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L-Glutamate-Dependent Medium Alkalinization by *Asparagus* Mesophyll Cells[1]

COTRANSPORT OR METABOLISM?

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ABSTRACT

Mechanically isolated *Asparagus sprengeri* Regel mesophyll cells cause alkalinization of the suspension medium on the addition of L-glutamate or its analog L-methionine-D,L-sulfoximine. Using a radiolabeled pH probe, it was found that both compounds caused internal acidification whereas L-aspartate did not. Fusicoccin stimulated H^+ efflux from the cells by 111% and the uptake of L-[U-^{14}C]glutamate by 55%. Manometric experiments demonstrated that, unlike L-methionine-D,L-sulfoximine, L-glutamate stimulated CO_2 evolution from nonilluminated cells. Simultaneous measurements of medium alkalinization and $^{14}CO_2$ evolution upon the addition of labeled L-glutamate showed that alkalinization was immediate and reached a maximum value after 45 minutes whereas $^{14}CO_2$ evolution exhibited a lag before its appearance and continued in a linear manner for at least 100 minutes. Rates of alkalinization and uptake of L-[U-^{14}C]glutamate were higher in the light while rates of $^{14}CO_2$ evolution were higher in the dark. The major labeled product of glutamate decarboxylation, γ-aminobutyric acid, was found in the cells and the suspension medium. Its addition to the cell suspension did not result in medium alkalinization and evidence indicates that it is lost from the cell to the medium. The data suggest that the origin of medium alkalinization is co-transport not metabolism, and that the loss of labeled CO_2 and γ-aminobutyric acid from the cell result in an overestimation of the stoichiometry of the H^+/L-glutamate uptake process.

A plasma membrane-located, ATP-driven, H^+-efflux process establishes a H^+ electrochemical gradient across the membrane of plant cells (22). Amino acid influx and accumulation is coupled to and driven by the downhill influx of H^+. It has been suggested that this H^+ cotransport process is mediated by plasma membrane proteins which bind H^+ and amino acids in a simple molar ratio of 1 or 2 H^+ per amino acid prior to their transfer into the cell (18). Evidence for a H^+ cotransport process is often obtained when addition of the transported amino acid results in an alkalinization of the suspension medium (7, 12, 13, 16, 18, 19, 25). However, alkalinization may also arise from metabolism of the transported amino acid (18). Protonation of a basic metabolite will result in alkalinization as will a decarboxylation process:

$$RCOO^- + H^+ \rightarrow RH + CO_2$$

On the basis of medium alkalinization and uptake studies, an H^+ cotransport system specific for L-Glu[3] and L-MSO has been proposed for photosynthetically competent *Asparagus* mesophyll cells (12). However, the apparent H^+ uptake rate was 5 to 8 times faster than the uptake of L-[^{14}C]Glu indicating a large molar excess of H^+ associated with the L-Glu uptake process. This investigation also demonstrated that addition of L-Glu results in the rapid release of CO_2 (12). These findings indicate that the consumption of H^+ may result from a metabolic process. Medium alkalinization would occur if L-Glu was decarboxylated by an extracellular enzyme prior to the entry of the decarboxylated product into the cell. This model is analogous to the hydrolysis of sucrose by an extracellular invertase prior to hexose cotransport into sugarcane cells (8). Medium alkalinization would also occur if glutamate cotransport into the cell resulted in the export and protonation of a basic product of L-Glu metabolism. Alternatively, the high H^+ to L-Glu ratio could arise from the loss of labeled CO_2 or other metabolites from the cell and a corresponding underestimation of the rate of L-Glu uptake.

The present article reports experiments designed to: (a) test further predictions of the H^+/L-Glu cotransport model of L-Glu uptake, (b) determine whether L-Glu dependent medium alkalinization may be attributed to the metabolic consumption of H^+, and (c) determine whether the uptake of L-[^{14}C]Glu is underestimated due to the rapid loss of labeled metabolites from the cell.

MATERIALS AND METHODS

The compounds MBTH, L-Glu, L-Asp, L-MSO, *n*-butyric acid, and Na_2SO_4 were purchased from the Sigma Chemical Co. FC was from Italchemia S.A. L-[^{14}C]Glu and [^{14}C]DMO were from New England Nuclear Corp. ACS scintillation fluid, HH, and [^{14}C]*n*-hexadecane were from Amersham.

Mechanically isolated *Asparagus sprengeri* Regel mesophyll

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[3] Abbreviations: L-Glu, L-glutamate; L-MSO, L-methionine-D,L-sulfoximine; FC, fusicoccin; DMO, 5,5 dimethyl oxazolidine-2,4-dione; HH, hyamine hydroxide; MBTH, methylbenzethonium hydroxide; GABA, γ-aminobutyric acid.

cells were prepared daily (1). Cells were normally suspended in unbuffered 1 mM $CaSO_4$, or 20 mM KPi buffer (pH 5.5) (12), when a constant pH was required. Cell numbers were determined using a light microscope and hemocytometer. Approximately 85% of the cells were found to be intact as indicated by their ability to exclude Evans blue (5). The volume of the cell suspension varied between experiments but the cell density was kept between 2 and 3 × 10^6 cells/mL. The cells were incubated at 30°C, stirred, and unless indicated otherwise, aerated at a rate of 500 mL/min to equilibrate dissolved CO_2 with atmospheric CO_2.

