Free amino acid levels and the regulation of nitrate uptake in maize cell suspension cultures

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Abstract

The ability of individual amino acids to regulate nitrate uptake and induction was studied in a Zea mays embryo cell line grown in suspension culture. The maize cells exhibited a marked preference for absorbing amino acids over nitrate when both were present in culture medium. The addition of an individual amino acid (2 mM glutamine, glycine, aspartic acid, or arginine) to the culture medium with 1 mM nitrate completely inhibited nitrate uptake and resulted in a cycle of low levels of nitrate influx followed by efflux to the growth medium. Glutamine was readily absorbed by the cells and was particularly effective in supporting optimum cell growth in the absence of an inorganic nitrogen source as compared to the three other amino acids evaluated. However, neither glutamine nor any of the remaining 19 proteinaceous amino acids appeared to be solely responsible for regulation of nitrate uptake and induction. The ability of amino acids to regulate nitrate uptake and assimilation appears to be more related to their overall levels in the cell rather than to an accumulation of a specific amino acid.

Key words: Amino acids, nitrate uptake, maize, regulation, cell suspension culture.

Introduction

Amino acids are the organic product of inorganic nitrate assimilation. Assimilation of nitrate requires the activity of several specific proteins: a membrane-bound transporter, nitrate reductase (NR), nitrite reductase (NiR), glutamine synthase (GS), glutamine oxoglutarate aminotransferase (GOGAT), and a number of transaminases involved in the synthesis of subsequent amino acids. Free amino acids are the currency in nitrogen metabolism, with which nitrogen is transferred between cells and organs (Atkins and Beevers, 1990). As such, it is not unreasonable to postulate that they would serve a pivotal role in the regulation of nitrate absorption and assimilation.

The elemental composition and structure of amino acids vary and include not only the 20 'structural' amino acids, but other non-protein amino acids such as allantoin, canavanine, citrulline, and ornithine which function in nitrogen storage or transfer. In addition to their structural role in proteins, amino acids are the starting point for the synthesis of pyrimidines (aspartate), purines (glutamine and glycine), growth regulators (tryptophan, methionine), and many secondary metabolites. Amino acids are also implicated in osmotic regulation, and provide the link between the nitrogen and carbon metabolic pathways (Häusler et al., 1994). Perhaps because of their mobility and wide-ranging functions in the plant, close correlation between free amino acid content of cells and nitrate uptake has not been widely published.

The interactions between the two inorganic nitrogen forms, NH4+ and NO3−, on plant growth and uptake characteristics have, however, been widely studied. The results of these studies have often been conflicting, with reports varying from no effect of NH4+ on NO3− uptake to strong inhibition (see Aslam et al., 1994, and references therein). The use of various nitrate analogues and tracers (36ClO−, 15NO−, 13NO−, and NO2−) has not resolved the issue. Results have shown that under some conditions, NH4+ inhibits NO3− influx alone (Lee and Drew, 1989; Ayling, 1993; King et al., 1992) and under others, NH4+ stimulates NO3− efflux while having little effect on the influx mechanism (Deane-Drummond and Glass, 1983; Deane-Drummond, 1985, 1986; Aslam et al., 1994; Chaillou et al., 1994). Differences in methodological
approaches may explain some of the conflicting results. The potential for artefactual data in NH\textsuperscript{+} versus NO\textsubscript{3} studies due to methodological flaws has been reiterated by Aslam \textit{et al.} (1994). For example, Glass \textit{et al.} (1985) suggested that the use of 36ClO\textsuperscript{3} as an analogue may be inappropriate for some species. Rapid changes in solution concentration of nitrate may also result in aberrant plant responses (Glass \textit{et al.}, 1985; Ingemarsson \textit{et al.}, 1987).

While NH\textsuperscript{+} may have a direct effect on NO\textsubscript{3} influx, efflux, and assimilation, some authors investigating NH\textsuperscript{+}/NO\textsubscript{3} interactions have suggested that the inhibitory effect of NH\textsuperscript{+} on NO\textsubscript{3} uptake may be due to the assimilation of NH\textsubscript{4} and the accumulation of assimilation products such as amino acids (Clarkson and Lüttege, 1991; Coronil and Lara, 1991; Jackson and Volk, 1992; King \textit{et al.}, 1992; Henriksen and Spanswick, 1993). However, Lee and Drew (1989) used mathematical estimates of NH\textsubscript{4} assimilation rates to demonstrate that the inhibitory effect of NH\textsubscript{4} was too rapid to be the result of amino acid accumulation alone.

