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

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ABSTRACT

The carbon atoms of glucose and malate in C_4 plants are 2 to 3‰ enriched in ^{12}C with respect to atmospheric CO_2; whereas these intermediates in C_3 plants are 15 to 18‰ enriched with ^{12}C with respect to atmospheric CO_2. The enzymatic synthesis of malate from phosphoenolpyruvate and bicarbonate in preparations of leaves of *Sorghum bicolor, Haygrazer* result in a carbon isotope fractionation of about 3‰. The enzymatic synthesis of phosphoglyceric acid from ribulose 1,5-diP and CO_2 in these preparations (contaminated with carbonic anhydrase) at 24 C and 37 C result in a carbon isotope fractionation of 33.7‰ and 18.3‰, respectively. These data are consistent with the conclusion that the small enrichment of ^{12}C in the carbon atoms of malate and glucose (with respect to atmospheric CO_2) in leaves of *Sorghum bicolor, Haygrazer* occurs at the phosphoenolpyruvate carboxylase step.

Park and Epstein (14) have shown that carbon assimilation during photosynthesis results in the fractionation of the stable carbon isotopes ($^{12}_{6}C$ and $^{13}_{6}C$). These workers showed that the enzymatic formation of PGA[2] from RuDP and bicarbonate by crude preparations of RuDP carboxylase resulted in a carbon isotope fractionation of 17‰ from bicarbonate. Thus, the primary step resulting in ^{12}C enrichment of organic compounds during photosynthesis is the fixation of CO_2 by RuDP carboxylase.

Smith and Epstein (16) and Bender (2) have shown the total carbon of C_4 plants has a relatively higher amount of ^{13}C than C_3 plants. Whelan *et al.* (18) have determined the δ ^{13}C values of metabolic intermediates in C_3 and C_4 plants. The δ ^{13}C value of malate and glucose in C_4 plants suggest the primary fractionation of carbon in the PEP pathway occurs at the CO_2 fixation step and not during the transfer of the β-carboxyl group of malate to ribulose-1,5-diP. We would like to report that the cell-free enzymatic synthesis of malate from PEP and bicarbonate result in a fractionation of carbon into malic acid by 3‰ from bicarbonate. These data suggest the small enrichment of ^{12}C in the metabolic intermediates of tropical grasses (18) with respect to atmospheric CO_2 occurs at the PEP carboxylase step.

MATERIALS AND METHODS

Materials. Sodium phosphoenolpyruvate, tetrasodium ribulose-1,5-diP and NADH were obtained from Sigma Chemical Company. Sodium bicarbonate was obtained from J. T. Baker Chemical Company.

Plants. Plants of *Sorghum bicolor Haygrazer* were grown in the greenhouse in a College Station atmosphere.

Preparation of Soluble Leaf Protein. Ten grams of sorghum leaves were harvested, rinsed with distilled H_2O, blotted with paper toweling, and ground in a chilled mortar in 0.1 M tris buffer, pH 8.2, containing 0.1 M $NaHCO_3$, 0.1 mM GSH and sand. The suspension was squeezed through two layers of cheesecloth and centrifuged 30 min at 27,000*g* in a Sorvall refrigerated centrifuge. A saturated solution of $(NH_4)_2SO_4$ was added to the soluble supernatant fraction. The protein which precipitated between 0 to 65% of $(NH_4)_2SO_4$ fractionation was collected by centrifugation. The protein pellet was dissolved in 0.1 M tris buffer, pH 7.5, containing 0.1 M $NaHCO_3$ prior to the enzyme assays.

Enzyme Assays. PEP carboxylase was assayed by the procedure of Ting (17). The reaction mixtures contained in μmoles: 100, tris buffer, pH 8.5; 125, $NaHCO_3$; 0.25, NADH in 0.5 M $NaHCO_3$; 5, $MgCl_2$; 10, Na phosphoenolpyruvate; 0.5 mg of crystalline malic dehydrogenase, 0.1 ml of leaf protein, and H_2O to 3 ml. The reaction was followed by observing the reduction in absorbance at 340 nm in a Beckman Model DK recording spectrophotometer. Malate was separated from 100 pooled reaction tubes.

