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Ammonium Assimilation and the Role of γ-Aminobutyric Acid in pH Homeostasis in Carrot Cell Suspensions[1]

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In vivo ^{15}N NMR spectroscopy was used to monitor the assimilation of ammonium by cell-suspension cultures of carrot (*Daucus carota* L. cv Chantenay). The cell suspensions were supplied with oxygen in the form of either pure oxygen ("oxygenated cells") or air ("aerated cells"). In contrast to oxygenated cells, in which ammonium assimilation had no effect on cytoplasmic pH, ammonium assimilation by aerated cells caused a decrease in cytoplasmic pH of almost 0.2 pH unit. This led to a change in nitrogen metabolism resulting in the accumulation of γ-aminobutyric acid. The metabolic effect of the reduced oxygen supply under aerated conditions could be mimicked by artificially decreasing the cytoplasmic pH of oxygenated cells and was abolished by increasing the cytoplasmic pH of aerated cells. The activity of glutamate decarboxylase increased as the cytoplasmic pH declined and decreased as the pH recovered. These findings are consistent with a role for the decarboxylation of glutamate, a proton-consuming reaction, in the short-term regulation of cytoplasmic pH, and they demonstrate that cytoplasmic pH influences the pathways of intermediary nitrogen metabolism.

GABA is present in virtually all plant tissues and often accumulates under conditions of environmental stress, including hypoxia (Streeter and Thompson, 1972a; Tsushida and Murai, 1987; Reggiani et al., 1988; Menegus et al., 1989; Fan et al., 1992; Roberts et al., 1992), low temperature (Wallace et al., 1984), heat shock (Mayer et al., 1990), and low pH (Lane and Stiller, 1970). It is mainly produced by the action of GDC on glutamate and it can be further metabolized to succinic semialdehyde and succinate via GAB-T and SSADH (Streeter and Thompson, 1972b; Satya Narayan and Nair, 1986). It has been suggested that the accumulation of GABA can be used as a marker for dead or dying hypoxic cells (Roberts et al., 1992). However, recent reports have shown that GABA is a major nitrogen sink for freshly assimilated ammonium in apparently nonstressed suspension-cultured carrot cells and protoplasts (Robinson et al., 1991; Carroll et al., 1992), suggesting that GABA plays an integral role in nitrogen metabolism. It has also been suggested that the high accumulation of GABA in many tissues could be the result of a change in the relative activities of GDC and GAB-T following a decrease in $\mathrm{pH_{cyt}}$ (Streeter and Thompson, 1972b; Wallace et al., 1984). Because GDC activity increases as pH decreases and because glutamate decarboxylation is a proton-consuming reaction, it follows that GABA production could act as a sink for excess protons in the cytoplasm and thus contribute to pH regulation (Murphy et al., 1983; Reid et al., 1985; Menegus et al., 1989; Roberts et al., 1992; Snedden et al., 1992).

In the study reported here, we used in vivo ^{15}N and ^{31}P NMR spectroscopy to investigate the effect of oxygen availability on ammonium assimilation by carrot cell suspensions. The aim was to establish whether the effect of the oxygen supply on GABA accumulation was controlled by changes in $\mathrm{pH_{cyt}}$ and to assess the importance of GABA production for $\mathrm{pH_{cyt}}$ regulation.

MATERIALS AND METHODS

Plant Material

Suspension cultures of carrot (*Daucus carota* L. cv Chantenay) were maintained on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.2 mg/L 2,4-D, 0.1 mg/L kinetin, and 20 g/L Suc at 25°C. Cells were subcultured every 14 d by inoculating 1 volume of suspension into 10 volumes of fresh medium. Protoplasts were prepared as described previously (Carroll et al., 1992).

Preparation of Cells for in Vivo NMR

Stationary phase cells (14 d) were harvested by gentle filtration through a 40-μm nylon mesh. Cells (approximately 12 g fresh weight) were resuspended in 10 mM Mes/0.1 mM

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Abbreviations: AOA, aminooxyacetate; GABA, γ-aminobutyric acid; GAB-T, γ-aminobutyric acid:α-oxo acid transaminase; GDC, glutamate decarboxylase; GOGAT, glutamate synthase; GS, glutamine synthetase; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; $\mathrm{pH_{cyt}}$, cytoplasmic pH; ppm, parts per million; SSADH, succinic semialdehyde dehydrogenase.

$CaSO_4$ at pH 6.0 to give a total volume of 25 mL, which corresponded to a packed cell volume of 50%. The suspension was transferred to a 20-mm-diameter NMR tube in which the cells were supplied with either pure oxygen ("oxygenated cells") or air ("aerated cells") in an airlift system operating with a gas flow rate of 50 mL/min (Fox et al., 1989). The cells were allowed to stabilize in the NMR tube for 3 h prior to the addition of ^{15}N-labeled ammonium chloride (98% ^{15}N) to an initial extracellular concentration of 20 mM (the same concentration as in the growth medium) and the acquisition of spectra. Control experiments showed that cell viability, assayed using fluorescein diacetate, generally decreased only slightly, typically from 94 to 92% in a period of 24 h in the NMR tube.

