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Dynamics of Nitrogenous Assimilate Partitioning between Cytoplasmic and Vacuolar Fractions in Carrot Cell Suspension Cultures[1]

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ABSTRACT

Bulk vacuole isolation, gas chromatography-mass spectrometry, and high-performance liquid chromatography have been used to investigate the accumulation and partitioning of assimilated nitrogen supplied as $^{15}NH_4Cl$ between vacuolar and extravacuolar (cytoplasmic) fractions of protoplasts from suspension cultures of carrot (*Daucus carota* L. cv Chantenay). Glutamine was the most abundant amino acid in the vacuole of protoplasts from late-exponential phase cells, whereas alanine, glutamate, and γ-aminobutyric acid were located primarily in the cytoplasmic fraction. In ^{15}N-feeding studies, newly synthesized glutamine partitioned strongly to the vacuole, whereas glutamate partitioned strongly to the cytoplasm, γ-aminobutyric acid was totally excluded from the vacuole, and alanine was distributed in both compartments. Comparison of the ^{15}N-enrichment patterns suggests that initial assimilation to glutamine occurs within a subcompartment of the cytoplasmic fraction. The protoplast-feeding technique may be extended to investigate cytoplasmic compartmentation further.

The importance of cellular compartmentation in the separation of metabolites from enzymes and in the regulation of metabolic processes was discussed by Oaks and Bidwell (17). Despite considerable advances in our understanding of the major metabolic pathways of nitrogen assimilation in higher plants (9, 12, 21, 23), there is still uncertainty as to the role played by intracellular partitioning of nitrogenous assimilates, particularly between the vacuolar and cytoplasmic compartments. Because of difficulties in measuring amino acid pools in vacuoles, approaches to this problem have been mostly indirect. Thus, analysis of changes in ^{15}N abundance of free amino acids with increasing time of exposure to [^{15}N]ammonium was compatible with a two-compartment model in *Lemna minor* (20). Using a ^{3}H-labeling technique, Cooke et al. (4) inferred the existence of an amino acid pool isolated from transaminase activity, and it was presumed to be vacuolar. Although modeling approaches may provide useful insights, they are dependent on many assumptions and need to be supplemented by more direct determination of metabolite compartmentation.

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Techniques for isolating vacuoles in bulk have made possible direct measurements of vacuolar amino acids and comparisons with cytoplasmic pools in a variety of species (1, 25, 27, 28). Such studies have provided information concerning storage capacity of vacuoles, with amino acid concentrations up to 150 mM (7), but as yet dynamic aspects of intracellular partitioning during nitrogen assimilation remain largely unexplored. Evidence is, however, accumulating that the vacuole plays an active role in several metabolic processes as a site for sequestering metabolites so as to maintain low cytosolic concentrations and avoid inhibition of enzymes or cytotoxic interactions. Examples of this include nitrate accumulation by barley mesophyll vacuoles (15), night storage of malate by vacuoles of CAM plants (14), and the active vacuolar sequestration of Ca^{2+} in carrot cell cultures (3).

In the present study, we investigated partitioning of amino acids in extravacuolar (cytoplasmic) and vacuolar fractions of suspension-cultured cells of carrot (*Daucus carota*) by incubation of protoplasts with ^{15}N-labeled NH_4Cl, bulk isolation of purified vacuoles, and amino acid determinations using HPLC and GC-MS. The findings highlight the complexity of cytoplasmic-vacuolar interactions and the importance of partition studies toward a more complete understanding of nitrogen assimilation in plants.

MATERIALS AND METHODS

Cell Culture and Protoplast Isolation

Suspension cultures of carrot (*Daucus carota* cv Chantenay) were maintained on Murashige and Skoog (16) medium (Flow Laboratories, Irvine, Scotland) supplemented with 2,4-D (0.2 mg/L), kinetin (0.1 mg/L), and sucrose (2%, w/v) at pH 5.7 and subcultured every 14 d by dilution of 1 volume of suspension with 10 volumes of fresh medium. Cultures were maintained on an orbital shaker at 25°C and 100 rpm in continuous low-intensity fluorescent light (5 $\mu E\ m^{-2}\ s^{-1}$). For protoplast isolation, cultures in the mid- to late-exponential phase (11–13 d) were aseptically harvested by filtration onto muslin, and protoplasts were released by digestion for 4 to 6 h at 25°C with 7 volumes of a solution containing 1% (w/v) cellulase R-10, 0.5% (w/v) macerozyme R-10 (Yakult Honsha Co., Tokyo, Japan), 0.01% (w/v) pectolyase Y23 (Seisham Pharmaceutical, Tokyo, Japan), 0.5 M mannitol, 2% (w/v) sucrose, and 50 mM Mes at pH 5.7 on an orbital shaker as