RuDP carboxylase was assayed by the procedure of Benedict and Kohel (3). The reaction mixture contained in μmoles: 100, tris buffer, pH 8.2; 525, $NaHCO_3$; 10, $MgCl_2$; 2.5, GSH; 2.0, ribulose-1,5-diP tetrasodium salt; 0.1 ml of leaf protein, and H_2O to 1.0 ml. The reaction was run 15 min. PGA was isolated from 25 pooled reaction tubes. The experiments involving the measurements of the enzymatic isotope effect were repeated two times, with 100 reaction tubes each time, for the PEP carboxylase

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[2] Abbreviations: PGA: 3-phosphoglyceric acid; RuDP: ribulose 1,5-diphosphate; PEP: phosphoenolpyruvate, PDB: The $^{13}C/^{12}C$ ratio in the samples is compared with a standard which is CO_2 from the fossil carbonate of *Belemnitella americana*.

reaction; and two times at 24 C, and two times at 37 C, (with 25 reaction tubes each time) for the RuDP carboxylase reaction.

The protein in each reaction tube was precipitated by immersing the tubes in boiling H_2O. The protein was collected by centrifugation. The clear reaction mixture was desalted by passing it through Dowex-50 (H^+) resin columns. The effluent was evaporated to dryness, and the residue dissolved in a few ml of H_2O, and the dissolved acids were absorbed on Dowex-1 columns. Malate and PGA was separated from the respective reaction tubes by elution of the acid from Dowex-1 (X-8) (formate) 200 mesh resin columns by a linear gradient of formic acid as described by Palmer (13) and Bartlett (1). A separate experiment was conducted to determine the amount of isotope fractionation of malate and PGA during the isolation procedure. Both acids were enriched 1.1‰ in ^{13}C during the isolation steps. A consideration is given to this error in calculating the enzymatic isotope effect.

Determination of Isotope Ratios. Carbon isotope ratios were made on a 60° sector, Nier type mass spectrometer similar to the one described by McKinney *et al.* (10). All of the samples were converted to CO_2 by combustion at 800 to 900 C over cupric oxide and in an excess O_2 atmosphere. The combustion products were circulated continuously by means of an electrically controlled Toepler pump. After removal of H_2O vapor and other condensable gases by passing through traps, cooled to Dry Ice temperatures, the CO_2 was distilled into a sample bulb at liquid N_2 temperatures.

The results are expressed in δ ^{13}C units as shown below:

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

The reference standard is the Chicago PDB limestone.

The active species of CO_2 in the PEP carboxylase and RuDP carboxylase has been shown to be bicarbonate (6) and CO_2, (5), respectively. The calculations of the enzymatic isotope effect are based on the δ ^{13}C value of dissolved CO_2 and bicarbonate in the reaction vessel.

RESULTS

Phosphoenolpyruvate Carboxylase. In the PEP pathway the incorporation of CO_2 into malate is catalyzed by phosphoenolpyruvate carboxylase and malate dehydrogenase. The isotope effect associated with this reaction in partially purified preparations of PEP carboxylase from sorghum leaves is shown in Table I. At pH 8.5 and 24 C, approximately 98% of the total CO_2 in the reaction mixture was in the form of HCO_3^-, about 2% was in the form of dissolved CO_2 and there was essentially no CO_3^{2-} (15). Thus, of the 17.5 mmoles of total inorganic carbon in the reaction mixture about 0.6 mmole are in the form of dissolved CO_2, and the remaining 16.9 mmoles are in the form of HCO_3^-. Thus for the 0.033 mmole of malate produced, 0.033 mmole of bicarbonate reacts with PEP. This is an insignificant fraction of the bicarbonate pool.

