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

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ABSTRACT

The enzymic fractionation of the stable carbon isotopes of CO_2 (Δ_{CO_2}) was determined using a purified preparation of ribulose-1,5-bisphosphate (RuBP) carboxylase isolated from cotton (a C_3 plant) leaves. The bicarbonate concentration in the reaction mixture saturated the enzyme and furnished an infinite pool of $^{12}CO_2$ and $^{13}CO_2$ for enzyme fractionation. The RuBP was 96 to 98% pure. The phosphoglycerate synthesized in the reaction mixtures was purified free of RuBP, phosphoglycolate, and other phosphate esters by column chromatography on Dowex 1-Cl⁻ resin. The average Δ_{CO_2} value of −27.1‰ was determined from five separate experiments. A discussion of the isotope fractionation associated with photosynthetic CO_2 fixation in plants shows that the enzymic fractionation of stable carbon isotopes of CO_2 by RuBP carboxylase is of major importance in determining the $\delta^{13}C$ values of C_3 plants.

Photosynthesis is accompanied by a fractionation of the stable carbon isotopes of CO_2. This discrimination favors the fixation of the $^{12}CO_2$ into plant material (5, 10, 15, 21, 22) and it is now known that the $\delta^{13}C$ values of plants is highly correlated with the presence of C_3 and C_4 photosynthesis (5, 23, 24).

Park and Epstein (22) were the first to show that ribulose-1,5-bisphosphate carboxylase preferentially fixed $^{12}CO_2$ into phosphoglycerate and accounted for the $^{13}C/^{12}C$ ratio of plants. This enzyme isotope fractionation (Δ_{CO_2}) of −17‰ plus the $\delta^{13}C$ value of −7‰ for atmospheric CO_2 gave a predicted $\delta^{13}C$ value of −24‰ for plants with a Calvin cycle. This predicted value is within the range of $\delta^{13}C$ values for C_3 plants. However, as pointed out by Whelan *et al.* (26), these workers were not aware that CO_2 was the active species of "CO_2" utilized by RuBP[2] carboxylase and actually calculated the enzyme fractionation of stable carbon isotopes of bicarbonate ($\Delta_{HCO_3^-}$). They did not correct their data for the equilibrium isotope effect for $HCO_3^- \rightleftharpoons CO_2 + H^+$. This equilibrium isotope effect results in CO_2 being 8.4‰ more depleted in $^{13}CO_2$ at 25 C (25) than HCO_3^-. Had the authors made the appropriate correction, the predicted value for C_3 plants would be −15.6‰. This value will not account for the ^{13}C content observed in C_3 plants. Subsequent work has shown that the Δ_{CO_2} 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 Δ_{CO_2} values further questions the importance of RuBP carboxylase in the overall isotopic fractionation of CO_2 during photosynthesis in C_3 plants. In much of this work, variations in the Δ_{CO_2} 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 $\delta^{13}C$ value of the PGA. Here, we redetermine the Δ_{CO_2} 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 $\delta^{13}C$ value of PGA. This work shows that RuBP carboxylase plays a predominant role in determining the $\delta^{13}C$ value of C_3 plants and confirms the Δ_{CO_2} values for RuBP carboxylase determined by Whelan *et al.* (26), Christeller *et al.* (8), and Estep *et al.* (16).

MATERIALS AND METHODS

Chemicals. Phosphoglycerate, RuBP tetrasodium salt, phosphoglycolate, and 2,3-diphosphoglycerate were purchased from Sigma Chemical. $NaH^{14}CO_3$ was purchased from New England Nuclear.

