Plant Physiol. (1992) 99, 291–297
0032-0889/92/99/0291/07/$01.00/0

Received for publication October 8, 1991
Accepted December 18, 1991

Quantitation of Rates of Transport, Metabolic Fluxes, and Cytoplasmic Levels of Inorganic Carbon in Maize Root Tips during K^+ Ion Uptake[1]

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_i), due to phospho*enol*pyruvate carboxylase activity, is limited by the availability of C_i in the cytoplasm of maize (*Zea mays* L.) root tips. Rates of C_i uptake and metabolism were measured during K_2SO_4 treatment, which stimulates dark C_i fixation. $^{13}C_i$ uptake was followed by ^{13}C-nuclear magnetic resonance (NMR); 5 millimolar K_2SO_4 had no significant effect on $^{13}C_i$ influx. The contribution of respiratory CO_2 production to cytoplasmic HCO_3^- was measured using *in vivo* ^{13}C-NMR and ^1H-NMR of cell extracts; K_2SO_4 treatment had no effect on respiratory CO_2 production. The concentration of cytoplasmic HCO_3^- was estimated to be approximately 11 millimolar, again with K_2SO_4 having no significant effect. These experiments allowed us to determine the extent to which extracellularly supplied $^{14}C_i$ was diluted in the cytoplasm by respiratory CO_2 and thereby measure phospho*enol*pyruvate (PEP) carboxylase activity *in vivo* using $^{14}C_i$. PEP carboxylase activity in root tips was enhanced approximately 70% over controls within 12 minutes of the addition of 5 millimolar K_2SO_4. The activity of carbonic anhydrase, which provides PEP carboxylase with C_i, was determined by saturation transfer ^{13}C-NMR to be more than 200 times that of PEP carboxylase *in vivo*. The regulation of PEP carboxylase in K_2SO_4-treated roots is discussed.

Fixation of C_i[2] into organic metabolites by plant roots was first reported more than 50 years ago (20). This dark C_i 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^+ 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 alkalinization during H^+ 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_3^-, the form of C_i 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_3^-, leading to enhanced dark C_i fixation.

The study of dark C_i fixation *in vivo* has relied on ^{11}C, ^{13}C, and ^{14}C isotope tracers, which permit uptake and incorporation of exogenous C_i to be followed without complete masking by competition from endogenous $^{12}C_i$, produced by respiration and other decarboxylation reactions. However, a quantitative description of dark C_i fixation requires assessment of the extent to which C_i uptake, respiratory CO_2 formation, and cytoplasmic CO_2-HCO_3^- exchange provide PEP carboxylase with its inorganic substrate, HCO_3^-. Thus, Jacoby and Laties (13) described "the inadequacy of $^{14}CO_2$ 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_i transport and the concentration of cytoplasmic HCO_3^- as determinants of PEP carboxylase activity in roots. Guern *et al.* (10) noted the lack of information concerning cytoplasmic HCO_3^- levels and rates of respiratory CO_2 hydration with respect to control of PEP carboxylase activity *in vivo*. Here, we present quantitative data regarding fluxes and concentrations of C_i in maize root tips during K^+-stimulated organic acid synthesis. The results are discussed with respect to the regulation of dark C_i 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 ^{13}C- and ^1H-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 ^{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* $H^{13}CO_3^-$ 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_i, inorganic carbon; PEP, phospho*enol*pyruvate.

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. ^{1}H-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 ± 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 $CaSO_4$, and 5 mM Tris-HCO_3^- (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 HCO_3^- (Tris-HCO_3 was prepared according to the method in ref. 6). K_2SO_4 treatment consisted of addition of 5 mM K_2SO_4 to the perfusion medium after 2 h. Experiments were performed at room temperature (24–25°C).

Measurements of Cytoplasmic [HCO_3^-] Derived from Extracellular C_i

$^{13}C_i$ Influx Measurements. Root tips were first equilibrated with perfusion medium for 2 h, then natural abundance Tris-HCO_3 was replaced with Tris-$H^{13}CO_3$ plus 0.1 mM $MnCl_2$ for 30 min (with or without 5 mM K_2SO_4), 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_2SO_4 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 $^{13}C_i$ was then calculated. Comparison of the ratio of $H^{13}CO_3^-$ to chloroform reference peak areas 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 $MnCl_2$ 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 $^{13}C_i$ at steady state was calculated from the intensity of the cytoplasmic $H^{13}CO_3^-$ resonance, as described above.

