Plant Physiol. (1992) 99, 659–664
0032-0889/92/99/0659/06/$01.00/0

Received for publication October 8, 1991
Accepted January 20, 1992

The Production and Efflux of 4-Aminobutyrate in Isolated Mesophyll Cells[1]

Induk Chung[2], Alan W. Bown*, and Barry J. Shelp

Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada, L2S 3A1 (I.C., A.W.B.); and Department of Horticultural Sciences, University of Guelph, Guelph, Ontario, Canada, N1G 2W1 (B.J.S.)

ABSTRACT

The pathway of 4-aminobutyric acid (GABA) production and efflux was investigated in suspensions of mesophyll cells isolated from asparagus (*Asparagus sprengeri* Regel) cladophylls. Analysis of free amino acids demonstrated that, on a molar basis, GABA represented 11.4, 19, and 6.5% of the xylem sap, intact cladophyll tissue, and isolated mesophyll cells, respectively. L-Glu, a GABA precursor, was abundant in intact cladophylls and isolated cells but not in xylem sap. When cells were incubated with L-[U-^{14}C]Glu, intracellular GABA contained less than 10% of the radioactivity found in intracellular Glu. However, GABA in the medium contained 78% of the radioactivity found in extracellular L-Glu metabolites. Incubation with L-[1-^{14}C]Glu resulted in the appearance of unlabeled GABA, demonstrating its production through decarboxylation at carbon 1. GABA released to the medium from cells incubated with L-[U-^{14}C]Glu had a specific activity of 18 nanocuries per nanomole, whereas GABA remaining in the cell had a specific activity of 2.25 × 10^{-1} nanocuries per nanomole. In the presence of exogenous L-Glu, amino acid analysis and cell volume measurements indicated intracellular Ala and GABA concentrations of 4.2 and 1.4 millimolar, respectively. In the medium, however, the corresponding concentrations were 2 and 57 micromolar. The data indicate that L-Glu entering the cell is decarboxylated to GABA, and that specific and passive efflux is from this pool of recently synthesized GABA and not from a previously synthesized unlabeled pool of GABA.

The nonprotein amino acid, GABA[3] appears to be ubiquitous in the plant kingdom. In many of the plant species and tissues studied, GABA concentrations exceed those of the free protein amino acids. GABA synthesis results predominantly from the decarboxylation of carbon-1 of L-Glu in a reaction catalyzed by GAD (EC 4.1.1.15), a cytosolic enzyme (2, 18). GABA synthesis also occurs through the hydrolysis of 4-guanidinobutyric acid to GABA and urea (8), and by the conversion of putrescine via pyrroline to GABA (21). Compartmental analysis demonstrates that the cytosol is enriched with GABA, suggesting that it may be the site of both synthesis and storage of GABA (26, 29). Other studies, however, have shown that GABA efflux is slower than that for other amino acids, suggesting that it is sequestered in an organelle (19). Although GABA is widely distributed and present at high levels, its physiological role in plants has not been clearly established (2, 18).

A H^+/L-Glu cotransport system has been characterized at the plasma membrane of *Asparagus sprengeri* mesophyll cells (1, 9, 10). Attempts to determine the stoichiometry of the H^+/L-Glu cotransport system by simultaneously measuring the initial rate of L-Glu-dependent alkalinization and the initial rate of L-[U-^{14}C]Glu uptake yielded unexpectedly high stoichiometries ranging from 3.0 to 7.7:1. Further experiments determined that a high proportion of the label entering the cells as L-[U-^{14}C]Glu was released to the medium as labeled GABA and $^{14}CO_2$ (1, 10). The efflux of GABA and CO_2 led to an underestimation of the rate of L-Glu entry and, consequently, to an overestimation of the H^+/L-Glu stoichiometry.

This article addresses four questions: (a) what are the concentrations of GABA in *A. sprengeri*; (b) what is the biochemical pathway of GABA production; (c) could GABA efflux occur through a passive mechanism; and (d) is the GABA that is synthesized during the uptake of L-Glu sequestered from previously existing GABA?