Rates of alkalinization or acidification were measured using a Radiometer (Copenhagen) recording pH apparatus and rates of H^+ efflux or influx were calculated in units of nmol $H^+/10^6$ cells·min after determining the buffering capacity of the cell suspension medium (12). Illumination, when required, was supplied by a 200W reflector lamp (Sylvania) which gave an irradiance at the surface of the vessel of 4.6×10^{-4} mol·m^{-2}·s^{-1}.

Experiments in which medium alkalinization, $^{14}CO_2$ evolution, and L-[^{14}C]Glu uptake were measured simultaneously, employed an aerated closed system containing 3 ml of cell suspension. Volumes of 50.17 mM (2946 dpm/nmol) or 150 mM (1182 dpm/nmol) L-[U^{14}C]Glu were added to give the final concentrations indicated. The resulting alkalinization was measured with a Radiometer pH electrode (GK 2321) attached to a Fisher model 310 Accumet pH meter which was in turn attached to a Fisher chart recorder. Full scale deflection corresponded to 1 pH unit. Evolution of $^{14}CO_2$ was determined by passing air through the closed system into HH or MBTH to trap any $^{14}CO_2$ evolved. At zero time and at 2 or 5 min intervals thereafter, 4 mL volumes of HH or MBTH were collected and added to scintillation vials containing 1 mL of 100% methanol and 10 mL of ACS scintillation fluid. At the end of the incubation, triplicate samples of the cell suspension were removed to determine L-Glu uptake (12). The efficiency of the Beckman LS 1800 scintillation counting system was determined using the internal standard [^{14}C]*n*-hexadecane. Rates of $^{14}CO_2$ evolution were calculated as nmol $CO_2/10^6$ cells·min.

The effect of FC on the uptake of L-[^{14}C]Glu was determined by pretreating the cells with 20 μM FC for 10 min then adding 1 mM L-[^{14}C]Glu. Rates of uptake were determined as previously described (12).

Changes in the internal pH of the cell were detected using the radiolabeled pH probe [^{14}C]DMO. To 1 mL of cell suspension was added 25 μL of 1.2 mM [^{14}C]DMO (1.2×10^5 dpm/nmol) to give a final concentration of 30 μM. At 5 min intervals 50 μL aliquots of the cell suspension were removed and placed as the top layer in a 400 μL Eppendorf tube. The bottom layer consisted of 100 μL of 6% (w/v) sorbitol and the middle layer consisted of 50 μL of silicone fluid (50% AR20:50% AR200) (Wacker-Chemie Munich). The tube was spun at 12,800*g* for 40 s in a microfuge. The tubes were then frozen and the tip containing the cell pellet was cut off and placed in a scintillation vial with 1 mL of 100% methanol and 14 mL of ACS scintillation fluid for counting (2).

Manometric determination of rates of CO_2 evolution and O_2 consumption were performed with an 8-channel Gilson Differential Respirometer. The contents of the eight manometer flasks are described in the appropriate legend. The flasks were maintained at 30°C in the dark. Readings were made every 5 min for 30 min and then the cells in the main well were mixed with the contents of the side arm and readings were made for another 75 min.

Investigation into L-[U-^{14}C]Glu metabolism involved addition of 0.1 mM L-Glu (42,000 dpm/nmol) to 5 mL of pH 5.5, 1 mM $CaSO_4$ containing 15 × 10^6 cells. Incubation in the presence and absence of illumination was for 10 min. The cells and cell suspension medium were separated by centrifugation and washing of the cell pellet. After solubilization of the pellet in 2.5 mL methanol, 5 mL of chloroform, then 2.5 mL of water were added and the mixture thoroughly agitated. Aqueous and chloroform soluble cell fractions were separated by centrifugation at 1,300*g*. Amino acids in the cell suspension medium and aqueous cell fractions were derivatized with *o*-phthalaldehyde/2-mercaptoethanol, and were separated with a Waters HPLC gradient system (Milford, MA) using reverse phase chromatography as previously described (21). A Waters HPLC system coupled with a UV detector operating at 210 nm was employed to elute organic acids from an Aminex HPX-87H column (Bio-Rad) using 0.008 N H_2SO_4 and ambient temperature. Radioactivity was determined by passing the eluates into the solid cell (500 μL volume) of a Ramona LS (Raytest Instruments, Quebec, Canada) flow-through liquid scintillation counter. Data processing was accomplished with the Ramona Radio-Chromatography system in conjunction with an IBM PC. Alternatively, amino acids in the cell suspension medium and aqueous cell fractions were analyzed by TLC using Silica Gel G and a phenol/H_2O (75/25 w/w) solvent. Amino acids were located with ninhydrin, and radioactive areas with radioautography. Radioactive compounds were eluted from the chromatogram prior to counting in ACS scintillation fluid.

RESULTS

Asparagus mesophyll cells, when incubated in a nonbuffered medium above pH 5.5, acidify the suspension medium (Fig. 1). This is due to energy-dependent H^+ efflux (12). Addition of 20 μM FC to the cell suspension medium stimulated the rate of H^+ efflux from 0.66 to 1.49 nmol $H^+/10^6$ cells·min. The stimulation occurred with a 2 to 5 min lag period, after which the acidification was linear. When cells were suspended in a buffered medium, pretreatment with 20 μM FC caused an increase in the uptake of L-[U-^{14}C]Glu from 0.37 (±0.02 SD) to 0.58 nmol (±0.04 SD) L-Glu/10^6 cells·min; a stimulation of 55% (data not shown). Thus, FC stimulated L-Glu uptake in the absence of any change in external pH.