Measurements of Cytoplasmic [HCO_3^-] 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 $HClO_4$ (4). The cleared, neutralized extracts were loaded onto prefilled anion-exchange columns (AG-1-X8; Bio-Rad, Richmond, CA), which were washed with deionized H_2O 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_2O (Sigma), and analyzed by ^{1}H-NMR. The ratio of $^{12}C/^{13}C$ 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 ^{1}H attached to [1-^{12}C] Glc-6-P and [1-^{13}C]Glc-6-P in the ^{1}H-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 ($^{12}C + ^{13}C$) 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:

$$HCO_3^- + H^+ \underset{k_2}{\overset{k_1}{\rightleftarrows}} CO_2 + H_2O \quad (1)$$

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:

$$\text{Rate of } HCO_3^- \text{ synthesis} = k_1 \times [HCO_3^-] = k_2 \times [CO_2]. \quad (2)$$

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

$$k_1 = \frac{1}{T_1 \cdot M^*} - \frac{1}{T_1} \quad (3)$$

and

$$\frac{1}{T_1'} = \frac{1}{T_1} + k_1 \quad (4)$$

where T_1 is the true longitudinal relaxation time of $H^{13}CO_3^-$; T_1' is the longitudinal relaxation time of the $H^{13}CO_3^-$ resonance, measured in the presence of selective saturation of the $^{13}CO_2$ resonance; and M^* is the ratio of the intensity of the $H^{13}CO_3^-$ signal during $^{13}CO_2$ irradiation, to the intensity of $H^{13}CO_3^-$ in the control spectrum, calculated after subtraction of the contribution of extracellular $H^{13}CO_3^-$ (which undergoes exchange at a rate too slow for measurement by saturation transfer NMR). The rate of synthesis of HCO_3^- (μmol/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, τ, 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 ±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 $\ln(M_\infty - M_\tau)$ *versus* τ, where M_∞ and M_τ are the intensities at delay time 10 s and the different delay times, τ, respectively. The $H^{13}CO_3^-$ NMR signals in these experiments contain contributions of similar magnitude from both cytoplasmic and extracellular $H^{13}CO_3^-$. Hence, in principal, if these two $H^{13}CO_3^-$ 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_2SO_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_i 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 $H^{14}CO_3^-$ (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 $H^{14}CO_3^-$. Samples were kept in a fume hood for 36 h to allow volatile radioactivity to escape and were counted in 3.5 mL of scintillation cocktail (Ecolume; ICN, Cleveland, OH) in a Beckman LS-3801 counter.

RESULTS

C_i Influx and the Contribution of Extracellular C_i 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 $^{13}C_i$ into root tips and the steady-state concentration of cytoplasmic $H^{13}CO_3^-$. 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.

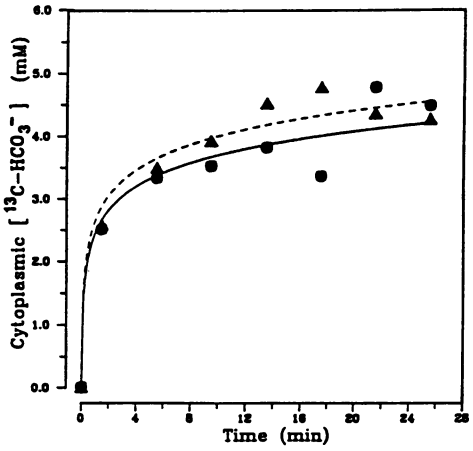

Figure 1. $^{13}C_i$ influx into the cytoplasm of maize root tips. Tissue was initially perfused with 5 mM natural abundance HCO_3^- for 2 h, then switched to 5 mM $H^{13}CO_3^-$ plus 0.1 mM $MnCl_2$ at time 0, either without (●——●) or with (▲----▲) 5 mM K_2SO_4 (see "Materials and Methods"). The lines are best logarithmic fits to each data set. An analysis of covariance of cytoplasmic $H^{13}CO_3^-$ *versus* log time, using the SAS program (SAS Institute, Inc., Cary, NC), indicated no significant difference between the control and K_2SO_4-treated tissue at the 95% confidence level.

