Quantitation of Rates of Transport, Metabolic Fluxes, and Cytoplasmic Levels of Inorganic Carbon in Maize Root Tips during K+ Ion Uptake

Keejong Chang and Justin K. M. Roberts
Department of Biochemistry, University of California, Riverside, California 92521

ABSTRACT

Our aim was to determine whether fixation of inorganic carbon (C\textsubscript{2}) due to phosphoenolpyruvate carboxylase activity, is limited by the availability of C\textsubscript{2} in the cytoplasm of maize (Zea mays L.) root tips. Rates of C\textsubscript{2} uptake and metabolism were measured during K\textsubscript{2}SO\textsubscript{4} treatment, which stimulates dark C\textsubscript{2} fixation. C\textsubscript{2} uptake was followed by \textsuperscript{13}C-nuclear magnetic resonance (NMR); 5 millimolar K\textsubscript{2}SO\textsubscript{4} had no significant effect on C\textsubscript{2} influx. The contribution of respiratory CO\textsubscript{2} production to cytoplasmic HCO\textsubscript{3}\textsuperscript{-} was measured using in vivo \textsuperscript{13}C-NMR and \textsuperscript{1}H-NMR of cell extracts; K\textsubscript{2}SO\textsubscript{4} treatment had no effect on respiratory CO\textsubscript{2} production. The concentration of cytoplasmic HCO\textsubscript{3}\textsuperscript{-} was estimated to be approximately 11 millimolar, again with K\textsubscript{2}SO\textsubscript{4} having no significant effect. These experiments allowed us to determine the extent to which extracellularly supplied C\textsubscript{2} was diluted in the cytoplasm by respiratory CO\textsubscript{2} and thereby measure phosphoenolpyruvate (PEP) carboxylase activity in vivo using \textsuperscript{13}C. PEP carboxylase activity in root tips was enhanced approximately 70% over controls within 12 minutes of the addition of 5 millimolar K\textsubscript{2}SO\textsubscript{4}. The activity of carbonic anhydrase, which provides PEP carboxylase with C\textsubscript{2}, was determined by saturation transfer \textsuperscript{13}C-NMR to be more than 200 times that of PEP carboxylase in vivo. The regulation of PEP carboxylase in K\textsubscript{2}SO\textsubscript{4}-treated roots is discussed.

Fixation of C\textsubscript{2} into organic metabolites by plant roots was first reported more than 50 years ago (20). This dark C\textsubscript{2} fixation has been related to many biological functions (reviewed in refs. 2 and 15), one of which is the synthesis of organic acids, principally malate, that accompanies excess cation over anion uptake (30; reviewed in ref. 18). Malate is synthesized by the sequential actions of PEP carboxylase and malate dehydrogenase (2, 28).

Several hypotheses have been proposed to provide a mechanism linking the accumulation of malate with excess cation uptake (reviewed in ref. 10). Among these hypotheses, the most popular has been that PEP carboxylase activity is stimulated by an increase in cytoplasmic pH due to H\textsuperscript{+} excretion that accompanies cation uptake (reviewed in ref. 7). However, evidence for this hypothesis is contradictory (reviewed in ref. 14), and it has been proposed that cytoplasmic alkalization during H\textsuperscript{+} extrusion may occur only in certain species or cell types under limited physiological conditions (3, 14). An alternative mechanism, initially proposed by Osmond and Laties (19) and later developed by Jacoby and Laties (13), is that HCO\textsubscript{3}\textsuperscript{-}, the form of C\textsubscript{2} used by PEP carboxylase, acts as the "prime mover" for organic acid synthesis. In this model, cation uptake is associated with elevation of cytoplasmic HCO\textsubscript{3}\textsuperscript{-}, leading to enhanced dark C\textsubscript{2} fixation.

The study of dark C\textsubscript{2} fixation in vivo has relied on \textsuperscript{13}C, \textsuperscript{14}C, and \textsuperscript{18}O isotope tracers, which permit uptake and incorporation of exogenous C\textsubscript{2} to be followed without complete masking by competition from endogenous \textsuperscript{13}C\textsubscript{2}, produced by respiration and other decarboxylation reactions. However, a quantitative description of dark C\textsubscript{2} fixation requires assessment of the extent to which C\textsubscript{2} uptake, respiratory CO\textsubscript{2} formation, and cytoplasmic CO\textsubscript{2}-HCO\textsubscript{3}\textsuperscript{-} exchange provide PEP carboxylase with its inorganic substrate, HCO\textsubscript{3}\textsuperscript{-}. Thus, Jacoby and Laties (13) described "the inadequacy of \textsuperscript{14}CO\textsubscript{2} incorporation into malate as a quantitative criterion of induced synthesis" during salt-induced organic acid synthesis. These authors pointed to the potential roles of C\textsubscript{2} transport and the concentration of cytoplasmic HCO\textsubscript{3}\textsuperscript{-} as determinants of PEP carboxylase activity in roots. Guern et al. (10) noted the lack of information concerning cytoplasmic HCO\textsubscript{3}\textsuperscript{-} levels and rates of respiratory CO\textsubscript{2} hydration with respect to control of PEP carboxylase activity in vivo. Here, we present quantitative data regarding fluxes and concentrations of C\textsubscript{2} in maize root tips during K\textsuperscript{+}-stimulated organic acid synthesis. The results are discussed with respect to the regulation of dark C\textsubscript{2} fixation.