The effect of different compounds on changes in the internal pH of the cells was studied using the [^{14}C]DMO technique (Fig. 2). Twenty min after the addition of 30 μM [^{14}C]DMO to the buffered cell suspension medium, the levels of [^{14}C]DMO in the cells and the suspension medium approached equilibrium. At this time, 5 mM L-Glu, L-MSO, and L-Asp (as the sodium salts),

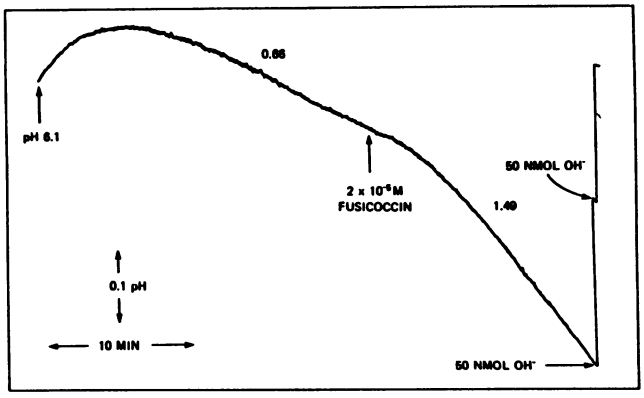

FIG. 1. FC stimulated H^+ efflux. A representative pH *versus* time tracing showing the effect of FC on the rate of H^+ efflux. Cells were stirred and aerated in 2 mL of 1 mM $CaSO_4$. The suspension medium contained KCl (20 mM) to stimulate H^+ efflux. The cells were allowed to acidify the medium for 20 min after which 20 μM FC was added. The pH of the medium was recorded for another 15 min. At the end of the experiment the cell suspension was titrated with 1 mM KOH to determine its buffering capacity. Rates of acidification are expressed in units of nmol $H^+/10^6$ cells·min.

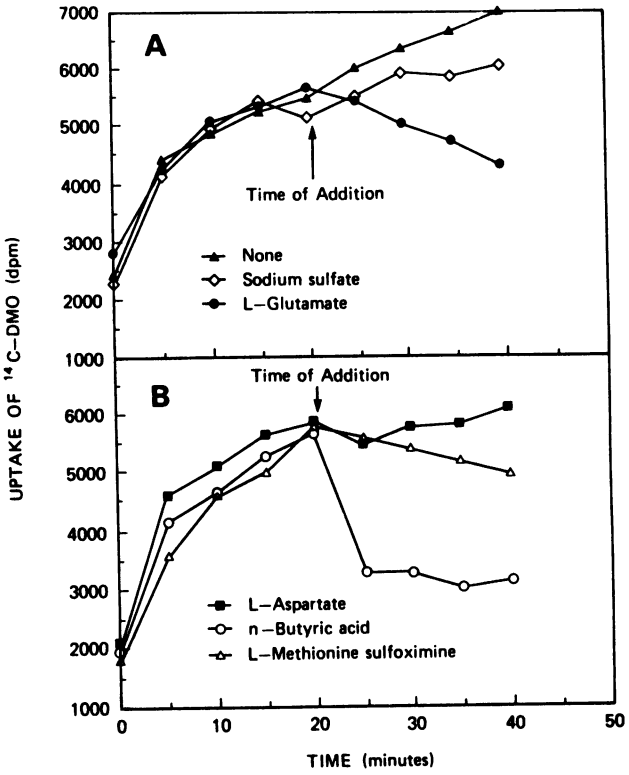

FIG. 2. L-Glu- and L-MSO-dependent internal acidification. Cells were incubated and stirred in 1 mL 20 mM KPi buffer (pH 5.5) in ambient light. To the suspension was added 50 μL 1.2 mM [^{14}C]DMO (1.1935×10^5 dpm/nmol). Levels of [^{14}C]DMO in the cell were determined after removing 50 μL aliquots every 5 min for 40 min. After 20 min, one of the following compounds was added: 5 mM L-Glu, L-MSO, L-Asp, *n*-butyric acid, or 2.5 mM Na_2SO_4. Each point is the mean of five trials.

n-butyric acid or 2.5 nM Na_2SO_4 were added. Addition of *n*-butyric acid caused an immediate and large decrease in the level of [^{14}C]DMO in the cell which lasted for at least 15 min. Addition of L-Glu or L-MSO caused a gradual decrease in the levels of [^{14}C]DMO in the cell. The decrease due to L-Glu was more pronounced than that due to L-MSO. Using the one-tailed Wilcoxon rank sum test, it was found that levels of [^{14}C]DMO were significantly lower than the control (Na_2SO_4) 20 min after the addition of *n*-butyric acid, L-Glu, and L-MSO ($n_1 = 5$, $n_2 = 5$, $T = 0$, $P < 0.05$). L-Asp addition did not significantly influence the distribution of [^{14}C]DMO. The data indicate that the addition of *n*-butyric acid, L-Glu, and L-MSO resulted in cytoplasmic acidification.

To determine if L-Glu and L-MSO were metabolized, the influence of these compounds on rates of O_2 consumption and CO_2 evolution were manometrically determined (Fig. 3, A–D). Rates of O_2 consumption and CO_2 evolution were obtained for 30 min after which time the cells were mixed with test solutions. Addition of L-Glu, Na_2SO_4, H_2O, and L-MSO stimulated O_2 consumption 70, 52, 28, and 27%, respectively, and stimulated CO_2 evolution 98, 38, 20, and 18%, respectively. Rates of O_2 consumption and CO_2 evolution were linear both before and after mixing. The mixing process appears to stimulate respiration. Unlike L-MSO, L-Glu stimulates CO_2 evolution and O_2 consumption.

