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Fractionation of Stable Carbon Isotopes by Phosphoenolpyruvate Carboxylase from C_4 Plants[1]

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ABSTRACT

The active species of "CO_2" and the amount of fractionation of stable carbon isotopes have been determined for a partially purified preparation of phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) from corn (*Zea mays*) leaves. The rates of the enzyme reactions, using substrate amounts of HCO_3^-, CO_2 or CO_2 plus carbonic anhydrase, show that HCO_3^- is the active species of "CO_2" utilized by PEP carboxylase. The K_m values for CO_2 and HCO_3^- are 1.25 mM and 0.11 mM, respectively, which further suggest the preferential utilization of HCO_3^- by PEP carboxylase. The amount of fractionation of stable carbon isotopes by PEP carboxylase from an infinite pool of $H^{12}CO_3^-$ and $H^{13}CO_3^-$ was −2.03 ‰. This enzyme fractionation (Δ), together with the fractionation associated with absorption of CO_2 into plant cells and the equilibrium fractionation associated with atmospheric CO_2 and dissolved HCO_3^- are discussed in relation to the fractionation of stable carbon isotopes of atmospheric CO_2 during photosynthesis in C_4 plants.

Whelan *et al.* (23) have shown that a preparation of PEP[2] carboxylase from sorghum leaves fractionates stable carbon isotopes of HCO_3^- by −2.7 ‰. Calculations show that the enzyme fractionation (Δ) is +5.4 ‰ relative to the CO_2 pool of carbon. Both CO_2 and HCO_3^- have been proposed as the active species of "CO_2" for PEP carboxylase (2, 4, 13, 20). A Δ CO_2 of +5.4 ‰ will not account for the observed fractionation between atmospheric CO_2 and metabolic intermediates of C_4 plants (22). The active species of "CO_2" of PEP carboxylase has to be known in order to determine the importance of Δ in the fractionation of stable carbon isotopes during C_4 photosynthesis. In this paper, we report data on the active species of "CO_2" utilized by PEP carboxylase and the amount of *in vitro* fractionation of stable carbon isotopes by this enzyme.

MATERIALS AND METHODS

Plants. Corn (*Zea mays* L. R. × 404 single cross) seed was obtained from Asgrow Seed Company, San Antonio, Tex. Seeds were germinated in flats of vermiculite in a growth chamber with a fluorescent light bank supplemented with incandescent bulbs. Seedlings were watered with a nutrient solution.

Enzyme Purification. PEP carboxylase was partially purified from month-old corn leaves. Leaves were homogenized with a Vir-Tis blender in ice-cold 0.1 M tris (pH 7.5) containing 0.1 mM GSH and 10% PVP. The homogenate was filtered through a nylon mesh to remove large debris. The filtrate was centrifuged in a Sorvall RC5 refrigerated centrifuge for 30 min at 27,000*g*. The supernatant was decanted and recentrifuged for 30 min. The soluble supernatant was fractionated with solid ammonium sulfate. PEP carboxylase activity was located in the 40 to 55% fraction. This fraction can be centrifuged and stored in saturated $(NH_4)_2SO_4$ at 0 C for several months without losing appreciable activity. The 40 to 55% $(NH_4)_2SO_4$ fraction was dialyzed in an Amicon ultrafiltration cell with a XM-100A membrane and 5 mM phosphate buffer (pH 7.5) under N_2. The dialysate was centrifuged at 27,000*g* for 30 min and then adsorbed onto a DEAE-cellulose column (Cellex-D, Bio-RaD, 1.5 × 15 cm) which had been previously equilibrated with 5 mM phosphate buffer (pH 7.5) according to the procedure of Ting (18). The protein was eluted with a stepwise gradient of phosphate buffer (pH 7.5) from 5 mM to 200 mM. The PEP carboxylase used in the active species of "CO_2" experiments was eluted from column with 200 mM phosphate buffer (pH 7.5) (Fig. 1).

Protein Determination. Protein was determined by the 280:260 method (10), and the method of Lowry (12).