MATERIALS AND METHODS

Plant Material

Maize (Zea mays L.) Funk hybrid G-4327 (Germain's Seeds, Los Angles, CA) root tips, 2 mm long, from 2-d-old seedlings were prepared as described previously (4).

NMR Spectroscopy

Fourier transform \textsuperscript{13}C- and \textsuperscript{1}H-NMR spectra were recorded with a General Electric GN-500 spectrometer operating at 125.7 and 500.1 MHz, respectively. The pulse sequence and parameters of the \textsuperscript{13}C-NMR experiment were as described by Chang and Roberts (4). Chloroform, sealed in a coaxial capillary, was used as an external reference at 77.9 ppm. The ratios of standard and in vivo \textsuperscript{13}CO\textsubscript{3}\textsuperscript{-} resonances to the chloroform reference were unchanged when the pulse interval

---

1 This work was supported by National Science Foundation grant DMB 8904091.
2 Abbreviations: C\textsubscript{2}, inorganic carbon; PEP, phosphoenolpyruvate.
was increased from the usual 2.4 to 9.6 s. The saturation transfer $^{13}$C-NMR experiment and $T_1^*$ measurement are described in the sections below. Twenty-hertz line broadening was applied to in vivo free induction decays before Fourier transformation. $^1H$-NMR spectra of cell extracts were obtained with a standard water presaturation pulse sequence. Scans were collected every 4.4 s, with a spectral width of $\pm 2500$ Hz and using 32,000 data points; 0.5-Hz line broadening was applied.

**Treatment Conditions for NMR Experiments**

Approximately 2 g of root tips were loaded into a glass tube modified for perfusion. In all experiments, root tips were perfused at 10 mL/min in oxygen-saturated medium containing 5 mM Glc, 0.1 mM CaSO₄, and 5 mM Tris-HCO₃⁻ (pH 7.5). $^{13}$C was either at natural abundance (1.1%) or enriched to 99% at carbon-1 of Glc (Isotech, Miamisburg, OH) or in HCO₃⁻ (Tris-HCO₃ was prepared according to the method in ref. 6). K$_2$SO₄ treatment consisted of addition of 5 mM K$_2$SO₄ to the perfusion medium after 2 h. Experiments were performed at room temperature (24–25°C).

**Measurements of Cytoplasmic [HCO₃⁻] Derived from Extracellular C$^{13}$C$^{18}$O$^{2-}$**

$^{13}$C influx Measurements. Root tips were first equilibrated with perfusion medium for 2 h, then natural abundance Tris-HCO₃ was replaced with Tris-$H^{13}$CO$_3$ plus 0.1 mM MnCl₂ for 30 min (with or without 5 mM K$_2$SO₄), and $^{13}$C-NMR spectra were obtained in 4-min blocks. The perfusion solution was switched back to the natural abundance solution for another 30 min, and the labeling cycle was then repeated. Spectra from both cycles of four replicate experiments for the control and K$_2$SO₄ treatments were summed. Mn$^{2+}$ at 0.1 mM eliminates the signal from extracellular H$^{13}$CO$_3$ without influencing signals from intracellular metabolites (data not shown). The concentration of cytoplasmic H$^{13}$CO$_3$ derived from extracellular $^{13}$C$^{18}$O$_2$ was then calculated. Comparison of the ratio of H$^{13}$CO$_3$ to chloroform reference peaks in in vivo spectra with the ratio for H$^{13}$CO$_3$ standard solutions in vitro gave the micromoles of H$^{13}$CO$_3$ per milliliter of sample chamber. These values were converted first to cytoplasmic H$^{13}$CO$_3$ content (μmol/g tissue) by dividing by the proportion of the NMR sample chamber occupied by root tips (approximately 0.83) (given by the ratio of root tip sample weight to sample volume; the latter exceeded the NMR-detectable volume; this ratio was essentially unchanged during the course of these experiments), and second to cytoplasmic H$^{13}$CO$_3$ concentration (mm) by further dividing by the proportion of cytoplasm in root tips (approximately 0.65) (cf. ref. 6).