MATERIALS AND METHODS

Cell Isolation

Mechanically isolated asparagus (*Asparagus sprengeri* Regel) mesophyll cells were prepared daily (3) and incubated in 5 mM Mes:1 mM $CaSO_4$ and adjusted to pH 6.0 with $Ca(OH)_2$. Depending on the experiment, the volume of the stirred cell suspensions varied from 1 to 5 mL. The 5-mL suspensions were aerated. The cell density varied from 4 to 6 × 10^6 cells·mL^{-1}. Cells were illuminated with a 200-W Sylvania lamp, giving an irradiance of 4.6 × 10^{-4} mol·m^{-2}·s^{-1}.

Amino Acid Analysis of Xylem Sap, Intact Cladophylls, Isolated Cells, and Incubation Medium

Xylem sap was collected from greenhouse-grown plants between 11 AM and 12 noon. Sixty-cm-long stems were cut near the base. The exudate was collected from the attached stem into a small glass tube by a low vacuum device (Nalgene, MitVac). The sap from five stems was pooled. This procedure was employed on 3 separate days generating three samples,

[1] Supported by grants A4453 and UO343 from the Natural Sciences and Engineering Research Council of Canada to A.W.B. and B.J.S.

[2] Present address: Department of Physiology, University of Toronto, Toronto, Ontario, Canada, M5S 1A1.

[3] Abbreviations: GABA, 4-aminobutyric acid; GAD, glutamate decarboxylase; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone.

which were dried in a vacuum concentrator (Savant speed vacuum).

Approximately 150 cladophylls were collected. Within 5 s of detachment, each cladophyll was placed into 20 to 25 mL of boiling 100% methanol to ensure a minimal time period between excision and the boiling methanol treatment. At the same time, another 150 cladophylls were collected for cell isolation. After 30 min of boiling, the methanol plus cladophylls were ground in 4 mL of 5 mM Mes in 1 mM $CaSO_4$ (pH 6.0). The slurry was mixed with 30 mL of chloroform and 15 mL of water and agitated for 1 h. The upper aqueous fraction was then separated from the lower chloroform fraction by centrifugation at 2900*g* for 10 min and dried in a filtered stream of air at 40°C.

Twenty to 30 × 10^6 isolated cells were incubated in 5 mL of the cell suspension medium for 10 min. After incubation, the cells were separated from the medium by centrifugation at 2500*g* for 2 min. The incubation medium was dried. The cell pellet was solubilized by sequential addition and shaking with 2.5 mL of 100% methanol for 30 min, 5 mL of chloroform for 30 min, and 2.5 mL of distilled water for a further 30 min. The aqueous cell fraction was separated from the chloroform fraction as indicated previously and dried.

The dried xylem sap and incubation medium, and the dried aqueous fractions from intact cladophylls and isolated cells were dissolved in 100 or 300 μL of deionized and filtered water. Amino acids were derivatized with 2-mercaptoethanol/*o*-phthalaldehyde and separated with a Waters HPLC gradient system (Milford, MA) by reverse-phase chromatography (20). Intracellular amino acid concentrations were calculated after determining cell volumes (22).

Analysis of L-[U-^{14}C]Glu or L-[1-^{14}C] Glu Metabolites

To a 5-mL suspension of 2 × 10^7 cells was added 0.1 mM L-[U-^{14}C]Glu (specific activity 22.5 nCi/nmol) or 0.1 mM L-[1-^{14}C]Glu (specific activity 17.5 nCi/nmol). The cell suspensions were incubated for 10 min. Cells were then separated from the medium by centrifugation at 2400*g* for 2 min. The medium was dried and prepared for HPLC analysis. The pellet was immediately resuspended in 15 mL of 4°C medium and centrifuged at 2400*g* for 2 min. The pellet was fractionated into an aqueous fraction and a chloroform fraction as indicated previously. The aqueous phase was removed, dried, and designated the cell fraction.

The dried aqueous fractions from the cells and the medium were dissolved in 500 μL of deionized and filtered water. A 300-μL aliquot of these dissolved fractions was mixed with phenylisothiocyanate to derivatize the amino acids. Both the derivatized amino acids and the nonderivatized organic acids were separated on a Waters HPLC system, and radioactivity was determined with an on-line Ramona Radiochromatography Scintillation system (10, 11).