above. Liberated protoplasts were collected by centrifugation at 250g for 10 min and washed three times with a solution containing 0.5 M mannitol, 2% sucrose, and 50 mM Mes, pH 5.7.

Vacuole Isolation

Washed protoplasts were pelleted by centrifugation, and 1 volume of pellet was resuspended in 5 volumes of lysis buffer containing 100 mM K_2HPO_4, 5 mM DTT, and 6% Percoll (Sigma), pH 8.0, at 20°C. After centrifugation at 250g for 5 min, vacuoles were collected as a floating band. The degree of contamination of vacuole preparations with intact protoplasts and cytoplasmic debris was determined microscopically and by assaying marker enzymes.

Enzyme and Protein Determinations

The vacuolar enzymes AP[3] (EC 3.1.3.2) and AMS (EC 3.2.1.24) were assayed as described in ref. 26; NADH-glutamate dehydrogenase, a mitochondrial marker (EC 1.4.1.2), was assayed as described in ref. 21; and the cytoplasmic/plastid marker glucose-6-P dehydrogenase (EC 1.1.1.49) was assayed as described in ref. 6. All enzyme assays were performed at 25°C, except that for AMS, which was assayed at 37°C, and were linear with respect to duration of incubation and quantity of enzyme assayed. Soluble protein was determined using the Bio-Rad protein assay (2) with BSA as the standard. Enzyme activities are given as nkat (nmol/s) per mg of protein. For TCA-insoluble total protein, protoplast pellets were washed three times with ice-cold 10% (w/v) TCA, extracted with 0.3 M NaOH at 40°C for 1 h, and assayed for total protein (13). Protoplast fresh weights were determined by pelleting aliquots in preweighed Eppendorf tubes.

^{15}N-Labeling Studies

Washed protoplasts were resuspended in Murashige and Skoog medium (16) similarly to the cell culture medium but modified by the omission of the inorganic nitrogen sources (NH_4NO_3 and KNO_3) and inclusion of mannitol (0.5 M) and Mes (50 mM) at pH 5.7. Protoplast density was adjusted to approximately 10^6/mL, and the suspension was incubated at 25°C on an orbital shaker. At time zero, 2 mM $^{15}NH_4Cl$ (99% ^{15}N, MSD Isotopes) was added. At 2-h intervals up to 8 h, 0.5-mL protoplast aliquots were taken for HPLC and GC-MS determinations, AMS activity, TCA-insoluble total and ^{15}N protein, and fresh weight. In addition, vacuoles were isolated at the same intervals, and aliquots were taken for HPLC/GC-MS analysis (100 μL) and AMS assay (20 μL). All experiments were repeated at least three times with good overall agreement.

HPLC and GC-MS Determinations

Protoplast or vacuole aliquots were transferred to HPLC-grade methanol. Filtered extracts were used directly for soluble amino acid determinations by HPLC. For GC-MS analysis of ^{15}N-amino acids, the methanol extracts were taken to dryness and redissolved in 2 mL of water. Methods for both procedures and for ANCA determinations of ^{15}N-protein were as described previously (21).

Expression of Results

Because AMS in protoplasts is restricted to the vacuole (8), all quantities determined for protoplast and vacuole preparations were normalized against this enzyme to permit stoichiometric comparisons and calculations of cytoplasmic quantities. For each amino acid, specific concentrations were calculated for the protoplast and vacuolar samples in terms of μmol of each amino acid per nkat of AMS activity, and the corresponding cytoplasmic (extravacuolar) value was obtained by subtraction. Quantities of each amino acid per g of protoplast equivalent were calculated from the HPLC and fresh weight data, and the distribution between vacuolar and cytoplasmic fractions was calculated from the specific concentrations. To determine vacuolar and cytoplasmic concentrations, the relative volumes of each compartment were determined, and molar concentrations were calculated assuming a specific gravity of 1 for pelleted protoplast preparations.