**Steady-State Measurements.** Samples were initially perfused with medium containing Tris-$H^{13}$CO$_3$ for 1.5 or 3.5 h (specified in “Results”). Just before the time of measurement, 0.1 mM MnCl₂ was added to the perfusion medium to eliminate the extracellular H$^{13}$CO$_3$ signal, and spectra were acquired during the next 30 min. The concentration of cytoplasmic H$^{13}$CO$_3$ derived from extracellular $^{13}$C at steady state was calculated from the intensity of the cytoplasmic H$^{13}$CO$_3$ resonance, as described above.

**Measurements of Cytoplasmic [HCO₃⁻] Derived from Respiration**

Samples were perfused with medium containing 5 mM [1-$^{13}$C]Glc. The concentration of cytoplasmic H$^{13}$CO$_3$ derived from [1-$^{13}$C]Glc was determined from the intensity of the in vivo cytoplasmic H$^{13}$CO$_3$ resonance, after subtraction of the contribution from $^{13}$C natural abundance, as described above. Immediately after $^{13}$C-NMR data collection, tissue samples were frozen in liquid nitrogen and extracted with HClO₄ (4). The cleared, neutralized extracts were loaded onto prefiltered anion-exchange columns (AG-1-X8; Bio-Rad, Richmond, CA), which were washed with deionized H$_2$O to remove carbohydrates and amino acids. The eluate between 2 and 2.4 N formic acid containing was evaporated to dryness, dissolved in 0.6 mL D$_2$O (Sigma), and analyzed by $^1H$-NMR. The ratio of $^{13}$C/$^{12}$C in carbon-1 of Glc-6-P, and hence the isotopic enrichment of respiratory substrate (see "Results") was obtained from the ratio of peak areas of $^1H$ attached to [1-$^{13}$C] Glc-6-P and [1-$^{13}$C]Glc-6-P in the $^1H$-NMR spectra. This information was used to convert the concentration of cytoplasmic H$^{13}$CO$_3$ derived from [1-$^{13}$C]Glc to the concentration of total ($^{13}$C + $^{12}$C) cytoplasmic HCO$_3$ derived from respiration (Table II).

**Measurement of Carbonic Anhydrase Activity in Vivo by Saturation Transfer $^{13}$C-NMR**

Root tips were perfused with medium containing Tris-$H^{13}$CO$_3$. Standard NMR experimental procedures were followed (9, 22, 23). Three different low-power irradiation frequencies were used to obtain three spectra from each sample: the resonance frequencies of H$^{13}$CO$_3^-$ and $^{13}$CO$_2$ and a frequency midway between these two resonances (to control for any imperfections in the selectivity of the irradiation). The three spectra were collected as interleaved blocks of 16 scans each; interleaving allows time-dependent variables to be distributed equally throughout the experimental period. Selective irradiation was turned off during spectrum acquisition. Scans were made every 2.4 s using a spectral width of $±15,000$ Hz and 16,000 data points.

Exchange of $^{13}$C between HCO$_3^-$ and CO$_2$ can be described as follows:

$$\text{HCO}_3^- + \text{H}^+ \overset{k_1}{\rightleftharpoons} \text{CO}_2 + \text{H}_2\text{O}$$  (1)

where $k_1$ is the pseudo-first-order rate constant for converting HCO$_3^-$ to CO$_2$, and $k_2$ is the rate constant for CO$_2$ conversion back to HCO$_3^-$. Saturation transfer experiments were performed under steady-state conditions so that:

$$\text{Rate of HCO}_3^- \text{ synthesis} = k_1 \times [\text{HCO}_3^-] = k_2 \times [\text{CO}_2].$$  (2)

$k_1$ was calculated by solution of the following simultaneous equations (9):

$$k_1 = \frac{1}{T_1 \cdot M^*} - \frac{1}{T_1}$$  (3)
\[ \frac{1}{T_1'} = \frac{1}{T_1} + k_1 \] 

where \( T_1 \) is the true longitudinal relaxation time of \( H^{13}C\text{O}_3^- \); \( T_1' \) is the longitudinal relaxation time of the \( H^{13}C\text{O}_3^- \) resonance, measured in the presence of selective saturation of the \( ^{13}C\text{O}_2 \) resonance; and \( M' \) is the ratio of the intensity of the \( H^{13}C\text{O}_3^- \) signal during \( ^{13}C\text{O}_2 \) irradiation, to the intensity of \( H^{13}C\text{O}_3^- \) in the control spectrum, calculated after subtraction of the contribution of extracellular \( H^{13}C\text{O}_3^- \) (which undergoes exchange at a rate too slow for measurement by saturation transfer NMR). The rate of synthesis of \( HCO_3^- \) (\( \mu \text{mol/min/g tissue} \)) was then obtained by inserting the values of \( k_1 \) and the tissue \( HCO_3^- \) content (described above), into Equation 2.

