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# The Synthesis of γ-Aminobutyric Acid in Response to Treatments Reducing Cytosolic pH[1]

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**γ-Aminobutyric acid (GABA) synthesis (L-glutamic acid + $H^+$ → GABA + $CO_2$) is rapidly stimulated by a variety of stress conditions including hypoxia. Recent literature suggests that GABA production and concomitant $H^+$ consumption ameliorates the cytosolic acidification associated with hypoxia or other stresses. This proposal was investigated using isolated asparagus (*Asparagus sprengeri* Regel) mesophyll cells. Cell acidification was promoted using hypoxia, $H^+$/L-glutamic acid symport, and addition of butyrate or other permeant weak acids. Sixty minutes of all three treatments stimulated the levels of both intracellular and extracellular GABA by values ranging from 100 to 1800%. At an external pH of 5.0, addition of 5 mM butyrate stimulated an increase in overall GABA level from 3.86 (0.56 ± SE) to 20.4 (2.16 ± SE) nmol of GABA/$10^6$ cell. Butyrate stimulated GABA levels by 200 to 300% within 15 s, and extracellular GABA was observed after 10 min. The acid load due to butyrate addition was assayed by measuring [$^{14}$C]butyrate uptake. After 45 s of butyrate treatment, $H^+$-consuming GABA production accounted for 45% of the imposed acid load. The cytosolic location of a fluorescent pH probe was confirmed using fluorescent microscopy. Spectrofluorimetry indicated that butyrate addition reduced cytosolic pH by 0.60 units with a half-time of approximately 2 s. The proposal that GABA synthesis ameliorates cytosolic acidification is supported by the data. The possible roles of $H^+$ and $Ca^{2+}$ in stimulating GABA synthesis are discussed.**

GABA is found in virtually all plant tissues and is normally a major component of the free amino acid pool. Its synthesis results primarily from an α-decarboxylation catalyzed by L-Glu decarboxylase (EC 4.1.1.15). There are numerous reports that rapid and large increases in GABA levels occur in response to a variety of stress conditions (Bown and Shelp, 1989; Satyanarayan and Nair, 1990, and refs. therein). GABA levels in soybean leaves increased from 0.05 to 1 to 2 μmol $g^{-1}$ fresh weight within 5 min of an imposed cold stress or mechanical stress (Wallace et al., 1984). However, neither the mechanism by which GABA levels rapidly increase nor the adaptive significance, if any, of these increases, is understood.

Recently, investigators have reported increased GABA levels in response to hypoxia (Reggiani et al., 1988; Menegus et al., 1989) and $H^+$/L-Glu symport (Chung et al., 1992; Snedden et al., 1992). In both cases it is proposed that increased GABA levels may result from a reduction in intracellular pH. Hypoxia is reported to reduce cytosolic pH by 0.4 to 0.8 pH units (Kurkdjian and Guern, 1989). Significantly, L-Glu decarboxylase exhibits a sharp pH optimum of approximately 6.0 (Tsushida and Murai, 1987; Snedden et al., 1992) and is located in the cytosol (Satyanarayan and Nair, 1985; Tsushida and Murai, 1987). Thus, a reduction in intracellular pH from normal physiological values could elevate GABA levels by stimulating L-Glu decarboxylase activity. This mechanism may be part of a metabolic pH-stat in which lower pH values activate the $H^+$-consuming decarboxylation of metabolites such as malate and L-Glu (Guern et al., 1986; Mathieu et al., 1986; Reggiani et al., 1988; Menegus et al., 1989; Snedden et al., 1992). However, the relationship between changes in intracellular pH and GABA level has not been investigated rigorously, since concurrent measures of both have not been obtained. In a recent investigation, however, changes in pH and GABA synthesis were measured in hypoxic maize root tips. Whereas a pH decline of 0.5 to 0.6 pH units was observed within 15 min, GABA accumulation was observed only after 30 min of hypoxia. Consequently, the authors suggested that GABA synthesis is not involved in pH regulation (Roberts et al., 1992).

Studies of $H^+$/L-Glu symport into isolated *Asparagus sprengeri* mesophyll cells demonstrated that the major product of L-[U-$^{14}$C]Glu metabolism was GABA. Most of the newly synthesized GABA was released to the medium (Chung et al., 1992; Snedden et al., 1992). Comparable investigations of GABA synthesis (Walker et al., 1984; Desmaison and Tixier, 1986) were conducted with intact tissues and consequently did not distinguish between intracellular (symplastic) and extracellular (apoplastic) GABA. Efflux of GABA may represent an aspect of its physiological function. It is not known, however, whether GABA efflux is specific to $H^+$/L-Glu symport or occurs whenever GABA synthesis is stimulated.