The influx of $^{13}C_i$ into root tips was followed by observing the intensity of the cytoplasmic $H^{13}CO_3^-$ resonance over time, after addition of 5 mM Tris-$H^{13}CO_3$ to the perfusion medium (Fig. 1). Influx was not influenced by K_2SO_4 treatment, which stimulates malate synthesis (6). This result suggests that K_2SO_4 treatment does not elevate cytoplasmic HCO_3^- by enhanced transport of C_i. Steady-state ^{13}C-NMR measurements of cytoplasmic $H^{13}CO_3^-$ confirmed this inference by showing that the concentration of exogenously derived cytoplasmic $H^{13}CO_3^-$ is independent of the presence of K_2SO_4 (Table I). Thus, under the conditions used in these experiments, ap-

Table I. *Concentration of Cytoplasmic $H^{13}CO_3^-$ in Maize Root Tips Derived from 5 mM Extracellular Tris-$H^{13}CO_3$*

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

Time	K_2SO_4[a] (5 mM)	n	$[H^{13}CO_3^-]$
h			*mM*
2–2.5	–	4	4.2 ± 0.4[b]
3.5–4	–	2	4.6 ± 0.8[c]
3.5–4	+	2	4.4 ± 0.6[c]

[a] Added at 2 h. [b] Mean ± SD. [c] Mean ± range.

proximately 4.4 mM of the total cytoplasmic HCO_3^- was derived from the perfusion medium.

Contribution of Respiratory CO_2 to Cytoplasmic HCO_3^-

Respiration also provides C_i to sustain organic acid synthesis. We measured this contribution to determine whether K_2SO_4 treatment elevated cytoplasmic HCO_3^- by stimulating metabolic CO_2 production. Root tips were labeled with [1-^{13}C]Glc, and the amount of cytoplasmic $H^{13}CO_3^-$ derived from this substrate was determined from *in vivo* ^{13}C-NMR spectra (Table II, column A). Whereas the concentration of cytoplasmic $H^{13}CO_3^-$ increased with time of perfusion in [1-^{13}C]Glc, indicating increased consumption of [1-^{13}C]Glc by glycolysis and respiration, the addition of K_2SO_4 had no significant effect (Table II, column A).

Conversions of the cytoplasmic $H^{13}CO_3^-$ concentration to the concentration of total (^{13}C + ^{12}C) cytoplasmic HCO_3^- derived from respiration required knowledge of the ^{13}C isotopic enrichment in the respiratory substrate. To this end, we determined the ^{13}C isotopic enrichment in carbon-1 of Glc-6-P in root tip extracts by ^{1}H-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-^{13}C]Glc uptake and compartmentation, and thus mixing with endogenous respiratory carbohydrate, to be quantitated. Whereas the ^{13}C enrichment in carbon-1 of Glc-6-P increased with time, K_2SO_4 treatment had no significant effect on ^{13}C enrichment (Table II, column B) and, thus, on HCO_3^- derived from Glc-6-P (Table II, column C). Therefore, under all conditions, respiration from Glc-6-P contributed approximately 6.8 mM HCO_3^- to the cytoplasmic HCO_3^- pool. Combining this value with the contribution of extracellular C_i to cytoplasmic HCO_3^- at steady state (Table I) gave a concentration of total cytoplasmic HCO_3^- of approximately 11 mM, which was constant with time and independent of K_2SO_4 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 HCO_3^- 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 $H^{13}CO_3^-$ NMR resonance was measured in root tips treated with HCO_3^- and Glc at natural abundance (1.1% ^{13}C). If root tip respiration depended significantly on noncarbohydrates, a significantly higher concentration of cytoplasmic HCO_3^- than the levels given in Table II (column D) would be observed. However, the concentration of cytoplasmic HCO_3^- in the natural abundance ^{13}C-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 ^{13}C-NMR signal from intracellular $^{13}CO_2$ that was approximately 0.038 times the intensity of the cytoplasmic $H^{13}CO_3^-$ signal (data not shown). This ratio of concentrations is similar to that predicted by the Henderson-Hasselbalch equation (*viz.* 0.042) for a HCO_3^- solution at pH 7.5, in equilibrium with respect to CO_2-HCO_3^- exchange. Such similarity suggests that cytoplasmic $^{13}CO_2$ and $H^{13}CO_3^-$ pools are in near equilibrium, *i.e.*, that the rate of carbon exchange between $^{13}CO_2$ and $H^{13}CO_3^-$ is much faster than either the rates of production or consumption of individual C_i species in root tip cytoplasm or the rates of influx or efflux of C_i species into or out of the cytoplasm. Such rapid exchange between $^{13}CO_2$ and $H^{13}CO_3^-$ 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 $^{13}CO_2$-$H^{13}CO_3^-$ exchange by saturation transfer ^{13}C-NMR. This method is capable of monitoring chemical reactions in