Table I. *Fractionation of Carbon Isotopes of Bicarbonate by Preparations of Phosphoenolpyruvate Carboxylase*

Each experiment was conducted at 24 C, pH 8.5.

Material	Experiment I			Experiment II		
	Begin	End	δ ^{13}C ‰ *vs.* PDB	Begin	End	δ ^{13}C ‰ *vs.* PDB
	mmole			*mmole*		
Phospho-enolpyru-vate	0.100	0.067	−21.2	0.100	0.069	−19.1
Malate	0.000	0.033	−23.9	0.000	0.031	−22.2
Bicarbonate	17.5	17.467	−29.1	17.5	17.469	−29.1

The procedure for calculating the enzymatic isotope effect is similar to that described by Park and Epstein (14) and is as follows: one-fourth of the carbon atoms in malate produced in the reaction mixture was derived from bicarbonate ions; the remaining three-fourths of the carbon atoms in malate are derived from PEP. Thus, the δ ^{13}C value for the carbon fixed into malate is given by the equations:

$$\tfrac{1}{4}\ \delta\ ^{13}C\ CO_2 \text{ fixed} + \tfrac{3}{4}\ \delta\ ^{13}C \text{ PEP} = \delta\ ^{13}C \text{ malate}$$

and substituting the data from experiment I (Table I)

$$\delta\ ^{13}C\ CO_2 \text{ fixed} = -32.0‰$$

and

$$\Delta = \delta\ ^{13}C \text{ fixed } CO_2 - \delta\ ^{13}C\ HCO_3^-$$
$$\Delta = -32.0 - (-29.1) = -2.9‰$$

Substitution of the data in experiment II in Table I

$$\delta\ ^{13}C\ CO_2 \text{ fixed} = -31.6‰$$

and

$$\Delta = -2.5‰$$

A sample of malate taken through the isolation procedure had an isotopic composition 1.1‰ different from the starting material. A 1.1‰ variability in the purified malate would result in a 4.4‰ variation in the calculated fractionation. Using the average value of the two experiments, the value for the carbon isotope fractionation associated with the fixation of carbon in the PEP Pathway is -2.7 ± 4.4‰.

Ribulose-1,5-diP Carboxylase. To obtain a complete picture of the enzymatic isotope effects of carboxylases in sorghum extracts, we determined the carbon isotope fractionation of the RuDP carboxylase reaction. Our results, along with the results of Park and Epstein (14) are shown in Table II. At pH 8.2 and 24 C, approximately 96% of the total CO_2 in the reaction mixture was in the form of HCO_3^-, about 4% was in the form of CO_2 and there was essentially no CO_3^{2-}. Thus, from 13.2 mmoles of total inorganic carbon, 528 μmoles of dissolved CO_2 was immediately available for reaction with RuDP to form PGA.

The δ ^{13}C value for the carbon fixed into phosphoglyceric acid is given by the following equations:

$$\tfrac{5}{6}\ \delta\ ^{13}C \text{ RuDP} + \tfrac{1}{6}\ \delta\ ^{13}C\ CO_2 \text{ fixed} = \delta\ ^{13}\text{PGA}$$

Substituting the data for Experiment I at 24 C (Table II),

$$\delta\ ^{13}C\ CO_2 \text{ fixed} = -72.0‰$$

and by definition

$$\Delta = \delta\ ^{13}C\ CO_2 \text{ fixed} - \delta\ ^{13}C\ HCO_3^- = \text{enzyme isotope fractionation}$$
$$\Delta = -42.9‰$$