Active Species Determinations. A spectrophotometric modification of the method of Cooper (3) was used to determine the active "CO_2" species for corn PEP carboxylase. Cooper's method is based on the rate of hydration of CO_2 by the following reaction:

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

The above reaction requires more than 60 sec to reach equilibrium at 10 C when the initial reactants are CO_2 and H_2O (9). Addition of carbonic anhydrase brings about a rapid equilibrium of CO_2 hydration. Figure 2 shows the estimated theoretical formation of oxaloacetate in a spectrophotometric assay of PEP carboxylase if the active species is CO_2 or HCO_3^- at 10 C (3). The rates of these theoretical curves apply to all carboxylation reactions. Our reactions were carried out at 10 C in an Acta III Beckman recording spectrophotometer equipped with a circulating H_2O bath surrounding the cuvettes. The reaction mixture contained in μmol: 100, tris (pH 7.5); 10, $MgCl_2$; 0.25, NADH; 10, tricyclohexylammonium salt of PEP; 250 units of Sigma pig heart malic dehydrogenase; 10, $KHCO_3$ or CO_2 with or without CA, 0.85 mg purified corn PEP carboxylase, and CO_2-free H_2O to 3 ml. The reactions were run under N_2. All reagents were CO_2-free and stored under N_2. The reactions were initiated by the addition of CO_2 or HCO_3^-. CO_2 was generated by mixing stoichiometric amounts of HCl and $KHCO_3$. A double reciprocal plot of the reaction rates *versus* substrate concentration was used to determine the Michaelis constants for CO_2 or HCO_3^-.

Malate Synthesis. Malate was enzymatically synthesized according to the procedure of Whelan (23). The reaction mixture contained in μmol: 100, tris(pH 7.5); 10, $MgCl_2$; 2.5, NADH; 250 units of Sigma pig heart malic dehydrogenase; 100, sodium PEP; 1500, $KH^{12}CO_3$, $KH^{13}CO_3$; 2.71 mg of PEP carboxylase from the dialyzed $(NH_4)_2SO_4$ fraction, and H_2O to 3 ml. Malate

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[2] Abbreviations: PEP: phosphoenolpyruvate; CA: carbonic anhydrase; Δ: enzyme fractionation.

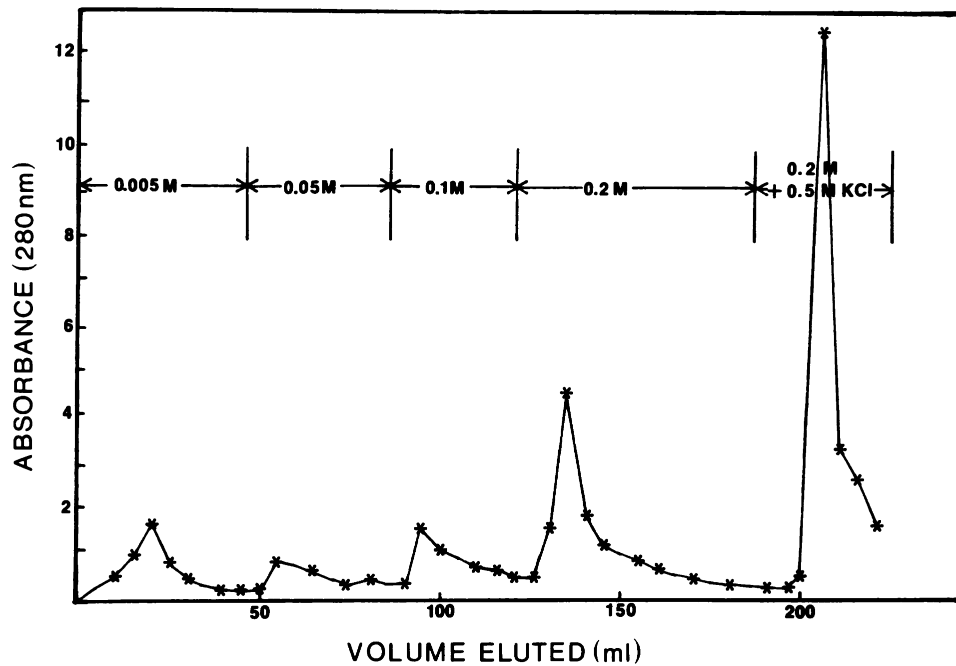

FIG. 1. Column chromatography of PEP carboxylase from corn leaves on DEAE-cellulose. Specific activities of the enzymes eluted in the peak tubes with 100 phosphate buffer (pH 7.5) and 200 phosphate buffer (pH 7.5) were: 178.1 μmol/mg protein·min and 157.6 μmol/mg protein·min, respectively. Enzyme eluted from the column with 200 mM phosphate buffer (pH 7.5) was used in the determination of the active species of "CO_2" experiments and was purified 31.3-fold over the enzyme in the crude fraction.