RESULTS

Vacuole Isolation and Characteristics

Protoplasts were routinely obtained at yields of approximately 25%, and their appearance (Fig. 1A) reflected the active metabolic state of cultured cells; several vacuoles of varying sizes were present in most protoplasts, with a minority of protoplasts containing a single large vacuole. During preliminary experiments, lysis occurred over a range of concentrations of lysis buffer, suggesting varying osmotic potentials within the protoplast population. The conditions finally adopted lysed >99% of the protoplasts but also caused many transiently released vacuoles to burst, presumably because of their lower osmotic potential. Under conditions optimized for minimum contamination with intact protoplasts, vacuole yields were approximately 10 to 15%. Contamination of isolated vacuole preparations with intact protoplasts, as revealed by microscopy investigation, was always <1%, and low levels of contamination with cytoplasmic debris were seen (Fig. 1B). Table I shows the relative activities of marker enzymes. Contamination of vacuole preparations with activities characteristic of cytosol/plastids and mitochondria was 2.1 and 2.9%, respectively, whereas the specific activity of AMS was 10.8 times higher in vacuoles than protoplasts. Low levels of contamination of vacuolar preparations were probably due to the use of stringent lysis conditions that virtually eliminated intact protoplasts. AP activity was largely, but not entirely, vacuolar; approximately 20% was found to be cytoplasmic when calculated relative to AMS.

[3] Abbreviations: AP, acid phosphatase; GABA, γ-aminobutyric acid; AMS, α-mannosidase; ANCA, automated $^{15}N/^{13}C$ analyzer.

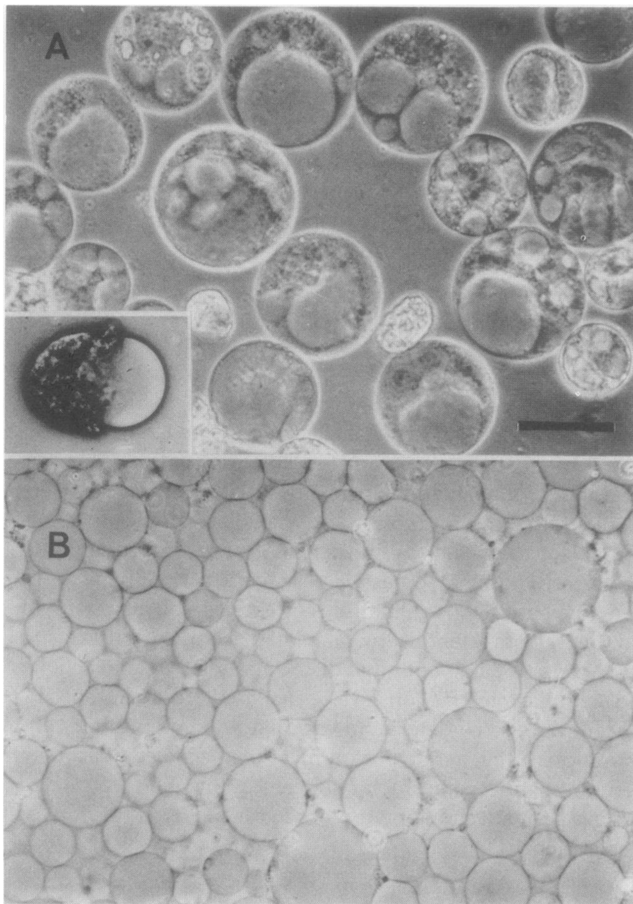

Figure 1. Phase contrast micrographs of protoplast (A) and purified vacuole (B) preparations from 12-d-old carrot cell suspension cultures. Inset in A shows vacuole release 3 min after addition of lysis buffer to the protoplast suspension. Bar = 15 μm.

Sasse et al. (22) reported that vacuoles of carrot protoplasts contained only 55% of total AP activity.