This investigation used isolated *A. sprengeri* mesophyll cells to address the following questions: (a) Do acidifying treatments other than hypoxia and $H^+$/L-Glu symport stimulate GABA synthesis? (b) Is the decline in cytosolic pH fast enough to account for a rapid stimulation of GABA synthesis? (c)

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Abbreviations: AM, acetoxymethyl ester; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; GABA, γ-aminobutyric acid; *I*, emission intensity.

What proportion of an increase in cytosolic $H^+$ is accounted for by $H^+$-consuming GABA synthesis? (d) Does GABA efflux occur only in response to $H^+$/L-Glu symport or whenever GABA synthesis is stimulated?

## MATERIALS AND METHODS

### Cell Isolation and Incubation

Mechanically isolated *Asparagus sprengeri* Regel mesophyll cells were prepared daily (Colman et al., 1979) and stored in 1 mM $CaSO_4$ adjusted to pH 5.5 with $Ca(OH)_2$. Cells ($4 \times 10^6$ $mL^{-1}$) were incubated in 5 mM Mes (pH 6.0 or pH 5.0 as indicated). Cell-suspension volumes varied from 1 to 30 mL, depending on the experiment, and were maintained by stirring and aeration at 25°C in a water-jacketed incubation vessel. Suspensions were kept in the dark or illuminated with a 200-W Sylvania lamp giving an irradiance of $4.6 \times 10^{-4}$ mol $m^{-2}$ $s^{-1}$. Depending on the experimental procedure, cells were incubated for periods ranging from 15 s to 60 min.

Treatments to induce cellular acidification included hypoxia, $H^+$/L-Glu co-transport, and addition of permeant weak acids. Hypoxic conditions were obtained by gently bubbling $N_2$ (3–5 min) through the cell suspension prior to excluding $O_2$ with a stopper inserted into the incubation vessel. Hypoxia was verified using a calibrated $O_2$ electrode while incubation proceeded in the absence of light. L-Glu (5 mM) was added to cell suspensions in both light and dark conditions. Sodium butyrate (5 mM), or other weak acids, adjusted to pH 5.0 and 6.0, were added to illuminated cell suspensions incubated at pH 5.0 or pH 6.0, respectively.

### GABA Extraction and Determination

After the allotted incubation period, aliquots of the suspension containing $20 \times 10^6$ cells were centrifuged at 2800*g* for 2 min, and the extracellular medium was collected. The cell pellet was resuspended and solubilized by sequential addition and stirring with 2.5 mL of methanol, 5 mL of chloroform, and 2.5 mL of water. The aqueous phase was separated from the chloroform phase by centrifugation at 2800*g* for 10 min and retained. The intracellular and extracellular fractions derived from the aqueous phase and the extracellular medium, respectively, were dried under a stream of air at 40°C. They were redissolved in 1 M potassium PPi buffer (pH 8.6) prior to GABA analysis. When rapid GABA production was measured, aliquots of the cell suspension were transferred directly into hot methanol. The procedure indicated above was then used to prepare an aqueous fraction for determination of total GABA (intracellular and extracellular GABA). GABA was measured using the commercially available coupled enzyme assay procedure for GABA (GABAse, Sigma).

$$\text{GABA} + \text{2-ketoglutarate} \rightarrow \text{L-Glu} + \text{succinate semialdehydesuccinate semialdehyde} + \text{NADP}^+ \rightarrow \text{succinate} + \text{NADPH}$$

Increased *A* in the 1-mL assay system was measured at 340 nm for 20 min. When standard GABA solutions were dried and dissolved as described above, the assay was found to be linear over the range of 0.03 to 0.11 μmol of GABA per cuvette and resulted in 90 to 100% of the expected *A* change. Similarly, the simultaneous addition of methanol and an amount of GABA comparable to that found in the cell suspension resulted in 90 to 100% of the expected signal. GABA levels were calculated in nmol of GABA/$10^6$ cells. Since daily variation in GABA levels and rates of increase were observed, control and experimental cells from the same stock suspension were always incubated simultaneously prior to GABA determination.