NMR Spectroscopy

^{14}N, ^{15}N, ^{13}C, and ^{31}P NMR spectra were recorded at 21.67, 30.42, 75.46, and 121.49 MHz, respectively, using a Bruker CXP300 spectrometer with an Oxford Instruments (Oxford, UK) 7.05 T superconducting magnet and 20-mm-diameter selective frequency and broadband probe heads. Cell suspensions were either oxygenated or aerated continuously with the airlift system, and the temperature was maintained at 25°C.

^{14}N NMR spectra were accumulated with a 60° pulse angle, a recycle time of 3 s, a total acquisition time of 30 min, and broadband ^{1}H-decoupling for 0.25 s during the acquisition.

^{15}N NMR spectra were accumulated with a 90° pulse angle, a recycle time of 2 s, a total acquisition time of either 30 min or 2 h, low-power broadband decoupling for 1.75 s prior to acquisition to produce the nuclear Overhauser enhancement, and high-power decoupling for 0.25 s during the acquisition. Chemical shifts were measured relative to the Gln and ammonium signals and are quoted relative to nitrate at 0 ppm.

^{13}C NMR spectra were accumulated with a 90° pulse angle, a recycle time of either 2 or 4 s, a total acquisition time of either 30 min or 2 h, low-power decoupling for 1.875 s prior to acquisition to produce the nuclear Overhauser enhancement, and high-power decoupling for 0.125 s during the acquisition.

^{31}P NMR spectra were accumulated with a 45° pulse angle, a recycle time of 0.5 s, a total acquisition time of 30 min, and broadband decoupling for 0.43 s during the acquisition. Chemical shifts were measured relative to the signal from a capillary containing a 2% (v/v) aqueous solution of the tetraethyl ester of methylene diphosphonic acid and are quoted relative to 85% orthophosphoric acid at 0 ppm (Kime et al., 1982). pH_{cyt} values were determined as described previously (Fox and Ratcliffe, 1990).

Determination of GDC Activity

GDC activity was assayed using ammonium-loaded protoplasts obtained from 14-d carrot cells. Protoplasts were used to overcome problems of inefficient and irreproducible enzyme extraction from cells. Ammonium chloride (2 mM) was added to a suspension of approximately 10^6 protoplasts/mL in an osmotically balanced (0.5 M mannitol), buffered (50 mM Mes, pH 5.7), nitrogen-free Murashige and Skoog growth medium. The protoplast suspension was aerated on an orbital shaker because it was not possible to maintain intact protoplasts using an airlift system in an NMR sample tube. Protoplasts were harvested at 2-h intervals as described previously (Carroll et al., 1992) and extracted into 3 volumes of ice-cold 100 mM K_2HPO_4/KH_2PO_4, 0.01% Triton X-100, 2 mM DTT at pH 5.8. The extract was centrifuged at 10,000g for 10 min at 4°C to remove cell debris and then held on ice until required. GDC activity was determined by measuring CO_2 release from glutamate. The reaction mixture (1 mL) contained 5 mM glutamate, 10 μM pyridoxal-5′-phosphate, 0.25% (v/v) Antifoam A emulsion (Sigma, Poole, Dorset, UK), 1 mM AOA, 100 mM K_2HPO_4, 100 mM KH_2PO_4 at pH 5.8. This mixture was contained within a gas-tight side arm test tube receiving CO_2-free air via an Analytical Development Co. (Hertfordshire, UK) air supply unit at 250 mL/min. The tube was connected via the side arm to an Analytical Development Co. LA2 portable IRGA, and CO_2 evolution was initiated by the addition of 100 μL of the crude enzyme extract (20–50 μg of soluble protein) via a needle and syringe piercing the bung. Steady-state CO_2 evolution was measured during 10-min intervals against a CO_2-free reference. The reaction was linear with respect to time and enzyme concentration. In the absence of glutamate, the rate of CO_2 evolution was always less than 4% of that observed in the presence of glutamate, indicating that the assay was specific for GDC activity. AOA was included in the assay to suppress transamination reactions, in the belief that it would have only a limited effect on the GDC activity. In fact, AOA reduced the total activity of the extracts by only 7%, and it could probably have been omitted from the assay mixture. Soluble protein was determined using the Bio-Rad protein assay (Bradford, 1976).

HPLC and GC-MS Analysis of Soluble Amino Acid Pools

Following certain NMR experiments, soluble amino acids were extracted into methanol. The amino acid composition of the extract was determined by HPLC analysis of the *o*-phthaldialdehyde derivatives, and ^{15}N incorporation was measured by GC-MS analysis of the *tert*-butyldimethylsilyl derivatives (Robinson et al., 1991).