GABA Flux Measurements

Measurements of net GABA flux were initiated by suspending 4.4 × 10^6 illuminated cells in 1.1 mL of medium. A 100-μL aliquot was removed and washed with 1 × 5 mL followed by 2 × 10 mL of 4°C cell suspension medium, and the cells were collected on Millipore filters (HA type, 0.45 μm). This sample was used to determine the background for flux measurements. To the remaining 1.0 mL of suspension was added 25 μL of 5 mM [U-^{14}C]GABA (final concentration 0.125 mM, specific activity 11.2 nCi/nmol). A 100-μL aliquot of the cell suspension was removed every 2 min for 6 min, and the cells were washed and collected as above. At 6 min, 20 μL of 50 mM L-Glu or 10 μL of 1 mM CCCP was added to the cell suspension to give final concentrations of 1.7 mM or 17 μM, respectively. Aliquots of 100 μL were then removed every 2 min for 12 min. Cells were washed and collected as described above. Filters with cells were transferred directly into a vial containing 1 mL of 100% methanol. Subsequently, 14 mL of counting scintillant (ACS, Amersham) was added, and radioactivity was determined in a Beckman LS 1800 scintillation system.

To measure the efflux of GABA, a similar procedure was used. However, at 6 min the cells were washed for 6 min with 2 × 10 mL of fresh cell suspension medium to remove external radioactivity. Then 0.8 mL of fresh suspension medium was added to the washed cells, and measurements of efflux were initiated. A 100-μL aliquot of cell suspension was removed immediately, and then 16 μL of 50 mM L-Glu (1.1 mM) or 8 μL of 1 mM CCCP (11 μM) was added. Aliquots of cell suspension were then removed every 2 min for 12 min, and cells were washed and collected on filters prior to scintillation counting.

RESULTS

HPLC analysis of the amino acid content of xylem sap, intact cladophyll tissue, and isolated mesophyll cells was performed to indicate the relative amounts of Glu and GABA in the respective amino acid pools (Fig. 1). In the xylem sap, the most abundant amino acid was Asn, comprising 68% of the total amino acid pool. GABA was the second most abundant at 11.4%. However, Glu comprised only 1% of the pool. The composition of xylem exudate obtained from the middle or top of the *Asparagus* stem indicated similar values (data not shown). In contrast, the most abundant amino acids in the intact cladophyll were Glu and GABA, making up 19.7 and 18.8% of the pool, respectively. Asp and Ala comprised 13.6 and 13.0% of the pool, respectively. In isolated mesophyll cells, Glu and Ala were the most abundant amino acids, making up 29.2 and 22.7% of the pool. GABA was the third most abundant amino acid, comprising 6.4% of the pool. Thus, whereas GABA is a major component of all three fractions, Glu is abundant in intact tissue and isolated cells but not in xylem sap. The abundance of GABA in cladophylls transferred within seconds from the plant to boiling methanol demonstrated that its presence is not a response to the cell isolation procedures.

To investigate L-Glu metabolism, cells were incubated with L-[U-^{14}C]Glu in the light for 10 min prior to HPLC analysis of an aqueous extract of the washed cells and the cell suspension medium (Table I). Of the total L-Glu metabolites analyzed, 12.7% remained in the cell, whereas 87.3% was found in the medium. In the cells, the most abundant radiolabeled compound was L-Glu. The label in GABA was less than 10% of that found in Glu. In the medium, however, the most