Indirect evidence for amino acid regulation of nitrate uptake can be found in studies of xylem and phloem loading as well as studies on the interactions between carbon and nitrogen metabolism. Amino acids comprise a large component of phloem sap in most species (Peuke and Jeschke, 1993; Peuke \textit{et al.}, 1994) particularly in plant species that do not reduce and assimilate nitrate in the root (Cooper and Clarkson, 1989). Plants, including many cereals, that exhibit low rates of NO\textsubscript{3} assimilation in root tissue export most of the absorbed NO\textsubscript{3} to shoots where it is reduced and assimilated. Reduced nitrogen is then translocated to roots from shoots in the form of amino acids in phloem sap (Andrews, 1986; Li \textit{et al.}, 1995). The relative proportion of nitrate and amino acids of both xylem and phloem sap can be manipulated by exogenous applications of NO\textsubscript{3} and NH\textsubscript{4} (Andrews, 1986; Cooper and Clarkson, 1989; Peuke and Jeschke, 1993; Peuke \textit{et al.}, 1994). However, the primary site of NH\textsubscript{4} assimilation in most plant species when NH\textsubscript{4} is the sole N source is thought to be in the roots. Ammonium-N in both xylem and phloem sap of castor bean was found to be less than 1% of the total nitrogen, regardless of the exogenous nitrogen source (Jeschke and Pate, 1992; Peuke and Jeschke, 1993). The amino acids (principally glutamine, glutamate, and asparagine, but some plants use non-structural amino acids) found in xylem sap may be the result of root assimilation of inorganic nitrogen, or may be the product of nitrogen cycling from shoot to root and back to shoot as was proposed by Cooper and Clarkson (1989). It is tempting to speculate that the communication between roots and shoots regarding nitrogen status of the plant is accomplished by free amino acids in the symplastic space. As the cellular demand increases, cycling free amino acids concentrations decline and that decline could trigger resumption (either by induction or derepression) of inorganic nitrogen absorption and assimilation. An excess of free amino acids could signal an ample nitrogen supply and, thus, inhibit nitrate uptake (Cooper and Clarkson, 1989; Imsande and Touraine, 1994).

A direct effect of NO\textsubscript{3} on carbon metabolism has been shown by Champigny and Foyer (1992) where two key enzymes, sucrose phosphate synthase and phosphoenolpyruvate carboxylase, were modulated by protein phosphorylation which was, in turn, modulated by NO\textsubscript{3}. The result of the regulation by phosphorylation or de-phosphorylation is to shunt carbon into amino acid synthesis and away from sucrose synthesis. A recent report by Kaiser and Huber (1994) indicated that protein phosphorylation modulates NR activities as well. Although a NO\textsubscript{3} effect on NR phosphorylation was not specifically tested to our knowledge, the authors pointed out the similarity between NR modulation and modulation of sucrose phosphate synthase and phosphoenolpyruvate carboxylase.

There are only a few reports of direct inhibition of nitrate transport by amino acids. Work by Breteler and Arnozis (1985) determined that pretreatment of dwarf bean roots with many different individual amino acids inhibited nitrate uptake to varying degrees dependent upon prior exposure of the plants to nitrogen and the specific amino acid treatment. No significant effect of amino acids on nitrate transport was detected when both NO\textsubscript{3} and amino acids were present in the bathing solution, and no correlation emerged between inhibition of nitrate uptake and inhibition of nitrate reductase relative to specific amino acids. A more detailed study, presented by Muller and Touraine (1992), demonstrated inhibition of uptake by 50% or greater by alanine, glutamine, asparagine, arginine, beta-alanine, serine, and glutamine when soybean seedlings were pretreated for 18 h prior to exposure to NO\textsubscript{3}. The mechanisms of inhibition by arginine and alanine appeared to differ, however. Arginine stopped NO\textsubscript{3} uptake immediately upon introduction to the uptake solution, kinetically similar to NH\textsuperscript{+} inhibition. The authors suggested that this may be the result of a non-metabolic response such as alteration of membrane potentials. Inhibition by alanine was slower to develop, suggesting a metabolic component to the regulation rather than a physical or chemical interference. Using wheat seedlings as the model, Rogers and Barneix (1993) also determined that N-status controlled the amino acid effects on nitrate uptake, however, aspartic acid was the only amino acid tested that showed a significant inhibitory effect. 14N-NMR, 15N, 15N labelling and standard uptake experiments were used by Lee \textit{et al.} (1992) to investigate the effect of potential assimilation inhibitors (Glu, Gln, Ala, and Asn) on NO\textsubscript{3} and NH\textsuperscript{+} uptake by maize roots. Although the results demonstrated some interesting dynamics in assimilation between amino acids, NH\textsuperscript{+},...
and NO$_3^-$, particularly between Glu and Asn, changes in pools of Glu and/or Asn did not correlate with stimulation or inhibition of transport of either NO$_3^-$ or NH$_4^+$. In general, all four amino acids inhibited both NH$_4^+$ and NO$_3^-$ transport. Results published by Henriksen and Spanswick (1993) using ion-selective microelectrodes demonstrated an apparent, but inconsistent, interaction between NO$_3^-$ and Glu. In a preliminary study (Padgett and Leonard, 1993), a slight but significant inhibition of nitrate uptake in excised maize roots was demonstrated when roots were incubated in equal molar NO$_3^-$ and individual Arg, Asp, Glu, or Gly.