Longitudinal relaxation times \( (T_1') \) were measured by the selective saturation recovery method (8). Seven different delays, \( r \), ranging from 10 ms to 10 s, were used between the saturating train of pulses and the observed pulses. Scans were made every 2.4 s using a spectral width of \( \pm 15,000 \) Hz and 16,000 data points. The seven spectra were acquired in interleaved blocks of 16 scans cycling over approximate 1.5 h. \( 1/T_1' \) is the slope of the plot of \( \ln(M_r - M_0) \) versus \( r \), where \( M_r \) and \( M_0 \) are the intensities at delay time 10 s and the different delay times, \( r \), respectively. The \( H^{13}C\text{O}_3^- \) NMR signals in these experiments contain contributions of similar magnitude from both cytoplasmic and extracellular \( H^{13}C\text{O}_3^- \). Hence, in principal, if these two \( H^{13}C\text{O}_3^- \) pools relaxed at different rates, plots to determine \( 1/T_1' \) would consist of a curve from superposition of at least two lines. However, actual plots of relaxation data were highly linear, with \( r^2 = -0.9955 \) and \(-0.9963 \) for control and \( K_2\text{SO}_4 \) treatment experiments, respectively (\( P > 0.95 \)). This result indicates that \( T_1' \) values for extracellular and cytoplasmic \( HCO_3^- \) are very similar.

**Measurements of Dark C, Fixation in Vivo**

Root tips having an initial fresh weight of 0.052 ± 0.003 g (mean ± sd, \( n = 10 \)) were loaded into capsules made from plastic pipet tips and containing a coarse plastic mesh plug. Up to 10 capsules were connected in series with plastic tubing and perfused as in the NMR experiments. At the specified times, capsules were disconnected from the series and perfused for 4 min with solution containing \( H^{14}C\text{O}_3^- \) (Amersham, Arlington Heights, IL) at 0.05 Ci/mol. Incorporation of radioactivity was linear for \( >10 \) min under all treatment conditions (data not shown). Boiling acetic acid (1 mL) was added to stop the reaction at the end of each labeling period and to remove the unreacted \( H^{14}C\text{O}_3^- \). Samples were kept in a fume hood for 36 h to allow radioactive activity to escape and were counted in 3.5 mL of scintillation cocktail (Ecolume; ICN, Cleveland, OH) in a Beckman LS-3801 counter.

**RESULTS**

**C, Influx and the Contribution of Extracellular C, to Cytoplasmic HCO_3^-**

We tested the hypothesis (13, 19) that salt-induced stimulation of organic acid synthesis (30) is due to elevation of cytoplasmic \( HCO_3^- \) by monitoring the flux of exogenous \( ^{13}C \) into root tips and the steady-state concentration of cytoplasmic \( H^{13}C\text{O}_3^- \). We consider essentially all intracellular \( HCO_3^- \) to be located in the cytoplasm; the contribution of vacuolar \( HCO_3^- \) to total intracellular \( HCO_3^- \) is negligible, because the acidic pH of the plant cell vacuole favors conversion of most \( HCO_3^- \) to \( CO_2 \).

The influx of \( ^{13}C \) into root tips was followed by observing the intensity of the cytoplasmic \( H^{13}C\text{O}_3^- \) resonance over time, after addition of 5 mm Tris-\( H^{13}C\text{O}_3^- \) to the perfusion medium (Fig. 1). Influx was not influenced by \( K_2\text{SO}_4 \) treatment, which stimulates malate synthesis (6). This result suggests that \( K_2\text{SO}_4 \) treatment does not elevate cytoplasmic \( HCO_3^- \) by enhanced transport of \( C \). Steady-state \( ^{13}C\text{-NMR} \) measurements of cytoplasmic \( H^{13}C\text{O}_3^- \) confirmed this inference by showing that the concentration of exogenously derived cytoplasmic \( H^{13}C\text{O}_3^- \) is independent of the presence of \( K_2\text{SO}_4 \) (Table I). Thus, under the conditions used in these experiments, ap-

![Figure 1. \( ^{13}C \) influx into the cytoplasm of maize root tips. Tissue was initially perfused with 5 mm natural abundance \( HCO_3^- \) for 2 h, then switched to 5 mm \( H^{13}C\text{O}_3^- \) plus 0.1 mm MnCl\(_2\) at time 0, either without \( (\cdot \cdot \cdot ) \) or with \( (\Delta --- \Delta) \) 5 mm \( K_2\text{SO}_4 \) (see "Materials and Methods"). The lines are best logarithmic fits to each data set. An analysis of covariance of cytoplasmic \( H^{13}C\text{O}_3^- \) versus log time, using the SAS program (SAS Institute, Inc., Cary, NC), indicated no significant difference between the control and \( K_2\text{SO}_4 \)-treated tissue at the 95% confidence level.