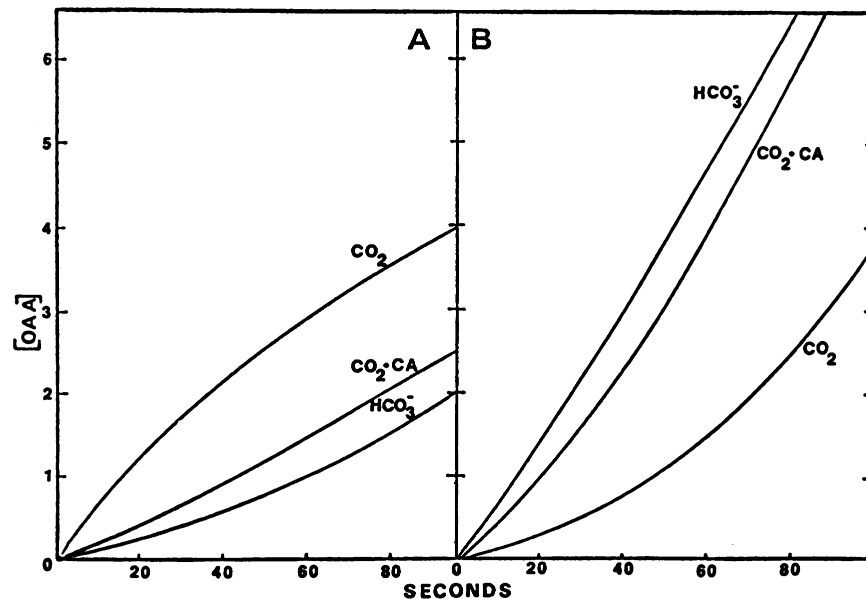

FIG. 2. Estimated theoretical formation of oxaloacetate for the spectrophotometric assay, A: If active species used is CO_2 and B: if active species is HCO_3^-. CO_2 means that CO_2 was initially added, HCO_3^- is when HCO_3^- was initially added, CO_2 + CA is when carbonic anhydrase was included in the assay mixture. Calculations have been made on the basis that the rate of fixation is directly proportional to the concentration of the active species. Absolute values for OAA concentration are not given, since amount is proportional to the concentration of the active species. (Adapted from Cooper *et al.*, 3).

was synthesized in 10 reaction mixtures with 10 additions of NADH. The reactions were monitored at 340 nm in a recording spectrophotometer at 25 C. The reactions were stopped by boiling the mixtures. The high concentration of HCO_3^- was used to insure an infinite reservoir of $H^{12}CO_3^-$ and $H^{13}CO_3^-$ for maximum enzyme fractionation. Walker and Brown (19) observed a CO_2 inhibition of PEP carboxylase at high CO_2 concentrations. The specific activity of the PEP carboxylase from corn leaves purified to the $(NH_4)_2SO_4$ stage with 10 or 1500 μmol of HCO_3^-/3 ml of reaction mixture was 33.5 μmol/mg protein·min and 35.4 μmol/mg protein·min. The mixtures were pooled and frozen for storage. The mixtures were centrifuged at 27,000*g* for 30 min to remove denatured protein. The supernatant fraction was passed through a column of Dowex 50-H^+ (2 × 20 cm) to remove cations. The eluant was evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of H_2O and adsorbed onto a column of Dowex 1-X2 formate (1 × 15 cm). The malic acid was eluted from the column with a linear gradient of 0 to 6 N formic

acid. The eluant was collected in 3-ml fractions. The formic acid was removed from the tubes by evaporation at 55 C with air from a manifold. Malic acid was located in the tubes by titration with NaOH to a phenolphthalein end point. The tubes containing malic acid were combined and passed through a column of Dowex 50-H^+ to remove Na^+. The eluant was evaporated to dryness *in vacuo*, dissolved in H_2O and applied stripwise to Whatman No. 3 filter papers alongside authentic malic acid. The chromatograms were developed in butanol-formic acid-H_2O (5:1:4, v/v/v). The enzymically synthesized malic acid was localized on the chromatograms by coincidence with the authentic malic acid. The malic acid was eluted from the chromatograms with H_2O and lyophilized prior to $\delta^{13}C$ analysis.

