic alkalosis. This requirement has been studied in detail for malate transport from cytosol to vacuole, in which channel-mediated malate transport is coupled to the pumping of a charge equivalence of protons into the vacuole by the tonoplast V-type ATPase and pyrophosphatase (i.e. both the malate anion and its two associated protons leave the cytosol in a symport process with overall electroneutrality; Gout *et al*. 1993; Lüttge *et al*. 1995, 2000; Cheffings *et al*. 1997). For this reason, the protons made available via carboxylation events in the cytosol cannot be used to neutralize any alkalinizing processes *in situ*, but are required as substrates for flux coupling across the tonoplast membrane; otherwise, the *trans*-tonoplast electrical potential would not be sustainable (see analysis by Gerendás & Schurr 1999). It is also important to point out that, while malate accumulation and transport to the vacuole are stimulated by nitrate (Lüttge *et al*. 2000), studies examining the relationship between malate pools and the flux through nitrate reduction do not support the proposed concerted synthesis of malate and reduction of nitrate. For instance, a 10-fold decrease in nitrate reduction resulted in no change in the malate pools of *Catharanthus roseus* cells (Marigo, Bouyssou & Belkoura 1985; also see Purvis, Peters & Hageman 1974). In another instance, mutant tobacco plants, growing on nitrate, but possessing no detectable root nitrate reductase activity, nevertheless accumulated more than twice as much malate in the root than wild-type plants (Stoimenova *et al*. 2003a).

PHOSPHOENOLPYRUVATE CARBOXYLASE REGULATION: IS IT COMPATIBLE WITH A PH-STAT ROLE FOR THE ENZYME?

The pH responses of PEP carboxylase and malic enzyme, the enzymes most commonly invoked as metabolic rectifiers of cytosolic pH perturbations, are a central feature of the biochemical pH-stat model. The pH-optima studies that have led to the model's proposal, however, as well as many studies comparing N-source variations in the activities of these enzyme systems, have been conducted under *in vitro* conditions that do not reflect the chemical complexities of the living cell. This is a particularly important concern in the case of PEP carboxylase, as extensive work has shown it to be a multipurpose, highly regulated enzyme, subject to phosphorylation and modulation by pools of a wide range of metabolites. These modulators include light, iron, PEP, shikimate, glucose-6-phosphate, bicarbonate, citrate, malate, aspartate, asparagine, glutamate, and glutamine, in addition to protons (Davies 1979; Mathieu *et al*. 1982; Van Quy, Foyer & Champigny 1991; Sugiharto & Sugiyama 1992; Leport *et al*. 1996; Vuorinen & Kaiser 1997; Chinthapalli *et al*. 2000; Espen *et al*. 2000; Murchie *et al*. 2000; Parvathi *et al*. 2000; Pasqualini *et al*. 2001; Ferrario-Mery *et al*. 2002; Lepiniec, Thomas & Vidal 2003). Given this plethora of regulatory agents for PEP carboxylase, it is difficult to conceive how their action could be subordinated

to the proposed crucial role of the enzyme in cytosolic pH regulation. The difficulty is compounded by observations that the pool sizes of many of these regulating compounds, and hence their effects on PEP carboxylase, vary considerably over time. This is in sharp contrast to the cytosolic H^+ pool size, changes of which are restrained by buffering and transport systems. We are not aware of any studies that have addressed the problems associated with this aspect of the proposed pH-stat.

In addition to being modulated by multiple regulatory agents, PEP carboxylase activity can also be substrate-limited. In a particularly striking example, Gout *et al.* (1993) show that when cytosolic pH in sycamore protoplasts drops from 7.5 to 7.0 as a result of the external provision of 5 mM bicarbonate, malate synthesis increases significantly. The authors attribute this increase to substrate limitation of PEP carboxylase, which clearly over-rides, and indeed contradicts, the proposed direction of the enzyme's activity relative to changes in cytosolic pH.