Calculation of mean relative volumes of vacuolar and cytoplasmic compartments is complicated by the multivacuolar nature of these protoplasts (Fig. 1A); two unrelated methods were used to give a reliable estimate. First, mean volumes of isolated protoplasts and vacuoles under isoosmotic conditions were calculated from microscopic measurement of diameters, giving a vacuole to protoplast volume ratio of 52% (Table I), subject to the assumption that larger vacuoles derive from univacuolate protoplasts. Second, comparisons were made of AMS activity per unit volume of isoosmotic preparations of vacuoles and protoplasts. Because 1 volume of vacuoles contains 1.75 times as much activity as 1 volume of protoplasts (Table I), calculation yields a vacuole volume equal to 57% of protoplast volume. The validity of this determination is subject only to assumptions that AMS is strictly vacuolar and that packing characteristics are similar in both preparations. Because both methods gave similar estimates, vacuolar volume was taken as 55% of that of the protoplast for calculations of amino acid concentrations. Vacuolar volumes on the order of 70 to 90% have been previously reported for studies of barley mesophyll (11) or other mature cell types such as fruit flesh (28). The relatively larger proportion occupied by the cytoplasm in our preparations likely reflects the higher metabolic activity of proliferating cultured cells as compared with mature cells in the plant.

Amino Acid Distribution

Amino acid contents of cells and protoplasts, calculated concentrations in vacuolar and cytoplasmic (extravacuolar) compartments, and percentages of the total protoplast content of each amino acid located in the vacuole are shown in Table II. The relative distribution of individual amino acids is broadly similar in cells and in protoplasts derived from them, indicating relatively little change resulting from protoplast preparation. The differences are less than those ob-

Table I. *Characteristics of Protoplast and Vacuole Preparations*

Protoplast and vacuole preparations resuspended in protoplast isolation medium were collected by Percoll flotation under isoosmotic conditions.

Parameter	Protoplasts	Vacuoles	Vacuolar Content
			%
Protein[a]	22.2 ± 1.9	3.6 ± 0.4	16.2
nkat AMS/mg[b]	6.94	75.0	1080
Volume[c]	5.9 ± 0.8	3.1 ± 0.4	52.5
nkat AMS/mL[d]	154 ± 6.5	270 ± 4.0	175
AMS activity[c]	1.0	1.0	100
AP activity[e]	10.2	8.1	79.4
NADH-glutamate dehydrogenase activity[e]	0.69	0.02	2.9
Glucose-6-phosphate dehydrogenase activity[e]	0.14	0.003	2.1

[a] Total TCA-insoluble protein (mg/mL or protoplasts). [b] AMS activity per mg of soluble protein. [c] Volumes ($m^{-3} \times 10^{-15}$) determined from mean diameter determinations at ×800 magnification, $n = 400$. [d] AMS activity per mL of protoplast or vacuole preparation. [e] Enzyme activities as nkat/nkat of AMS.

served during the course of a single growth passage of the cells; for example, during the exponential growth phase (day 6), Ala is the most abundant amino acid. Increased concentrations in protoplasts relative to cells reflect the reduced volumes of protoplasts due to plasmolysis.

Amino acids were primarily cytoplasmic in their distribution, with no amino acid being present in a substantially higher concentration in the vacuole than in the cytoplasm. Several amino acids present at relatively low concentrations (His, Met, Arg, Leu, Ile, Asp) were found at similar concentrations in both compartments. Of the more abundant amino acids, Ala, GABA, and Glu were largely restricted to the cytoplasmic fraction, with only 11, 14, and 16% respectively, localized in the vacuole. By contrast, about 40% of total Gln was vacuolar, and Gln was the most abundant vacuolar amino acid, making up 27% of total vacuolar amino acid content. Other reports on Glu and Gln distribution are in general agreement with these findings; in sweet clover, 22% of the Glu pool was vacuolar (1); in *Lemna* fronds, Gln was inferred to be largely vacuolar and Glu largely cytosolic (10); and in barley mesophyll protoplasts, Glu was excluded from vacuoles, although most other amino acids were present (8). All studies of this kind reflect a static or equilibrium position in which individual pool sizes are derived from a complex series of events involving synthesis, catabolism, and interconversion of amino acids. Kinetic studies were, therefore, undertaken to study partitioning of newly synthesized amino acids.