RESULTS AND DISCUSSION

Effect of Oxygen Supply on Ammonium Assimilation

The incorporation of $^{15}NH_4Cl$ into amino acids of 14-d carrot cells was analyzed using in vivo NMR and GC-MS. Figure 1 shows the in vivo ^{15}N NMR spectra obtained from oxygenated cells in the presence of 20 mM [^{15}N]ammonium. Despite the activities of GS and GOGAT being at their lowest and the activity of glutamate dehydrogenase being at its highest in the stationary phase of the culture cycle (Robinson et al., 1991, 1992), the labeling pattern was consistent with assimilation via the GS/GOGAT cycle (Fig. 2). Thus, label from ammonium appeared in the amide-N of Gln first (Fig. 1a) and only subsequently in the amino-N of glutamate. The labeling of Gln and glutamate increased steadily during the first 8 h and then declined. Sometimes Ala and GABA could also be detected but only just above the noise level. Previ-

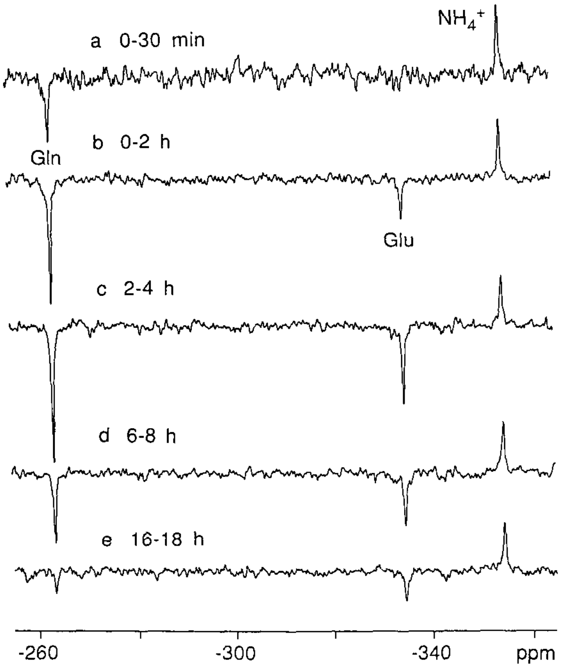

Figure 1. ^1H-decoupled ^{15}N NMR spectra of oxygenated carrot cells following the addition of 20 mM [^{15}N]ammonium chloride. The spectra were obtained in either 30 min or 2 h during the intervals indicated. The peaks were assigned as described previously (Robinson et al., 1991).

ously published spectra of younger (11 d) carrot cells had more readily detectable Ala and GABA resonances (Robinson et al., 1991), probably reflecting a difference in metabolism between growing cells and those that have already entered stationary phase.

Passing air, as opposed to oxygen, through the airlift system limited the amount of oxygen available to the cells in the NMR tube (Fox et al., 1989), and under these conditions, there was a change in the labeling pattern during ammonium assimilation (Fig. 3). Label still appeared first in the amide-N of Gln (data not shown), but the initial accumulation of Gln was markedly less than that in the oxygenated cells. Glutamate became labeled earlier and more intensely, and there was also a rapid and strong incorporation of label into Ala. The intensity of the Ala resonance increased over 12 h, and then the label incorporated in both glutamate and Ala decreased concurrently with an increase in the intensity of the GABA resonance, which became the dominant feature of the spectrum by the end of the experiment.

The airlift system is a closed system, and therefore, the NMR signals detected would have been derived from the whole sample, including the external medium. After treating aerated cells with ^{15}N-labeled ammonium for 20 h, we found that approximately 20% of the intensity of the GABA resonance originated from the external medium, indicating that the cells exported some of the newly synthesized ^{15}N-labeled GABA. This proportion was much less than that observed for asparagus mesophyll cells, which export approximately 80% of the GABA synthesized following the addition of glutamate (Chung et al., 1992). Intracellular GABA would have been located predominantly in the cytoplasm. In carrot protoplasts, the vacuolar content has been shown to be only 14% of the total pool, and newly synthesized [^{15}N]GABA was found only in the cytoplasm during the first 8 h (Carroll et al., 1992).

In general, ^{15}N NMR does not allow an accurate determination of amino acid pools with a good time resolution (Fox et al., 1992), and because changes in ^{15}N NMR peak intensity can reflect either a genuine change in pool size or just a change in fractional labeling, HPLC and GC-MS were used to determine total amino acid pools and the extent of label incorporation (Table I). The labeling patterns were generally consistent with the NMR data, although as observed previously (Robinson et al., 1991), labeled GABA was not readily detectable by in vivo ^{15}N NMR in oxygenated cells even though HPLC and GC-MS indicated that the cell extracts contained comparable amounts of labeled Gln, glutamate, and GABA after 18 h (Fig. 1e; Table I). The stress imposed on the cells during filtration and extraction could conceivably have induced GABA synthesis, leading to the increased level detected in the extract, and this possibility needs to be explored in future work.

After 18 h of incubation with ^{15}N-labeled ammonium, aerated cells contained approximately half the amount of Gln, twice as much glutamate, and more than 5-fold more Ala than oxygenated cells (both ^{15}N-labeled and total pool sizes). The ^{15}N-labeled GABA pool was 5-fold greater in the