Plants. Cotton plants were grown in the 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, deveined, and rinsed with deionized H_2O. 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 mM GSH, 1% Triton X-100, and 12% Dowex 1-X8 Chloride (100–200 mesh). The Dowex 1 resin was prepared by washing with deionized H_2O until the effluent was clear followed by equilibrating with 0.1 M Tris-HCl (pH 7.5) containing 0.1 mM 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 $(NH_4)_2SO_4$. The protein which precipitated between 40 to 50% of saturation with $(NH_4)_2SO_4$ was collected by centrifugation and dissolved in a minimal amount of 0.1 M Tris-HCl (pH 7.5) containing 0.1 mM GSH. The protein was desalted by passing through a column (2.3 × 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 mM GSH. The protein eluted in the void volume was adsorbed onto a column (2 × 20 cm) of DEAE-cellulose which had been equilibrated with 0.01 M Tris-HCl (pH 7.5) containing 0.1 mM 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 mM GSH. RuBP carboxylase was eluted from the column with 0.20 M NaCl.

Disc Gel Electrophoresis. The homogeneity of the RuBP carboxylase eluted from the DEAE-cellulose column with 0.20 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 Criddle (17),

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[2] Abbreviations: RuBP: ribulose-1,5-bisphosphate; PGA: 3-phosphoglycerate; Δ_{CO_2}: enzymic fractionation of stable carbon isotopes of CO_2; BSC: bundle sheath cells of C_4 plants.

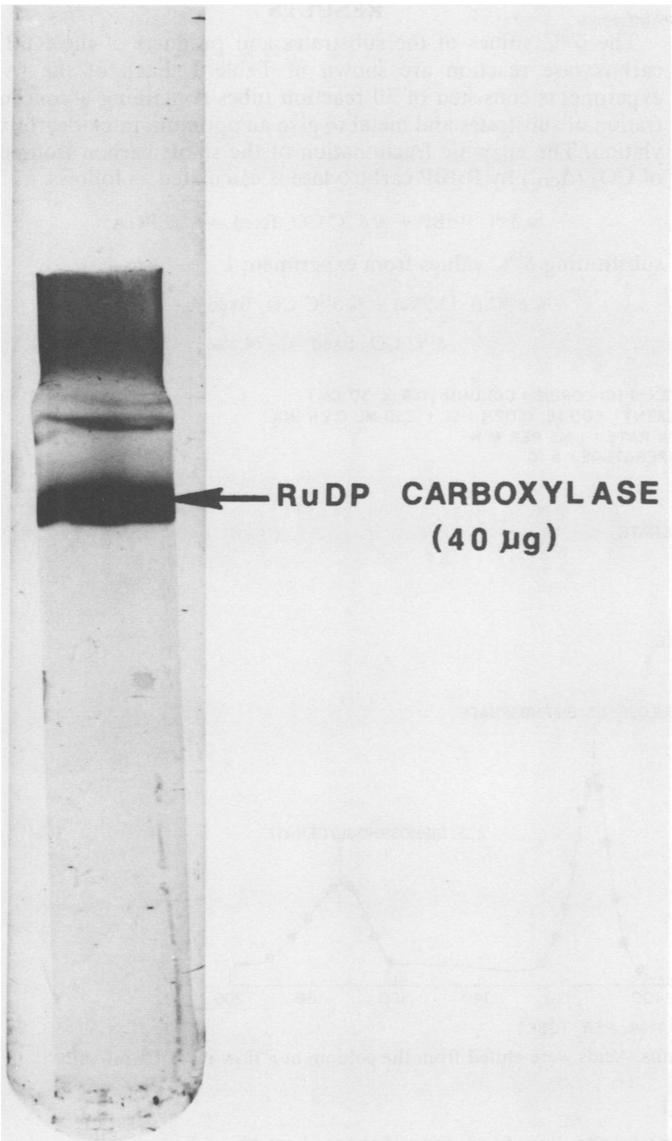

FIG. 1. Electrophoretic pattern of RuBP carboxylase purified from cotton leaves in 7% polyacrylamide gel. Electrophoretic technique was similar to that described in Davis (12).

and Howell *et al.* (18) for the single electrophoretic component of purified RuBP carboxylase.

Protein Determination. Protein was determined by the methods of Lowry *et al.* (19) and Bradford (7). BSA was used as the standard protein for the calibration curves.