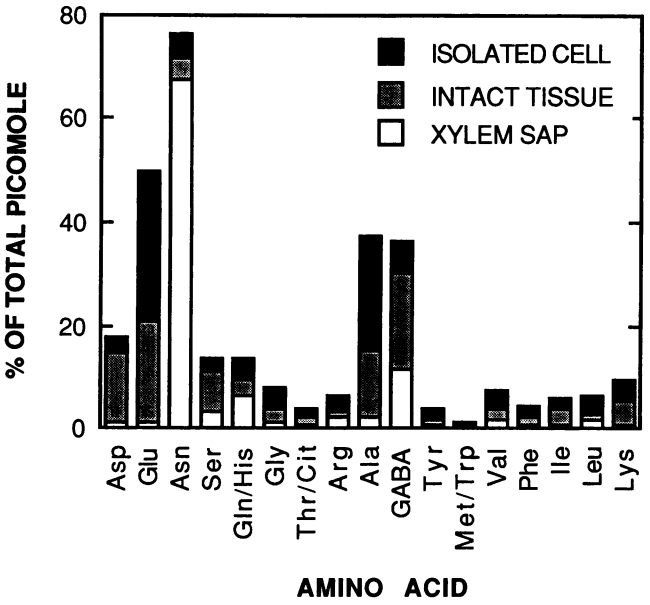

Figure 1. A stack histogram of the relative amino acid content of xylem sap exudate, intact cladophyll tissue, and isolated mesophyll cells. Dried aqueous fractions from the three sources were analyzed by HPLC. Standard amino acid solutions and corresponding integration values were used to convert sample amino acids into pmol units. Individual amino acids are expressed as a percentage of the total pmol in each fraction. The means from three separate samples are indicated.

Table I. *Percentage of ^{14}C Distribution of L-[U-^{14}C] Glu Metabolites*

To 20 × 10^6 illuminated cells suspended in 5 mL of 5 mM Mes in 1 mM $CaSO_4$ (pH 6.0) was added 0.1 mM L-[U-^{14}C]Glu (22.5 nCi/nmol). Metabolites were separated and analyzed as indicated in "Materials and Methods." Each metabolite is expressed as a percentage of the total metabolite radioactivity. Each value indicates the percent mean (±SE) of three to eight trials.

Metabolite	Medium		Cell	
	% Mean	SE	% Mean	SE
α-Ketoglutarate	3.40	0.32	1.08	0.47
Succinate	4.64	2.94	0.40	0.07
L-Glu	—[a]	—	4.79	0.13
GABA	67.88	2.21	0.39	0.19
Minor constituents	0.00	0.00	5.31	2.13
Unknown	11.43	3.44	0.70	0.57
Sum	87.35		12.67	

[a] L-[U-^{14}C]Glu added to the medium; therefore, data not shown here.

abundant radiolabeled compound was GABA, which made up 78% of the pool of L-[U-^{14}C]Glu metabolites. The data demonstrate that GABA is the most abundant radiolabeled metabolite of L-Glu and that most of it is released to the medium. Similar results were obtained with nonilluminated cells. GABA synthesis was shown to be a cell-dependent process. It was not a contaminant of the stock of L-[U-^{14}C]Glu, nor was it produced by the breakdown of L-[U-^{14}C]Glu during incubation and sample preparation (data not shown).

The compartmentation and biochemical pathway of GABA production was investigated in cell suspensions incubated with either L-[U-^{14}C]Glu or L-[1-^{14}C]Glu for 10 min. The specific activity of GABA was then calculated after HPLC analysis of the aqueous cell fractions and the cell suspension medium. In the medium, the specific activity of GABA after incubation with L-[U-^{14}C]Glu was approximately 18 nCi/nmol, whereas the specific activity of GABA for cells incubated with L-[1-^{14}C]Glu was 0. Although the amount of GABA released to the medium was similar in both cases, the cells incubated with L-[1-^{14}C]Glu released nonlabeled GABA. This demonstrates that GABA production involves the decarboxylation of L-Glu at carbon 1. Similar results were obtained with illuminated or nonilluminated cells (Table II). In addition, the results with L-[U-^{14}C]Glu demonstrated that the specific activity of the GABA released to the medium was at least 80 times greater than that of the endogenous GABA (Table II). Thus, the sites of GABA production and storage are separate. This suggests that GABA efflux occurs from a pool of newly synthesized GABA, and that the low specific activity for endogenous GABA results during extraction when previously synthesized unlabeled GABA is mixed with a pool of recently synthesized labeled GABA.

GABA influx and equilibration were faster in illuminated than in nonilluminated cells. Equilibration occurred after approximately 6 min (Fig. 2B). The effect of L-Glu and CCCP on the unidirectional and net efflux of [^{14}C]GABA was studied to investigate the possible presence of a Glu/GABA antiport (Fig. 2). In both cases, 1 mM L-Glu had no effect on the rate of efflux of [^{14}C]GABA, whereas 10 μM CCCP stimulated efflux. The data do not support the presence of an antiport. Addition of 10 mM K_2SO_4, 5 mM butyric acid, or 1 mg/mL oligomycin also had no effect on GABA efflux (data not shown).