### Determination of Cytoplasmic Acid Load

The cytoplasmic acid load (mol of $H^+$) in response to butyrate addition was determined by measuring radiolabeled butyric acid uptake. Protonated permeant butyric acid (HA, pKa 4.8) enters and dissociates fully at cytosolic pH values ($HA \rightarrow H^+ + A^-$). Cells ($4 \times 10^6$) were suspended in 950 μL of 5 mM Mes (pH 5.0 or pH 6.0) maintained at 25°C. Fifty microliters of [1-$^{14}$C]butyrate (0.5 mCi $mmol^{-1}$) was added to give a final concentration of 5 mM. Aliquots of 75 μL were removed at periodic intervals, washed with ice-cold 5 mM Mes (pH 5.0 or 6.0) containing 5 mM unlabeled butyrate, and collected on Millipore filters (HA type 0.65 μm). After scintillation counting the uptake of butyric acid was calculated and expressed as nmol/$10^6$ cells.

### Determination of the Cytoplasmic Location of the pH Probe

BCECF-AM (Molecular Probes, Eugene, OR) is a membrane-permeant lipophilic ester that after hydrolysis to BCECF by endogenous esterases, exhibits pH-dependent fluorescence. Five million cells were incubated with gentle stirring in 1 mM $CaSO_4$ containing 3 or 6 μM BCECF-AM (Molecular Probes). After 30 min of incubation with ambient light and temperature, cells were washed three times by centrifugation and resuspension to remove any extracellular BCECF-AM or BCECF.

Cells incubated with 6 μM BCECF-AM for 30 min were observed with a Leitz Diaplan microscope equipped with epifluorescence optics (×100 objective lens, oil immersion, ×10 eyepiece lens). An HBO 50-W super pressure mercury lamp was used with a BP 450- to 490-nm exciting filter, an RKP 510-nm beam splitter, and an LP 515-nm suppression filter. An additional green glass filter (peak transmission 535 nm), placed on top of the suppression filter, was used to block the red autofluorescence of the chloroplasts. Photographs were taken using 400 ASA Kodak Ektachrome color film.

### Determination of Cytoplasmic pH Changes

Fluorescence measurements of cytosolic pH were made in a Perkin-Elmer LS-50 spectrofluorimeter fitted with a thermoregulated cell compartment. BCECF-loaded cells were stirred at 25°C in 2 mL of pH 5.0 or 6.0 buffer at a density of $0.1 \times 10^6$ cells $mL^{-1}$. The cells were excited at 501 and 435 nm, and *I* was monitored at 527 nm, with slits set at 5 nm. The ratio of the emitted fluorescence intensity at 527 nm with excitation at 501 and 435 nm was measured as a function

of time. Fluorescence with excitation at 435 nm is pH independent, whereas fluorescence declines with pH when excitation occurs at 501 nm. Consequently, the $I_{501}/I_{435}$ ratio is lowered with lower pH values.

The $I_{501}/I_{435}$ signal was calibrated in pH units. BCECF-loaded cells were incubated in a medium containing 10 μM nigericin and 80 mM $K^+$. Nigericin allows equilibration of the outside (o) to inside (i) concentration gradients of $H^+$ and $K^+$, such that $H^+_o/H^+_i = K^+_o/K^+_i$ (Thomas et al., 1979). After 10 min of incubation the suspension was titrated through a physiological pH range and $I_{501}/I_{435}$ measurements were made at each external [$H^+$]. Knowing internal and external $K^+$ concentrations and external [$H^+$], a calibration curve ($I_{501}/I_{435}$ versus $pH_{intracellular}$) can be constructed. Using atomic emission spectroscopy and samples of cell sap, we determined the cytoplasmic $K^+$ concentration to be 77.5 mM. However, cellular compartmentation means that the cytosolic $K^+$ concentration is uncertain. Consequently, a series of parallel calibration curves was generated using a series of arbitrary cytosolic $K^+$ concentrations. These curves allow calculation of changes in pH but not the absolute pH value.

## RESULTS

During a 60-min period hypoxia, 5 mM L-Glu, or 5 mM butyrate at pH 5.0 increased extracellular or intracellular GABA levels by 100 to 1800%. Hypoxia resulted in an increase of 120% in the total GABA level from 2.16 nmol of GABA/$10^6$ cells in the control to 4.73 nmol of GABA/$10^6$ cells in the experimental sample. Using the Wilcoxon rank sum test, we found that this was a significant increase ($P < 0.025$). Butyrate addition resulted in a total increase of more than 400%, and the comparable figure for L-Glu addition was approximately 900%. These results indicate that a variety of acidifying treatments stimulate GABA synthesis. In contrast, addition of butyrate at pH 6.0 did not stimulate GABA synthesis (Table I). However, an increase in pH from 5.0 to 6.0 will reduce the concentration of the permeant species butyric acid from 2.0 to 0.3 mM. In addition, the increase in medium pH will reduce the pH gradient across the plasma membrane and reduce the energy required for ATP-driven $H^+$ efflux.