To determine if any of the L-Glu-dependent CO_2 evolved came from the carbon skeleton of L-Glu, and whether there was any correlation between CO_2 evolution and medium alkalinization, both processes were measured simultaneously (Fig. 4). When 1 mM L-[U-^{14}C]Glu was added to a cell suspension, $^{14}CO_2$ was evolved. Following an initial 5 to 10 min lag period the rate was linear for at least 90 min. The alkalinization response was immediate and peaked after 50 min. Thus, $^{14}CO_2$ evolution and the alkalinization response were not temporally correlated.

The effect of light and dark and the external L-[U-^{14}C]Glu concentration on the H^+/L-Glu cotransport system was investigated by measuring medium alkalinization, uptake of labeled L-Glu and $^{14}CO_2$ evolution simultaneously (Fig. 5). As the external concentration of L-[U-^{14}C]Glu increased from 0.5 to 10 mM, the rate of alkalinization increased 1076% (light, L) and 935% (dark, D), the rate of uptake increased 562% (L) and 344% (D), and,

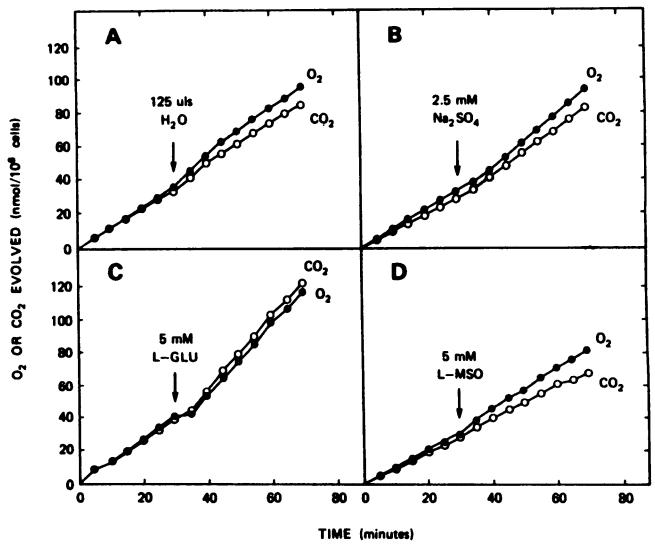

FIG. 3. The effect of L-Glu and L-MSO on rates of O_2 consumption and CO_2 evolution. Cells were incubated in 5 mL of 20 mM KPi buffer (pH 5.5) in the dark at 30°C in each of eight manometer flasks. The center well of flasks 1, 3, 5, and 7 remained empty while that of flasks 2, 4, 6, and 8 contained a piece of filter paper saturated with 0.2 mL 1M KOH. The side arm of flasks 1 and 2 contained 125 μL 200 mM L-Glu, flasks 3 and 4 contained 125 μL 200 mM L-MSO, flasks 6 and 7 contained 125 μL 100 mM Na_2SO_4, and flasks 7 and 8 contained 125 μL of distilled H_2O. Once the experiment was started, readings were made every 5 min for 30 min. The contents of the side arm were then tipped into the main well and readings were obtained for another 45 min. O_2 consumption and CO_2 evolution is expressed in units of nmol/10^6 cells. Each point is the mean of four trials.

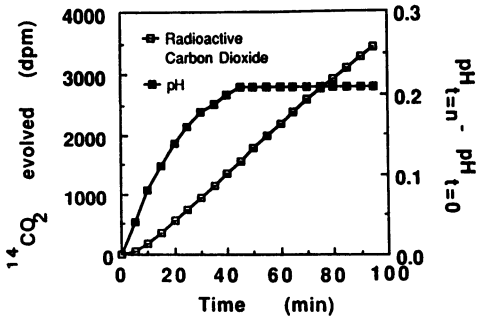

FIG. 4. Simultaneous measurement of medium alkalinization and $^{14}CO_2$ evolution. Cells were stirred and incubated in 3 mL 1 mM $CaSO_4$ in the dark in a closed system. The experiment was started by the addition of 20 μL 150 mM L-[^{14}C]Glu (1182 dpm/nmol). The pH of the medium was continually monitored. $^{14}CO_2$ evolution was determined by collecting 4 mL samples of HH every 5 min. Each point is the mean of three trials.