Direct evidence from studies of unicellular plants and microbes strongly indicates that amino acids, particularly glutamine, have a significant regulatory role in nitrate uptake and assimilation (Clarkson and Lüttge, 1991). Microbial cells are easily manipulated experimentally, but may or may not have regulatory mechanisms similar to higher plants. Plant cell suspension cultures are a compromise between whole plant studies and studies of single-cell organisms. Cultured cells are relatively easy to manipulate experimentally, and they contain the same genetic background as would be found in intact plants. Expression of a particular biochemical or metabolic activity, however, may not necessarily reflect the activity found in an intact plant.

Early studies by Filner (1966) tested the effect of amino acids on nitrate reductase in tobacco cell suspension cultures with limited success. Later experiments (Heimer and Filner, 1970) determined that casein hydrolysate (mixture of amino acids) inhibited nitrate accumulation. Nitrate and other nitrogen sources have a profound, but may or may not have regulatory mechanisms similar to higher plants. Plant cell suspension cultures are a compromise between whole plant studies and studies of single-cell organisms. Cultured cells are relatively easy to manipulate experimentally, and they contain the same genetic background as would be found in intact plants. Expression of a particular biochemical or metabolic activity, however, may not necessarily reflect the activity found in an intact plant.

In the work presented here, a non-photosynthetic Zea mays cell suspension culture (Padgett and Leonard, 1994a) was used to evaluate the effect of arginine, aspartic acid, glutamine, and glycine on nitrate transport. The maize cells demonstrated a decided preference for all four amino acids over nitrate when grown in the presence of both NO$_3^-$ and amino acids. Nitrate uptake and induction was inhibited by amino acids, but there appeared to be no specific amino acid responsible for inhibiting NO$_3^-$ transport. Evidence is presented to support the theory that NO$_3^-$ uptake is regulated, at least in part, by enhancement of an efflux mechanism.

Materials and methods

Culture solution

Unless otherwise specified, cells were grown and maintained in the modified Murashige and Skoog (MS) medium reported earlier (Padgett and Leonard, 1994a). The solution consisted of 30 g sucrose; 40 mg NaFeEDTA; 0.1 g inositol; 2.5 g KCl; 100 mg aspartic acid; 150 mg arginine; 100 mg glycine; 10 ml of MS sulphate stock (37 g MgSO$_4$, 1.69 g MnSO$_4$, 0.89 g ZnSO$_4$, 2.5 mg CuSO$_4$ per litre); 10 ml of MS halide stock (44 g CaCl$_2$, 2H$_2$O; 83 mg KI, 2.5 mg CoCl$_2$ per litre); and 10 ml of MS P-B-Mo stock (17 g H$_3$PO$_4$, 0.62 g H$_2$BO$_3$, 25 mg NaMoO$_4$ per litre) per litre. Ten millilitres of vitamin stock (100 mg thiamine-Cl, 100 mg pyridoxine-HCl, and 50 mg nicotinic acid per 100 ml) was mixed with 10 ml auxin stock (200 mg 2,4-D per 100 ml), and 1 g glutamine and brought to 100 ml. The organic stock solution was filter-sterilized through a 0.2 μm pore cellulose acetate disposable filter unit (Nalgene, Rochester, NY) and was added to the autoclaved culture medium at a rate of 10% (v/v) stock to sterile culture medium. Solutions were dispensed into 125 ml DeLong flasks (20 ml), 500 ml Erlenmeyer flasks (60 ml) or 11 Erlenmeyer flasks (150 ml) and autoclaved for 20 min at 121 °C prior to the addition of vitamin, glutamine and 2,4-D supplement. Amino acids were added from filter-sterilized stock solutions after the medium was autoclaved.

