Enzymatic Fractionation of Carbon Isotopes by Phosphoenolpyruvate Carboxylase from C₄ Plants

Received for publication January 29, 1973

TOM WHELAN and W. M. SACKETT
Department of Oceanography, Texas A&M University, College Station, Texas 77843

C. R. BENEDICT
Department of Plant Sciences, Texas A&M University, College Station, Texas 77843

ABSTRACT

The carbon atoms of glucose and malate in C₄ plants are 2 to 3% enriched in ¹³C with respect to atmospheric CO₂; whereas these intermediates in C₃ plants are 15 to 18% enriched with ¹³C with respect to atmospheric CO₂. The enzymatic synthesis of malate from phosphoenolpyruvate and bicarbonate in preparations of leaves of Sorghum bicolor, Haygrazer results in a carbon isotope fractionation of about 3%. The enzymatic synthesis of phosphoglyceric acid from ribulose-1,5-diP and CO₂ in these preparations (contaminated with carbonic anhydrase) at 24°C and 37°C results in a carbon isotope fractionation of 23.6% and 18.3%, respectively. These data are consistent with the conclusion that the small enrichment of ¹³C in the carbon atoms of malate and glucose (with respect to atmospheric CO₂) 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 (¹³C and ¹²C). These workers showed that the enzymatic formation of PGA² 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 ¹³C enrichment of organic compounds during photosynthesis is the fixation of CO₂ by RuDP carboxylase.

Smith and Epstein (16) and Bender (2) have shown the total carbon of C₄ plants has a relatively higher amount of ¹³C than C₃ plants. Whelan et al. (18) have determined the δ¹³C values of metabolic intermediates in C₃ and C₄ plants. The δ¹³C value of malate and glucose in C₄ plants suggest the primary fractionation of carbon in the PEP pathway occurs at the CO₂ 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 results in a fractionation of carbon into malic acid by 3% from bicarbonate. These data suggest the small enrichment of ¹³C in the metabolic intermediates of tropical grasses (18) with respect to atmospheric CO₂ 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 a greenhouse at a College Station atmosphere.

Preparation of Soluble Leaf Protein. Ten grams of sorghum leaves were harvested, rinsed with distilled H₂O, blotted with paper towels, and ground in a chilled mortar in 0.1 M tris buffer, pH 8.2, containing 0.1 M NaHCO₃, 0.1 M GSH and sand. The suspension was sieved through two layers of cheesecloth and centrifuged 30 min at 27,000g. A saturated solution of (NH₄)₂SO₄ was added to the soluble supernatant fraction. The protein which precipitated between 0 to 65% of (NH₄)₂SO₄ fractionation was collected by centrifugation. The protein pellet was dissolved in 0.1 M tris buffer, pH 7.5, containing 0.1 M NaHCO₃ 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 8.2; 125, NaHCO₃; 0.25, NADH in 0.5 M NaHCO₃; 5, MgCl₂; 10, Na phosphoenolpyruvate; 0.5 mg of crystalline malic dehydrogenase, 0.1 ml of leaf protein, and H₂O 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, NaHCO₃; 10, MgCl₂; 2.5, GSH; 2.0, ribulose-1,5-diP tetrasodium salt; 0.1 ml of leaf protein, and H₂O 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

Acknowledgement is made to the donors of the Petroleum Research Fund, administered to the American Chemical Society, for partial support of this research and to the Robert A. Welch Foundation for partial support of this research by grants to W.M.S. and C.R.B.

¹ Abbreviations: PGA: 3-phosphoglyceric acid; RuDP: ribulose-1,5-diphosphate; PEP: phosphoenolpyruvate, PDB: The ¹³C/¹²C ratio in the samples is compared with a standard which is CO₂ 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 H2O. 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 H20, 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 CO2 by combustion at 800 to 900 C over cupric oxide and in an excess O2 atmosphere. The combustion products were circulated continuously by means of an electrically controlled Tcooiler pump. After removal of H2O vapor and other condensable gases by passing through traps, cooled to Dry Ice temperatures, the CO2 was distilled into a sample bulb at liquid N2 temperatures.

The results are expressed in $\delta^{13}$C units as shown below:

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

The reference standard is the Chicago PDB limestone.

The active species of CO2 in the PEP carboxylase and RuDP carboxylase has been shown to be bicarbonate (6) and CO3, (5), respectively. The calculations of the enzymatic isotope effect are based on the $\delta^{13}$C value of dissolved CO2 and bicarbonate in the reaction vessel.