Since the isolation procedure for PGA resulted in a 1.1‰ difference between starting material and product a ± 6.6‰ error must be included in the enzymatic isotope effect, $\Delta = -42.9 \pm 6.6$‰ at 24 C, whereas $\Delta = -17.4$‰ from Park and Epstein's data. Mook (11) has shown that the dissolved CO_2 in isotopic equilibrium with a bicarbonate solution is enriched in ^{12}C by 9.2‰. Therefore $\Delta = -33.7 \pm 6.6$‰ at 24 C with respect to the dissolved CO_2 in the reaction mixture, and $\Delta = -8.2$‰ from Park and Epstein's data. For experiment II (Table II) the carbon isotope effect at 37C is: Δ ^{13}C HCO_3^- fixed $= -26.1$‰ and using a 7.8‰ fractionation between CO_2 (aq) and HCO_3^- (11) Δ $^{13}C = -18.3$‰ at 37 C with respect to the dissolved CO_2 in the reaction mixture.

Table II. *Fractionation of Carbon Isotopes of CO_2 by Preparations of Ribulose-1,5-diP Carboxylase*

Material	Experiment I (24 C, pH 8.2)			Experiment II (37 C, pH 8.2)			Park and Epstein's (14) Data (24 C, pH 7.4)
	Begin	End	δ ^{13}C ‰ vs. PDB	Begin	End	δ ^{13}C ‰ vs. PDB	δ ^{13}C ‰ vs. PDB
	mmole			*mmole*			
Ribulose-1,5-diP	0.050	0.043	−12.0	0.050	0.041	−12.0	−13.7
Total CO_2	13.120	13.005	−29.1	13.120	13.001	−29.1	−5.4
Phosphoglycerate	0.000	0.015	−22.0	0.000	0.019	−19.2	−15.2

DISCUSSION

Park and Epstein (14) have found that the δ ^{13}C values of carbon compounds of C_3 plants are −25 to −35. These plants have a higher abundance of ^{12}C than ^{13}C. This study demonstrated further that isolated preparations of RuDP carboxylase fractionated bicarbonate into PGA by 17‰ at 24 C. These workers postulated a 7‰ fractionation of CO_2, as the gas is absorbed by the wet cell walls of mesophyll or palisade cells. Thus, the total fractionation of the carbon isotopes of CO_2 during photosynthesis would be −7‰ + −17‰ = −24‰ which agrees with the δ ^{13}C of −25 to −35 of the carbon compounds of C_3 plants.

Since CO_2 is the active species of the RuDP carboxylase reaction (5), the calculation of isotope fractionation of CO_2 into PGA catalyzed by RuDP carboxylase must be calculated from dissolved CO_2 and not HCO_3^- as was originally done by Park and Epstein (14). If the isotope fractionation of Park and Epstein's data is recalculated, the amount of fractionation is 8.2‰ and not 18‰. It has also been demonstrated that no significant carbon isotope fractionation exists between atmospheric CO_2 and dissolved CO_2 (7, 11, 19). The large 7‰ enrichment in cytoplasmic CO_2 from gaseous CO_2 which was postulated in the Park and Epstein model (14) is doubtful. The 8.2‰ fractionation of CO_2 into PGA in the absence of any kinetic effect will not account for the observed δ ^{13}C composition of C_3 plants. The results in this paper show that the fractionation of CO_2 into PGA by preparations of RuDP carboxylase at 24 C and 37 C is 33.7 and 18‰, respectively. The isotope fractionation at this enzymatic step probably accounts for the δ ^{13}C value of most C_3 plants, which grow at summer temperatures over 30 C, since the amount of carbon fractionation from gaseous CO_2 (δ ^{13}C = −7) to carbon intermediates (δ ^{13}C = −25) is 18‰. These data also substantiate that the isotope fractionation of 18‰ at the RuDP carboxylase CO_2 fixation step at 37 C does not account for the small amount of fractionation of carbon isotopes of CO_2 found in C_4 plants.

