Enzymic Fractionation of the Stable Carbon Isotopes of Carbon Dioxide by Ribulose-1,5-bisphosphate Carboxylase

Received for publication September 7, 1978 and in revised form November 15, 1978

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ABSTRACT

The enzymic fractionation of the stable carbon isotopes of CO₂ (Δ₁₂C) was determined using a purified preparation of ribulose-1,5-bisphosphate (RuBP) carboxylase isolated from cotton (a C₃ plant) leaves. The bicarbonate concentration in the reaction mixture saturated the enzyme and furnished an infinite pool of ¹³CO₂ and ¹²CO₂ for enzyme fractionation. The RuBP was 96 to 98% pure. The phosphoglycerate synthesized in the reaction mixtures was purified free of RuBP, phosphoglycerol, and other phosphate esters by column chromatography on Dowex 1-Cl⁻ resin. The average Δ₁₂C value of -27.1% was determined from five separate experiments. A discussion of the isotope fractionation associated with photosynthetic CO₂ fixation in plants shows that the enzymic fractionation of stable carbon isotopes of CO₂ by RuBP carboxylase is of major importance in determining the δ¹³C values of C₃ plants.

Photosynthesis is accompanied by a fractionation of the stable carbon isotopes of CO₂. This discrimination favors the fixation of the ¹³CO₂ into plant material (5, 10, 15, 21, 22) and it is now known that the δ¹³C values of plants is highly correlated with the presence of C₃ and C₄ photosynthesis (5, 23, 24).

Park and Epstein (22) were the first to show that ribulose-1,5-bisphosphate carboxylase preferentially fixed ¹³CO₂ into phosphoglycerate and accounted for the ¹³C/¹²C ratio of plants. This enzyme isotope fractionation (Δ₁₂C) of -17% minus the δ¹³C value of -7% for atmospheric CO₂ gave a predicted δ¹³C value of -24% for plants with a Calvin cycle. This predicted value is within the range of δ¹³C values for C₃ plants. However, as pointed out by Whelan et al. (26), these workers were not aware that CO₂ was the active species of “CO₂” utilized by RuBP carboxylase and actually calculated the enzyme fractionation of stable carbon isotopes of bicarbonate (Δ₁₂Cbic). They did not correct their data for the equilibrium isotope effect for HCO₃⁻ ↔ CO₂ + H⁺. This equilibrium isotope effect results in CO₂ being 8.4% more depleted in ¹³CO₂ at 25 C (25) than HCO₃⁻. Had the authors made the appropriate correction, the predicted value for C₃ plants would be -15.6%. This value will not account for the ¹³C content observed in C₃ plants. Subsequent work has shown that the Δ₁₂C values for RuBP carboxylase are: -18.3% (26), -28.3% (8), -38.8 to -89.2% (13) and -28.3 to -41.7% (16). This wide range of Δ₁₂C values further questions the importance of RuBP carboxylase in the overall isotopic fractionation of CO₂ during photosynthesis in C₃ plants. In much of this work, variations in the Δ₁₂C values may be due to the use of impure RuBP and/or the use of the total organic acid fraction from the enzyme experiments for the determination of the δ¹³C value of the PGA. Here, we re-determine the Δ₁₂C values using a purified RuBP carboxylase, 96 to 98% pure RuBP, and separating the synthesized PGA free of P-glycolate and RuBP before determining the δ¹³C value of PGA. This work shows that RuBP carboxylase plays a predominant role in determining the δ¹³C value of C₃ plants and confirms the Δ₁₂C values for RuBP carboxylase determined by Whelan et al. (26), Christi et al. (8), and Estep et al. (16).

MATERIALS AND METHODS

Chemicals. Phosphoglycerate, RuBP tetrasodium salt, phosphoglycerol, and 2,3-diphosphoglycerate were purchased from Sigma Chemical. NaH¹³CO₃ was purchased from New England Nuclear.

Plants. Cotton plants were grown in a greenhouse in a mixture of Vermiculite and sand. Prior to flowering, the leaves were harvested for the isolation of RuBP carboxylase.