Table II. *The Specific Activity of GABA after Feeding L-[U-^{14}C]Glu or L-[1-^{14}C]Glu to a Suspension of Mesophyll Cells*

Conditions were the same as in Table I. The specific activity of GABA was derived from HPLC analysis after L-[U-^{14}C]Glu (22.5 nCi/nmol) or L-[1-^{14}C]Glu (17.5 nCi/nmol) metabolism for 10 min. Each value is the mean of three to six trials. L, Light; D, dark.

Illumination	^{14}C Label in L-Glu	Specific Activity in Medium	Specific Activity in Cell
		nCi/nmol	
L	U-^{14}C	18.0	0.23
D	U-^{14}C	19.4	0.14
L	1-^{14}C	0	0.20
D	1-^{14}C	0	0.06

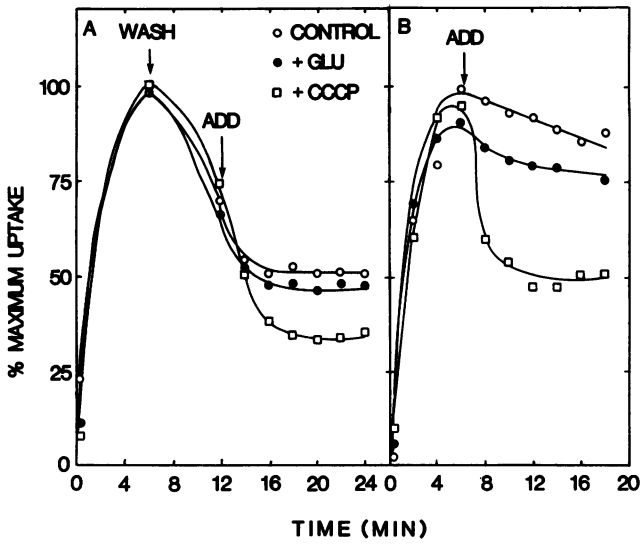

Figure 2. The effect of L-Glu and CCCP on unidirectional and net GABA efflux. A, Cells (4×10^6) in a 1-mL suspension were incubated with [U-^{14}C]GABA for 6 min. Exogenous GABA was then removed by washing, and L-Glu or CCCP was added as indicated to give final concentrations of 1.1 mM and 11 μM, respectively. Aliquots (100 μL) of cell suspension were removed periodically to determine radioactivity inside the cell. The mean of three trials is indicated. B, Cells (4×10^6) in a 1-mL suspension were incubated with [U-^{14}C]GABA. At 6 min, L-Glu or CCCP was added as indicated to give final concentrations of 1.7 mM and 17 μM, respectively. Aliquots (100 μL) of the cell suspension were removed periodically to determine radioactivity inside the cell. The mean of three trials is indicated.

Concentrations of amino acids in the cells and medium were obtained to determine whether efflux was specific to certain amino acids and whether efflux could occur through passive diffusion down a concentration gradient. Cells were suspended in the presence and absence of L-Glu for 10 min in the light (Table III). In either case, Ala was the most abundant amino acid in the cell. It was at least twofold higher than the levels of Glu and GABA, which were the next most abundant amino acids. However, when Glu was absent, GABA concentrations in the medium were twice those of Ala. When Glu was present, GABA was the only amino acid whose concentration in the medium rose significantly. Its concentration was 28 times higher than that for Ala. These results demonstrate a specificity for enhanced GABA efflux over the more abundant Ala and other amino acids. In addition, concentrations of GABA in the cells always exceeded those in the medium by at least 20-fold, demonstrating that passive diffusion could drive GABA efflux (Table III). Similar data were obtained with nonilluminated cells. No evidence for a H^+/GABA symport was obtained when the medium pH was monitored in response to GABA addition (data not shown).

DISCUSSION

GABA levels in plant tissues often increase in response to various forms of stress (15, 24, 28). However, in this study, the xylem sap was sampled immediately after cutting the stem, and the detached cladophylls were placed within 5 s into boiling methanol (Fig. 1). Thus, high GABA levels are most probably not a response to the stress of experimental manipulation. High levels of GABA in xylem have been reported previously (12, 13). Compared with intact cladophylls, the level of GABA in isolated cells was relatively low (Fig. 1). This result is most readily explained by GABA efflux from isolated cells, a finding consistently obtained when cells were incubated in the presence or absence of L-Glu (Fig. 2, Table III).