Determination of Isotope Ratios. In order to determine carbon isotope ratios, samples were converted to CO_2 by combustion at 800 C in an excess of O_2. The gases were circulated over cupric oxide for 20 min by means of an automatic Toepler pump to insure complete conversion of carbon to CO_2. Water vapor was removed by isopropyl alcohol-dry ice traps and the CO_2 was collected by a liquid N_2 trap. In the bicarbonate samples, the bicarbonate was converted to CO_2 by the addition of 85% phosphoric acid after the evacuation of atmospheric gases from the reaction vessels. Water vapor was again removed by isopropyl alcohol-dry ice traps, whereas the CO_2 was collected in a sample bulb at liquid N_2 temperature. The carbon isotope ratios were determined with a Nuclide Corporation, model RMS, 6 in, 60° sector mass spectrometer similar to the one described by McKinney *et al.* (14). Corrections for gas mixing, mass 44 tailing, background peaks, and ^{17}O contribution to mass 45 were made according to the procedure of Craig (7). Isotope ratios are expressed as $\delta^{13}C$ values where:

$$\delta^{13}C\ (‰) = \left[\frac{(^{13}C/^{12}C)\ \text{sample}}{(^{13}C/^{12}C)\ \text{standard}} - 1\right] \times 10^3$$

The working standard was Norit which has a $\delta^{13}C$ value of −24.8 ‰ *versus* the Chicago PDB-1 Standard (7). The PDB Standard is a Cretaceous belemnite from the Peedee formation of South Carolina. For ease of comparison, all isotope analyses measured relative to the Norit Standard were converted to $\delta^{13}_{PDB}C$ values ($\delta^{13}_{PDB}C$ = $\delta^{13}C$ *versus* PDB) according to the method of Craig (7).

RESULTS

The PEP carboxylase obtained from the DEAE columns was used to determine the active species of "CO_2." The PEP carboxylase eluted from the column with 200 mM phosphate buffer had a specific activity of 102.2 μmol malate produced/mg protein·min. The reaction rates of PEP carboxylase are shown in Figure 3. The initial velocity with HCO_3^- is 10 times the rate with CO_2. The initial velocity in the presence of CO_2 plus CA was greater than CO_2 alone. This latter rate was not as great as the theoretical rate (Fig. 2) if HCO_3^- is the active species. The data in Figures 2 and 3 indicate that HCO_3^- is the active species of "CO_2" for the PEP carboxylase from corn leaves. Determinations of *Km* values for CO_2 or HCO_3^- were done with CO_2-free reagents, CO_2-free H_2O, and identical reaction conditions to the active species experiments. Figure 4 shows the Lineweaver-Burk double reciprocal plots of the velocity curves of PEP carboxylase in the presence of increasing amounts of CO_2 or HCO_3^-. The *Km* for HCO_3^- was 0.11 mM and the *Km* for CO_2 was 1.25 mM. This suggests that the affinity of PEP carboxylase for HCO_3^- is 10 times the affinity of the enzyme for CO_2. These data agree with the above active species data and show that HCO_3^- is the active species of "CO_2" for PEP carboxylase.

The amount of fractionation of stable carbon isotopes by preparations of PEP carboxylase was determined by the method of Whelan *et al.* (23). The malate was synthesized enzymically