Enzyme Isolation and Purification. Cotton leaves were harvested, deined, and rinsed with deionized H₂O. The leaves were homogenized in a Sears blender, model Insta Blend, at a “liquefy” speed for 60 s in a 0.1 M Tris-HCl (pH 7.5) containing 0.1 M GSH, 1% Triton X-100, and 12% Dowex 1-X8 Chloride (100–200 mesh). The Dowex 1 resin was prepared with Triton X-100 and used as a detergent was prepared with deionized H₂O until the effluent was clear followed by equilibrating with 0.1 M Tris-HCl (pH 7.5) containing 0.1 M GSH. The homogenizing medium contained 8 ml buffer/g fresh weight leaves. Following homogenization the brei was filtered through four layers of cheesecloth and centrifuged at 15,000 rpm for 30 min in a Sorvall refrigerated centrifuge. The soluble supernatant was fractionated with solid (NH₄)₂SO₄. The protein which precipitated between 40 to 50% of saturation with (NH₄)₂SO₄ was collected by centrifugation and dissolved in a minimal amount of 0.1 M Tris-HCl (pH 7.5) containing 0.1 M GSH. The protein was desalted by passing through a column (2 x 30 cm) of Sephadex G-25 (coarse, 100- to 300-μm particle size) which had been equilibrated with 0.01 M Tris-HCl (pH 7.5) containing 0.1 M GSH. The protein eluted in the void volume was adsorbed onto a column (2 x 20 cm) of DEAE-cellulose which had been equilibrated with 0.01 M Tris-HCl (pH 7.5) containing 0.1 M GSH. The protein eluted in the void volume was adsorbed onto a column (2 x 20 cm) of DEAE-cellulose which had been equilibrated with 0.01 M Tris-HCl (pH 7.5) containing 0.1 M GSH. The protein was eluted from the column by stepwise increases in NaCl in 0.01 M Tris-HCl (pH 7.5) containing 0.1 M GSH. RuBP carboxylase was eluted from the column with 0.2 M NaCl.

Disc Gel Electrophoresis. The homogeneity of the RuBP carboxylase eluted from the DEAE-cellulose column with 0.2 M NaCl was determined by the electrophoretic technique described by Davis (12). The purified RuBP carboxylase consisted of a single electrophoretic component (Fig. 1). The width of the protein band and the mobility of the carboxylase in the gel were similar to those described by Andrews et al. (1), Givan and Criddel (17),

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1 This research was supported in part by the Texas Agricultural Experiment Station and The Robert A. Welch Foundation Research Grant A-482.
2 Abbreviations: RuBP: ribulose-1,5-bisphosphate; PGA: 3-phosphoglycerate; Δ₁₂C: enzymic fractionation of stable carbon isotopes of CO₂; BSC: bundle sheath cells of C₃ plants.
The RuBP used to synthesize PGA in these experiments was further purified free of ribose-5-P, ribulose-5-P, and xylulose-5-P by column chromatography. The tetrasodium salt of RuBP was passed through a Dowex 50-H⁻ resin column. The eluate was lyophilized, the residue dissolved in H₂O, and the RuBP adsorbed onto a Dowex 1-Cl⁻ resin column (1 × 30 cm). The RuBP was chromatographically separated from the other phosphate esters with a concave gradient of HCl described in the section on the chromatographic purification of PGA. The tubes containing the RuBP were collected and pooled and the HCl removed by lyophilization. The RuBP was stored 1 to 2 days at −20°C before use.

The RuBP eluted from the column was located by assaying an aliquot of the eluate for organic phosphate by the method of Bartlett (4). The δ¹³C value of the RuBP before and after column chromatography was −14.8‰ and −14.2‰, respectively. There was a +0.6‰ fractionation of the RuBP accompanying the purification. This would result in an error of +2.4‰ in the determination of the ΔCO₂ for RuBP carboxylase.

Enzymic Synthesis of PGA. PGA was enzymically synthesized in five separate experiments followed by chromatographic purification of the PGA and determination of the ¹³C/¹²C content. Each of the five experiments consisted of 50 individual reaction mixtures containing: 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2.5 mM GSH, 50 mM KHCO₃, and 37 to 107 μg of purified RuBP carboxylase to a final volume of 0.5 ml. Each reaction mixture was preincubated for 10 min at 35°C and 1 mM RuBP was added followed by an additional 10-min incubation at 35°C. The reaction was stopped with concentrated HCl. The specific activity of the purified cotton RuBP carboxylase used in the experiments varied between 582 to 1571 nmol PGA formed/mg protein-min. For each of the five experiments, 50 reaction tubes were run and following the addition of HCl these were pooled and the reaction mixtures lyophilized.