Table III. *Changes in Total Amino Acids and Protein following Addition of $^{15}NH_4Cl$ to N-Depleted Protoplasts*

Protoplast suspensions were incubated with 2 mM $^{15}NH_4Cl$, and quantities of combined unlabeled amino acids, combined ^{15}N-labeled amino acids, total protein, and ^{15}N-protein were determined at 2-h intervals during 8 h in vacuolar (V) and cytoplasmic (C) fractions. The ^{15}N content of protein was calculated from atom percentage excess values of TCA-extracted protoplasts assuming an average protein content of 16% nitrogen.

Time	Amino Acids				Protein	
	Unlabeled		^{15}N-labeled		Total	^{15}N
	C	V	C	V		
h	*μmol/g*				*mg/g*	*μmol/g*
0	78.3	26.2	0	0	25.4	0
2	86.4	50.6	5.1	5.3	19.7	0
4	86.6	89.5	17.2	6.4	20.7	0
6	92.6	94.7	15.4	7.4	21.3	0.075
8	109.2	62.6	17.5	7.8	26.1	0.15

Total Amino Acid and Protein Changes following $^{15}NH_4Cl$ Addition

Changes in the combined amino acid and protein contents during an 8-h period following incubation of protoplast suspensions with 2 mM $^{15}NH_4Cl$ are shown in Table III. Data from a representative experiment are shown, but similar patterns were observed in three experiments. The vacuolar content of unlabeled amino acids increased during the first 6 h by a factor of 3.6 before declining, whereas the cytoplasmic content increased steadily throughout. Catabolism of preexisting proteins may be the source of the increase in unlabeled amino acids; the observed decline of 22% in TCA-insoluble total protein between 0 and 2 h is sufficient to account for the increased amino acids, assuming an average protein nitrogen content of 16%. Carryover of nitrate (7.7 mmol/g)

Table II. *Amino Acid Contents of Cells, Protoplasts, and Vacuolar and Cytoplasmic Fractions*

Amino Acid	Concentration				Vacuolar Content[a]
	Cells[b]	Protoplasts	Vacuolar	Cytoplasmic	
	μmol/g[c]				%
GABA	10.7 ± 2.9	31.4 ± 13.9	8.0 ± 1.4	60.0 ± 21.2	14.0
Gln	8.2 ± 2.3	19.3 ± 4.3	14.4 ± 4.5	25.3 ± 4.1	41.0
Ala	3.3 ± 1.1	14.8 ± 3.4	3.0 ± 0.8	29.8 ± 6.8	11.1
Glu	8.4 ± 3.2	7.1 ± 1.2	2.1 ± 1.1	13.1 ± 1.3	16.3
Val	1.6 ± 0.8	4.1 ± 0.2	2.2 ± 1.6	6.5 ± 0.4	29.5
His	4.4 ± 2.3	4.0 ± 1.7	3.9 ± 1.2	4.2 ± 2.6	53.6
Ser	3.5 ± 1.7	3.5 ± 0.9	2.1 ± 0.7	5.8 ± 1.1	33.0
Thr	3.4 ± 1.1	3.4 ± 1.6	1.0 ± 0.3	6.3 ± 3.3	16.2
Gly	1.5 ± 0.5	3.1 ± 1.2	1.3 ± 0.6	5.3 ± 1.8	23.1
Lys	1.9 ± 0.7	2.7 ± 0.8	1.2 ± 0.4	4.6 ± 1.5	24.4
Met	0.7 ± 0.5	2.7 ± 0.4	2.7 ± 0.5	2.6 ± 0.3	55.0
Asn	1.2 ± 0.8	2.6 ± 0.3	1.5 ± 0.4	4.0 ± 0.2	32.2
Phe	0.3 ± 0.2	2.5 ± 0.9	1.6 ± 0.5	3.7 ± 1.2	35.2
Leu	0.7 ± 0.4	2.4 ± 1.0	1.8 ± 1.1	3.2 ± 1.0	41.3
Tyr	0.4 ± 0.3	2.3 ± 0.8	2.2 ± 1.0	2.4 ± 0.8	52.6
Arg	0.4 ± 0.3	2.2 ± 0.2	1.9 ± 0.6	2.5 ± 0.4	47.5
Ile	0.9 ± 0.3	1.9 ± 0.6	1.5 ± 0.6	2.8 ± 1.0	43.4
Asp	0.7 ± 0.4	1.2 ± 0.3	1.0 ± 0.4	1.5 ± 0.2	45.8