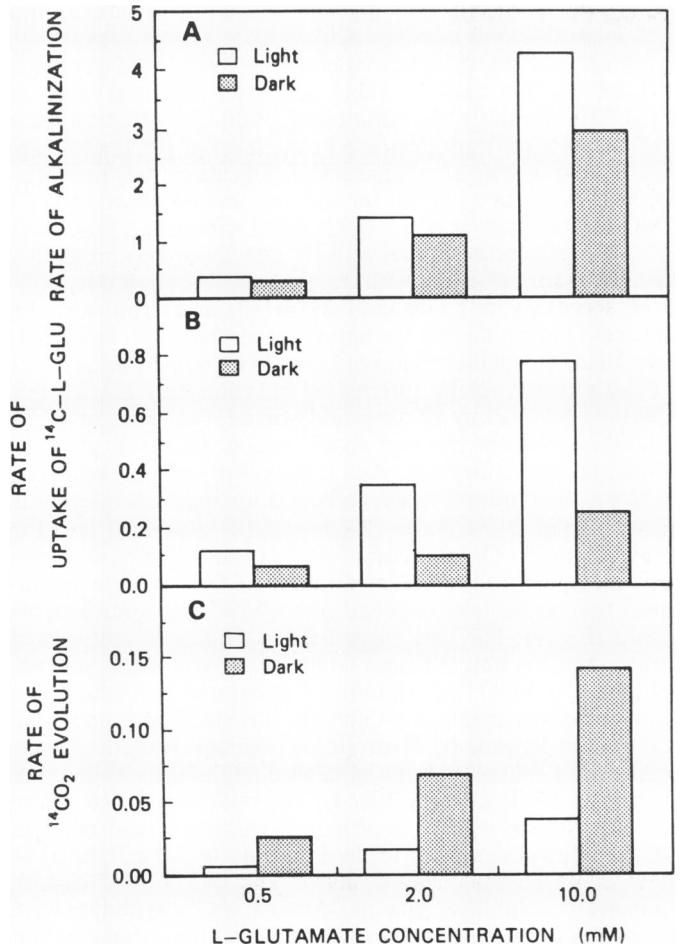

FIG. 5. Simultaneous measurement of rates of medium alkalinization, L-Glu uptake, and CO_2 evolution at three different external L-[U-^{14}C]Glu concentrations in the light and dark. Cells were stirred in a closed system in 3 mL 1 mM $CaSO_4$ in either the light or dark. The pH of the medium was continuously monitored. The evolution of $^{14}CO_2$ was determined by collecting CO_2 in the aeration stream in 4 mL samples of MBTH every 2 min for 10 min. The uptake of L-[U-^{14}C]Glu was determined by removing 200 μL aliquots at time zero and at the end of the experiment. All rates are expressed in units of nmol/10^6 cells·min. Each value is the mean of four trials.

the rate of $^{14}CO_2$ evolution also increased 600% (L) and 480% (D). Rates of uptake were consistently higher in the light while rates of $^{14}CO_2$ evolution were higher in the dark. A stoichiometry for the H^+/L-Glu cotransport system could be calculated from the data in Figure 5 by dividing the rate of alkalinization by the rate of total L-Glu uptake (Table I). Total uptake was defined as the rate of uptake of L-[U-^{14}C]Glu plus the rate of $^{14}CO_2$ evolution. The lowest H^+/L-Glu stoichiometry obtained was 3:1. The stoichiometry was higher in the dark than in the light, and increased as the external L-Glu concentration increased. The H^+/L-Glu stoichiometry obtained for nonilluminated cells exposed to 10 mM L-Glu was 7.7.

L-Glu metabolism was investigated with HPLC to determine whether these unexpectedly high H^+/L-Glu stoichiometries could be due to the loss of other labeled glutamate metabolites from the cell (Table II). The results demonstrate that labeled GABA was detected in both the cell and the medium. The sum of the endogenous and exogenous labeled GABA accounted for 63 and 66% of the labeled L-Glu metabolites produced, respectively, by the illuminated and nonilluminated cells. In addition, exogenous labeled GABA represented over 90% of the GABA produced and was in excess of the L-Glu found in the cells by at least 100%. Thus, the loss of L-Glu metabolites will result in a significant underestimation of L-Glu uptake rates. The organic acids produced from L-Glu could not be unambiguously identified. One fraction contained oxoglutarate, citrate, or pyruvate; a second fraction contained malate, succinate, or fumarate. Because of this uncertainty, the combined radioactivity from the two fractions is presented (Table II). No evidence for the release of a basic metabolite was obtained.

Rates of L-Glu uptake and GABA appearance in the medium were measured in the absence and presence of inhibitors of L-Glu uptake using thin layer chromatographic analysis (Table III). Results indicate that after 10 min, the total ^{14}C label in the cell was exceeded by ^{14}C label in external GABA by 700%. In addition, 20 mM K^+ inhibited uptake of L-Glu by 16% and GABA appearance by 18%. Corresponding figures for 10 μM CCCP were 95 and 51%. Thus, it appears that the appearance of GABA in the medium is dependent on L-Glu uptake into the cell. Further evidence for the specific release of GABA from the cell to the medium was obtained when cells were incubated in the absence and presence of unlabeled 1 mM L-Glu. GABA appeared in the medium in the absence of added exogenous L-Glu. However, in the presence of L-Glu, the GABA concentration was much higher. Other amino acids were not found in the medium. GABA addition to the cells did not result in medium alkalinization (data not shown).

Table I. *Stoichiometry of the H^+/L-Glu Cotransport System*

Conditions are the same as defined in the legend to Figure 5. The stoichiometry was determined by dividing the rate of alkalinization by the rate of total uptake. The rate of total uptake is defined as the sum of the rate of uptake of L-[U-^{14}C]Glu plus the rate of ^{14}CO evolution. These values were calculated from the data in Figure 5. Each value is the mean of five trials. Illuminated cells (L); nonilluminated cells (D).