Chromatographic Purification of PGA. The above lyophilized powders were dissolved in a few ml of H₂O and passed through a column (2.3 × 15 cm) of Dowex 50-H⁻ resin. The eluate was lyophilized and adsorbed onto a column (1 × 30 cm) of Dowex 1-Cl⁻ resin. PGA was separated from other phosphate esters in the reaction mixture by developing the column with a concave gradient created by putting 250 ml of 0.02 N HCl in a first and second reservoir and 250 ml of 0.20 N HCl in a third reservoir. The chromatographic separation of PGA, P-glycolate, RuBP, and 2,3-diphosphoglyceric acid by this method is shown in Figure 2. Authentic PGA and other phosphate esters were located in the tubes containing the eluate by the phosphorous method of Bartlett (4). PGA in the fraction tubes was further identified by a colorimetric assay for phosphorlated glycic acid (3). The fraction tubes containing the synthesized PGA were pooled, lyophilized, and stored at −20°C until it was combusted to CO₂. To test the amount of isotopic fractionation associated with the purification of enzymically synthesized PGA by this procedure, the δ¹³C values of authentic PGA were determined to be −21.1‰ and −20.8‰ before and after purification. This would lead to an error of +1.8‰ in the determination of the ΔCO₂ for RuBP carboxylase. There was little isotope fractionation of PGA during this procedure for the extraction and chromatographic separation of enzymically synthesized PGA.

Mass Spectrometric Analysis. PGA and RuBP were converted to CO₂ by combustion at 800 to 900°C in an excess of O₂ in a precombusted organic-free combustion boat. The combustion apparatus was similar to that described by Craig (9). Water vapor was removed by isopropyl alcohol dry ice traps. Nitrogen oxides were converted to molecular nitrogen by passing the combustion products through copper turnings at 400°C. The nitrogen was pumped away, and CO₂ was collected in a sample bulb at a liquid N₂ temperature. Bicarbonate was converted to CO₂ with the addition of 85% H₃PO₄ after the evacuation of the atmospheric gases from the reaction vessel. Again, the CO₂ was collected in a...
sample bulb at a liquid N\textsubscript{2} temperature. The purified CO\textsubscript{2} was analyzed with a Nuclide Corporation, model RMS, 15.24-cm 60° sector field mass spectrometer. The results are expressed in \(\delta^{13}C\) values. Corrections for gas mixing, background peaks, mass 44 tailing, and \(^{17}O\) contribution to mass 45 were made according to the method of Craig (11). \(\delta^{13}C\) was defined as follows:

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

The working standard was a powdered charcoal sample of Norit which has a \(\delta^{13}C\) value of -24.8%o versus the Chicago PDB-1 standard. The PDB standard is a cretaceous belemnite from the Peedee formation of South Carolina (11).

FIG. 2. Chromatography of organic acids on Dowex 1-formate columns. Acids were eluted from the column at a flow rate of 1 ml/min.

Table I
Fractionation of \(^{12}\text{CO}_2 - ^{13}\text{CO}_2\) by RuBP Carboxylase Purified from Cotton Leaves

All experiments were carried out at 35 C. The specific activities of the RuBP carboxylase varied from 582 to 1571 nmol HC\textsubscript{3}O\textsubscript{3} / mg protein \texttimes\textmin.

\[
\begin{array}{|c|c|c|c|c|}
\hline
& \text{I} & \text{II} & \text{III} & \text{IV} & \text{V} \\
\hline
\delta^{13}C & -13.9 & -13.9 & -13.9 & -14.3 & -14.3 \\
\hline
\text{Enzymatic Assay} & 96 & 96 & 96 & 98 & 98 \\
\text{Phosphorus Assay} & 95 & 95 & 95 & 98 & 98 \\
\text{PGA Recovery} (\%) & 88 & 103 & 106 & 81 & 73 \\
\text{HCO}_3^- & -34.0 & -30.8 & -30.8 & -33.4 & -33.4 \\
\text{CO}_2 Disolved & -42.0 & -38.8 & -38.8 & -41.4 & -41.4 \\
\text{3-PGA} & -24.0 & -22.4 & -21.9 & -23.5 & -23.1 \\
\text{CO}_2 Fixed & -74.5 & -64.9 & -61.9 & -65.0 & -67.3 \\
\delta^{13}C, \%o & -32.5 & -26.1 & -23.1 & -28.1 & -25.8 \\
\text{Average} \delta^{13}C & -27.1 \\
\hline
\end{array}
\]