**Table I. Concentration of Cytoplasmic \( H^{13}C\text{O}_3^- \) in Maize Root Tips Derived from 5 mm Extracellular Tris-\( H^{13}C\text{O}_3^- \)**

*In vivo \( ^{13}C\text{-NMR} \) spectra were collected during the times indicated (see "Materials and Methods").

<table>
<thead>
<tr>
<th>Time</th>
<th>K(_2\text{SO}_4)^* (5 mm)</th>
<th>( n )</th>
<th>[( H^{13}C\text{O}_3^- )]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–2.5</td>
<td>–</td>
<td>4</td>
<td>4.2 ± 0.4^a</td>
</tr>
<tr>
<td>3.5–4</td>
<td>–</td>
<td>2</td>
<td>4.6 ± 0.8^b</td>
</tr>
<tr>
<td>3.5–4</td>
<td>+</td>
<td>2</td>
<td>4.4 ± 0.6^c</td>
</tr>
</tbody>
</table>

* Added at 2 h. ^a Mean ± sd. ^b Mean ± range.
approximately 4.4 mM of the total cytoplasmic HCO₃⁻ was derived from the perfusion medium.

**Contribution of Respiratory CO₂ to Cytoplasmic HCO₃⁻**

Respiration also provides C₂ to sustain organic acid synthesis. We measured this contribution to determine whether K₂SO₄ treatment elevated cytoplasmic HCO₃⁻ by stimulating metabolic CO₂ production. Root tips were labeled with [1-¹³C]Glc, and the amount of cytoplasmic H¹³CO₃⁻ derived from this substrate was determined from in vivo ¹³C-NMR spectra (Table II, column A). Whereas the concentration of cytoplasmic H¹³CO₃⁻ increased with time of perfusion in [1-¹³C]Glc, indicating increased consumption of [1-¹³C]Glc by glycolysis and respiration, the addition of K₂SO₄ had no significant effect (Table II, column A).

Conversions of the cytoplasmic H¹³CO₃⁻ concentration to the concentration of total (¹³C + ¹²C) cytoplasmic HCO₃⁻ derived from respiration required knowledge of the ¹³C isotope enrichment in the respiratory substrate. To this end, we determined the ¹³C isotopic enrichment in carbon-1 of Glc-6-P in root tip extracts by ¹³C-NMR (Fig. 2, Table II, column B). Unlike Glc and sucrose, Glc-6-P is located exclusively in the cytoplasm; therefore, isotope analysis of carbon-1 of Glc-6-P allowed changes in [1-¹³C]Glc uptake and compartmentation, and thus mixing with endogenous respiratory carbonhydrate, to be quantitated. Whereas the ¹³C enrichment in carbon-1 of Glc-6-P increased with time, K₂SO₄ treatment had no significant effect on ¹³C enrichment (Table II, column B) and, thus, on HCO₃⁻ derived from Glc-6-P (Table II, column C). Therefore, under all conditions, respiration from Glc-6-P contributed approximately 6.8 mM HCO₃⁻ to the cytoplasmic HCO₃⁻ pool. Combining this value with the contribution of extracellular C₂ to cytoplasmic HCO₃⁻ at steady state (Table I) gave a concentration of total cytoplasmic HCO₃⁻ of approximately 11 mM, which was constant with time and independent of K₂SO₄ treatment (Table II, column D). These results are consistent with the absence of any detectable salt-induced stimulation of respiration in maize root tips (6).

The above estimate of cytoplasmic HCO₃⁻ derived from respiration assumes that carbohydrate is the exclusive respiratory substrate in this tissue. The strong dependence of respiration in maize root tips upon exogenous sugars (24) lends credence to this assumption. We tested the validity of this assumption in separate experiments in which the intensity of the cytoplasmic H¹³CO₃⁻ NMR resonance was measured in root tips treated with HCO₃⁻ and Glc at natural abundance (1.1% ¹³C). If root tip respiration depended significantly on noncarbohydrates, a significantly higher concentration of cytoplasmic HCO₃⁻ than the levels given in Table II (column D) would be observed. However, the concentration of cytoplasmic HCO₃⁻ in the natural abundance ¹³C-NMR experiments was found to be 13.5 mM (SE ±2.0, n = 7). This result is not significantly different from the data in Table II (at the 95% confidence level by Student’s t test), indicating that carbohydrate is the predominant respiratory substrate in this tissue.