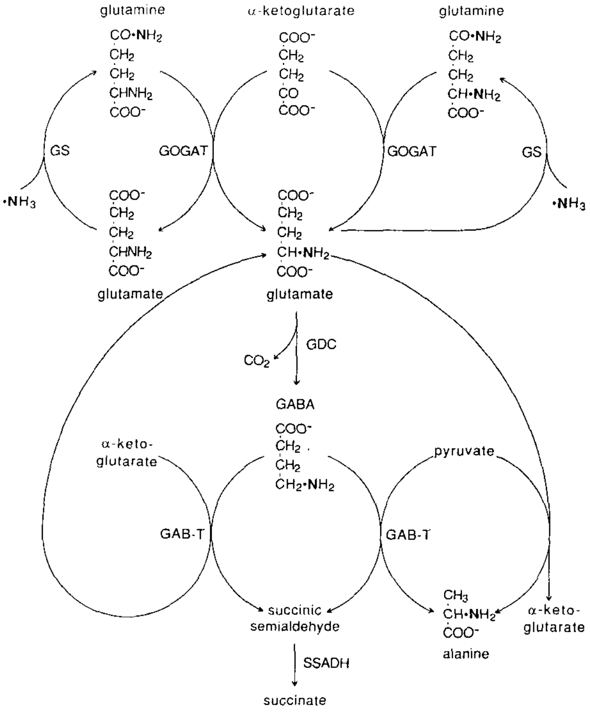

Figure 2. The assimilation of ^{15}N-labeled ammonia by the GS/GOGAT cycle and the subsequent metabolism of glutamate by the GABA shunt. The transamination of glutamate to Ala by Ala aminotransferase is also shown. The ^{15}N-labeled nitrogen atoms are highlighted with a bullet (•).

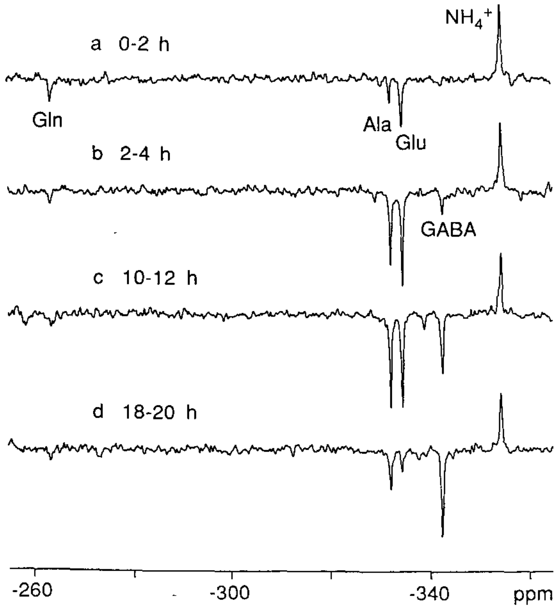

Figure 3. ^{1}H-decoupled ^{15}N NMR spectra of aerated carrot cells following the addition of 20 mM [^{15}N]ammonium chloride. The spectra were obtained in 2 h during the intervals indicated.

aerated cells, whereas the total pool size was only about 2.5-fold greater, indicating that most of the GABA had been produced from recently synthesized glutamate and not from pools of unlabeled glutamate already present. Overall, however, the generally low level of labeling at the end of the 18-h experiment can be attributed to the dilution of the [^{15}N]-ammonium by the endogenous pools and sources of unlabeled ammonium. In vivo ^{14}N NMR experiments showed that the cells contained a substantial ammonium pool at the start of the labeling period and that the size of this pool varied throughout the 18-h time course (data not shown).

Increases in GABA, ranging from 5-fold in 24 h in rice shoots (Reggiani et al., 1988) to 100-fold (with a 7-fold increase in Ala) in 4 h in radish leaves (Streeter and Thompson, 1972a) have been observed previously in a wide range of plants under hypoxia (Streeter and Thompson, 1972a; Tsushida and Murai, 1987; Reggiani et al., 1988; Menegus et al., 1989; Fan et al., 1992; Roberts et al., 1992). However, in these studies oxygen-free nitrogen was supplied to ensure complete anaerobiosis, whereas in the experiments reported here the oxygen supply was only mildly restricted. Increases in Ala (Streeter and Thompson, 1972a; Roberts et al., 1992) and glutamate (Roberts et al., 1992) have also been observed following the onset of hypoxia, and in the case of hypoxic maize root tips the accumulation of Ala, glutamate, and GABA was enhanced by pretreatment with ammonium (Roberts et al., 1992). The accumulation of these amino acids is consistent with pH$_{cyt}$ being regulated, at least in part, by changes in primary metabolism because the synthesis of Ala, arising from the decarboxylation of malate as a result of malic enzyme activation by cytoplasmic acidosis, the conversion of Gln to glutamate, and the decarboxylation of glutamate to GABA are all proton-consuming reactions.

GABA Accumulation and pH$_{cyt}$ Regulation

^{31}P NMR was used to assess the physiological status of the carrot cells under oxygenated and aerated conditions (Fig. 4). In aerated cells the chemical shift of the cytoplasmic Pi signal indicated that pH$_{cyt}$ was only marginally lower, at 7.5, than the value of 7.6 obtained for oxygenated cells. These high pH values are typical of well-oxygenated plant tissues (Pfeffer et al., 1986), and they suggest that switching from oxygen to air had only a marginal effect on the supply of oxygen to the tissue. However, whereas the β-NDP signal at −5.45 ppm, which serves as a ^{31}P NMR marker for hypoxia, was absent from the spectra recorded under oxygenated conditions (Fig. 4a), a weak signal was sometimes observed under aerated conditions (Fig. 4b). This result, coupled with the observation of the higher cytoplasmic Pi:NTP intensity ratio in the spectra of the aerated cells (Fig. 4), indicated that the aerated cells were mildly hypoxic.