Cells

Zea mays cv. P3377 embryo cell suspension cultures (Duncan and Widholm, 1991a, b) were used for all experiments. Cells were maintained as described earlier (Padgett and Leonard, 1994a, b). Cells were subcultured weekly by dividing the contents of a single flask into two flasks containing fresh medium. The cells were transferred in suspension by sterile 25-ml-wide bore pipettes. The cultures were maintained in constant logarithmic growth (Felker et al., 1989) by subculturing prior to the onset of the stationary phase. Flasks were incubated at 28 °C on a rotary shaker (100–125 rpm) under low light. Cells were harvested and used in experiments between day 5 and day 7 after subculturing. Growth of the cell cultures were assessed non-destructively in 125 ml flasks by drawing the entire culture into sterile 25 ml graduated pipettes and allowing the cells to settle by gravity for 10 min. The 'settled volume' was recorded, and the cells were released from the pipette for later measurements.

Uptake assay

Ten millilitres of well-suspended cells (approximately 1 g) were transferred into 125 ml flasks of fresh standard induction medium, for a total volume of 30 ml. Cells and solution were swirled, then allowed to settle to the bottom of the flask before a 0.8 ml sample of the solution was removed. During the uptake period, flasks were placed on a rotary shaker set at 120 rpm under continuous low light. When sampled, flasks were removed from the shaker, the cells were allowed to settle for 5 min before the ml solution samples were removed aseptically and frozen for later analysis. Sampling periods and duration of the experiments varied depending upon the nature of the experiment and were noted in figure captions and text but, in general, the uptake period was 24 h and flasks were sampled every 3–4 h. Control flasks containing media, but no cells, were evaluated for microbial contamination during the course of the experiment. Contamination, when it occurred, was rapidly detectable by visible cloudiness in the media usually within 24–48 h and was generally the result of poorly sterilized solutions prior to initiation of the experiments. Innoculation of foreign organisms

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rarely occurred as a result of opening flasks and sampling during the experiments. When contamination was detected, the experiment was discarded. All the data presented here were from experiments where there was no evidence of contamination artefacts. Uptake was calculated from solution depletion. At the conclusion of all uptake experiments, cells were removed from the medium by vacuum filtration and weighed (FW). Cells were frozen (—20°C) and lyophylized for dry weight measurements and tissue extraction.

Amino acid inhibition
To investigate the interaction between amino acid uptake and nitrate uptake, cells were grown in the standard culture medium (containing 100 mg Gly, 100 mg Asp, 150 mg Arg, 100 mg Gln per litre) described above with the addition of 1 mM NO3-. Solutions were prepared as described and approximately 5 g of 7-d-old cells were transferred in suspension into two 500 ml Erlenmeyer flasks of medium. Duplicate 0.8 ml samples of solutions were removed daily and analysed for nitrate and amino acid concentrations.

To study the effects of individual amino acids on nitrate uptake, standard induction medium was prepared and the individual amino acids were added from filter-sterilized stock solutions for a final concentration of 2 mM. Amino acid concentrations in these experiments were selected based on depletion rates and the necessity of keeping similar molarities between N-sources. One mM NO3- was determined to be the optimum concentration for induction of nitrate transport (Padgett and Leonard, 1994a), but preliminary experiments indicated 1 mM concentrations of the more readily absorbed amino acids were depleted from solutions within 24 h. After 48 h, solutions containing 2 mM of the individual amino acids still contained small amounts of the amino acid in the medium. Triplicate 125 ml flasks were established for each amino acid treatment. Solution samples were taken every 3–5 h over a 49 h uptake period. The experiment was repeated once.

To establish the effect of cellular free amino acids on nitrate uptake, 7-d-old cells were transferred into 500 ml flasks of standard induction medium containing one of each of the four amino acids evaluated and 1 mM NO3-. Each amino acid treatment was replicated three times. At the time points indicated in the figures, 10 ml aliquots of well-suspended cells and solutions were harvested from each flask to maintain a constant cell to solution ratio. The cells from each sample were allowed to settle prior to removal of the medium. The medium was stored at —20°C for later analysis. Cells were gently washed in 3% sucrose. The wash solution was aspirated off, and the cells frozen in liquid nitrogen and stored at —80°C prior to lyophilization. Preliminary experiments showed that vacuum filtration and washing increased cellular free amino acid content suggesting that the rough handling increased proteolytic activity (data not shown).