Enzyme Assay. The reaction mixture for the assay of RuBP carboxylase contained: 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2.5 mM GSH, 50 mM $KHCO_3$ (containing 10 μCi $NaH^{14}CO_3$), and 50 μg purified enzyme. The mixture was preincubated for 10 min at 35 C and 1 mM RuBP was added to a final volume of 0.5 ml. The reaction mixture was incubated for 15 min at 35 C and the reaction was terminated with 1.0 ml concentrated HCl. The mixture was brought to 10.0 ml with deionized H_2O and N_2 was bubbled through for 15 min. A 0.1-ml aliquot was assayed for radioactivity in a Beckman liquid scintillation spectrometer.

Chromatographic Purification of RuBP. The RuBP used in experiments I, II, and III (Table I) was 96% pure as determined enzymically and chemically by Sigma Chemical. The $\delta^{13}C$ value of the purchased RuBP was −13.9‰.

The RuBP used in experiments IV and V was initially 98% pure. 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 H_2O, 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 $\delta^{13}C$ 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 Δ_{CO_2} 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 $^{13}C/^{12}C$ content. Each of the five experiments consisted of 50 individual reaction mixtures containing: 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2.5 mM GSH, 50 mM $KHCO_3$, 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 H_2O 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 the 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 phosphorylated glyceric acid (3). The fraction tubes containing the synthesized PGA were pooled, lyophilized, and stored at −20 C until it was combusted to CO_2. To test the amount of isotopic fractionation associated with the purification of enzymically synthesized PGA by this procedure, the $\delta^{13}C$ 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 Δ_{CO_2} 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 CO_2 by combustion at 800 to 900 C in an excess of O_2 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 CO_2 was collected in a sample bulb at a liquid N_2 temperature. Bicarbonate was converted to CO_2 with the addition of 85% H_3PO_4 after the evacuation of the atmospheric gases from the reaction vessel. Again, the CO_2 was collected in a sample bulb at a liquid N_2 temperature. The purified CO_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:

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

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

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_2 (Δ_{CO_2}) by RuBP carboxylase is calculated as follows:

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

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

$$\tfrac{5}{6}\ \delta^{13}C\ (-13.9‰) + \tfrac{1}{6}\ \delta^{13}C\ CO_2\ \text{fixed} = -24.0‰$$

$$\delta^{13}C\ CO_2\ \text{fixed} = -74.5‰$$

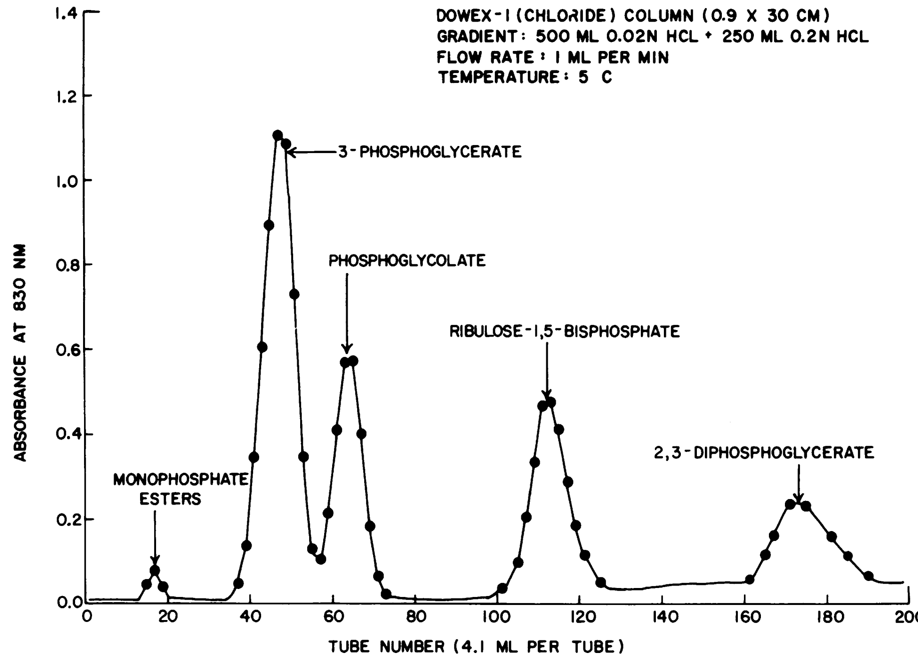

FIG. 2. Chromatography of organic acids on Dowex 1-formate columns. Acids were eluted from the column at a flow rate of 1 ml/min.