Nevertheless, it remains possible that an *in vivo* pH dependence of PEP carboxylase can coexist, perhaps in a regulatory hierarchy, with these other regulatory processes. Indeed, various reports have shown that changes in PEP carboxylase activity, in apparent response to cytosolic pH changes, are consistent with the biochemical pH stat model (e.g. Gout, Bligny & Douce 1992; Rajagopalan, Gayathri & Raghavendra 1998; Sakano, Kiyota & Yazaki 1998; Gerendás & Ratcliffe 2000). However, these papers do not refer to differences in PEP carboxylase activities that result from differences in nitrogen source, which is the central subject here. Moreover, in two key studies it was concluded that the proposed pH-stat mechanism was too slow, or too low in capacity, to be of significance to pH rectification (Gout*et al.* 1992; Gerendás & Ratcliffe 2000), a conclusion also supported by others (Savchenko *et al.* 2000). In addition, there are other reports that indicate that pH stresses on the cytosol failed to modulate PEP carboxylase activity in a direction appropriate for a true pH-stat (Gout *et al.* 1993; Meinhard & Schnabl 2001; Outlaw *et al.* 2002).

Notwithstanding the regulatory complexity of PEP carboxylase, abundant evidence indicates that the enzyme's activity is especially strongly associated with the assimilatory flux of inorganic nitrogen into amino acids. In particular, changes in PEP carboxylase activity have been shown to be positively correlated with the activity of glutamine synthetase (Arnozis, Nelemans & Findenegg 1988; Vanlerberghe *et al.* 1990; Sugiharto & Sugiyama 1992; Sugiharto *et al.* 1992; Manh *et al.* 1993; Díaz, Lacuesta & Muñoz-Rueda 1996; Koga & Ikeda 2000), the enzyme that catalyses the entry of inorganic N (irrespective of N source) into the organic N pool (see Fig. 3). This relationship is highlighted by the potent stimulatory effects of glutamine itself, as well as the glutamine : glutamate ratio, on the activity and synthesis of PEP carboxylase (Vanlerberghe *et al.* 1990; Sugiharto *et al.* 1992; Sugiharto & Sugiyama 1992; Manh *et al.* 1993; Foyer *et al.* 1994; Díaz *et al.* 1996; Li, Zhang & Chollet 1996; Koga & Ikeda 2000; Murchie *et al.* 2000; Ferrario-Mery *et al.* 2002; Britto & Kronzucker 2004). The reason for this close correspondence between PEP carboxylase activity and nitrogen assimilation, irrespective of inorganic N source, appears to be clear: the carbon skeletons aminated to form amino acids in primary N assimilation are organic acids (more specifically, 2-oxo acids, especially 2-oxoglutaratic and oxaloacetic acid) that also function as intermediates in the tricarboxylic acid cycle, and therefore their depletion must be counteracted. Hence, they need to be synthesized in anaplerotic reactions such as that catalysed by PEP carboxylase (see Fig. 4). The control of PEP