^{31}P NMR was also used to assess the physiological status of the carrot cells during ammonium treatments and to monitor pH$_{cyt}$. In keeping with the minimal decline in cell viability during the NMR experiments, Figure 5 shows that prolonged exposure to ammonium under aerated conditions had no adverse effect on such metabolic indicators as pH$_{cyt}$, the NTP level, and the phosphomonoester pool. In fact, comparisons

Table I. *Total and ^{15}N-labeled pool sizes of amino acids after 18 h of treatment with 20 mM [^{15}N]ammonium chloride*

The pool sizes are given only for those amino acids detected by in vivo ^{15}N NMR spectroscopy. Each value is the mean ± SE of three experiments for the oxygenated cells or four experiments for the aerated cells.

Amino Acid	Oxygenated Cells		Aerated Cells	
	Total	^{15}N labeled	Total	^{15}N labeled
		µmol/g fresh wt		
Gln	1.50 ± 0.28	0.28 ± 0.04[a] 0.06 ± 0.02[b]	0.78 ± 0.10	0.13 ± 0.01[a] 0.03 ± 0.01[b]
Glutamate	0.98 ± 0.22	0.20 ± 0.06	1.59 ± 0.44	0.41 ± 0.12
Ala	0.28 ± 0.05	0.07 ± 0.02	1.42 ± 0.46	0.46 ± 0.18
GABA	3.06 ± 0.65	0.24 ± 0.07	7.21 ± 0.38	1.25 ± 0.07

[a] Singly labeled Gln. [b] Doubly labeled Gln.

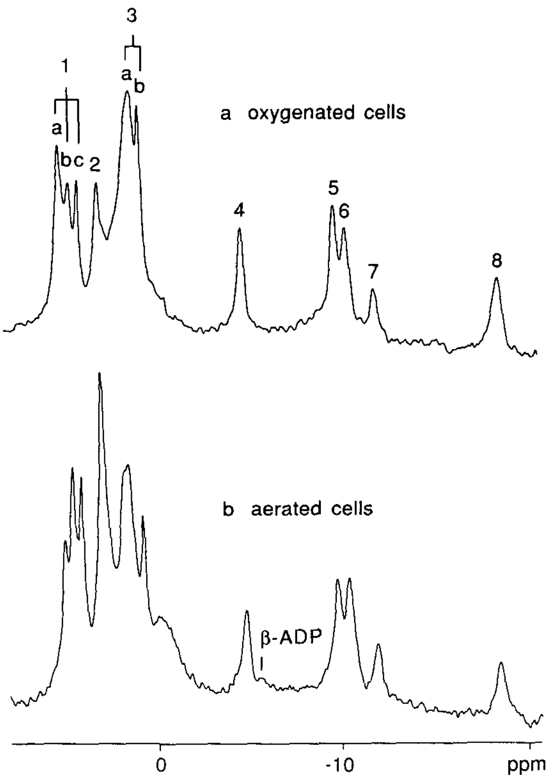

Figure 4. ^{31}P NMR spectra of carrot cells after 10 h in the NMR tube under oxygenated (a) and aerated conditions (b). The spectra were obtained in 2 h. The assignment of the labeled resonances is: 1, several phosphomonoesters, including Glc-6-P (1a) and phosphocholine (1c); 2, cytoplasmic Pi; 3, vacuolar Pi with several underlying phosphodiesters (3a) and glycerophosphorylcholine (3b); 4, the γ-phosphate of NTP, principally ATP; 5, α-NTP; 6, NDP-hexose and nicotinamide adenine dinucleotides [NAD(P)(H)]; 7, NDP-hexose; 8, β-NTP.

between control and ammonium-treated cells, under both aerated and oxygenated conditions, showed no long-term differences (data not shown). However, whereas there was no change in pH_{cyt} during 18 h in control experiments, irrespective of the oxygen supply, ammonium treatment caused a transient decrease in pH_{cyt} in the aerated cells (Fig. 6). The pH change indicates that the pH regulatory mechanisms were operating less efficiently under aerated conditions, and the transient decrease in pH presumably reflects a proton load generated either by the uptake of ammonium or by its subsequent assimilation. If ammonia is the permeable form, even at pH 6, then uptake would tend to increase pH_{cyt}; whereas if ammonium is also permeable, then in principle pH_{cyt} could decrease, particularly at low external pH. There is uncertainty in the literature about the significance of ammonium transport in plant tissues (Ullrich, 1992), but in contrast to the situation in mammalian cells, in which exposure to ammonium at high concentrations can be used to equilibrate internal and external pH (Franchi et al., 1986), it seems unlikely that ammonium transport could dominate the ammonium-loading process in the carrot cells. Thus, it seems that ammonium assimilation, which generates protons (Raven, 1985), is responsible for the pH change in the aerated cells. Unfortunately, Met sulfoximine and phosphinothricin, which inhibit GS, were ineffective under the conditions imposed on the aerated cells (data not shown), and therefore, it was not possible to use metabolic inhibitors to prove that ammonium assimilation caused the transient decrease in pH.