It is not clear whether the high levels of GABA in the intact tissue (Fig. 1) are due to GABA delivery via the xylem or conversion of xylem-delivered Glu to GABA in the cells. These suggestions are not mutually exclusive. The conversion of xylem Glu to GABA is dependent on (a) the delivery of Glu to the cells, (b) the entry of Glu into the cell, and (c) Glu decarboxylation. Although Glu levels are usually low in the xylem sap (Fig. 1) (6, 14, 20), the specific delivery of Asp and Glu to mature mesophyll cells has been demonstrated (13, 25). In *Asparagus,* the cotransport of H^+ and L-Glu into mesophyll cells has been demonstrated (1, 9, 10), as has GAD activity, which catalyzes the synthesis of GABA from Glu (22). Alternatively, GABA may arise from Glu synthesized within the cells.

The major product of L-[U-^{14}C]Glu metabolism was GABA (Table I). Nearly all of the newly synthesized GABA was released into the medium. There is little or no published data indicating that newly synthesized GABA leaves the cell. Previous determinations of GABA levels and rates of GABA production from labeled Glu have been conducted with intact

Table III. *Amino Acid Concentrations in Cells and Medium in the Presence and Absence of L-Glu*

Illuminated cells (30×10^6) were stirred and incubated in 5 mL of incubation medium in the presence or absence of 1 mM L-Glu for 10 min. Concentrations in the cell were calculated by dividing the moles of each amino acid per 10^6 cells by the cell volume (4.68 μL/10^6 cells). Concentrations in the medium were calculated by dividing the moles of each amino acid per 10^6 cells by the volume of the surrounding medium (167 μL/10^6 cells). Values are the mean of two trials.

Amino Acid	Cells		Medium	
	−L-Glu	+L-Glu	−L-Glu	+L-Glu
		mM		
Asp	0.068	0.096	0.000	0.001
Glu	1.090	1.586	0.001	0.318[a]
Asn	0.269	0.387	0.000	0.000
Ser	0.299	0.346	0.000	0.000
Gln/His	0.413	0.406	0.000	0.000
Gly	0.419	0.366	0.000	0.001
Thr	0.265	0.344	0.000	0.000
Arg	0.152	0.086	0.000	0.000
Ala	2.800	4.252	0.001	0.002
GABA	1.225	1.362	0.002	0.057
Tyr	0.314	0.378	0.000	0.000
Met	0.026	0.006	0.000	0.000
Val/Trp	0.269	0.301	0.000	0.000
Phe	0.143	0.406	0.000	0.000
Ile	0.118	0.355	0.000	0.000
Leu	0.173	0.199	0.000	0.000
Lys	0.389	0.413	0.001	0.000

[a] Glu added.

tissues. These studies do not distinguish between an intracellular (symplast) or an extracellular (apoplast) location of GABA. Consequently, the occurrence or significance of GABA efflux was not addressed (4, 7, 19, 27). Other labeled L-Glu metabolites were Krebs cycle acids. Label could enter the cycle through deamination of L-Glu to α-ketoglutarate or conversion of GABA to succinate by the GABA shunt (17). Entry was supported by an increase in O_2 consumption and CO_2 evolution when cells were incubated with L-Glu (9).

The production of unlabeled GABA after feeding L-[1-^{14}C] Glu clearly indicates that GABA production occurs via decarboxylation at carbon 1 (Table II) (18, 27). The data do not support alternative pathways of GABA production (8, 21). The production of GABA through L-Glu decarboxylation is supported by the widespread occurrence of GAD and the *in vivo* conversion of endogenous or exogenous Glu to GABA (23, 24, 27, 28). However, the small amount of label found in intracellular GABA after feeding L-[1-^{14}C]Glu appears inconsistent with decarboxylation at carbon 1. Because this label was much greater when the cells were illuminated, the result may be attributed to refixation of released CO_2 (Table II). In addition, the higher specific activity of labeled CO_2 released from L-[1-^{14}C]Glu will result in the fixation of more label than with L-[U-^{14}C]Glu.