Table II. *Concentration of Cytoplasmic HCO_3^- Derived from Respiration and Total Cytoplasmic HCO_3^-*
Results are from four replicates at each time point.

Time	K_2SO_4[a] (5 mM)	(A) $[H^{13}CO_3^-]$[b]	(B) $^{12}C/^{13}C$ in C-1 of Glc-6-P[c]	(C) $[HCO_3^-]$ from Glc-6-P[d]	(D) Total $[HCO_3^-]$[e]
h		*mM*		*mM*	*mM*
1–2	–	0.22 ± 0.07	4.2 ± 0.2	6.9 ± 2.2	11.1 ± 2.2
3–4	–	0.44 ± 0.07	1.5 ± 0.1	6.6 ± 1.1	11.2 ± 1.7
3–4	+	0.42 ± 0.07	1.7 ± 0.1	6.8 ± 1.2	11.2 ± 1.9

[a] Added at 2 h. [b] Determined from intensity of the $H^{13}CO_3^-$ resonance in NMR spectra of tissue perfused with [1-^{13}C]Glc, obtained during the last hour before extraction (see "Materials and Methods"). Error values are ranges. [c] Determined from ^{1}H-NMR spectra of extracts (see Fig. 2 legend and "Materials and Methods"), prepared at the end of the indicated time. Error values are SD. [d] Calculated by multiplying $[H^{13}CO_3^-]$ by the ratio (^{12}C + ^{13}C)/^{13}C in carbon-1 of Glc-6-P and by 6 (number of carbons in Glc). Errors values are SD. [e] Determined as the sum of column C, plus $[HCO_3^-]$ derived from extracellular HCO_3^- (Table I). Error values are ranges.