[a] Total vacuolar content as percentage of total protoplast content taking the relative volume of each compartment into account. [b] Cells cultured for 12 d and incubated in N-free medium for 6 h. [c] Means (±SE) of three experiments.

and ammonium (0.2 mmol/g) ions in the protoplasts could account for less than 10% of the amino acid increase. The contributions of the individual amino acids to the overall increase in unlabeled compounds (data not shown) were broadly proportional to their initial concentrations (Table II). Thus, GABA gave the largest increase, followed by Gln, Glu, and Ala. Vacuolar contents of all amino acids increased more than the corresponding cytoplasmic content relative to the initial value in a manner similar to the overall totals.

Newly synthesized amino acids, represented by total ^{15}N-labeled compounds, are equally distributed between cytoplasmic and vacuolar fractions after 2 h, but subsequent increases are primarily cytoplasmic (Table III). After 4 h, the quantity of ^{15}N present in combined amino acids closely approximated the total supplied initially as $^{15}NH_4Cl$ (25 μmol/g). Consequently, assimilation is complete by this time, and the kinetics describe a pulse of ammonia assimilation that is completed within the first half of the experimental period. The incorporation of ^{15}N into protein was estimated by ANCA determinations on TCA-extracted protoplasts. No incorporation occurred up to 4 h (Table III). After 6 and 8 h, ^{15}N incorporation into protein accounted for only 0.3 and 0.6%, respectively, of the total supplied initially.

Individual ^{15}N-Labeled Amino Acids

Changes in the accumulation, distribution, and ^{15}N enrichment of the four major newly synthesized amino acids are shown in Table IV. Marked differences in the distribution between cytoplasmic and vacuolar fractions were observed. Newly synthesized GABA accumulated exclusively in the cytoplasm, whereas Gln was largely sequestered in the vacuole. Glu partitioned strongly toward the cytoplasmic fraction with increasing time, but Ala was more evenly distributed. ^{15}N enrichment data (atom percentage excess, Table IV) emphasize the differences in compartmentation behavior, particularly between Gln and Glu. Vacuolar Gln was strongly enriched, whereas the cytoplasmic species showed low and declining enrichment; this situation was reversed in the case of Glu.

Eight other ^{15}N-labeled amino acids were present at a total content in both compartments of less than 1.0 μmol/g protoplasts. Gly, Leu, Asn, Ser, and Asp were accumulated in both cytoplasmic and vacuolar fractions, whereas labeled Phe and Ile were detected in the vacuolar, and Arg in the cytoplasmic, fraction only. No ^{15}N enrichment was detected in Val, His, and Thr.

The use of *tert*-butyldimethylsilyl derivatives for GC-MS permits the proportion of singly (amino or amido) or doubly (amino and amido) labeled Glu to be determined (21). The ratio of single ^{15}N-labeled to double ^{15}N-labeled Glu was 14.3, 5.0, 2.5, and 11.1 after 2, 4, 6, and 8 h, respectively, in the vacuolar fraction and 3.0, 0.15, 0.12, and 0.006 at the same time intervals in the cytoplasmic fraction. These data indicate a strong functional separation between a stable vacuolar pool and a metabolically labile cytoplasmic pool of Glu.

DISCUSSION

Isolation of vacuoles in quantities sufficient for direct analysis of amino acid content can only be achieved by protoplast lysis. The validity of the methods described herein as a means to study the intracellular compartmentation of assimilated ammonia depends on bulk isolation of highly purified vacuolar preparations whose contents can be related to those of the parent protoplasts, and also on the metabolic integrity of isolated protoplasts, particularly during feeding experiments. The stringent lysis conditions developed in this study resulted in vacuolar preparations free of intact protoplasts and with minimal contamination with cytoplasmic (extravacuolar) components. The use of AMS as a strict vacuolar reference allowed stoichiometric comparison of vacuole and protoplast contents and consequent determination of cytoplasm content by subtraction.