1 Percent of theoretical enzymatically synthesized PGA recovered in the reaction mixtures.

2 \(\delta^{13}C\) of CO\textsubscript{2} dissolved = \(\delta^{13}C\) of HCO\textsubscript{3} dissolved - (10.2 - 0.064 \times T)

3 \(\delta^{13}C\) of CO\textsubscript{2} fixed = 6(\(\delta^{13}C\) of 3-PGA) - 5(\(\delta^{13}C\) of RuBP)

4 \(\delta^{13}C\) (\%o) = \(\delta^{13}C\) of CO\textsubscript{2} fixed - \(\delta^{13}C\) of CO\textsubscript{2} dissolved

RESULTS
The \(\delta^{13}C\) values of the substrates and products of the RuBP carboxylase reaction are shown in Table I. Each of the five experiments consisted of 50 reaction tubes containing a concentration of substrates and metal to give an optimum rate of carboxylation. The enzymic fractionation of the stable carbon isotopes of CO\textsubscript{2} (\(\Delta^{13}C\)) by RuBP carboxylase is calculated as follows:

\[
\% \delta^{13}C \text{ RuBP} + \% \delta^{13}C \text{ CO}_2 \text{ fixed} = \delta^{13}C \text{ PGA}
\]

substituting \(\delta^{13}C\) values from experiment I

\[
\% \delta^{13}C (\%o) + \% \delta^{13}C \text{ CO}_2 \text{ fixed} = \delta^{13}C \text{ PGA}
\]

\(\delta^{13}C \text{ CO}_2 \text{ fixed} = -74.5\%o

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\[ \Delta_{\text{CO}_2} = \delta^{13}\text{C} \text{ CO}_2 \text{ fixed} - \delta^{13}\text{C} \text{ dissolved CO}_2 \]

\[ \delta^{13}\text{C} \text{ dissolved CO}_2 = \delta^{13}\text{C} \text{ HCO}_3^- - (10.2 - 0.064 \times T) \]

where \( T \) is the temperature in centigrade (20, 25)

\[ \delta^{13}\text{C} \text{ dissolved CO}_2 = -42.0\%o \]

substituting

\[ \Delta_{\text{CO}_2} = (-74.5\%o) - (-42.0\%o) = -32.5\%o \]

The average \( \Delta_{\text{CO}_2} \) value calculated in a similar manner for all five experiments is \(-27.1\%o\).

In the above experiments, the HCO\(_3^-\) concentration (50 mM) insured enzyme saturation and provided an infinite pool (2.55 mM) of dissolved \(^{12}\text{CO}_2\) and \(^{13}\text{CO}_2\) so that maximal enzymatic fractionation of stable carbon isotopes of CO\(_2\) would occur during the carboxylation reaction. The \( \delta^{13}\text{C} \) value of the HCO\(_3^-\) shown in each experiment (Table I) was determined by acidifying an aliquot of the reaction mixtures. Since the HCO\(_3^-\) in the buffer of unknown isotopic composition would mix with the HCO\(_3^-\) from the reagent bottle, this assured that the \( \delta^{13}\text{C} \) value for HCO\(_3^-\) represented the HCO\(_3^-\) in the reaction mixture and not the HCO\(_3^-\) from the reagent bottle as is often reported. The purity of the RuBP used in these experiments varied between 96 and 98%. In experiments IV and V, the 98% pure RuBP obtained from Sigma Chemical was further purified by column chromatography on Dowex 1-Cl\(^-\) resins prior to its use in the reaction mixtures. The high purity of the RuBP in these experiments assured the accuracy of the \( \delta^{13}\text{C} \) value of the substrate RuBP. From the specific activity of the purified RuBP carboxylase and the time of incubation of the reaction mixtures, the theoretical amount of PGA enzymically synthesized was calculated for each experiment. The per cent recovery of the theoretical amount of PGA synthesized is shown in Table I and varies between 73 and 106% with an average recovery of 90.2%. A high percentage of PGA recovery is critical in determining the \( \delta^{13}\text{C} \) value of the synthesized PGA. In experiments IV and V, the unreacted RuBP was recovered from the reaction mixtures by chromatography on Dowex 1-Cl\(^-\) resins and the \( \delta^{13}\text{C} \) value was determined to be \(-14.0\%o\). This value compared to the starting \( \delta^{13}\text{C} \) value for RuBP of \(-14.3\%o\) shows that there is little enzymic fractionation of RuBP during the carboxylation reaction.