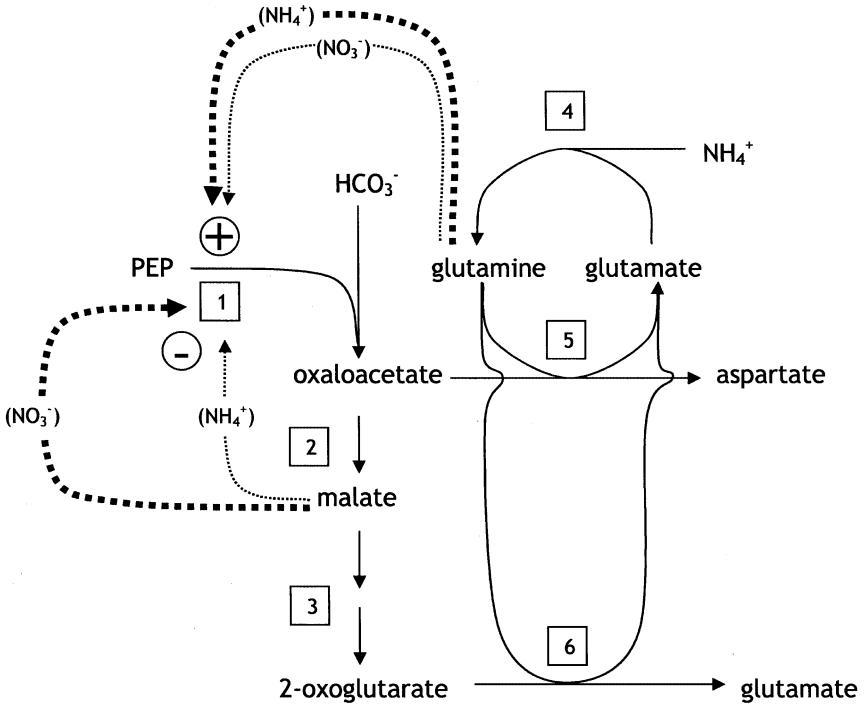

Figure 4. Model outlining a revised role of PEP carboxylase in inorganic N assimilation. Key enzymes or enzyme groups are indicated in boxed numbers as follows: (1) PEP carboxylase; (2) malate dehydrogenase; (3) tricarboxylic acid cycle enzymes; (4) glutamine synthetase (GS); (5) aspartate aminotransferase; (6) glutamine 2-oxoglutarate aminotransferase (GOGAT). Note that (as in the legend to Fig. 1), bicarbonate (HCO_3^-) is produced by the activity of carbonic anhydrase. Dashed lines indicate feedback activities on PEP carboxylase by metabolic intermediates. Circled plus or minus signs indicate positive or negative feedback, respectively. Thickness of dashed lines indicates intensity of feedback, due to nitrogen-source-dependent variations in pool sizes of feedback agents (N source given in parentheses).

carboxylase by the glutamine : glutamate ratio is indicative of the enzyme's anaplerotic role, in that this ratio reflects the extent to which nitrogen has been incorporated into organic forms by drawing from organic acid pools. The role of PEP carboxylase in inorganic N assimilation is further indicated by the well-documented rise in this enzyme's activity that results from provision of either NO_3^- or NH_4^+ (Hammel, Cornwell & Bassham 1979; Müller, Baier & Kaiser 1991; Sugiharto *et al*. 1992; Díaz *et al*. 1995, 1996; Scheible *et al*. 1997; Esposito, Carillo & Carfagna 1998; Koga & Ikeda 2000; Murchie *et al*. 2000; Lepiniec *et al*. 2003). Interestingly, the conclusion that PEP carboxylase has a major anaplerotic function in N assimilation has been drawn in a large number of studies examining nitrate and/or ammonium nutrition, despite its incongruence with a proposed pH-stat role of PEP carboxylase in the context of N acquisition, namely the enzyme's proposed down-regulation during NH_4^+ assimilation (Popp & Summons 1983; Dahlbender & Strack 1986; Melzer & O'Leary 1987; Arnozis *et al*. 1988; Guy, Vanlerberghe & Turpin 1989; Vanlerberghe *et al*. 1990; Cramer, Lewis & Lips 1993; Foyer *et al*. 1994; Díaz *et al*. 1995, 1996; Gao & Lips 1997; Golombek *et al*. 1999; Koga & Ikeda 2000; Murchie *et al*. 2000; Norici, Dalsass & Giordano 2002; Rademacher *et al*. 2002).

Perhaps the most powerful challenge to the proposed role of a PEP carboxylase-based, N-source-dependent, pH stat, however, is the large number of studies indicating that PEP carboxylase activity can be substantially higher in NH_4^+-grown, relative to NO_3-grown, plants (Schweizer & Erismann 1985; Arnozis *et al*. 1988; Arnozis & Barneix 1989; Ikeda, Mizoguchi & Yamakawa 1992; Villa *et al*. 1992; Cramer & Lewis 1993; Cramer *et al*. 1993; Díaz *et al*. 1996; Koga & Ikeda 1997, 2000; Lasa *et al*. 2002; Norici *et al*. 2002; Viktor & Cramer 2005). This most likely reflects the higher GS activities, glutamine contents, glutamine : glutamate ratios, and higher rates of amino acid synthesis that are often observed in NH_4^+-grown plants (Yemm & Willis 1956; van Beusichem *et al*. 1988; Magalhães & Huber 1989; Sugiharto & Sugiyama 1992; Cramer *et al*. 1993; Díaz *et al*. 1996; Claussen & Lenz 1999; Pasqualini *et al*. 2001; Lasa *et al*. 2002). Where observed, the lower PEP carboxylase activities of NO_3^--grown plants may be due to their much larger malate pools and the potential negative feedback exerted on the enzyme by such pools (Pasqualini *et al*. 2001; Rademacher *et al*. 2002). Although the predominant subcellular location of malate is the vacuole (see above), while PEP carboxylase is a cytosolic enzyme, the higher accumulation of malate in vacuoles of NO_3^--grown plants may translate into higher cytosolic malate levels relative to those of cells of NH_4^+-grown plants. Moreover, while NO_3^- nutrition has been shown to reduce malate inhibition of PEP carboxylase activity to some degree (Duff & Chollet 1995; Murchie *et al*. 2000), this release from inhibition appears not to generally result in an increased *in vivo* PEP carboxylase activity in NO_3^--grown plants, possibly due to the hyperaccumulation of malate to concentrations an order of magnitude greater than in NH_4^+-grown plants. Indeed, it has been suggested that the increased malate pools under nitrate nutrition indicate a lower demand for anaplerotic carbon fixation, in direct contradiction to the often assumed positive correlation between malate pool size and ongoing organic acid synthesis via PEP carboxylase and malate dehydrogenase (Gao & Lips 1997).