Studies have shown that 50% of Glu, GABA, and Ala may be located outside of the vacuole (26, 29), a result consistent with the cytosolic location of GAD (16, 23, 24). Thus, GABA production and GABA accumulation may occur in the cytosol. However, in isolated soybean cells, GABA was a major component of the amino acid pool, yet, compared with other amino acids, it exhibited a slow net efflux. This suggested that either GABA is sequestered within an organelle and not available for efflux from the cytosol, or GABA efflux was limited by a selective mechanism (19). In the present study, the specific activity of the GABA released to the medium was 80 times greater than the intracellular GABA (Table II), and GABA was the most abundant amino acid in the medium (Table III). Thus, the newly synthesized GABA is readily available for efflux and is not in equilibrium with a pool of previously synthesized GABA. Consequently, for *Asparagus* mesophyll cells, the same location for GABA production and GABA accumulation appears highly unlikely.

The data in Figure 2 show that L-Glu had no effect on net or unidirectional efflux of [U-^{14}C]GABA. This does not support the presence of a Glu/GABA antiport operating at the plasma membrane. CCCP, however, stimulated the efflux of GABA in both cases. Metabolism of labeled GABA was not detected by amino acid or organic acid analyses after GABA uptake (data not reported). Stimulation of amino acid efflux by CCCP or a reduction in intracellular pH has been shown with other tissues (5, 19). Incubation with *p*-chloromercuribenzyl sulfonic acid resulted in a specific stimulation of GABA efflux from isolated soybean cells (19). Similarly, GABA was the major amino acid released from *Asparagus* cells even though it was not the most abundant amino acid in the cell (Table III). Both these studies suggest that GABA efflux may occur via specific mechanisms. In addition, efflux appears to be passive because the overall intracellular concentration of GABA was approximately 24 or 600 times its external concentration in the presence or absence of L-Glu, respectively (Table III). GABA efflux could indicate the occurrence of stress to adjacent cells.

It may be argued that the production and efflux of GABA is a nonphysiological and wasteful response to cell isolation. However, it is clear that *Asparagus* cells *in situ* contain (a) a H^+/L-Glu cotransport system (1, 9, 10), (b) GAD (22), and (c) high levels of GABA (Fig. 1, Table III). Thus, in addition to GABA, the cells possess prior to isolation the molecular processes required for the influx and conversion of Glu to GABA. Two recent reviews (2, 18) suggest that such an abundant and ubiquitous compound will fulfill a significant physiological role. Consequently, it is important to characterize the mechanisms regulating GABA production and efflux.

ACKNOWLEDGMENT

The authors wish to thank Steve McCutcheon for his invaluable assistance in the preparation of the manuscript.