Cellular amino acid analysis
Amino acids were analysed as described by Mitchell et al. (1992). Twenty milligrams of lyophilized cells were weighed into a glass homogenizer. Cells were extracted in 5 ml 80% ethanol. The homogenate was centrifuged for 10 min at 450 x g in a table-top centrifuge. One millilitre of the homogenate was passed through a cation exchange column containing 1 ml bed volume 100–200 mesh AG 50W-X8 resin (Bio-Rad Laboratories, Inc., Hercules, CA). The column was washed with 20 ml DDI water and amino acids were eluted with two 1 ml aliquots of 4 M NH₄OH. The eluent was taken to dryness and resuspended in 100 ml drying reagent (1:1:1, triethylamine: absolute ethanol: water) and redried. Detection of amino acids was accomplished by pre-column phenylisothiocyanate (PITC) derivatization as modified by Mitchell et al. (1992). Derivatized samples were dried, resuspended in 1 ml buffer (15 mM sodium acetate, 3% acetonitrile and 0.025% triethylamine, adjusted to pH 7.4 with phosphoric acid), and filtered through 0.2 µm syringe filters. Twenty-microlitre samples were injected into a Rainin Dynamax ODS column (4.5 x 25 mm) held at 48°C. The column was connected to a Beckman binary gradient chromatography system. Separation of PITC-derivatized amino acids was as described by Mitchell et al. (1992) using a binary gradient buffer system. Individual amino acids were detected by UV absorption at 254 nm. Identification and quantification was effected by comparison to PITC-amino acid standards (Sigma, St Louis, MO).

Media analysis
Amino acid concentrations in media were analysed by the same method as cell samples minus the homogenization step. Nitrate concentrations were measured using a Technicon Continuous Flow Analyzer with a standard range of 0–2 mM. Nitrate and amino acids uptake was calculated by solution depletion.

Results
Cells grown in complete nutrient solution containing both amino acids and nitrate quickly depleted the medium of amino acids. Uptake of nitrate was initiated only after organic nitrogen was unavailable (Fig. 1). There is a 1–2 d lag between absorption of nitrate and depletion of extracellular amino acids. During this lag period between amino acid depletion and NO3- uptake, cells were assimilating the amino acids accumulated earlier in the growth phase (Padgett and Leonard, 1994a).

When added individually, each of the amino acids used in the cell suspension medium was equally effective in suppressing absorption of nitrate (Fig. 2). Induction of accelerated nitrate transport by cells transferred into medium containing nitrate as a sole nitrogen source (Fig. 2, inset) was as reported earlier (Padgett and Leonard, 1994b) and similar to the pattern observed for intact roots. Cells transferred to complete culture medium containing both 2 mM of a single amino acid and 1 mM NO3-, however, did not demonstrate accelerated NO3- uptake. Frequent sampling of the solution revealed that some influx of nitrate did occur under these conditions, but that each period of influx was followed by a period of efflux. The periodicity of the response appeared to broaden with time (Fig. 2). The wave-like pattern was not correlated to any known abiotic signals, such as light or temperature, as both were constant during the uptake period.

Uptake of amino acids by cultured maize cells was also not simply linear over a 24 h period (Fig. 3). Glutamine and glycine were absorbed to a greater extent than aspartic acid and arginine (Figs 3, 4). Glutamine was absorbed at high rates and was a superior nitrogen source in sustaining cell growth. In contrast, glycine did not
Regulation of nitrate uptake

Fig. 1. Uptake of total amino acids (Asp, Arg, Gln, Gly) and nitrate by *Zea mays* cell suspension cultures over the course of a 1 week growth period. Solution samples were taken under sterile conditions on the days indicated and analysed for nitrate and amino acid content. Plateaus in the uptake curves are the result of solution depletion. Uptake was calculated from changes in solution concentration. Error bars equal SEM. (Data replotted from *Plant and Soil* 155/156, 159-61, 1994.)

Fig. 2. Uptake of nitrate by *Zea mays* cell suspension cultures over a 48 h period. Cells were transferred 4 d after subculturing into complete medium containing 1 mM NO$_3^-$ as the sole nitrogen source (inset) or containing both 1 mM NO$_3^-$ and 2 mM of the individual amino acids indicated. Solution samples were taken under sterile conditions at the times indicated and analysed for nitrate content. Uptake was calculated from changes in solution concentration. Error bars equal SEM.

Support cell growth in proportion to its relative rate of absorption in the first 24 h. Arginine, despite being a rich source of nitrogen did not lead to rapid cell growth and it was taken up in relatively low amounts. Arginine gave inconsistent results with respect to its ability to suppress nitrate uptake and induction, possibly because it was weakly absorbed by the cells (data not shown).

Analysis of amino acid concentrations in the cells confirmed the poor uptake properties of arginine by maize cell cultures. Cells placed in medium containing arginine
Fig. 3. Uptake of individual amino acids by *Zea mays* cell suspension cultures over a 24 h period. Cells were transferred 4 d after subculturing into complete medium containing either one of the amino acids indicated. Cells and solutions were sampled under sterile conditions at the time points indicated and solutions were analysed for amino acid content. Uptake was calculated from solution depletion. Error bars equal SEM.

and nitrate had cellular concentrations of total amino acids only marginally different from cells grown in medium containing nitrate alone (Fig. 5). Cellular free amino acid concentrations of cells grown in nitrate plus aspartic acid, glutamine or glycine were much higher. Nitrate reductase activities were also very low under these conditions (data not shown).