The trend of higher PEP carboxylase activity associated with NH_4^+ nutrition is more apparent in roots than shoots (Schweizer & Erismann 1985; Arnozis *et al*. 1988), reflecting the general rule that primary NH_4^+ assimilation takes place mostly in roots, whereas the location of primary NO_3^- assimilation is predominantly in shoots (Andrews 1986). In other words, PEP carboxylase activity appears to be colocalized with nitrogen assimilation and its associated anaplerotic requirement. An alternative explanation, that leaves assimilating nitrogen must use biochemical pH-stat pathways because (unlike roots) they lack the ability to dispose of protons into an extracellular medium (Raven & Smith 1976; Marschner 1995), is challenged by the observation that leaves exposed to acid stressors such as SO_2 translocate resultant acids to the root, which then excretes excess protons (Thomas & Runge 1992; Kaiser, Hofler & Heber 1993). More fundamentally, it is falsified by the proton neutrality of integrated N transport and assimilation (see above).

CONCLUDING REMARKS

While attractive in its simplicity, the malate-based biochemical pH stat model agrees neither with current views of ion transport, nor with regulatory information about the key enzyme PEP carboxylase. This lack of agreement applies in particular to nitrogen acquisition, when the following considerations are fully taken into account: the proton involvement in nitrogen transport and metabolism, the subcellular compartmentation of ions, and the numerous exceptions to the proposed increased PEP carboxylase activity in nitrate-fed plants. Although such plants unquestionably build up higher malate concentrations than NH_4^+-grown plants, these pools exist almost entirely in the vacuole, where they cannot influence cytosolic pH. Rather, the high malate pools reflect the larger vacuolar excess of cations over anions, and may indeed reflect a lesser anaplerotic requirement, and therefore a lower PEP carboxylase activity, in NO_3^--grown plants.

Past emphases on this biochemical pH stat model may have detracted from the importance and potency of other pH maintenance and rectification systems in plants. These include systems that are analogous to those well established in microbial and animal physiology, and which are likely to act much more rapidly than metabolic processes (Savchenko *et al*. 2000). For instance, the operation of open-system buffers (in addition, and in contrast, to stationary buffers such as proteins) has received little or no attention in plant systems. Nevertheless, such systems, in which changes in partial pressures of CO_2 and concentrations of bicarbonate (for instance) can resist pH changes very effectively in the human body (Putnam 1998), and

should be expected to do so in plants, given the widespread occurrence of carbonic anhydrase, which can dramatically speed up the velocity of the equilibration reactions. Rectification of pH changes in the cytosol involving the antiport or symport of protons with ions such as K^+, Na^+, or Cl^-, across the plasma membrane, tonoplast, or plastidic membranes (Felle 1991; Siebke *et al*. 1992; Raghavendra, Yin & Heber 1993; Bligny *et al*. 1997; Venema *et al*. 2003; Song *et al*. 2004), may have also been underestimated. Recent evidence for the potency of such mechanisms is indicated by the observaion that a mutation in sodium-proton antiport at the thylakoid membrane has consequences for cytosolic pH (Song *et al*. 2004). Transient shifts in steady-state concentrations of nutrient ions, to compensate for proton deficits or excesses, should be readily tolerable by the plant, given that baseline proton concentrations are many orders of magnitude smaller than the concentrations of most nutrient ions. The enormous and well-documented capacity of plant cells to effectively maintain cytosolic calcium homeostasis, following the sudden, and intense, changes in cytosolic $[Ca^{2+}]$ that are associated with cellular signalling events, provides striking evidence of the feasibility of rapid, and highly refined, ion homeostasis based solely on membrane transport mechanisms.