Glutamate Concentration		Rate of Alkalinization	Rate of Total Uptake	Stoichiometry (H^+/L-Glu)
mM		*nmol/10^6 cells·min*		
0.5	(L)	0.364	0.121	3.0:1
	(D)	0.285	0.079	3.6:1
2.0	(L)	1.395	0.357	3.9:1
	(D)	1.078	0.153	7.0:1
10	(L)	4.282	0.803	5.3:1
	(D)	2.951	0.383	7.7:1

Table II. *Metabolism of L-[U-^{14}C]Glu in Illuminated and Nonilluminated Cells*

Cells were incubated for 10 min in 5 ml of pH 5.5 1 mM $CaSO_4$ in the absence or presence of illumination. Subsequently, 0.1 mM L-[U-^{14}C]Glu (42,000 dpm/nmol; 21 × 10^6 dpm) was added for an additional 10 min. The cells and cell medium were then analyzed for the presence of labeled products of glutamate metabolism using HPLC ("Materials and Methods").

Sample	DPM			
	Glu	Gln	GABA	Organic acids
Light				
Cell extract	140,110	Trace	50,040	127,440
Cell medium	19,854,000	Trace	931,500	451,440
Dark				
Cell extract	545,770	40,700	102,080	129,600
Cell medium	17,409,170	Trace	1,119,888	475,060

Table III. *L-Glu Uptake and GABA Release to the Medium*

Cells were incubated with illumination for 10 min in 5 mL of pH 6.0 5 mM Mes, 1 mM $CaSO_4$. Subsequently, 0.5 mM L-[U-^{14}C]Glu (3100 dpm/nmol, 7.75 × 10^6 dpm) was added for an additional 10 min. Samples of 200 μl were removed at 2 min intervals to determine uptake. The remaining cells and cell medium were then analyzed for labeled L-Glu metabolites using TLC ("Materials and Methods"). K_2SO_4 (10 mM), CCCP (10 μM).

Experimental Conditions	L-Glu Uptake	GABA in Medium
	nmol/10^6 cells·10 min	
Control		
Experiment:		
1	0.92	9.19
2	1.69	7.17
3	0.94	8.54
Mean (SD)	1.18 (0.44)	8.30 (0.9)
K_2SO_4		
Experiment:		
1	0.86	6.36
2	1.48	7.04
3	0.64	7.11
Mean (SD)	0.99 (0.58)	6.84 (0.41)
CCCP		
Experiment:		
1	0.05	4.18
2	0.09	4.32
3	0.04	3.79
Mean (SD)	0.06 (0.02)	4.1 (0.27)

DISCUSSION

Medium alkalinization on addition of a metabolite to a cell suspension may derive from a H^+/metabolite cotransport uptake system or a metabolic process such as decarboxylation (18). The results presented support the previous contention that L-[U-^{14}C] Glu uptake into *Asparagus* mesophyll cells is driven by H^+ cotransport and that L-Glu-dependent medium alkalinization results from the transport of H^+ from the medium into the cell (12). FC is a known specific stimulator of the plasma membrane H^+ ATPase which catalyses H^+ efflux from plant cells. Thus, FC should elevate the proton electrochemical gradient, which is the driving force for H^+/amino acid cotransport processes (18, 22), and stimulate H^+ cotransport uptake mechanisms (11, 13, 14). L-[^{14}C]Val uptake into *Commelina* mesophyll cells and L-[^{14}C] Ala uptake into *Lemna gibba* plants was stimulated by 10 and 15 μM FC, respectively (10, 23). In the present study, 20 μM FC stimulated H^+ efflux by 125%, demonstrating the presence of the normal FC-stimulated plasma membrane/H^+ ATPase in *Asparagus* mesophyll cells. A 10 min pretreatment of these cells with FC stimulated uptake of L-[^{14}C]Glu by 55%, indicating a H^+ co-transport mediated uptake process.

Another prediction of the co-transport model is that the addition of substrate should lead to both external alkalinization and internal acidification. Medium alkalinization has been observed when a variety of amino acids have been added to various plant tissues (7, 12, 13, 16, 18, 19, 25). The rate of internal acidification will depend on (a) the rate of the H^+ influx due to co-transport, (b) rates of other H^+ fluxes into and out of the cytoplasm, (c) the buffering capacity of the cytoplasm, and (d) any metabolic production of consumption of H^+. Using [^{14}C] DMO as a pH probe, it was shown that sucrose transport into soybean cotyledon protoplasts led to internal acidification (9). The [^{14}C]DMO technique has been used to determine the cytoplasmic pH of *Asparagus* mesophyll cells (2) and met the criteria required of a radiolabeled pH probe (9). In the present study, steady state levels of [^{14}C]DMO were established within 20 min (Fig. 2). Addition of *n*-butyric acid which is known to permeate cells and acidify the cytoplasm was used to validate the technique (17). Its addition caused a rapid decrease in internal levels of [^{14}C]DMO. Similarly, the addition of L-Glu or L-MSO caused a gradual but significant decrease in the level of [^{14}C]DMO suggesting a slower rate of cytoplasmic acidification. Addition of L-Asp or Na_2SO_4 as a control for sodium, had no effect on [^{14}C] DMO levels. These results suggest that L-Glu and L-MSO, but not L-Asp, are cotransported with H^+ into *Asparagus* mesophyll cells. They are consistent with external alkalinization data indicating specificity for L-Glu and L-MSO (12), and results with the same cells indicating that the uptake of labeled L-Glu was much faster than the uptake of labeled L-Asp (data not shown).