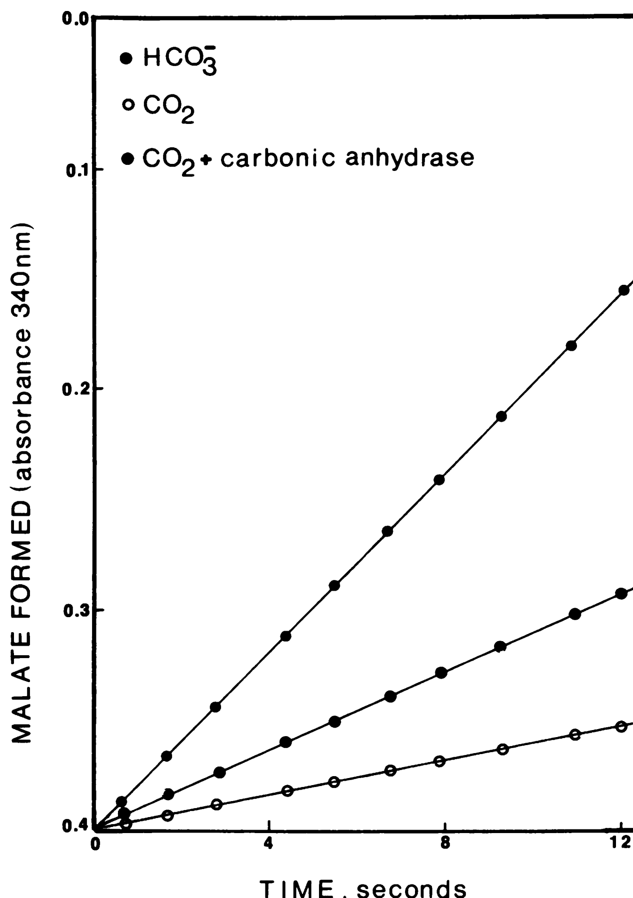

FIG. 3. Determination of the active species of "CO_2" for PEP carboxylase. Formation of malate was determined by measuring the decrease in absorbancy at 340 nm using HCO_3^-, CO_2, or CO_2 plus CA, as substrates.

from the sodium salt of PEP and $KHCO_3$, both of known $\delta^{13}C$ content. The bicarbonate reservoir was infinitely large to observe a maximum enzyme fractionation. The experimental procedure was designed to produce sufficient malate for $\delta^{13}C$ analysis yet to prevent equilibration of oxaloacetate and PEP. If equilibration between oxaloacetate and PEP existed, the enzyme fractionation of $H^{12}CO_3^-$ and $H^{13}CO_3^-$ might be masked by an equilibrium isotope effect. The limitation of this method is that high concentrations of malate (8 mM) accumulate in the reaction cuvette. Several workers (5, 11, 18) have shown that malate inhibits PEP carboxylase. This inhibition is competitive (11), partially competitive (5), or allosteric inhibition (5, 18). The high concentrations of PEP and HCO_3^- used in the reaction mixtures may partially overcome allosteric inhibition by malate and therefore minimize the effect of malate inhibition on the fractionation process. The equations for the calculation of the fractionation of stable carbon isotopes of HCO_3^- by PEP carboxylase are as follows:

$$3/4\ \delta^{13}C\ \text{PEP} + 1/4\ \delta^{13}C\ HCO_3^-\ \text{fixed} = \delta^{13}C\ \text{malate}$$

$$\Delta = \text{enzyme fractionation} = \delta^{13}C\ HCO_3^-\ \text{fixed} - \delta^{13}C\ HCO_3^-\ \text{source}$$

Substituting the values from experiment 1 Table I results in Δ = 0.51 ‰

$$3/4\ (-19.17\ ‰) + 1/4\ \delta^{13}C\ HCO_3^-\ \text{fixed} = -22.31\ ‰$$

$$\delta^{13}C\ HCO_3^-\ \text{fixed} = -31.76\ ‰$$

$$\Delta = \text{enzyme fractionation} = -31.76\ ‰ - (-31.25\ ‰)$$

$$\Delta\ HCO_3^- = -0.51\ ‰$$

Substituting the values of experiment 2 Table I in these equations gives a value of Δ HCO_3^- = −3.55 ‰. An average of experiments 1 and 2 gives a value of Δ = −2.03 ‰ which agrees with the Δ HCO_3^- = −2.7 ‰ determined by Whelan *et al.* (23) for PEP carboxylase from sorghum leaves.

DISCUSSION

The range of $\delta^{13}C$ values for C_4 plants is −10 to −17 ‰ (17). Malate isolated from C_4 plants has a $\delta^{13}C$ value of −9.0 ‰ (22). A fractionation of −2 ‰ has occurred between atmospheric CO_2 and the fixed carbon compounds in the leaves. Possible mechanisms which cause the carbon isotope fractionation associated with photosynthesis in C_4 plants include: (*a*) fractionation associated with the absorption of CO_2 into the mesophyll cells; (*b*) equilibrium fractionation associated with atmospheric CO_2 and dissolved HCO_3^-; and (*c*) fractionation associated with the PEP carboxylase reaction.