Control values in Table I were subtracted from experimental values to allow calculation of the percentage of the newly synthesized GABA, which was intracellular or extracellular. Anaerobiosis resulted in a total increase of 120%, 34% of which was extracellular. The comparable figures for butyrate at pH 5.0 were 428 and 50%, and for L-Glu they were approximately 900 and 95%. Thus, it appears that with increased GABA synthesis a greater proportion is found in the extracellular milieu (Table I).

This trend was also seen when the time course of intracellular and extracellular GABA appearance was measured in response to butyrate addition to cells maintained at pH 5.0 (Fig. 1). After 5 min of incubation the intracellular GABA level had increased more than 1000% and was close to its maximum value. In contrast, the extracellular GABA levels, which were not different from the control values at 5 min, increased in an approximately linear manner for the subsequent 60 min. These data suggest that GABA efflux occurs when a variety of acid loads stimulate the intracellular GABA concentration beyond a threshold value.

The time required to initiate the large accumulation of GABA observed 5 min after butyrate addition (Fig. 1) was investigated. Butyrate-stimulated GABA synthesis was determined during a time course ranging from 15 to 900 s. The time required to separate the medium and cells precluded measurement of intracellular and extracellular GABA. Consequently, total GABA values were determined. After 15 s of butyrate treatment the GABA level had increased above control values by 200 to 300% (Fig. 2). Similar results were obtained with acetate and propionate (data not shown).

The uptake of 5 mM [1-$^{14}$C]butyric acid was measured at pH 5.0 and 6.0. At pH 5.0 uptake of label equilibrated after approximately 45 s and the entry of 14 nmol/$10^6$ cells. Corresponding figures for pH 6.0 were 200 s and 13 nmol/$10^6$ cells (data not shown). Subsequently, using cells obtained from the same stock suspensions, we measured GABA production and butyric acid uptake at pH 5.0, 45 s after adding unlabeled or labeled 5 mM butyric acid, respectively. Different sensitivities of the two assays necessitates different cell numbers for each. The data demonstrate that at 45 s the mean GABA production was 4.5 nmol/$10^6$ cells and the butyric acid uptake was 10.0 nmol/$10^6$ cells. Thus, at the point of equilibration the $H^+$-consuming decarboxylation of L-Glu is approximately 45% of the $H^+$-generating butyric acid entry (Table II). The data indicate a significant role for GABA synthesis in pH regulation.

Cells were incubated with BCECF-AM for 30 min prior to washing to remove extracellular fluorescence and were then

**Table I.** *The synthesis of GABA in response to cellular acidification*

Cells (20 × $10^6$) were incubated in 5-mL volumes of 5 mM Mes (pH 6.0, except where pH 5.0 is indicated). Five millimolar L-Glu or butyrate were added to the experimental samples. After 60 min of incubation extracellular and intracellular GABA levels were measured. Each experiment was repeated three to five times with duplicate assays of each sample. The SE for each value is indicated in parentheses.

| Treatment | Extracellular | | Intracellular | | Total | | Increase |
|---|---|---|---|---|---|---|---|
| | Control | Experimental | Control | Experimental | Control | Experimental | |
| | *nmol GABA/$10^6$ cells* | | | | | | % |
| Hypoxia | 0.64 (0.17) | 1.52 (0.25) | 1.51 (0.14) | 3.21 (0.32) | 2.16 (0.39) | 4.73 (0.20) | 120 |
| L-Glu (light) | 1.65 (0.15) | 31.36 (2.13) | 1.79 (0.22) | 3.47 (0.31) | 3.44 (0.48) | 34.83 (3.65) | 914 |
| L-Glu (dark) | 1.79 (0.34) | 28.47 (1.67) | 1.47 (0.28) | 3.15 (0.51) | 3.26 (0.96) | 31.62 (3.00) | 870 |
| Butyrate (pH 5.0) | 1.73 (0.15) | 10.08 (0.90) | 2.09 (0.66) | 10.28 (1.08) | 3.86 (0.56) | 20.36 (2.16) | 428 |
| Butyrate | 1.89 (0.13) | 1.15 (0.13) | 1.90 (0.31) | 2.18 (0.25) | 3.79 (0.61) | 3.33 (0.46) | 0 |