The carbohydrate status of the tissue had an important bearing on the extent of ammonium assimilation, with assimilation being greatly reduced in carbon-limited cells under both oxygenated and aerated conditions. In aerated cells, very little GABA accumulated, and there was no detectable decrease in pH_{cyt} following the addition of ammonium. Natural abundance ^{13}C NMR spectra of these cells revealed that the carbohydrate content had decreased dramatically and in some cases was virtually undetectable (data not shown). Addition of at least 5 g/L Suc to the external medium of such cells resulted in an increase in ammonium assimilation, under both oxygenated and aerated conditions, and restored the accumulation of GABA and the transient decrease in pH_{cyt} in the aerated cells. Supplying cells with 20 g/L Suc usually had a similar effect but occasionally resulted in pH_{cyt} decreasing slightly lower to pH 7.2 and sometimes prolonged the pH recovery by several hours. Clearly, when there was insufficient carbohydrate to provide carbon skeletons via glycolysis and the Krebs cycle for the production of Gln and glutamate, ammonium assimilation was limited. When the rate of ammonium assimilation was reduced, there was no transient decrease in pH_{cyt} and little accumulation of GABA, suggesting

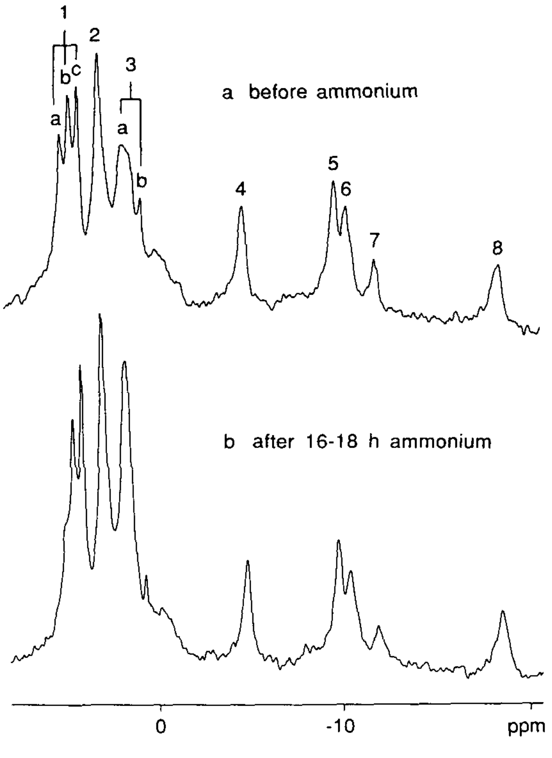

Figure 5. ^{31}P NMR spectra of aerated carrot cells before the addition of 20 mM ammonium chloride (a) and after 16 to 18 h of exposure to ammonium (b). The spectra were obtained in 2 h. The assignment of the labeled resonances is the same as in Figure 4.

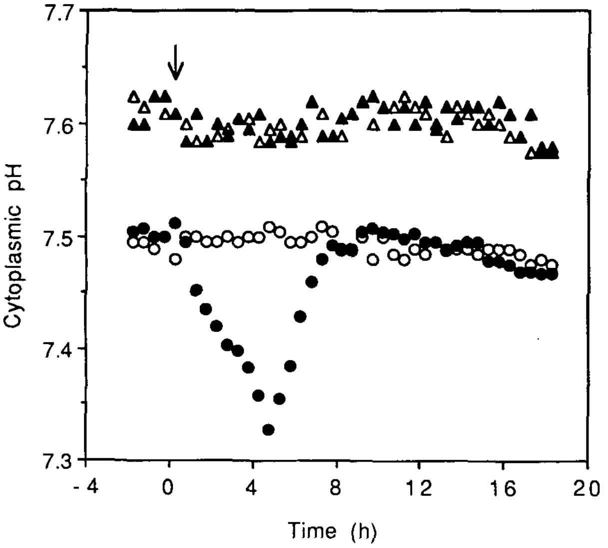

Figure 6. Changes in pH_{cyt} of untreated aerated (○), ammonium-treated aerated (●), untreated oxygenated (△), and ammonium-treated oxygenated (▲) carrot cells. Ammonium chloride was added at time 0 h as indicated by the arrow. The graph is a plot of the mean of three experiments for the ammonium-treated aerated cells and of two experiments for the other treatments. The exact nature of the pH profile of the ammonium-treated aerated cells was sensitive to many factors, such as cell density, oxygen supply, and carbohydrate status of the tissue, and therefore, the data given here are from experiments carried out under the most comparable conditions.

first that the transient decrease in pH seen in "carbon-replete" cells was indeed caused by the proton-generating reactions involved in ammonium assimilation and second that a decrease in pH_{cyt} was required for GABA accumulation to occur.