Depletion of amino acids from the culture solution is rapid (Fig. 1) and leads to large accumulations of free amino acids within the cell. Pools of accumulated free amino acids slowly decline over the growth period as amino acids are incorporated into proteins and other nitrogenous compounds. Cellular total protein concentrations reached a peak 2–3 d after transfer and subsequently declined (Padgett and Leonard, 1994a). Conversion of the absorbed amino acid to other amino acids must occur during this time. Therefore, it is possible that the inhibition of influx and/or the stimulation of efflux of nitrate is not due specifically to the exogenous amino acid, but to other amino acids synthesized from the exogenous precursor. To investigate this possibility, cells were treated with various amino acids and cellular content of the individual amino acids was determined.

**Cellular free amino acid levels**

The transfer, storage and remobilization of nitrogenous compounds between the vacuole and the cytoplasm and its significance to \( \text{NO}_3^- \) uptake and assimilation in root cells has been controversial, particularly with regard to the highly vacuolated cortical cells (Beck and Tenner, 1989). The P3377 maize suspension cultures contain cells that are primarily meristematic in nature (Felker, 1987; Padgett and Leonard, 1994a). Like meristematic cells, microscopic investigation of the P3377 cell line revealed that rapidly dividing cells are densely cytoplasmic with no central vacuole (Padgett and Leonard, 1994a).

Although a thorough investigation of vacuolar volume and function for the P3377 cell line has not been completed, if typical literature values for vacuolar development in apical meristems are used (Patel et al., 1990; Davies et al., 1992) the P3377 cells would contain vacuolar volumes ranging from 0% to 15% of the total volume enclosed by the plasmalemma. Although this does not exclude the possibility of compartmentation of free amino acid into provacuoles or other organelles, it does increase the validity of using whole cell measurements for correlations between uptake and amino acid concentrations.

Cellular concentrations of individual amino acids shifted rapidly over the course of the 24 h experiment (Fig. 6). Internal concentrations of the same amino acids added to the external solution increased in proportion to their rate of uptake (Figs 3, 6). Aspartic acid levels in cells transferred into medium containing nitrate and Asp were similar to other treatments until 6 h after transfer.
Fig. 4. Growth of Zea mays cell suspension cultures using individual amino acids as sole nitrogen sources. Cells were transferred into media containing approximately 15 mM of a single amino acid. Changes in culture volume were measured by the 'settled volume' method under sterile conditions.

Fig. 5. Changes in cellular concentration of total free amino acids of Zea mays suspension cultures over 24 h of growth in various amino acid solutions. Cells were harvested from the treatments described in Fig. 3 and lyophilized. Total amino acids were extracted from dried cells and analysed by HPLC. Error bars equal SEM.
when elevated levels of free Asp were observed throughout the remainder of the 24 h period (Fig. 6A). Arginine levels in cells treated with arginine and nitrate (Fig. 6B) showed no consistent accumulation pattern until 9 h after transfer, possibly because it was slowly absorbed from the medium (Fig. 3). By 21–24 h, arginine levels were below detection limits in all treatments. Uptake and accumulation of glutamine was the most consistent of the four amino acids treatments (Fig. 6C). Cellular concentrations of Gln were relatively constant from 6 h after transfer until the end of the experiment. Glycine was rapidly taken up by cells treated with glycine and nitrate (Fig. 3) and cellular concentrations of glycine remained much higher than any of the other treatments (Fig. 6D).

No single amino acid appeared to accumulate in response to treatment with the various amino acids. Hence, of the amino acids tested, none appear to regulate nitrate uptake alone. Glutamine has been proposed as an important nitrogen status signal and regulator of nitrate uptake and assimilation, but the data showed no consistent pattern to support such a conclusion. Glutamic acid levels in Asp- and Gin-treated cells were nearly double the levels found in induced cells, but the glycine-treated cells which were not induced showed low Glu levels similar to that of the inducible nitrate controls (Fig. 7A). Asparagine itself did not accumulate in Asp-treated cells until 6 h after transfer by which time induction had occurred. Cellular Asn concentrations were below detection limits in all other treatments until 9 h after transfer (Fig. 7B).