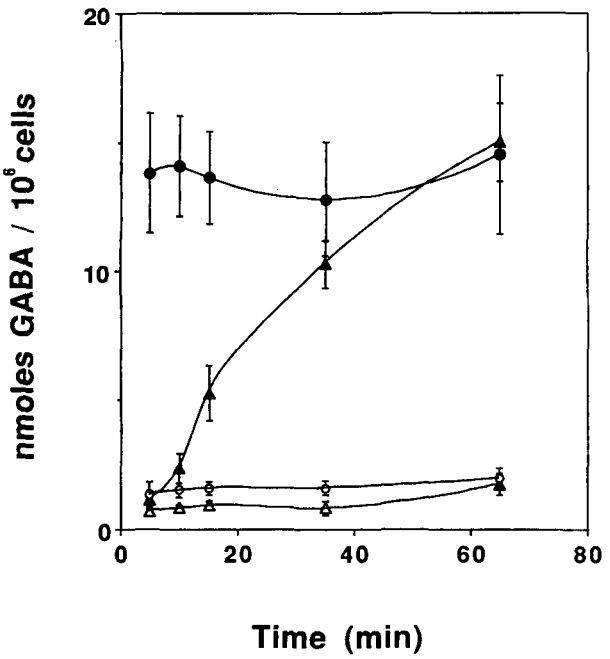


**Figure 1.** The time course of GABA synthesis and efflux in response to butyrate. Cells ($1 \times 10^8$) were incubated in 25-mL volumes of 5 mM Mes (pH 5.0). Five millimolar butyrate, adjusted to pH 5.0, was added at time zero to the experimental samples. Five-milliliter aliquots of the cell suspension were removed at the times indicated for GABA analysis. Each point represents the mean (± SE) of five separate experiments with duplicate assays of each sample. ●, Intracellular GABA after butyrate addition; ▲, extracellular GABA after butyrate addition; ○, intracellular GABA in control; △, extracellular GABA in control.

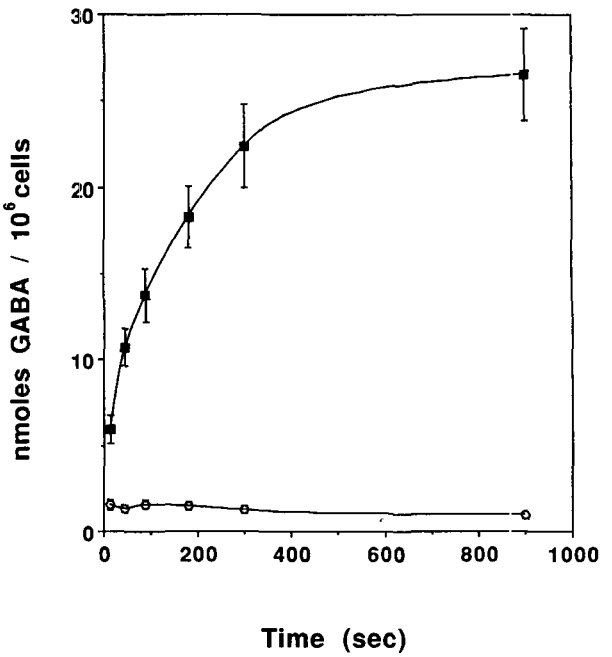


**Figure 2.** Analysis of rapid GABA synthesis in response to butyrate. Cells ($1.2 \times 10^8$) were incubated in 30-mL volumes of 5 mM Mes (pH 5.0). Five millimolar butyrate, adjusted to pH 5.0, was added at time zero to the experimental samples. Five-milliliter aliquots of the cell suspension were removed at the times indicated and placed directly into hot methanol prior to extraction into an aqueous phase and analysis of total (intracellular plus extracellular) GABA. Each point represents the mean (± SE) of five separate experiments, with duplicate assays of each sample. ■, Total GABA after butyrate addition; ○, total GABA in control.

observed with epifluorescence optics (Fig. 3). Fluorescence microscopy revealed no fluorescence from the vacuole (Fig. 3B) but pronounced yellowish-green fluorescence from the cytosolic strands around the chloroplasts (Fig. 3D).

The $I_{501}/I_{435}$ excitation ratio could not be calibrated in units of absolute pH because the specific cytosolic $K^+$ concentration was not known (see "Materials and Methods"). The mean value for the cell obtained using extracted sap and atomic emission spectroscopy was determined to be 77.5 mM (data not shown). However, a range of arbitrary cytosolic $K^+$ concentrations resulted in a series of parallel curves when $I_{501}/I_{435}$ excitation ratios were plotted against pH. The plots are linear between $I_{501}/I_{435}$ ratios of 2 and 10 (Fig. 4). Consequently, changes in cytosolic pH can be determined but not absolute pH.