To prove that pH_{cyt} was an important factor in the accumulation of GABA, pH_{cyt} of the cells was altered by adding acid or alkali to the external medium (Fig. 7). The influence of the external pH on pH_{cyt} of carrot cells has been extensively studied, and sudden changes in external pH, caused by the addition of sodium hydroxide or hydrochloric acid, have been shown to cause a transient alkalinization or acidification of the cytoplasm (Fox and Ratcliffe, 1990). The addition of alkali to aerated cells prevented the decrease in pH_{cyt} during ammonium loading and also prevented the subsequent accumulation of ^{15}N-labeled GABA; whereas the addition of acid to oxygenated cells mimicked the decrease in pH_{cyt} observed in the aerated cells during the ammonium treatment, resulting in the accumulation of ^{15}N-labeled GABA to a level approaching that seen in the aerated cells. Although the results in Figure 7 show that the decrease in pH_{cyt} is a necessary condition for the accumulation of ^{15}N-labeled GABA, it is not the only factor influencing GDC activity because (a) GABA labeling in the acid-treated, oxygenated cells did not reach the same level as in the aerated cells and (b) recovery of pH_{cyt} did not prevent the continued accumulation of ^{15}N-labeled GABA.

Significant accumulation of GABA occurred during acidosis of the cytoplasm and its subsequent recovery and, therefore, could have contributed to the regulation of pH_{cyt} as proposed in earlier studies (Murphy et al., 1983; Reid et al., 1985; Menegus et al., 1989). The continued production of GABA after pH_{cyt} had recovered presumably reflects the continuing need to counteract the pH-perturbing effect of ammonium assimilation. This result can be compared with the conclusion of Roberts et al. (1992), who argued that most of the GABA accumulation in hypoxic maize roots, particularly in the absence of an external ammonium supply, occurred after the predominant acid-producing reactions had ceased and, thus, was too late to be involved in pH_{cyt} regulation. However, this does not alter the fact that, as a proton-consuming reaction, GABA production must influence pH and thus have a bearing on pH regulation.

GDC Activity

If GABA accumulation is involved in pH_{cyt} regulation, then the activities of the enzymes that synthesize and metabolize GABA (the GABA shunt; Satya Narayan and Nair, 1986, 1990) should be affected by pH in such a way that a decrease in pH activates the synthesis of GABA and an increase in pH activates its degradation. GDC, GAB-T, and SSADH have pH optima of 5.7, 8.9, and 9.0, respectively (Streeter and

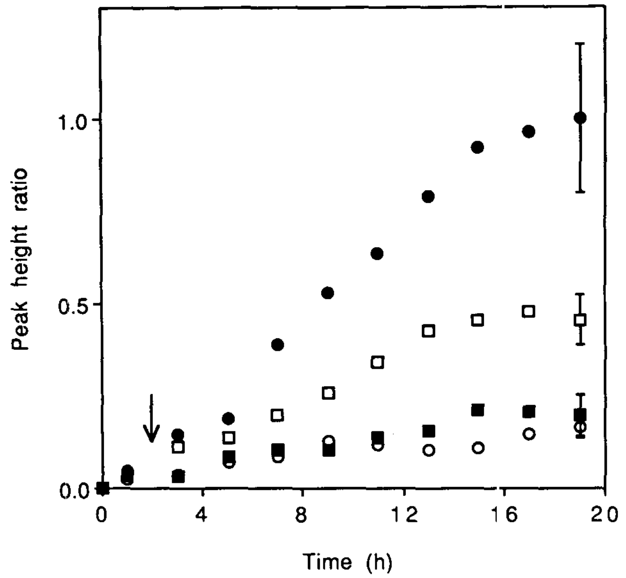

Figure 7. ^{15}N NMR peak height ratios of the [^{15}N]GABA signal of untreated oxygenated (○), untreated aerated (●), acid-treated oxygenated (□), and alkali-treated aerated (■) carrot cells following the addition of [^{15}N]ammonium chloride at time 0 h. HCl (0.5 mL of 0.8 M) or NaOH (0.5 mL of 1 M) was injected into the NMR tube at time 2 h as indicated by the arrow. Addition of acid caused a transient decrease in pH_{cyt} of approximately 0.3 pH unit, before recovering slowly to its original value. Addition of alkali increased pH_{cyt} to above pH 8.5 (the upper limit of pH_{cyt} determination by ^{31}P NMR), before returning to its original value in 2 h. The graph is a plot of the height of the resonances relative to that of the GABA resonance of the untreated aerated cells at the end of the experiment. The values are the means (± SE for the final points) of four experiments for the untreated aerated cells and of three experiments for the other treatments.

Thompson, 1972b), and therefore, the GABA shunt could act as a biochemical pH-stat in a way similar to that described for malic enzyme and PEP carboxylase (Davies, 1986). In addition, SSADH is believed to be inactivated under anaerobic conditions contributing to the accumulation of GABA (Satya Narayan and Nair, 1990).

GDC is exclusively a cytosolic enzyme, whereas GAB-T is found in the cytosol and mitochondria in a 3:1 ratio (Wallace et al., 1984; Satya Narayan and Nair, 1986). The pH-activity curves of GDC and GAB-T (Streeter and Thompson, 1972b) show that GAB-T activity is approximately 45% of its maximum value at pH_{cyt} of 7.5, whereas GDC activity is only about 4% of its maximum value (Streeter and Thompson, 1972b). Decreasing the pH to 7.3 reduces GAB-T activity only slightly but increases the GDC activity 3-fold. GDC activity has also been shown to be 20 to 30 times greater than that of GAB-T (Streeter and Thompson, 1972b; Wallace et al., 1984; Satya Narayan and Nair, 1986), and therefore, it is likely that the control of GABA accumulation would be mediated primarily via GDC.