Cellular concentrations of valine, lysine, and proline were relatively equivalent in all treatments during the first 12 h (Fig. 8) with the exception of valine concentrations in glutamine-treated cells. The sulphur containing amino acids, cysteine, and methionine increased in concentration over the course of the experiment in a pattern similar to that demonstrated by total amino acid concentrations (data not shown).

While no single amino acid appeared to signal nitrogen status of the maize cells, differences in total amino acids accumulating in the cells correlated well with inducibility of the cells (Fig. 5). This suggests that regulation of nitrate transport by maize solution-cultured cells is not a simple 'switch-type' mechanism, where the pool size of one particular amino acid determines nitrate assimilation, but rather a more complex 'steady-state' mechanism where the relationship between concentrations of several, or all, amino acids is significant in regulating the uptake and assimilation of nitrate.

**Discussion**

The use of either a mixture of amino acids, such as casein hydrolysate or defined concentrations of individual amino acids is common practice in plant cell and tissue culture. Several authors have documented the stimulatory or inhibitory effect of amino acids on the growth of cells in solution culture (e.g. Gamborg, 1970; Heimer and Filner, 1970; Bayley *et al*., 1972; Behrend and Mateles, 1975). The effect of nitrogen source, $\text{NO}_3^-$, $\text{NH}_4^+$, urea, or amino acids on culture growth appears to be species dependent, with some species being more tolerant of a wide variety of sources than others (Behrend and Mateles, 1975; Skokut and Filner 1980). To our knowledge, no one has compared the actual uptake characteristics of the individual nitrogen sources. The *Zea mays* cell cultures demonstrated significant differences in patterns of absorption for specific amino acids and nitrate.

The growth characteristics of the *Zea mays* embryo cell cultures in nitrate and amino acids has been documented elsewhere (Padgett and Leonard, 1994b). Clearly, amino acids are preferentially absorbed in the early stages of culture growth (Fig. 1). Although small amounts of $\text{NO}_3^-$ appear to enter the cells in the first 3 d, active uptake does not occur until amino acids are depleted from the solution and cellular free amino acid concentrations are below some threshold level. When individual amino acids were tested for their ability to support growth over the normal culture life-span, glutamine exhibited both higher uptake rates (Fig. 3) and growth when compared to Asp, Arg, and Gly (Fig. 4), presumably because of its pivotal role in nitrogen metabolism. To use the nitrogen for synthesis of other amino acids or nitrogenous containing compounds, the cells must be able to remove the amino groups. Arginine may be the most difficult to utilize, as the catabolism of arginine produces urea in addition to free ammonium (Atkins and Beevers, 1990). Without the enzymes required to metabolize urea, this compound may reach toxic levels (Wedding *et al*., 1992). The ability of solution-cultured cells to utilize urea is poor without time to adapt under an appropriate selection pressure (Skokut and Filner, 1980). Urease activities in the P3377 maize cell cultures have not been investigated. Changes in nitrogen metabolism have been documented that correlate to lag, log, and stationary phases of cell culture growth (Street *et al*., 1976; Padgett and Leonard, 1994a). It is possible that the ability to utilize urea may change with other variations in nitrogen utilization. Lack of catabolic assimilation of the nitrogen in Arg was
apparent by the small changes in both the total and individual amino acids (Figs 5–7) in cells grown in Arg as compared to changes in amino acid concentrations exhibited by cells grown in Asp, Gln or Gly.

Glycine was rapidly absorbed from the nutrient solution, but failed to support rapid cell growth over the 8 d period, although Gly was effective in preventing uptake of nitrate. Catabolism of glycine yields TCA cycle intermediates (Atkins and Beevers, 1990). If the cells are dependent upon the nitrogen in Gly, accumulations of the catabolic product glyoxylate may negatively impact respiratory or other pathways and reduce culture growth.

The uptake and growth response to Asp was somewhat more puzzling. Although growth in Asp for the first 3 d was comparable to that of Gln (Fig. 4), the growth rate was not sustained. Even though induction of nitrate uptake was inhibited by Asp (Fig. 2), total amino acid levels in cells grown in Asp were less than those grown in Gln or Gly, but significantly higher than those grown in NO$_3^-$ alone.