**Measurement of Carbonic Anhydrase Activity in Vivo**

In the experiments leading to the results in Table I, we were able to observe a ¹³C-NMR signal from intracellular ¹³CO₂ that was approximately 0.038 times the intensity of the cytoplasmic H¹³CO₃⁻ signal (data not shown). This ratio of concentrations is similar to that predicted by the Henderson-Hasselbalch equation (viz. 0.042) for a HCO₃⁻ solution at pH 7.5, in equilibrium with respect to CO₂-HCO₃⁻ exchange. Such similarity suggests that cytoplasmic ¹³CO₂ and H¹³CO₃⁻ pools are in near equilibrium, i.e., that the rate of carbon exchange between ¹³CO₂ and H¹³CO₃⁻ is much faster than either the rates of production or consumption of individual C₂ species in root tip cytoplasm or the rates of influx or efflux of C₂ species into or out of the cytoplasm. Such rapid exchange between ¹³CO₂ and H¹³CO₃⁻ would require significant carbonic anhydrase activity in vivo.

We therefore determined the activity of carbonic anhydrase in maize root tips by measuring the unidirectional rate of ¹³CO₂-H¹³CO₃⁻ exchange by saturation transfer ¹³C-NMR. This method is capable of monitoring chemical reactions in

**Table II. Concentration of Cytoplasmic HCO₃⁻ Derived from Respiration and Total Cytoplasmic HCO₃⁻**

<table>
<thead>
<tr>
<th>Time</th>
<th>K₂SO₄⁺ (5 mM)</th>
<th>(A) [¹⁴C]HCO₃⁻</th>
<th>(B) ¹²C/¹³C in</th>
<th>(C) [HCO₃⁻] from</th>
<th>(D) Total</th>
<th>[HCO₃⁻]⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C-1 of</td>
<td>Glc-6-P⁺</td>
<td>Glc-6-P⁺</td>
<td>HCO₃⁻⁺</td>
</tr>
<tr>
<td>h</td>
<td>mm</td>
<td>mm</td>
<td>Glc-6-P⁺</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>-</td>
<td>0.22 ± 0.07</td>
<td>4.2 ± 0.2</td>
<td>6.9 ± 2.2</td>
<td>11.1 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>3-4</td>
<td>-</td>
<td>0.44 ± 0.07</td>
<td>1.5 ± 0.1</td>
<td>6.6 ± 1.1</td>
<td>11.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>3-4</td>
<td>+</td>
<td>0.42 ± 0.07</td>
<td>1.7 ± 0.1</td>
<td>6.8 ± 1.2</td>
<td>11.2 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

* Added at 2 h.  
* Determined from intensity of the H¹⁴CO₃⁻ resonance in NMR spectra of tissue perfused with [1-¹³C]Glc, obtained during the last hour before extraction (see "Materials and Methods"). Error values are ranges.  
* Determined from ¹³C-NMR spectra of extracts (see Fig. 2 legend and "Materials and Methods"), prepared at the end of the indicated time. Error values are so.  
* Calculated by multiplying [¹⁴HCO₃⁻] by the ratio (¹²C + ¹³C)/¹²C in carbon-1 of Glc-6-P and by 6 (number of carbons in Glc). Errors values are so.  
* Determined as the sum of column C, plus [HCO₃⁻] derived from extracellular HCO₃⁻ (Table I). Error values are ranges.

---

*Copyright © 1992 American Society of Plant Biologists. All rights reserved.*

Downloaded from on January 6, 2018 - Published by www.plantphysiol.org
INORGANIC CARBON UPTAKE AND METABOLISM IN MAIZE ROOT TIPS

determined to be 2.5 ± 0.2 and 2.4 ± 0.4 s, and the ratio $M^*$ was found to be 0.77 ± 0.06 and 0.73 ± 0.04. Insertion of these values and the cytoplasmic HCO$_3^-$ content into Equations 2 to 4 gives a carbonic anhydrase activity of 43 ± 14 (control) and 52 ± 21 (+K$_2$SO$_4$) µmol/min/g tissue. Thus, K$_2$SO$_4$ treatment had no significant effect on carbonic anhydrase activity. This result supports the view that flux of C$_i$ through carbonic anhydrase is much faster than rates of C$_i$ transport (as indicated in Fig. 1, which shows a unidirectional rate of influx of approximately 2 µmol/min/g fresh weight).