It would have been useful to have been able to measure the GDC activity in carrot cell extracts to test the hypothesis that mild hypoxia caused an increase in activity following the addition of ammonium. However, it proved difficult to do this reproducibly, and consequently, carrot cell protoplasts were used for these measurements. Unfortunately, the protoplasts, although suitable for extracting GDC, were too fragile for NMR measurements in the airlift system, making a direct comparison between the enzyme data and the NMR measurements difficult. However, when the extractable GDC activity was monitored in aerated carrot protoplasts following the addition of ammonium, it was found to increase by almost 80% after 2 h (Fig. 8). This elevated activity was maintained for an additional 2 h before decreasing during the next 4 h to about one-third of its original level. As described elsewhere (Carroll et al., 1992), ^{15}N incorporation into GABA increased over the same time scale, indicating that changes in GDC activity in the protoplasts are indeed associated with changes in GABA labeling.

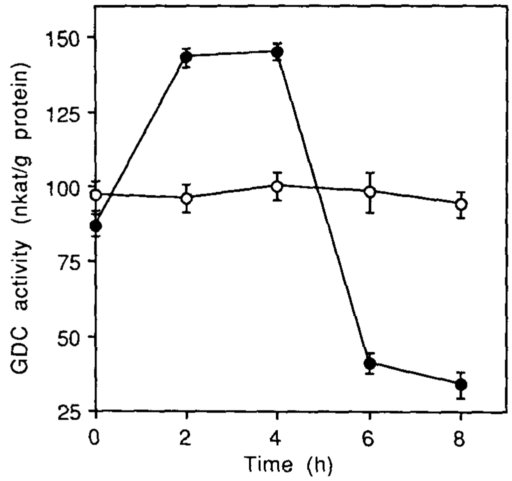

Figure 8. The activity of GDC in untreated (○) and ammonium-loaded (●) aerated carrot protoplasts. The graph is a plot of the means ± SE of three experiments.

Despite the inherent difficulty in comparing the protoplast and NMR data, the fact that GDC activity increased in the protoplasts as pH_{cyt} decreased in the cells, and decreased as pH_{cyt} recovered, suggests that GABA synthesis might be controlled by pH_{cyt} and that this is not just the result of a shift toward the acidic pH optimum for GDC. It is important to note that GDC was assayed in vitro at a fixed pH; therefore, the changes in extractable GDC activity did not include the direct effect of pH on that activity. It is therefore necessary to multiply the observed changes in GDC activity by the increase in enzyme activity caused by the decrease in pH_{cyt}. Thus, the GDC activity could have increased by more than 5-fold when pH_{cyt} had decreased to its lowest value. Note that it is not clear at this stage whether the changes in extractable GDC activity represent changes in its synthesis, reflecting a transcriptional or translational event, or a post-translational modification such as an effector-mediated activation or deactivation of the enzyme.

Another factor to be considered in these experiments is the potential effect of subcellular compartmentation on the flux into GABA. Carroll et al. (1992) showed that all of the Gln that accumulates in carrot cell protoplasts after the first 2 h of exposure to ammonium is found in the vacuole. In the experiments reported here, there was little buildup in the Gln pool under aerated conditions (Fig. 3), suggesting that relatively little Gln was transferred to the vacuole under these conditions and that more Gln would have been available for conversion to glutamate. In agreement with this interpretation, the aerated cells showed earlier and greater accumulation of glutamate (Fig. 3). Because the K_m of GDC is thought to be in the range 3 to 25 mM (Satya Narayan and Nair, 1990) and because the cytoplasmic concentration of glutamate is within this range (Carroll et al., 1992), it is possible that GDC activity is limited by the availability of glutamate in vivo and that the higher glutamate level in the aerated cells could lead to increased GABA production.

CONCLUSION

The results presented here show that GABA synthesis is stimulated by a decrease in pH_{cyt}. Ammonium assimilation under aerated conditions caused a transient decrease in pH_{cyt} that led to an increase in GDC activity and the accumulation of GABA. GDC activity decreased dramatically after pH_{cyt} had recovered. The results provide direct evidence for the effect of pH_{cyt} on GABA accumulation and the pathways of intermediary nitrogen metabolism, and they are consistent with a role for GABA accumulation in pH regulation. Further support for these conclusions can be found in the results of an independent study that was published while this paper was being reviewed (Crawford et al., 1994). Working with isolated asparagus mesophyll cells, Crawford et al. correlated GABA production, measured in cell extracts by an enzymic assay, with changes in cytosolic pH, measured by spectrofluorimetry. These experiments showed that acidification of the cytosol stimulated GABA synthesis and that this process offset the decrease in pH. Taken together, both sets of results point to a dynamic role for GABA synthesis in the maintenance of pH, and, therefore, contrary to a recent proposal, GABA accumulation would be an unreliable marker for dead or dying hypoxic cells.

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