FC-stimulated uptake, internal acidification data (Fig. 2), and published data (12) are all consistent with a H^+ cotransport origin of L-Glu-dependent medium alkalinization. However, alkalinization may also arise from L-Glu metabolism (18). In particular extracellular or intracellular decarboxylation reactions may result in net alkalinization if the CO_2 released is dissipated into the aeration stream. The addition of L-Glu to suspensions of *Asparagus* mesophyll cells stimulated rates of CO_2 evolution significantly beyond the rates obtained with L-MSO and control experiments (Fig. 3). The data suggest that L-Glu is decarboxylated while L-MSO is not. Whereas L-Glu has several possible metabolic fates, L-MSO, an inhibitor of glutamine synthetase, is a nonmetabolised analog of L-Glu (20). It is clear that some or all of the L-Glu dependent CO_2 evolution originates from the carbon skeleton of L-Glu as addition of labeled L-Glu to the cells led to the release of labeled CO_2 (Fig. 4). The initial lag period before the release of labeled CO_2 is presumably due to time-dependent metabolic processes prior to decarboxylation. Since labeled L-MSO is not commercially available, and the price of custom synthesis is prohibitive, data on L-MSO and CO_2 evolution were obtained manometrically (Fig. 3). The data indicate that medium alkalinization does not result from decarboxylation. L-MSO which also causes medium alkalinization, and appears to compete with L-Glu for entry into the cell (12) did not stimulate decarboxylation (Fig. 3). In addition, the time course of L-[^{14}C] Glu-dependent alkalinization and $^{14}CO_2$ release were very different. It is difficult to attribute medium alkalinization to decarboxylation when the latter appears to start after alkalinization (Fig. 4). The evidence for L-MSO and L-Glu stimulated internal acidification (Fig. 2) eliminates the possibility that an internal alkalinization process results in external alkalinization. In contrast, the evidence suggests that medium alkalinization involves a H^+ cotransport mechanism.

The unexpected finding that label in GABA released from the cell greatly exceeds label in the cell (Tables II and III) answered two questions associated with this study. Because production of GABA from L-Glu involves a one-step decarboxylation catalyzed by glutamate decarboxylase, the rapid evolution of CO_2 on L-Glu addition (Figs. 3 and 4) is explained in whole or in part by the formation of GABA. In addition, the high H^+/L-Glu stoichiometries found in the present (Table I) and previous (12) studies is explained by an underestimation of labeled L-Glu uptake when label in the form of GABA leaves the cell. The H^+/L-Glu stoichiometry can be recalculated for illuminated cells exposed to 0.5 mM L-Glu using the data in Figure 5 and Table III. Given that the label in GABA released from the cell exceeds the total label in the cell by a factor of 7 (Table III) the rate of L-Glu uptake for these conditions is increased from 0.12 (Fig. 5) to 0.96 nmol/10^6 cells·min. The resulting stoichiometry is changed from 3.0 (Table I) to 0.37 H^+/L-Glu. The loss of label as CO_2 is not significant under these conditions (Fig. 5). However, the apparent loss of organic acids from the cell (Table III) may result in an overestimation of this value. A similar stoichiometry has been reported for the cotransport of L-Gln and sucrose. In both

cases the authors suggested that the deviation from a 1/1 ratio could involve the recirculation of H^+ via the plasma membrane ATPase (3, 19). A H^+/L-Glu stoichiometry of 2/1 was obtained with sugar cane suspension cells using a short 4 min incubation period (25). However, the present data suggests that this value may be an overestimate since the loss, if any, of labeled metabolites was not determined.

Plasma membrane depolarization in response to amino acids has been observed with oat coleoptiles and is believed to result from the uptake system transporting net positive charge into the cell (6, 7). Similarly it has been suggested that an amino acid stimulated K^+ efflux from sugar cane cells is an electrophoretic response to depolarization (25). A process transporting anionic L-Glu with one H^+ would be electroneutral and would not cause depolarization. The lack of L-Glu stimulated K^+ efflux from *Asparagus* mesophyll cells suggests an electroneutral process (12) or an electrogenic process in which the movement of an ion, other than K^+, maintains electroneutrality. In sugar cane cells K^+ efflux in response to a variety of amino acids is convincingly demonstrated except in the case of L-Glu addition (25). A simulation model for amino acid cotransport correctly predicted the effect of neutral and basic amino acids on membrane potential changes. However, in the case of the acidic amino acids (L-Glu and L-Asp) models with one or two H^+ cotransported with the amino acid did not predict experimentally observed membrane potential changes. Transport of the anionic form of these acidic amino acids with one H^+ and another unspecified cation resulted in theoretical changes which were closest to the observed changes (7). Further work on the acidic amino acid cotransport mechanism is required to determine the ions involved and their stoichiometry.

Investigations into L-Glu metabolism have reported that significant levels of labeled GABA are produced when L-[U-^{14}C] Glu is fed to illuminated or nonilluminated bean leaves (4) or wheat leaves (24). In these C3 plants GABA appears to be produced by a cytosolic glutamate decarboxylase and is then incorporated into intermediates of the tricarboxylic acid cycle (4). Various organic acid products of glutamate metabolism were also detected in this study (Table II). The role of GABA production and release from the cell and the relationship of these events to the photorespiratory nitrogen cycle and movements of nitrogen in the plant remains to be investigated. The decarboxylation of L-Glu may constitute a cytosolic pH-stat mechanism which consumes H^+ and limits the internal acidification (Fig. 2) when H^+ and L-Glu are cotransported into the cytosol. Alternatively, the loss of GABA from the cell, which has not been reported previously, may be a manifestation of the whole plant nitrogen cycle (15) in which excess nitrogen supplied in the form of L-Glu is fated for export by conversion to GABA. The existence of a transport protein which can export GABA either alone or in exchange for L-Glu and H^+ remains to be explored.