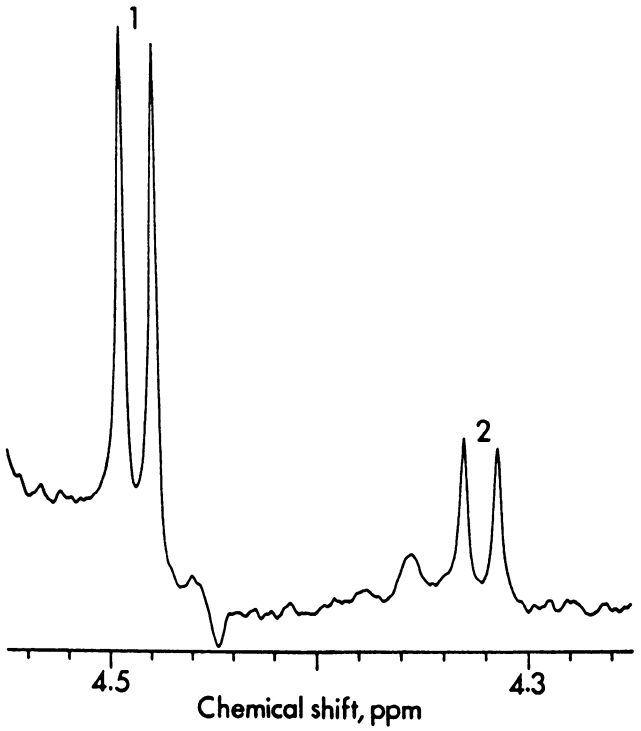

Figure 2. Determination of ^{13}C isotopic enrichment in carbon-1 of Glc-6-P. ^{1}H-NMR spectrum (2 h acquisition) of ^{1}H attached to carbon-1 of Glc-6-P in an extract of [1-^{13}C]Glc-labeled maize root tips. Peak assignments: 1, ^{1}H attached to carbon-1 of [1-^{12}C]Glc-6-P; 2, ^{1}H attached to 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 $^{12}C/^{13}C$ 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 $^{13}CO_2$-$H^{13}CO_3^-$ exchange in aqueous bicarbonate solutions (data not shown), because the rate of exchange is too slow under these conditions; k'_h (the first-order rate constant for hydration of CO_2) is approximately 4.8×10^{-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 $H^{13}CO_3^-$ in maize root tips, seen as a decrease in the intensity of the $H^{13}CO_3^-$ 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 $^{13}CO_2$ (compare spectra A and C in Fig. 3), which is qualitatively consistent with $^{13}CO_2$-$H^{13}CO_3^-$ 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_2SO_4-treated ($n = 5$) samples, respectively, T_1' was 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_2SO_4) μmol/min/g tissue. Thus, K_2SO_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)

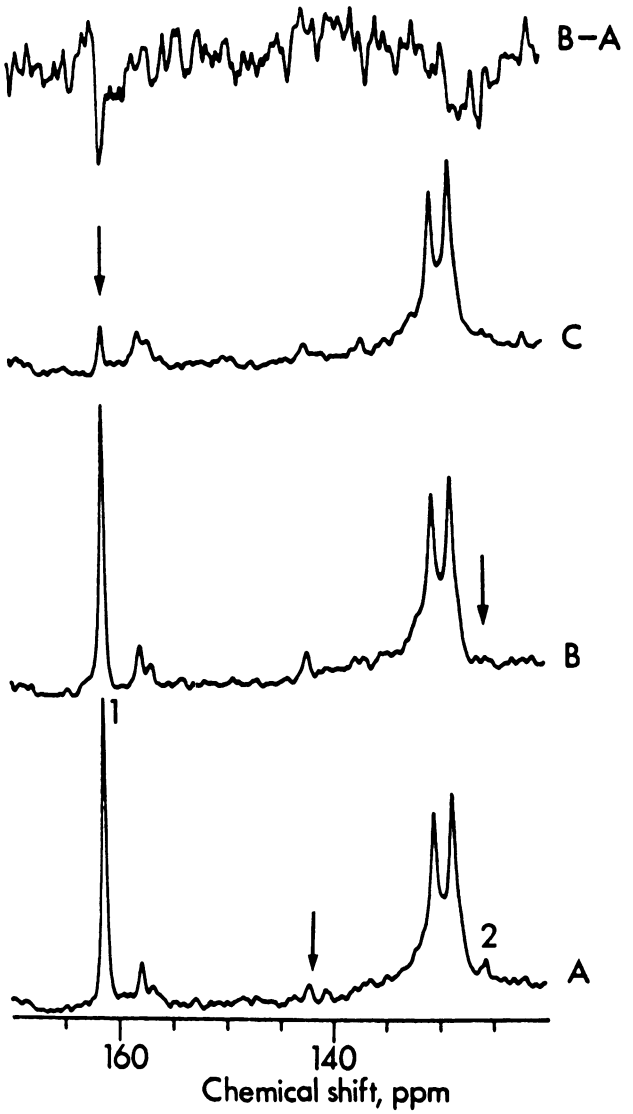

Figure 3. Saturation transfer NMR between $^{13}CO_2$ and $H^{13}CO_3^-$ in maize root tips. A, Control ^{13}C-NMR spectrum with irradiation at position marked by arrow; B, spectrum obtained with selective irradiation of the $^{13}CO_2$ resonance (peak 2) indicated by arrow; C, spectrum acquired with selective irradiation of the $H^{13}CO_3^-$ 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.