The control of nitrate uptake as a function of regulation of influx, efflux or both is presently a matter of debate (Aslam et al., 1994). As most uptake experiments are conducted by measuring changes in concentrations of the bulk solution or microelectrodes at the external surface of the root, the use of intact roots limits the ability to account for changes in solution NO$_3^-$ concentration at the plasma membrane. Estimation of the solution concentration at the cell membrane is hampered by the extensive apoplastic space of the root cortex. Changes in NO$_3^-$

Fig. 7. Cellular concentrations of glutamate and asparagine for *Zea mays* suspension cultures over 24 h of growth. Cells were harvested from media containing NO$_3^-$ alone or nitrate in combination with a single amino acid (Asp, Arg, Gln, Gly). Amino acids were extracted from cells and analysed by HPLC. Treatments are shown in the legend and the cellular concentration of the individual amino acids is noted in the body of each panel: (A) glutamate, (B) asparagine. Error bars equal SEM.
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Fig. 8. Cellular concentrations of lysine, proline, and valine in Zea mays suspension cultures over 24 h of growth. Cells were harvested from media containing NO\textsuperscript{3-} alone or nitrate in combination with a single amino acid (Asp, Arg, Gln, Gly). Amino acids were extracted from cells and analysed by HPLC. Treatments are shown in the legend and the cellular concentration of the individual amino acids noted in the body of each panel: (A) lysine, (B) proline, (C) valine. Error bars equal SEM.

Concentrations of the bulk solution reflect only what has penetrated the root surface, not necessarily what has entered the cell. In order for efflux to be detected, the NO\textsuperscript{3-} ions must cross back through the apoplastic space to the root surface and re-enter the bulk solution external to the root. Solution-cultured cells have cell wall spaces, and interspaces in clumps, but to a limited extent when compared to intact roots. This allows for a greater sensitivity, and a better estimation of how much NO\textsuperscript{3-} enters or exits the individual cell. In the presence of any of the four amino acids investigated (Fig. 2), induction of NO\textsuperscript{3-} transport was inhibited. The pattern of repeated slight influx followed by efflux is consistent with a role for nitrate efflux in the regulation of nitrate transport. It would appear that without induction, the cells are capable of absorbing NO\textsuperscript{3-} from the solution, but in the presence of the amino acids nitrate efflux was sufficient to remove the absorbed NO\textsuperscript{3-} from the cells, thereby regulating nitrate accumulation. At a later point, when the amino acid concentrations were depleted, the cycling was no longer apparent and influx would become the dominant factor. As both light and temperature were held constant and the cells are non-photosynthetic, there seems to be no other explanation for the apparent influx and efflux of nitrate.

Glutamine was the most effective amino acid in both accumulation in cells and the ability to sustain growth (Figs 3, 4). Given the pivotal role of Gln in nitrogen metabolism, this was an expected result. Glutamine is generally considered to be a likely candidate for the key amino acid in the feedback inhibition of the induction of nitrate reductase activity in addition to uptake. In a recent report by Li et al. (1995), the addition of Gln to the hydroponic nutrient solution reduced accumulation of nitrate reductase mRNA and protein in roots, but not in shoots, of 6-d-old maize seedling. This was correlated with a dramatic increase in Gln levels in roots, as compared to shoots, and is consistent with a role for Gln in regulation of induction of nitrate reductase.

While Gln was an effective regulator of NO\textsuperscript{3-} uptake and assimilation, these results indicate that accumulation of other amino acids in cells inhibited NO\textsuperscript{3-} uptake without resulting in an increase in Gln levels. For example, Gly prevented the induction of NO\textsuperscript{3-} uptake in cultured maize cells (Fig. 2) without leading to an accumulation of Gln in these cells (Fig. 6D). Whatever the mechanism of amino acid regulation on NO\textsuperscript{3-} metabolism, it does not appear to involve just one specific amino acid. This is contrary to the evidence in single-celled organisms, but in accordance with the work of Lee et al. (1992). While this does not necessarily eliminate Gln as a regulatory molecule, it would appear to eliminate a simple accumulation model for regulation of nitrate uptake. Since the suspension-cultured cells used in our studies do not contain a single large vacuole (Padgett and Leonard, 1994a) as is typical of root cortical cells, the potential still exists for regulation via vacuolar compartmentation as was suggested by Lee et al. (1992).

The observation that cellular free amino acid levels inhibit nitrate uptake and induction has potential implications for developing fertilizer management strategies and/or molecular techniques to improve nitrate fertilizer utilization in some crop situations. Presently, methods to determine nitrogen status and requirements in field-grown
crops are often difficult to calibrate. The development of methods utilizing metabolic signals such as cellular free amino acid levels may improve fertilizer timing and rate applications. Understanding the molecular mechanism of amino acid feedback inhibition of nitrate uptake will lead to identification of genes involved in this regulation. In turn, it should be possible to use appropriate molecular techniques to uncouple amino acid levels from control of nitrate absorption. Unregulated absorption and storage of nitrate may potentially lead to utilization of a greater proportion of applied nitrate fertilizer. As a first step, it will be necessary to determine if amino acid regulation of nitrate uptake is a significant factor limiting nitrate use efficiency during the course of crop growth.

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