Table I

Fractionation of $^{12}CO_2$ - $^{13}CO_2$ 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 ηmol HCO_3^- / mg protein·min.

	$\delta^{13}C$ vs PDB, °/oo				
	I	II	III	IV	V
RUBP	-13.9	-13.9	-13.9	-14.3	-14.3
Purity of RUBP: (%)					
1. Enzymatic Assay	96	96	96	98	98
2. Phosphorus Assay	95	95	95	98	98
PGA Recovery (%)[1]	88	103	106	81	73
HCO_3^-	-34.0	-30.8	-30.8	-33.4	-33.4
CO_2 Dissolved[2]	-42.0	-38.8	-38.8	-41.4	-41.4
3-PGA	-24.0	-22.4	-21.9	-23.5	-23.1
CO_2 Fixed[3]	-74.5	-64.9	-61.9	-69.5	-67.1
ΔCO_2, °/oo[4]	-32.5	-26.1	-23.1	-28.1	-25.8
Average ΔCO_2, °/oo			-27.1		

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

[2] $\delta^{13}C$ of CO_2 dissolved = $\delta^{13}C$ of HCO_3^- dissolved - (10.2 - 0.064 x T)

[3] $\delta^{13}C$ of CO_2 fixed = 6($\delta^{13}C$ of 3-PGA) - 5($\delta^{13}C$ of RuBP)

[4] ΔCO_2 (°/oo) = $\delta^{13}C$ of CO_2 fixed - $\delta^{13}C$ of CO_2 dissolved

$$\Delta_{CO_2} = \delta^{13}C\ CO_2\ \text{fixed} - \delta^{13}C\ \text{dissolved}\ CO_2$$

$$\delta^{13}C\ \text{dissolved}\ CO_2 = \delta^{13}C\ HCO_3^- - (10.2 - 0.064 \times T)$$

where T is the temperature in centrigrade (20, 25)

$$\delta^{13}C\ \text{dissolved}\ CO_2 = -42.0‰$$

substituting

$$\Delta_{CO_2} = (-74.5‰) - (-42.0‰) = -32.5‰$$

The average Δ_{CO_2} value calculated in a similar manner for all five experiments is −27.1‰.

In the above experiments, the HCO_3^- concentration (50 mM) insured enzyme saturation and provided an infinite pool (2.55 mM) of dissolved $^{12}CO_2$ and $^{13}CO_2$ so that maximal enzymic fractionation of stable carbon isotopes of CO_2 would occur during the carboxylation reaction. The $\delta^{13}C$ value of the HCO_3^- shown in each experiment (Table I) was determined by acidifying an aliquot of the reaction mixtures. Sine the HCO_3^- in the buffer of unknown isotopic composition would mix with the HCO_3^- from the reagent bottle, this assured that the $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ value was determined to be −14.0‰. This value compared to the starting $\delta^{13}C$ value for RuBP of −14.3‰ 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 −27.1‰. Park and Epstein (22), Whelan *et al.* (26), Deleens *et al.* (13), Christeller *et al.* (8), and Estep *et al.* (16) have determined Δ_{CO_2} values for RuBP carboxylase of −7.4‰, −18.3‰, −38.8 to −89.2‰, −28.3‰, and −28.3 to −41.7‰, respectively. The work in this paper agrees with the work of Whelan *et al.* (26), Christeller *et al.* (8), and Estep *et al.* (16). However, the range of Δ_{CO_2} values of −7.4 to −89.2‰ will give predicted $\delta^{13}C$ values for C_3 plants of −14.4 to −96.2‰ which is too large to account for the $\delta^{13}C$ values of C_3 plants which range from −21 to −34‰ (5, 23, 24). Two reasons for variations in the Δ_{CO_2} values may be the use of impure RuBP in the reaction mixtures, and failure to purify the enzymically synthesized PGA analytically. The purity of the RuBP in the experiments of Park and Epstein (22) and Deleens *et al.* (13) was not given. The RuBP used in the experiments of Estep *et al.* (16) was 45% pure and the major contaminants were ribulose-5-P, inorganic PO_4, H_2O, and possibly some Tris buffer. The calculation of Δ_{CO_2} involves the $\delta^{13}C$ of RuBP which is affected by carbon impurities; however, Estep *et al.* (16) stated that RuBP is unstable and converts to ribulose-5-P. The calculations of Δ_{CO_2} of RuBP carboxylase were not affected by the presence of ribulose-5-P. The $\delta^{13}C$ values of the PGA synthesized from RuBP carboxylase in the experiments of Deleens *et al.* (13) varied between −19.4 and −27.8‰. This is a variation of ±8.2‰. The $\delta^{13}C$ value of the PGA in these experiments was determined on the acidified reaction mixtures and there was no further purification of the PGA from other phosphate esters in the reaction mixture. A ±8‰ variation in the $\delta^{13}C$ value of PGA, will account, however, for a wide variation in determined Δ_{CO_2} values.