LITERATURE CITED

1. **Bown AW, Chung I, Snedden WA, Shelp BJ** (1989) Specific glutamate cotransport into mesophyll cells and efflux of the major metabolite 4-aminobutyric acid. *In* J Dainty, ed, Plant Membrane Transport. Elsevier, New York, pp 329–334
2. **Bown AW, Shelp BJ** (1989) The metabolism and physiological roles of 4-aminobutyric acid. Biochem (Life Sci Adv) **8:** 21–25
3. **Colman B, Mawson BT, Espie GS** (1979) The rapid isolation of photosynthetically active mesophyll cells from *Asparagus* cladophylls. Can J Bot **57:** 1505–1510
4. **Desmaison AM, Tixier M** (1986) Amino acids content in germinating seeds and seedlings from *Castanae sativa* L. Plant Physiol **81:** 692–695
5. **Felle H, Johannes E** (1990) The regulation of proton-amino acid symport in *Riccia fluitans* L. by cytosolic pH and proton pump activity. J Exp Bot **41:** 587–592
6. **Hocking PJ** (1983) The dynamics of growth and nutrient accumulation by fruits of *Grevillea leucopteris* Meissn., a proteaceous shrub, with special reference to the composition of xylem and phloem sap. New Phytol **93:** 511–529
7. **Jordan BR, Givan CV** (1979) Effects of light and inhibitors on glutamate metabolism in leaf discs of *Vicia faba* L. Plant Physiol **64:** 1043–1047
8. **Kato T, Yamagata M, Tsukahara S** (1985) Metabolism of arginine-ϵ-$^{15}N_2$, urea-$^{15}N_2$ and ammonium-^{15}N in citrus trees (*Citrus unshiu* Marc.). J Jpn Hortic Sci **54:** 26–32
9. **McCutcheon SL, Bown AW** (1987) Evidence for a specific glutamate/H^+ cotransport in isolated mesophyll cells. Plant Physiol **83:** 691–697
10. **McCutcheon SL, Ciccarelli BW, Chung I, Shelp BJ, Bown AW** (1988) L-Glutamate-dependent medium alkalinization by *Asparagus* mesophyll cells. Plant Physiol **88:** 1024–1047
11. **Micallef BJ, Shelp BJ, Ball RO** (1989) Quantification of ^{14}C-labeled amino acids by reverse-phase high performance liquid chromatography. J Liquid Chromatogr **12:** 1281–1300
12. **Pate JS** (1986) Xylem-to-phloem transfer-vital component of the nitrogen-partitioning system of a nodulated legume. *In* J Cronshaw, WJ Lucas, RT Giaquinta, eds, Phloem Transport. Alan R. Liss, New York, pp 445–462
13. **Pate JS, Atkins CA** (1983) Nitrogen uptake, transport and utilization. *In* JW Broughton, ed, Ecology of Nitrogen Fixation, Vol 3. Clarendon Press, Oxford, UK, pp 245–298
14. **Peoples MB, Pate JS, Atkins CA** (1985) The effect of nitrogen source on transport and metabolism of nitrogen in fruiting plants on cowpea. J Exp Bot **366:** 567–582
15. **Reggiani R, Cantu CA, Brimballa I, Bertani A** (1988) Accumulation and interconversion of amino acids in rice roots under anoxia. Plant Cell Physiol **29:** 981–987
16. **Satyanarayan V, Nair PM** (1985) Purification and characteriza-

tion of glutamate decarboxylase from *Solanum tuberosum*. Eur J Biochem **150:** 53–60

17. **Satyanarayan V, Nair PM** (1986) The 4-aminobutyrate shunt in *Solarum tuberosum*. Phytochemistry **25:** 997–1001
18. **Satyanarayan V, Nair PM** (1990) Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. Phytochemistry **29:** 367–375
19. **Secor J, Schrader LE** (1984) Characterization of amino acid efflux from isolated soybean cells. Plant Physiol **74:** 26–31
20. **Shelp BJ** (1987) The composition of phloem exudate and xylem sap from broccoli (*Brassica oleracea* var *italica*) supplied with NH_4^+, NO_3^- or NH_4NO_3. J Exp Bot **38:** 1619–1636
21. **Slocum RD, Kaur-Sawhney R, Galston AW** (1984) The physiology and biochemistry of polyamines in plants. Arch Biochem Biophys **235:** 283–303
22. **Snedden WA, Chung I, Pauls RH, Bown AW** (1992) Proton/L-glutamate symport and the regulation of intracellular pH in isolated mesophyll cells. Plant Physiol **99:** 665–671
23. **Streeter JA, Thompson JF** (1972) *In vivo* and *in vitro* studies on α-aminobutyric acid metabolism with the radish plant (*Raphanus sativus* L.). Plant Physiol **49:** 579–584
24. **Tsushida T, Murai T** (1987) Conversion of glutamic acid to α-aminobutyric acid in tea leaves under anaerobic conditions. Agric Biol Chem **51:** 2805–2871
25. **Vogelmann TC, Dickson RE, Larson RR** (1985) Comparative distribution and metabolism of xylem-borne amino compounds and sucrose in shoots of *Populus deltoides*. Plant Physiol **77:** 418–428
26. **Wagner GJ** (1979) Content and vacuole/extravacuole distribution of neutral sugars, free amino acids, and anthocyanin in protoplasts. Plant Physiol **64:** 88–93
27. **Walker KA, Givan CV, Keys AJ** (1984) Glutamic acid metabolism and the photorespiratory nitrogen cycle in wheat leaves. Plant Physiol **75:** 60–66
28. **Wallace W, Secor J, Schrader LE** (1984) Rapid accumulation of 4-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol **75:** 170–175
29. **Yamaki S** (1982) Distribution of sorbitol, neutral sugars, free amino acids, malic acid and hydrolytic enzymes in vacuoles of apple cotyledons. Plant Cell Physiol **23:** 881–889