Smith and Epstein (16) and Bender (2) have shown the total carbon of C_4 plants have a δ ^{13}C values of −9 to −14. Whelan *et al.* (18) have shown that the δ ^{13}C values of metabolic intermediates of C_4 plants are −9 to −14. The δ ^{13}C values of metabolic intermediates of C_4 plants in respect to PDB is −9 to −14 and in respect to gaseous CO_2 −2 to −7. The results in this paper show that preparations of PEP carboxylase fractionate bicarbonate into malate by 2.5 to 3.3‰. There is a known fractionation of the isotopes of carbon as gaseous CO_2 is absorbed by H_2O and converted to HCO_3^- of 6‰ to 9‰ (7, 11, 19). Thus the total fractionation of isotopes of carbon of atmospheric CO_2 during photosynthesis in C_4 plants would be about −7‰ + (−2.5‰) or −7‰ + (−3.3‰) = −9.5‰ to −10.3‰ which agrees with the δ ^{13}C value of −9 to −14 of the carbon intermediates of these plants. These results show that the relatively small fractionation of ^{12}C into the metabolic intermediates of C_4 plants could be accounted for by the fractionation during the conversion of CO_2 to HCO_3^- and the small fractionation of bicarbonate into malic acid catalyzed by PEP carboxylase.

This view, concerning the step which predominantly determines the δ ^{13}C value of carbon intermediates in C_4 plants is not without difficulties. First, Edwards and Black (8) have shown that 15% of atmospheric CO_2 entering the leaf air spaces of crabgrass enters the bundle sheath cells and is assimilated through the photosynthetic reductive pentose phosphate cycle. Second, O'Neal *et al.* (12) have also challenged Hatch and Slack's view (9) that PEP is the sole primary recipient of atmospheric CO_2 in tropical grasses. In isolated chloroplasts of young corn leaves, the primary product of CO_2 fixation was found to be PGA. Radioactive malate was found to accumulate from $^{14}CO_2$ fixation in intact leaves, but these workers view the role of C_4 acids as storehouses for CO_2 in tropical grasses. The CO_2 in malate may be subsequently released by decarboxylation and refixed into PGA by RuDP carboxylase.

If the primary CO_2 fixation step in tropical grasses is RuDP carboxylase, then all of the carbon intermediates including glucose and malate should have δ ^{13}C values similar to plants possessing the reductive pentose phosphate cycle. This, of course, has not been found to be the case (2, 16, 18). If, on the other hand, the route of carbon in C_4 plants is $CO_2 \xrightarrow{PEP}$ malate $\xrightarrow{C_3}$ $CO_2 \xrightarrow{RuDP}$ PGA there should be two primary CO_2 fixation steps for the discrimination of carbon isotopes of CO_2 namely: PEP carboxylase and RuDP carboxylase. Since, as we have shown in this paper, the carbon isotope fractionation by preparations of PEP and RuDP carboxylase is 3‰ and 18‰, it follows that RuDP carboxylase in this sequence of reactions should be the step which determines the isotopic composition of the carbon intermediates. Again, if this latter sequence of reactions were operable in C_4 plants the δ ^{13}C values for the carbon intermediates would resemble C_3 plants. However, PEP carboxylase would determine the δ ^{13}C value of the intermediates in C_4 plants in the above reactions, if all of the CO_2 which is fixed into malate is transferred (or decarboxylated to CO_2 and refixed) to RuDP carboxylase. There would be no isotope discrimination of the CO_2 from the β-COOH group of malate at the RuDP carboxylase step. The δ ^{13}C values of all the carbon intermediates would be determined at the PEP carboxylase step. Some support for the idea that carbon flow through RuDP carboxylase and PEP carboxylase earmark the δ ^{13}C content of carbon intermediates, is the work of Black's (4) on Crassulacean acid metabolism plants. The δ ^{13}C values of a single Crassulacean acid metabolism plant can be shifted, from those of a C_4 plant to those of a C_3 plant by simply changing the environment. The shift in δ ^{13}C content indicates that CO_2 fixation occurs at the PEP carboxylase or RuDP carboxylase step.

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