Table II shows a theoretical example of the amount of variation in Δ_{CO_2} for RuBP carboxylase as the $\delta^{13}C$ values of RuBP and PGA deviate from known values by ±2‰. As the $\delta^{13}C$ value of RuBP independently varies from the determined value of −14‰ by ±2‰ the calculated Δ_{CO_2} values range from −36.6‰ to −16.6‰. As the $\delta^{13}C$ value of the PGA independently varies from the determined value of −24‰ by ±2‰ the calculated Δ_{CO_2} values range from −19.1 to −41.4‰. Thus, a small change of only a few parts per mil in the substrate or product of the RuBP carboxylase can lead to widely different Δ_{CO_2} values. In the case of the $\delta^{13}C$ value of the synthesized PGA between −19.4‰ and −27.8‰ (13) this will lead to a variation in the Δ_{CO_2} values between −38.8 and −89.2‰. These examples show the importance of determining the $\delta^{13}C$ values of RuBP and PGA on highly pure compounds in determining the Δ_{CO_2} of RuBP carboxylase.

The Δ_{CO_2} values reported in this paper are calculated from RuBP which is analytically 96 to 98% pure as tested enzymically and by organic phosphate analysis. To assure a higher degree of

TABLE II.
VARIATION IN Δ_{CO_2} VALUES ASSOCIATED WITH VARIATIONS IN $\delta^{13}C$ VALUES OF PGA OR RUBP

	$\delta^{13}C$, °/oo				
	deviation(-2°/oo)	deviation(-1°/oo)	known values	deviation(+1°/oo)	deviation(+2°/oo)
CO_2 DISSOLVED			-41.4		
PGA[1]	-22.0(-2°/oo)	-23.0(1°/oo)	-24.0	-25.0(+1°/oo)	-26.0(+2°/oo)
Δ_{CO_2}, °/oo	-19.1	-25.1	-31.1	-37.1	-41.4
RUBP[2]	-12.0(-2°/oo)	-13.0(-1°/oo)	-14.0	-15.0(+1°/oo)	-16.0(+2°/oo)
Δ_{CO_2}, °/oo	-36.6	-31.6	-26.6	-21.6	-16.6

[1]PGA has a $\delta^{13}C$ value of -24.0°/oo vs PDB.

[2]RuBP has a $\delta^{13}C$ value of -14.0°/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 carboxylase reaction resulted in Δ_{CO_2} values of −28.1‰ and −25.8‰. These values deviate from the average Δ_{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 Δ_{CO_2} of RuBP carboxylase is −27.1‰. This value compares favorably to the Δ_{CO_2} values reported by Whelan *et al.* (26), Christeller *et al.* (8), and Estep *et al.* (16).