The data provide further evidence for H^+/L-Glu cotransport into *Asparagus sprengeri* cells. They demonstrate that previously calculated stoichiometries (12) were overestimated because of the loss of the L-Glu metabolites CO_2 and GABA from the cell and the consequent underestimation of rates of L-[U-^{14}C]Glu uptake. The origin of L-Glu dependent alkalinization appears to be cotransport not metabolism.

LITERATURE CITED

1. COLMAN B, BT MAWSON, GS ESPIE 1979 The rapid isolation of photosynthetically active mesophyll cells from *Asparagus* cladophylls. Can J Bot 57: 1505–1510
2. ESPIE GS, B COLMAN 1981 The intracellular pH of isolated, photosynthetically active *Asparagus* mesophyll cells. Planta 153: 210–216
3. HUTCHINGS V 1978 Sucrose and proton cotransport in *Ricinus* cotyledons. I. H^+ influx associated with sucrose uptake. Planta 138: 229–235
4. JORDAN BR, CV GIVAN 1979 Effects of light and inhibitors on glutamate metabolism in leaf discs of *Vicia faba* L. Plant Physiol 64: 1043–1047
5. KANAI R, GE EDWARDS 1973 Purification of enzymatically isolated mesophyll protoplasts from C3, C4 and crassulacean acid metabolism plants using an aqueous dextron-polyethylene glycol two phase system. Plant Physiol 52: 484–490
6. KINRAIDE TB, B ETHERTON 1980 Electrical evidence for different mechanisms of uptake for basic neutral and acidic amino acids in oat coleoptiles. Plant Physiol 65: 1085–1089
7. KINRAIDE TB, IA NEWMAN, B ETHERTON 1984 A quantitative simulation model for H^+-amino acid cotransport to interpret the effects of amino acids on membrane potential and extracellular pH. Plant Physiol 76: 806–813
8. KOMOR E, M THOM, A MARETZKI 1981 The mechanism of sugar uptake by sugarcane suspension cells. Planta 153: 181–192
9. LIN W 1985 Energetics of sucrose transport into protoplasts from developing soybean cotyledons. Plant Physiol 78: 41–45
10. LUTTGE U, K-D JUNG, CI ULLRICH-EBERIUS 1980 Evidence for amino acid-H^+ cotransport in *Lemna gibba* given by effects of fusicoccin. Z Pflanzenphysiol 102: 117–125
11. MARRE E 1979 Fusicoccin: a tool in plant physiology. Annu Rev Plant Physiol 30: 273–288
12. MCCUTCHEON S, AW BOWN 1987 Evidence for a specific glutamate/H^+ cotransport in isolated mesophyll cells. Plant Physiol 83: 691–697
13. MOUNOURY G, S DELROT, J-L BONNEMAIN 1984 Energetics of threonine uptake by pod wall tissues of *Vicia faba* L. Planta 161: 178–185
14. OTSIOGO-OYABI H, G ROBLIN 1985 Uptake of glycine by excised pulvini of *Mimosa pudica* in relation to proton pump activity. Physiol Plant 65: 40–44
15. PATE JS, CA ATKINS 1983 Nitrogen uptake, transport and utilization. *In* WJ Broughton, ed, Nitrogen Fixation, Vol 3, Legumes. Clarendon Press, Oxford, pp. 245–298
16. PETZOLD U, I DAHSE, E MULLER 1985 Light-stimulated electrogenic uptake of neutral amino acids by leaf cells of *Egeria densa* and *Vicia faba*. Biochem Physiol Pflanzen 180: 655–666
17. REID RJ, LD FIELD, MG PITMAN 1985 Effects of external pH, fusicoccin and butyrate on the cytoplasmic pH in barley root tips measured by ^{31}P-nuclear magnetic resonance spectroscopy. Planta 166: 341–347
18. REINHOLD L, A KAPLAN 1984 Membrane transport of sugars and amino acids. Annu Rev Plant Physiol 35: 45–83
19. ROBINSON SP, H BEEVERS 1981 Evidence for amino-acid: proton cotransport in *Ricinus* cotyledons. Planta 152: 527–533
20. RONZIO R, W ROWE, A MEISTER 1969 Studies on the mechanism of inhibition of glutamine sythetase by methionine sulfoximine. Biochemistry 8: 1066–1075
21. SHELP BJ 1987 The composition of phloem exudate and xylem sap from broccoli (*Brassica oleracea* va italica) supplied with NH_4^+, NO_3^-, or NH_4NO_3. J Exp Bot 38: 1619–1636
22. SZE H 1985 H^+-translocating ATPases: advances using membrane vesicles. Annu Rev Plant Physiol 36: 175–208
23. VAN BEL AJE, AC BORSTLAP, A VAN PINXTEREN-BAZUINE, A AMMERLAAN 1982 Analysis of valine uptake by *Commelina* mesophyll cells in a biphasic active and a diffusional component. Planta 155: 335–341
24. WALKER KA, CV GIVAN, AJ KEYS 1984 Glutamic acid metabolism and the photorespiratory nitrogen cycle in wheat leaves. Plant Physiol 75: 60–66
25. WYSE R, E KOMOR 1984 Mechanism of amino acid uptake by sugarcane suspension cells. Plant Physiol 76: 865–870