Figure 2. Determination of $^{13}$C isotopic enrichment in carbon-1 of Glc-6-P. $^1$H-NMR spectrum (2 h acquisition) of $^1$H attached to carbon-1 of Glc-6-P in an extract of [1-$^{13}$C]Glc-labeled maize root tips. Peak assignments: 1, $^1$H attached to carbon-1 of [1-$^{13}$C]Glc-6-P; 2, $^1$H attached to carbon-1 of [1-$^{13}$C]Glc-6-P (the $^{13}$C-coupled, $^1$H-resonance having a higher chemical shift than peak 1 overlapped with water signal and is not shown). The ratio $^{13}$C/$^{12}$C at carbon-1 of Glc-6-P is given by the intensity of doublet 1 over twice the intensity of doublet 2.

which the average lifetimes of reacting species are within approximately an order of magnitude of nuclear magnetic relaxation times ($T_1$, generally approximately 1 s) (see ref. 21). Saturation transfer NMR is incapable of measuring $^{13}$CO$_2$–H$^1$CO$_3^-$ exchange in aqueous bicarbonate solutions (data not shown), because the rate of exchange is too slow under these conditions; $k'_b$ (the first-order rate constant for hydration of CO$_2$) is approximately $4.8 \times 10^{-2}$ s$^{-1}$ at pH 7.6 and 25°C (16). However, we do observe transfer of magnetization from intracellular $^{13}$CO$_2$ to cytoplasmic H$^1$CO$_3^-$ in maize root tips, seen as a decrease in the intensity of the H$^1$CO$_3^-$ resonance when the $^{13}$CO$_2$ resonance is saturated (Fig. 3, compare spectra A, B, and B-A), which is indicative of carbonic anhydrase activity in vivo. Transfer of magnetization is also evident in the reverse direction from H$^{13}$CO$_3^-$ to $^{12}$CO$_2$ (compare spectra A and C in Fig. 3), which is qualitatively consistent with $^{13}$CO$_2$–H$^{12}$CO$_3^-$ exchange described by Equation 1 (“Materials and Methods”).

Spectra such as Figure 3, A and B, when combined with measurements of the longitudinal relaxation time ($T_1'$) of cytoplasmic H$^{13}$CO$_3^-$ and the cytoplasmic HCO$_3^-$ content (µmol/g tissue) permit estimation of in vivo carbonic anhydrase activity (see “Materials and Methods”). For control ($n = 4$) and K$_2$SO$_4$-treated ($n = 5$) samples, respectively, $T_1'$ was

Figure 3. Saturation transfer NMR between $^{13}$CO$_2$ and H$^{12}$CO$_3^-$ in maize root tips. A, Control $^{13}$C-NMR spectrum with irradiation at position marked by arrow; B, spectrum obtained with selective irradiation of the $^{13}$CO$_2$ resonance (peak 2) indicated by arrow; C, spectrum acquired with selective irradiation of the H$^{12}$CO$_3^-$ resonance (peak 1) (arrow); B-A, difference spectrum obtained by subtracting spectrum A from spectrum B. Spectra are summations from four experiments collected as interleaved blocks (see “Materials and Methods”) during 2 h.

Copyright © 1992 American Society of Plant Biologists. All rights reserved.
and CO₂ production from respiration (estimated from ref. 23 at approximately 1 µmol CO₂/min/g tissue). This issue is considered further in “Discussion.”

Measurement of PEP Carboxylase Activity in Vivo

The results in Tables I and II indicate that extracellularly derived C₄ is diluted in the cytoplasm two- to threefold by respiratory CO₂. This dilution is of practical significance with regard to the in vivo measurement of PEP carboxylase activity using radiolabeled C₄ (cf. 28). Previous estimates of in vivo PEP carboxylase activity in plants have used the specific radioactivity of extracellular C₄ to convert disintegrations per minute ¹⁴C, fixed into micromoles C₄ fixed (1, 31). However, the dilution of extracellular C₄ by respiratory CO₂ means that, for maize root tips, such a conversion would lead to a significant underestimation of in vivo PEP carboxylase activity. We calculated the specific radioactivity of intracellular H⁺¹⁴CO₃⁻ from the specific radioactivity of extracellular H⁺¹⁴CO₃⁻ using the data in Figure 1 and Tables I and II to determine the dilution factor at various times after addition of exogenous C₄ isotope. We then determined the effect of K₂SO₄ treatment on the in vivo activity of PEP carboxylase in maize root tips by measuring the unidirectional rate of incorporation of H⁺¹⁴CO₃⁻ into acid-stable metabolites and correcting for the specific radioactivity of intracellular H⁺¹⁴CO₃⁻ (Fig. 4). Potassium sulfate treatment led to a stimulation of PEP carboxylase activity at the earliest time measured (4 min) and was maximal (approximately 70% higher than control tissue) within 12 min of K₂SO₄ addition.