In assessing the importance of Δ_{CO_2} by RuBP carboxylase as a factor in determining the $\delta^{13}C$ values of C_3 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 of C_3 plants: (*a*) the absorption of CO_2 by the leaf cytoplasm; (*b*) the isotopic equilibrium of the reaction $CO_2 + H^+ \rightleftharpoons HCO_3^-$; (*c*) CO_2 fixation by RuBP carboxylase; and (*d*) the removal of the ^{13}C-enriched CO_2 (produced as a result of the preferential fixation of $^{12}CO_2$ by RuBP carboxylase) from the chloroplast.

In 1960, Park and Epstein (22) originally proposed a model to explain the parameters which determine the $\delta^{13}C$ values of plants. Epstein (15) discussed this model in relation to the known variation of $\delta^{13}C$ values in marine and terrestrial plants. Since these discussions, the physiology and biochemistry of C_4 plants have been elucidated and an understanding of the operation of the Calvin cycle in the bundle sheath cells (BSC) is now known. Berry and Troughton (6) have discussed the operation of the RuBP carboxylase and the enzymic fractionation of stable carbon isotopes in BSC which is essentially a closed system, *i.e.* the CO_2 in the BSC is not in equilibrium with the atmosphere. They contrasted this system to stable carbon isotope fractionation by RuBP carboxylase in C_3 plants which in an open system, *i.e.* the chloroplast CO_2 is in equilibrium with atmospheric CO_2. The operation of RuBP carboxylase in an open or closed system will profoundly affect the amount of enzymic fractionation and the original model should be modified to include carbon isotope fractionation within C_3 and C_4 plants.

Atmospheric CO_2 has a general $\delta^{13}C$ value of −7‰. A carbon isotope fractionation of −2 to −7‰ is associated with dissolving CO_2 into a solution of base or cell sap (2, 10, 22). This isotope effect between atmospheric CO_2 and dissolved CO_2 in $Ba(OH)_2$, NaOH, and leaf cytoplasm has been measured by Baertschi (2) Craig (10) and Park and Epstein (22). The $\delta^{13}C$ value for the dissolved CO_2 in leaf cytoplasm is −9.5 to −17.5‰ (22). It is also possible that an isotopic exchange can occur between dissolved CO_2 and atmospheric CO_2. Deuser and Degens (14) and Wendt (25) have established a $\delta^{13}C$ value of approximately 0‰ for dissolved HCO_3^- in equilibrium with atmospheric CO_2. The $\delta^{13}C$ value of dissolved CO_2 in the leaf cytoplasm can therefore range between 0 and −17.5‰ depending on how rapidly the atmospheric CO_2 is fixed by the plant. In the open system of C_3 plants, *i.e.* the dissolved cytoplasmic CO_2 is in equilibrium with the atmospheric CO_2, the $^{13}CO_2$ remaining in the chloroplast from the preferential use of $^{12}CO_2$ by RuBP carboxylase would diffuse into the atmosphere and there would be a maximum fractionation by the RuBP carboxylase. The stable carbon isotope fractionation steps in an open system, like a C_3 plant, would be additive and the $\delta^{13}C$ value would be a result of the fractionations associated with dissolving CO_2 in the cell cytoplasm, isotopic equilibrium of atmospheric CO_2 and dissolved CO_2 and RuBP carboxylase. Fractionations by these steps would give a range of $\delta^{13}C$ values for C_3 plants between −27.1 and −44.6‰, using a Δ_{CO_2} for RuBP carboxylase of −27.1‰. Therefore, fixation of chloroplast CO_2 by RuBP carboxylase can account for the range of $\delta^{13}C$ values of −21 to −34‰ (5, 23, 24) found in C_3 plants. In a closed system such as occurs in the BSC of C_4 plants the chloroplast CO_2 would be completely fixed by RuBP carboxylase. Since the total CO_2 is fixed, there is no fractionation associated with the RuBP carboxylase step. In this case the $\delta^{13}C$ value of C_4 plants would be determined by the fractionations associated only by CO_2 dissolving in the mesophyll cells, isotopic equilibrium of atmospheric CO_2 and dissolved Co_2, and the fixation of CO_2 by PEP carboxylase.

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