Changes in cytosolic pH in response to 5 mM butyrate addition at pH 5.0 and 6.0 were investigated. An initial increase in the $I_{501}/I_{435}$ ratio was an artifact resulting from cuvette exposure to ambient light when the spectrofluorimeter lid was raised for butyrate addition. At pH 5.0 the mean reduction in cytosolic pH was 0.60 (±0.03 SE) units, with half of this reduction occurring within approximately 2 s. At pH 6.0 the comparable values were 0.20 (±0.05 SE) pH units and 2.5 s (Fig. 5). Similar pH changes were obtained with acetate and propionate addition (data not shown). These results support the proposal that a reduction in cytosolic pH is implicated in the rapid stimulation of GABA synthesis (Fig. 2).

## DISCUSSION

GABA synthesis in response to hypoxia (Table I; Reggiani et al., 1988; Menegus et al., 1989) or $H^+$/L-Glu symport (Table

**Table II.** *Concurrent measures of GABA production and butyric acid uptake*

Measurements were made using cells from the same stock suspensions 45 s after butyric acid addition. For GABA determinations $20 \times 10^6$ cells in 5 mL were incubated with 5 mM butyrate acid. For uptake determinations $4.2 \times 10^6$ cells in 1 mL were incubated with 5 mM [1-$^{14}$C]butyrate. Control values have been subtracted from the values indicated.

| Experiment | GABA Production | Butyric Acid Uptake | Production as Percentage of Uptake |
|---|---|---|---|
| | *nmol/10$^6$ cells* | | |
| 1 | 4.58 | 11.60 | 39.5 |
| 2 | 4.85 | 7.81 | 62.1 |
| 3 | 5.42 | 11.68 | 46.4 |
| 4 | 3.25 | 9.16 | 35.5 |
| Mean | 4.52 | 10.06 | 45.9 |

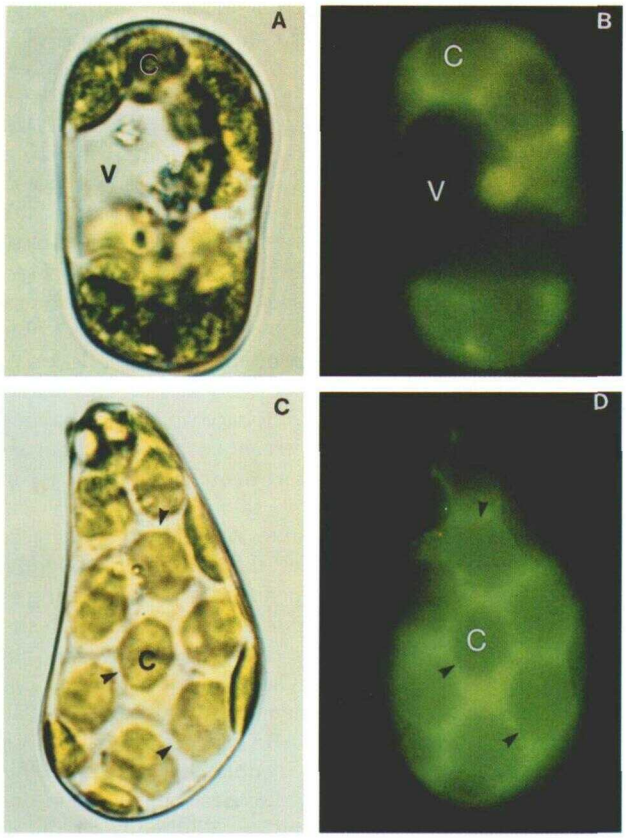


**Figure 3.** The intracellular location of a fluorescent pH probe (BCECF). A and B, Bright-field and fluorescence photography, respectively, of the same cell (magnification ×3500). C and D, As above (magnification ×2300). V, Vacuole; C, chloroplast; and cytosolic strands (arrowhead) are indicated. The fluorescence originates primarily from the cytosolic strands.