DISCUSSION

The results presented here allow critical evaluation of the hypothesis (13, 19) that the enhanced C₄ fixation accompanying cation transport is due to elevation of cytoplasmic HCO₃⁻ levels. It is apparent that the maximal unidirectional rates of dark C₄ fixation (approximately 0.17 µmol/min/g tissue, Fig. 4) are much lower than the rates of reactions that provide PEP carboxylase with its inorganic substrate, HCO₃⁻: C₄ uptake (approximately 2 µmol/min/g tissue, Fig. 1), carbonic anhydrase activity (>40 µmol/min/g tissue), and respiration (approximately 1 µmol/min/g tissue from ref. 23, assuming a respiratory quotient of 0.8). Hence, we conclude that the K₂SO₄-induced synthesis of organic acids in maize root tips (6) is not due to salt effects on C₄ availability in the cytoplasm. This view is reinforced by observation of approximately 11 mm HCO₃⁻ in root tip cytoplasm (Table II), which is about two orders of magnitude higher than the Kₑ of PEP carboxylase for HCO₃⁻ (17). Only in roots exhibiting much lower rates of respiration than maize root tips and under conditions of low extracellular C₄ would it appear possible for rates of cytoplasmic HCO₃⁻ production to be reduced to rates of dark C₄ fixation. And those circumstances would most likely lead to additional limitations on dark C₄ fixation, such as availability of PEP.

The in vivo activity of carbonic anhydrase is >200-fold greater than the unidirectional rate of dark C₄ fixation (Fig. 4). By comparison, Holtum et al. (12) found that the excess of carbonic anhydrase over PEP carboxylase activity in various plants displaying crassulacean acid metabolism ranged from 7- to >100-fold. This contrasts with C₄ mesophyll cells in rapidly photosynthesizing maize leaves, where carbonic anhydrase activity may be only just sufficient to provide PEP carboxylase with HCO₃⁻ and so sustain high rates of photosynthesis (11).

In vitro, malate acts as a potent inhibitor of PEP carboxylase activity (27, 32). However, although PEP carboxylase in vitro may be inhibited by cytoplasmic malate, the inhibition does not appear to be critical, because PEP carboxylase activity and cytoplasmic malate levels are positively, not negatively, correlated. Thus, the K₂SO₄-induced stimulation of dark C₄ fixation (i.e. PEP carboxylase activity), which peaks approximately 20 min after addition of K₂SO₄ (Fig. 4), is accompanied by an increase in cytoplasmic malate levels, which double within approximately 45 min (6). At later times, both the rate of C₄ fixation and cytoplasmic malate levels decline slightly. Hence, any inhibitory effect of malate on PEP carboxylase in vitro must be saturated at the concentration of malate found in root tip cytoplasm (3.5–7.5 mm) (6) and at the cytoplasmic concentrations of other effectors of PEP carboxylase, which have been identified in studies of PEP carboxylase in vitro (see ref. 17 for review).

It appears that the regulation of PEP carboxylase in vivo during salt-stimulated organic acid synthesis is quite distinct from the pattern of regulation evident during anaplerotic malate synthesis associated with ammonium assimilation. In studies of dark ammonium assimilation in the green alga Selenastrum, regulation of PEP carboxylase appears to be dominated by the induced synthesis and accumulation of the PEP carboxylase activator glutamine (25) and accompanied by a decline in levels of the PEP carboxylase inhibitors glutamate, 2-oxoglutarate, aspartate, and malate (25, 26, 29). This is the converse of metabolite behavior in maize root tips during K₂SO₄-induced dark C₄ fixation, in which levels of cytoplasmic malate, glutamate, and aspartate increase (5, 6),
and glutamine remains low (K. Chang and J.K.M. Roberts, unpublished observations). Thus, the activation of PEP carboxylase during ion uptake must involve a unique regulatory mechanism that does not depend on removal of the products of dark C fixation from the cytoplasm.

ACKNOWLEDGMENTS

We thank Dr. Tai-houn Tsuo and Mr. Jackson Chow (Department of Statistics, University of California, Riverside) for assisting with the statistical analyses and Dr. Cecelia Webster for criticism during the preparation of this manuscript.

LITERATURE CITED

6. Chang K, Roberts JKM (1991) Cytoplasmic malate levels in maize root tips during K\textsuperscript{+} ion uptake determined by \textsuperscript{13}C-NMR spectroscopy. Biochim Biophys Acta 1092: 29-34
16. Magid E, Turbeck BO (1968) The rates of the spontaneous hydration of CO\textsubscript{2} and the reciprocal reaction in neutral aqueous solutions between 0° and 38°. Biochim Biophys Acta 165: 515-524
20. Overstreet R, Ruben S, Broyer TC (1940) The absorption of bicarbonate ion by barley roots as indicated by studies with radioactive carbon. Proc Natl Acad Sci USA 26: 688-695
22. Roberts JKM (1990) Observation of uridine triphosphate: glucose-1-phosphate uridylyltransferase activity in maize root tips by saturation transfer \textsuperscript{31}P-NMR. Estimation of cytoplasmic PP. Biochim Biophys Acta 1051: 29-36