**DISCUSSION**

The average enzymic fractionation of the stable carbon isotopes of CO\(_2\) by RuBP carboxylase determined in this paper is

**TABLE II.**

VARIATION IN \( \Delta_{\text{CO}_2} \) VALUES ASSOCIATED WITH VARIATIONS IN \( \delta^{13}\text{C} \) VALUES OF PGA OR RuBP

<table>
<thead>
<tr>
<th>( \delta^{13}\text{C}, %o )</th>
<th>deviation ((-2^o/oo))</th>
<th>deviation ((-1^o/oo))</th>
<th>known values</th>
<th>deviation ((+1^o/oo))</th>
<th>deviation ((+2^o/oo))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CO}_2 \text{ DISSOLVED} )</td>
<td>-22.0 ((-2^o/oo))</td>
<td>-23.0 ((1^o/oo))</td>
<td>-24.0</td>
<td>-25.0 ((+1^o/oo))</td>
<td>-26.0 ((+2^o/oo))</td>
</tr>
<tr>
<td>PGA (^1)</td>
<td>-22.0 ((-2^o/oo))</td>
<td>-23.0 ((1^o/oo))</td>
<td>-24.0</td>
<td>-25.0 ((+1^o/oo))</td>
<td>-26.0 ((+2^o/oo))</td>
</tr>
<tr>
<td>( \delta_{\text{CO}_2}, %o )</td>
<td>-19.1</td>
<td>-25.1</td>
<td>-31.1</td>
<td>-37.1</td>
<td>-41.4</td>
</tr>
<tr>
<td>( \text{RuBP} ) (^2)</td>
<td>-12.0 ((-2^o/oo))</td>
<td>-13.0 ((-1^o/oo))</td>
<td>-14.0</td>
<td>-15.0 ((+1^o/oo))</td>
<td>-16.0 ((+2^o/oo))</td>
</tr>
<tr>
<td>( \delta_{\text{CO}_2}, %o )</td>
<td>-36.6</td>
<td>-31.6</td>
<td>-26.6</td>
<td>-21.6</td>
<td>-16.6</td>
</tr>
</tbody>
</table>

\(^1\) PGA has a \( \delta^{13}\text{C} \) value of \(-24.0^o/oo\) vs PDB.

\(^2\) RuBP has a \( \delta^{13}\text{C} \) value of \(-14.0^o/oo\) vs PDB.
purity from contaminating phosphate esters or metals, the 98% pure RuBP was chromatographed on Dowex 1-Cl- resin columns. Use of this pure RuBP in the carboxylation reaction resulted in $\Delta_{CO_2}$ values of $-28.1\%$ and $-25.8\%$ . These values deviate from the average $\Delta_{CO_2}$ values by only 1 to 2\% . The synthesized PGA in these experiments was chromatographically pure. Based on the use of a purified enzyme, highly purified substrate, and highly purified product, in the experiments reported here, we conclude the $\Delta_{CO_2}$ of RuBP carboxylase is $-27.1\%$ . This value compares favorably to the $\Delta_{CO_2}$ values reported by Whelan et al. (26), Christeller et al. (8), and Estep et al. (16).

In assessing the importance of $\Delta_{CO_2}$ by RuBP carboxylase as a factor in determining the $\delta^{13}C$ values of C3 plants, the enzymic fractionation has to be discussed together with other parameters which affect the over-all fractionation. Isotope fractionation associated with the following steps is important in determining the $\delta^{13}C$ values by RuBP carboxylase. Since the total $\Delta_{CO_2}$ is fixed, there is no fractionation associated with the RuBP carboxylase step. In this case the $\delta^{13}C$ value of C4 plants would be determined by the fractionations associated only by CO2 dissolving in the mesophyll cells, isotopic equilibrium of atmospheric CO2 and dissolved CO2, and the fixation of CO2 by PEPP carboxylase.

**LITERATURE CITED**

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