The likely existence of these alternative pH-regulating mechanisms in plants, in conjunction with well-established pH stats and buffering systems, calls into question the necessity of postulating a biochemical 'fine tuning' mechanism. Even if such a mechanism does exist, its postulated functioning in the context of N nutrition, via differential synthesis of malic acid, is clearly contradicted by a wide range of studies. Other metabolic pH stat mechanisms may nevertheless be of importance in some contexts, such as the shift from lactic acid to ethanol production in plants undergoing extended periods of hypoxia or anaerobiosis (Roberts *et al*. 1984; Gerendás & Ratcliffe 2002; Stoimenova *et al*. 2003b), although, again, such mechanisms have not been demonstrated to occur within the context of nitrogen source differences, or even under non-adverse physiological conditions.

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APPENDIX

List of additional notes. Numbers refer to those given in the main text.

(1) A more complicated metabolic scheme, in the form of an 'equation for growth with ammonium as the nitrogen source': $3NH_4^+ + 60(CH_2O) + 15O_2 \rightarrow 15CO_2 + 28H_2O + C_{45}H_{72}N_3O_{32}^- + 4H^+$, is sometimes used to convey the hypothetical trend of NH_4^+-dependent cellular acidification (Raven 1985; also cited in Marschner 1995). However, the consideration of unlikely and somewhat arbitrarily constructed compounds ('$C_{45}H_{72}N_3O_{32}^-$') in these schemes underscores their speculative character and obscures the mechanisms of N uptake and assimilation, which are at the very centre of the discussion; moreover, untenable assumptions are built into such schemes, such as the premises that C/N ratios are invariable (cf. Wedin & Tilman 1996), that all plant N is organic (cf. Kirkby & Mengel 1967), and that all carbon for N assimilation derives from carbohydrate, discounting the role of anaplerotic C fixation in this process (cf. Melzer & O'Leary 1987; also see below). While the source of C for amino acid skeletons and for reducing power is important in the context of metabolic H^+ production and consumption during N assimilation, especially in the context of aerobic versus anaerobic conditions (Gerendás & Ratcliffe 2000; Stoimenova *et al*. 2003b; also see below), it should be noted that GS/GOGAT is the entry point for most inorganic N into the amino acid pool, irrespective of N source, and therefore the H^+ balance associated with the production of carbon skeletons, and with the oxidation of carbohydrate, is also independent of N source (except insofar as NH_4^+-grown plants often synthesize more amino acids).

(2) Although this pH rise is often attributed to NH_3 diffusion through the lipid bilayer of the plasma membrane, much evidence supports the idea that NH_4^+ is the permeating species, especially in cases where the external pH is well below the pK_a for NH_3/NH_4^+ (e.g. pH 7 in the study by Kosegarten *et al*. 1997); for a further analysis of this subject, see Britto *et al*. 2001a. Interestingly, whether NH_4^+ uptake (accompanied by the associated membrane potential rectification) or NH_3 uptake occurs, the consequences for cytosolic pH are identical.

(3) Interestingly, as pointed out by Stoimenova *et al*. (2003b), the first step of NO_3^- reduction, catalysed by NR, localized in the cytosol, and producing nitrite (NO_2^-), is not a proton-consuming process, as can be seen in the equation $NO_3^- + [NAD(P)H + H^+] \rightarrow NO_2^- + [NAD(P)^+] + H_2O$. Conversely, the subsequent, proton-consuming,

reduction step from NO_2^- to NH_4^+, is localized in the plastid; in leaf tissue, this localization should intensify the *trans*-thylakoid ΔpH, and should therefore have consequences for photosynthetic processes. This possibility has been rarely, if ever, considered.

(4) Note that, as with all summary equations of this nature (see note (1)), while deceptively more complete and satisfying than piecemeal approaches, nevertheless fail to account for some well-known physiological observations. In particular: (1) a significant fraction of the carbon budget in amino acid anabolism derives not directly from glucose, but is introduced anaplerotically via PEP carboxylase (see below); and (2) contrary to the apparent outcomes of Eqns 6 and 8, the rates of overall respiration, and of O_2 consumption and CO_2 evolution, are typically significantly higher in NH_4^+-grown than in NO_3^--grown plants (see Britto *et al*. 2001b), indicating the importance of physiological processes superimposed upon the primary events of N acquisition. In addition to these shortcomings, and as pointed out in note (3), metabolic schemes such as the one given here do not often take into account the effects of subcellular compartmentation of metabolism.