**RESULTS**

**Phosphoenolpyruvate Carboxylase.** In the PEP pathway the incorporation of CO2 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 CO2 in the reaction mixture was in the form of HCO3-, about 2% was in the form of dissolved CO2 and there was essentially no CO32- (15). Thus, of the 17.5 mmole of total inorganic carbon in the reaction mixture about 0.6 mmole are in the form of dissolved CO2 and the remaining 16.9 mmole are in the form of HCO3-. 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.

The procedure for calculating the enzymatic isotope effect is similar to that described by Park and Epstein (14) and as is 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 $\delta^{13}$C value for the carbon fixed into malate is given by the equations:

$$\delta^{13}C_{\text{Malate}} = \delta^{13}C_{\text{CO2}} + \delta^{13}C_{\text{PGA}}$$

and substituting the data from experiment I (Table I)

$$\delta^{13}C_{\text{CO2}} = \frac{-32.0}{\%	ext{e}}$$

and

$$\Delta = \delta^{13}C_{\text{CO2}} - \delta^{13}C_{\text{PGA}}$$

Substitution of the data in experiment II in Table I

$$\delta^{13}C_{\text{CO2}} = \frac{-31.6}{\%	ext{e}}$$

and

$$\Delta = \frac{-2.5}{\%	ext{e}}$$

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 $-27 \pm 4.4\%$.

**Ribulose-1,5-diPhosphate 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 CO2 in the reaction mixture was in the form of HCO3-, about 4% was in the form of CO2 and there was essentially no CO32-. Thus, from 13.2 mmole of total inorganic carbon, 528 mmole of dissolved CO2 was immediately available for reaction with RuDP to form PGA.

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

$$\delta^{13}C_{\text{PGA}} = \delta^{13}C_{\text{RuDP}} + \delta^{13}C_{\text{CO2}}$$

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

$$\delta^{13}C_{\text{CO2}} = \frac{-72.0}{\%	ext{e}}$$

and by definition

$$\Delta = \delta^{13}C_{\text{CO2}} - \delta^{13}C_{\text{PGA}}$$

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 CO2 in isotopic equilibrium with a bicarbonate solution is enriched in $^{13}$C by 9.2%. Therefore $\Delta = -33.7 \pm 6.6\%$ at 24 C with respect to the dissolved CO2 in the reaction mixture, and $\Delta = -8.2\%$ from Park and Epstein's data. For experiment II (Table II) the carbon isotope effect at 37 C is: $\Delta^{13}C_{\text{HCO3-}}$ fixed = $-26.1\%$ and using a 7.8% fractionation between CO2 (aq) and HCO3- (11) $\Delta^{13}C = -18.3\%$ at 37 C with respect to the dissolved CO2 in the reaction mixture.

<table>
<thead>
<tr>
<th>Material</th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Begin</td>
<td>End</td>
</tr>
<tr>
<td>Phospho-enolpyruvate</td>
<td>0.100</td>
<td>0.067</td>
</tr>
<tr>
<td>Malate</td>
<td>0.000</td>
<td>0.033</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>17.5</td>
<td>17.467</td>
</tr>
</tbody>
</table>
fractionation of $^{13}C$ 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 $\delta^{13}C$ 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 $^{13}$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 $\delta^{13}C$ 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$_2$ $\rightarrow$ PEPC $\rightarrow$ PGA there should be two primary CO$_2$ fixation steps for the discrimination of carbon isotopes of CO$_2$, namely: PEPC carboxylase and RuDP carboxylase. Since, as we have shown in this paper, the carbon isotope fractionation by preparations of PEPC and RuDP carboxylase is $3^{13}C$ and $18^{13}C$, 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 $\delta^{13}C$ values for the carbon intermediates would resemble C$_4$ plants. However, PEPC carboxylase would determine the $\delta^{13}C$ 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 isotopic discrimination of the CO$_2$ from the $\beta$-COOH group of malate at the RuDP carboxylase step. The $\delta^{13}C$ values of all the carbon intermediates would be determined at the PEPC carboxylase step. Some support for the idea that carbon flow through RuDP carboxylase and PEPC carboxylase earmark the $\delta^{13}C$ content of carbon intermediates, is the work of Black's (4) on Crasulacean acid metabolism plants. The $\delta^{13}C$ values of a single Crasulacean 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 $\delta^{13}C$ content indicates that CO$_2$ fixation occurs at the PEPC carboxylase or RuDP carboxylase step.

### LITERATURE CITED