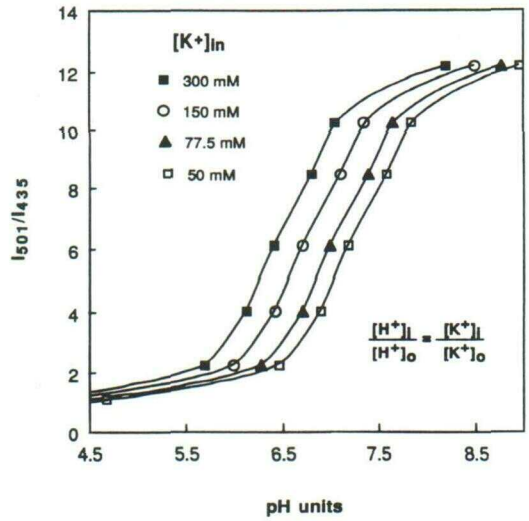


**Figure 4.** The calibration of changes in the fluorescence of intracellular BCECF in units of pH. The excitation ratio $I_{501}/I_{435}$ is plotted against pH. The plot is calculated after experimental determination of $I_{501}/I_{435}$ values at varying external pH and knowledge of the external $K^+$ concentration (80 mM). Four different curves result when the cytosolic $K^+$ concentration is presumed to be 300 mM (■), 150 mM (○), 77.5 mM (△), and 50 mM (□). Essentially similar curves resulted when the calibration procedure was repeated three times.

I; Chung et al., 1992; Snedden et al., 1992) could reflect the specific consequences associated with these treatments and not a reduction in pH as suggested. The present investigation, however, demonstrates that addition of a permeant weak acid results in a reduction of cytosolic pH of 0.60 units with a half-time for the response of approximately 2 s (Fig. 5). This response is fast enough to account for the associated rapid stimulation of GABA synthesis (Figs. 1 and 2). These results support the hypothesis that a reduced cytosolic pH stimulates L-Glu decarboxylase activity (Reggiani et al., 1988; Menegus et al., 1989; Snedden et al., 1992). The hypothesis is further supported by a cytosolic location for L-Glu decarboxylase (Tsushida and Murai, 1987; Satyanarayan and Nair, 1990), a sharp pH optimum for enzymic activity of approximately 6.0 (Tsushida and Murai, 1987; Snedden et al., 1992), and estimates of the cytosolic pH in asparagus mesophyll cells between pH 7.0 and 8.0 (Espie and Colman, 1981). However, a decrease in cytosolic pH of approximately 0.20 pH units (Fig. 5) did not result in a measurable increase in GABA level (Table I). This suggests that cytosolic pH must be reduced to a threshold pH value before L-Glu decarboxylase activity is stimulated.

The data indicate that cytosolic acidification (Fig. 5) initiates

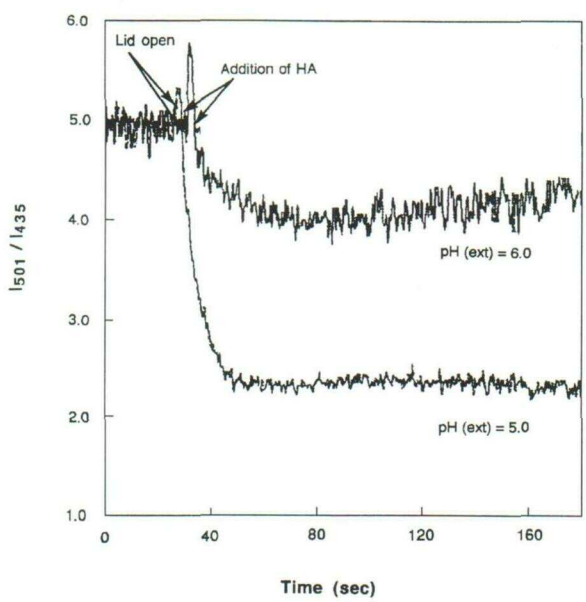


**Figure 5.** Continuous recordings of cytosolic acidification in response to butyrate. Typical recordings of changes in $I_{501}/I_{435}$ are shown for 5 mM butyrate addition to BCECF-loaded cells. Cells were incubated in 5 mM Mes (pH 5.0 or 6.0) as indicated. The experiments were repeated five times.