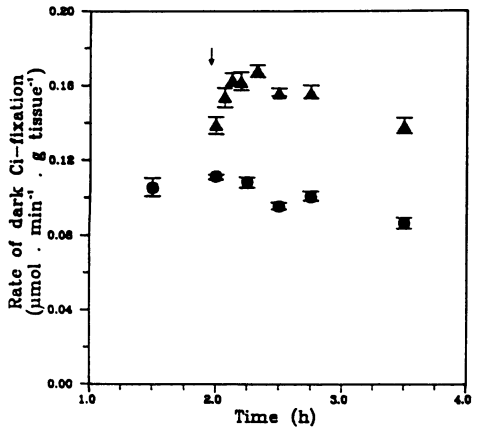

Figure 4. Effect of K_2SO_4 on unidirectional rates of dark C_i fixation *in vivo*. The flux of HCO_3^- through dark C_i fixation (μmol·min^{-1}·g tissue^{-1}) was determined from the rate of $^{14}C_i$ fixation (dpm·min^{-1}·g tissue^{-1}) (see "Materials and Methods") and the specific radioactivity of intracellular $H^{14}CO_3^-$ (see text). Samples were perfused either without (●) or with (△) 5 mM K_2SO_4 (beginning at 2 h,). Data points are means ± SD; control data are from four replicates and K_2SO_4 treatment from five replicates.

and CO_2 production from respiration (estimated from ref. 23 at approximately 1 μmol CO_2/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 C_i is diluted in the cytoplasm two- to threefold by respiratory CO_2. This dilution is of practical significance with regard to the *in vivo* measurement of PEP carboxylase activity using radiolabeled C_i (*cf.* 28). Previous estimates of *in vivo* PEP carboxylase activity in plants have used the specific radioactivity of extracellular C_i to convert disintegrations per minute $^{14}C_i$ fixed into micromoles C_i fixed (1, 31). However, the dilution of extracellular C_i by respiratory CO_2 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 $H^{14}CO_3^-$ from the specific radioactivity of extracellular $H^{14}CO_3^-$ using the data in Figure 1 and Tables I and II to determine the dilution factor at various times after addition of exogenous C_i isotope. We then determined the effect of K_2SO_4 treatment on the *in vivo* activity of PEP carboxylase in maize root tips by measuring the unidirectional rate of incorporation of $H^{14}CO_3^-$ into acid-stable metabolites and correcting for the specific radioactivity of intracellular $H^{14}CO_3^-$ (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 K_2SO_4 addition.

DISCUSSION

The results presented here allow critical evaluation of the hypothesis (13, 19) that the enhanced C_i fixation accompanying cation transport is due to elevation of cytoplasmic HCO_3^- levels. It is apparent that the maximal unidirectional rates of dark C_i 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, HCO_3^-: C_i 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 1). Hence, we conclude that the K_2SO_4-induced synthesis of organic acids in maize root tips (6) is not due to salt effects on C_i availability in the cytoplasm. This view is reinforced by observation of approximately 11 mM HCO_3^- in root tip cytoplasm (Table II), which is about two orders of magnitude higher than the K_m of PEP carboxylase for HCO_3^- (17). Only in roots exhibiting much lower rates of respiration than maize root tips and under conditions of low extracellular C_i would it appear possible for rates of cytoplasmic HCO_3^- production to be reduced to rates of dark C_i fixation. And those circumstances would most likely lead to additional limitations on dark C_i fixation, such as availability of PEP.

The *in vivo* activity of carbonic anhydrase is >200-fold greater than the unidirectional rate of dark C_i 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 C_4 mesophyll cells in rapidly photosynthesizing maize leaves, where carbonic anhydrase activity may be only just sufficient to provide PEP carboxylase with HCO_3^- 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 vivo* 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 K_2SO_4-induced stimulation of dark C_i fixation (*i.e.* PEP carboxylase activity), which peaks approximately 20 min after addition of K_2SO_4 (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 C_i fixation and cytoplasmic malate levels decline slightly. Hence, any inhibitory effect of malate on PEP carboxylase *in vivo* 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 anapleurotic 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 K_2SO_4-induced dark C_i 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_i 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.

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