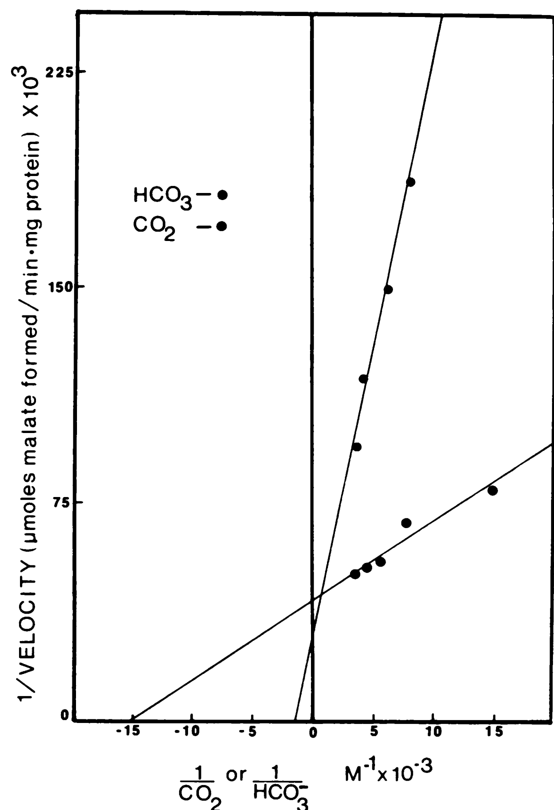

FIG. 4. Lineweaver-Burk double reciprocal plot of the reaction rates *versus* substrate concentrations of PEP carboxylase.

Table I. The $\delta^{13}C$ Values and Enzyme Fractionation by PEP Carboxylase from Corn Leaves

	Sample	$\delta^{13}C_{PDB}$	Enzyme Fractionation
1.	Malate	-22.31	
	PEP	-19.17	HCO_3^- = -0.51°/oo (from eqs. 1 & 2)
	HCO_3^-	-31.25	
2.	Malate	-23.31	
	PEP	-19.20	HCO_3^- =-3.55°/oo (from eqs. 1 & 2)
	HCO_3^-	-31.10	

Equation (1) 3/4 $\delta^{13}C$ PEP + 1/4 $\delta^{13}C$ HCO_3^- fixed = $\delta^{13}C$ malate

Equation (2) Δ HCO_3^- = enzyme fractionation = $\delta^{13}C$ HCO_3^- fixed − $\delta^{13}C$ HCO_3^- source

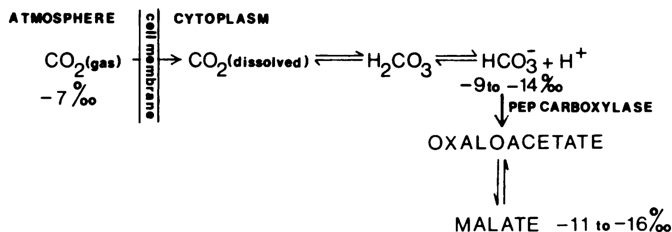

FIG. 5. Model for the fractionation of stable carbon isotopes of atmospheric CO_2 during CO_2 fixation in C_4 plants.

Atmospheric CO_2 has a general $\delta^{13}C$ value of −7 ‰. A fractionation of −2 to −7 ‰ is associated with dissolving CO_2 in a solution of hydroxide or cell sap (1, 6, 15, 16). This isotope effect between atmospheric CO_2 and dissolved HCO_3^- in $Ba(OH)_2$, NaOH, and leaf cytoplasm has been measured by Baertschi (1), Craig (6), and Park and Epstein (15, 16). The $\delta^{13}C$ value for dissolved HCO_3^- in leaf cytoplasm is −9 to −14 ‰ (15). In dissolving CO_2 into leaf cytoplasm, it is possible that exchange between dissolved HCO_3^- and atmospheric CO_2 can occur, resulting in an enrichment in ^{13}C in the dissolved HCO_3^-. Deuser and Degens (8) and Wendt (21) have established a $\delta^{13}C$ value of approximately 0 ‰ for dissolved HCO_3^- in equilibrium with atmosphere CO_2. The $\delta^{13}C$ value of dissolved HCO_3^- in leaf cytoplasm can therefore range between 0 to 14 ‰ depending on how rapidly CO_2 is fixed into malate. In steady-state photosynthetic CO_2 fixation in C_4 plants (with little equilibration of HCO_3^- and atmosphere CO_2), the $\delta^{13}C$ value of dissolved HCO_3^- is probably close to −9 to −14 ‰ which is the measured value of Park and Epstein (15).

The steps involved in the fractionation of stable carbon isotopes of atmospheric CO_2 during CO_2 fixation in C_4 plants are seen in Figure 5. There is a −2 to −7 ‰ fractionation associated with the absorption of atmospheric CO_2 and conversion to HCO_3^- in the cell cytoplasm. The dissolved HCO_3^- would have a $\delta^{13}C$ value between −9 to −14 ‰. A Δ HCO_3^- of −2.03 ‰ by PEP carboxylase would give malate with a $\delta^{13}C$ value of −11 to −16 ‰. These values are within the range of $\delta^{13}C$ values of C_4 plants (17).

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