a rapid and sustained synthesis of GABA (Figs. 1 and 2). A similar rapid accumulation of GABA has been observed in soybean leaves in response to cold or mechanical stress (Wallace et al., 1984). This capacity for rapid and sustained GABA synthesis is required for a metabolic pH-stat based on $H^+$-consuming L-Glu decarboxylation. The data support previous suggestions that GABA synthesis is involved in active pH regulation (Reggiani et al., 1988; Menegus et al., 1989; Snedden et al., 1992). After 45 s of butyrate treatment (pH 5.0), the increase in GABA was 4.5 nmol/$10^6$ cells, and the acid load was 10.0 nmol/$10^6$ cells (Table II). Thus, at this point GABA synthesis accounted for 45% of the acid load. In addition, the contribution of passive buffering to pH regulation can be calculated using a cytoplasmic buffering capacity of 14.9 $\mu$mol of $H^+$ $mL^{-1}$ of cytoplasm pH $unit^{-1}$ (Chung et al., 1992), a cell volume of 4.68 $\mu L/10^6$ cells (Chung et al., 1992), and measurements indicating that the cytosolic volume represents 10% of the cytoplasmic volume (Espie and Colman, 1981). The buffering capacity in terms of cytoplasmic volume is, therefore, 69.8 nmol/$10^6$ cells pH $unit^{-1}$, and in terms of cytosolic volume it is 6.98 nmol/$10^6$ cells pH $unit^{-1}$. Consequently, 4.2 nmol/$10^6$ cells are required to move the cytosolic pH 0.6 units (Fig. 5). The accuracy of this calculation is subject to the additive impact of error limits associated with the values used. It assumes that, on a volume basis, the cytosolic buffering capacity is equal to the cytoplasmic buffering capacity. The calculations suggest that at 45 s GABA synthesis and passive pH buffering account for approximately 8.7 nmol of $H^+$ associated with an acid load of 10.0 nmol $H^+$. After 45 s GABA accumulation continues, but at a diminishing rate (Fig. 2). Equilibration across the plasma membrane of the excess extracellular butyric acid presumably precludes recovery of the cytosolic pH. The conclusion that GABA synthesis is involved in pH regulation conflicts with that reached by workers using root tips, who found that GABA synthesis was stimulated several minutes after hypoxia induced a pH decline of 0.5 to 0.6 pH units (Roberts et al., 1992). This result contrasts with the very rapid stimulation of GABA synthesis seen in this study (Fig. 2). Different conclusions may derive from the use of permeant weak acids instead of hypoxia to induce acidification or from differences between mature vacuolated mesophyll cells and immature nonvacuolated root tips.

GABA efflux in response to $H^+$/L-Glu symport has been reported previously (Chung et al., 1992; Snedden et al., 1992). The present investigation demonstrates that it also occurs in response to anaerobiosis (Table I) and permeant weak acids (Table I, Fig. 1). Measurement of intracellular and extracellular GABA concentrations demonstrated that specific GABA efflux could occur via a passive process (Chung et al., 1992). Similarly, the data in Table I and Figure 1 suggest that efflux begins when a threshold concentration is reached and that, as GABA synthesis continues, a greater proportion leaves the cell. Amino acid analysis indicates that more than 50% of L-Glu and GABA is found outside the vacuole, suggesting a cytosolic location for these amino acids (Wagner, 1979; Yamaki, 1982). Neither the mechanism nor the function of GABA efflux is clear. It may function as an intercellular messenger denoting stress and regulate some plasma membrane function in adjacent cells. In animal cells, extracellular GABA regulates plasma membrane-located $Cl^-$ channels.

Stimulation of GABA synthesis by reduced cytosolic pH values is supported by the literature and the data presented here. However, this mechanism does not explain the rapid accumulation of GABA in response to a variety of stress conditions including mechanical and cold stress (Wallace et al., 1984; Bown and Shelp, 1989). Cold stress also stimulates GABA synthesis in asparagus mesophyll cells (data not reported). Stimulation may involve $Ca^{2+}$, which regulates cellular processes via $Ca^{2+}$-binding proteins such as calmodulin, and $Ca^{2+}$-dependent kinases (Roberts and Harmon, 1992). Rapid and transient increases in intercellular $Ca^{2+}$ levels in response to mechanical stress, cold stress, and fungal elicitors were demonstrated in transgenic tobacco plants containing aequorin, a $Ca^{2+}$-sensitive luminescent protein (Knight et al., 1991). In addition, a recent report demonstrates that L-Glu decarboxylase from petunia binds calmodulin (Baum et al., 1993). Reduced cytosolic pH values are known to increase cytosolic $Ca^{2+}$ levels (Gehring et al., 1990). Consequently, increased $Ca^{2+}$ may be the immediate signal stimulating L-Glu decarboxylase activity. Despite repeated attempts, a significant reduction in asparagus cell cytosolic pH was not observed on the addition of L-Glu (data not shown). This result was unexpected given demonstrations of $H^+$/L-Glu symport and concurrent rapid GABA synthesis (McCutcheon et al., 1988; Chung et al., 1992; Snedden et al., 1992). In this case, stimulation of L-Glu decarboxylase may involve increased concentrations of $Ca^{2+}$ and/or L-Glu. Thus, a reduced cytosolic pH appears to be a sufficient but not a necessary requirement for stimulated GABA synthesis.

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