Comparisons between the relative proportions of amino acids in cultured cells and protoplasts indicate that shifts do occur during the process of plasmolysis and treatment with cell wall-degrading enzymes. The major changes are increased concentrations of all compounds in protoplasts, an inevitable consequence of osmotic shrinkage, and a relative increase in GABA. Although GABA accumulation has been reported in tissues exposed to anaerobic conditions (24), in this study all protoplast suspensions were well aerated by rotary shaking. In a previous study (21), ^{15}N-labeled GABA

Table IV. *Changes in Incorporation of ^{15}N Label into Individual Amino Acids*

Protoplasts were incubated with 2 mM $^{15}NH_4Cl$ and quantities of ^{15}N-amino acids, and atom percentage excess values in the vacuolar (V) and cytoplasmic (C) fractions were determined at 2-h intervals during 8 h.

Amino Acid	Fraction	^{15}N Incorporation							
		2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
		µmol/g protoplast equivalent				*atom % excess*			
GABA	V	0	0	0	0	0	0	0	0
	C	4.5	12.6	10.8	12.6	11.4	28.3	26.7	28.8
Gln	V	3.4	4.2	4.5	4.2	32.3	23.9	24.9	36.2
	C	0	1.4	0.7	0.9	0	7.7	4.3	3.9
Glu	V	0.4	0.3	0.5	0.2	5.5	3.6	5.1	3.3
	C	0.4	0.5	2.7	2.3	7.7	10.9	18.1	16.4
Ala	V	0.5	0.9	1.8	2.2	13.5	9.0	15.4	44.0
	C	0	1.4	0	0.9	0	13.0	0	10.8

was the major labeled compound following supply of $^{15}NH_4Cl$ to osmotically unstressed carrot cells. Thus, GABA accumulation appears to be a characteristic of the cultured carrot cells rather than a stress response to protoplast preparation.

The presence of ammonium ion as the sole source of ^{15}N in feeding experiments may induce characteristic metabolic responses due to proton release. In preliminary experiments with unbuffered suspensions of either cells or protoplasts, the pH of the culture medium decreased by up to 2 units within 4 h of the addition of 2 mM NH_4Cl. The inclusion of Mes in the protoplast incubation medium stabilized the medium pH, but cytosolic acidification remains a possibility. Increased protease activity (18) and glutamate decarboxylase activity (5) have been reported in response to cytosolic acidification, and it has been suggested that Glu decarboxylation may act as a metabolic buffer by absorbing excess protons (19). These reports are compatible with our findings and may account for the initial increases in unlabeled amino acids and the accumulation of GABA as a substantial sink for newly assimilated nitrogen.

Against this metabolic background, the partitioning of newly synthesized Gln and that of newly synthesized Glu show striking differences. From previous studies (21), it is known that in carrot suspension cultures ammonia is assimilated exclusively through Gln synthetase into Gln, which is consequently the direct precursor of glutamate. In the present study, Gln was rapidly accumulated in the vacuole, where it was isolated from the metabolic activity evident in the cytoplasm, whereas Glu accumulated mostly in the cytoplasm. The basis for this specificity of compartmentation is not known, although uptake studies with isolated vacuoles may give insights into the mechanism. The selective nature of vacuolar partitioning was further emphasized by the complete exclusion of newly synthesized GABA from the vacuole and the more even distribution of Ala between the two cellular compartments. The partitioning of compounds into the vacuole was not driven by concentration gradients; for example, total GABA vacuolar concentrations were less than 50% of cytoplasmic concentrations throughout the feeding period, whereas total Glu concentrations were similar in the two compartments throughout.

A striking feature of the cytoplasmic labeling patterns was the low ^{15}N enrichment of Gln relative to that of Glu and GABA. Because Gln is known to be the precursor of glutamate, and hence GABA, this suggests the existence of a Gln pool with ^{15}N enrichment considerably greater than the bulk cytoplasmic pool. Vacuolar Gln is more highly enriched, at a level that can account for Glu and GABA production. These observations are compatible with a model in which Gln is synthesized within a subcompartment of the cytoplasmic fraction, from which it can either be sequestered in the vacuole or metabolized onward through glutamate. The nature of any such subcompartment is speculative; however, the protoplast-feeding technique can be used to investigate this possibility, by extending the fractionation procedure to cytoplasmic components such as plastids and mitochondria.

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