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Carbon Isotope Fractionation by Ribulose-1,5-Bisphosphate Carboxylase from Various Organisms[1]

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MARILYN F. ESTEP, F. ROBERT TABITA,[2] PATRICK L. PARKER, AND CHASE VAN BAALEN
University of Texas, Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373

ABSTRACT

Carbon isotope fractionation by structurally and catalytically distinct ribulose-1,5-bisphosphate carboxylases from one eucaryotic and four procaryotic organisms has been measured under nitrogen. The average fractionation for 40 experiments was −34.1 ‰ with respect to the $\delta^{13}C$ of the dissolved CO_2 used, although average fractionations for each enzyme varied slightly: spinach carboxylase, −36.5 ‰; *Hydrogenomonas eutropha*, −38.7 ‰; *Agmenellum quadruplicatum*, −32.2 ‰; *Rhodospirillum rubrum*, −32.1 ‰; *Rhodopseudomonas sphaeroides* peak I carboxylase, −31.4 ‰; and *R. sphaeroides* peak II carboxylase, −28.3 ‰. The carbon isotope fractionation value was largely independent of method of enzyme preparation, purity, or reaction temperature, but in the case of spinach ribulose-1,5-bisphosphate carboxylase fractionation, changing the metal cofactor used for enzyme activation had a distinct effect on the fractionation value. The fractionation value of −36.5 ‰ with Mg^{2+} as activator shifted to −29.9 ‰ with Ni^{2+} as activator and to −41.7 ‰ with Mn^{2+} as activator. These dramatic metal effects on carbon isotope fractionation may be useful in examining the catalytic site of the enzyme.

Carbon isotope fractionation between atmospheric CO_2 and terrestrial plant carbon was shown by Park and Epstein (20) to occur primarily as a result of CO_2 fixation via RuBP[3] carboxylase (EC 4.1.1.39). Since RuBP carboxylase has been shown to fix "CO_2," not HCO_3^- (6), fractionation values here will be reported with respect to dissolved CO_2, or Δ dissolved CO_2. Park and Epstein reported Δ dissolved CO_2 values of −8.2 and −5.5 ‰ using enzyme isolated from tomato leaves. Subsequently, the fractionation of stable carbon isotopes by RuBP carboxylase has been reported by several other laboratories. All isotope discriminations reported have varied considerably. Whelan *et al.* (33), using a crude preparation of sorghum carboxylase, reported on two experiments: Δ dissolved CO_2 of −18.3 ‰ at 37 C, and a Δ dissolved CO_2 of −33.7 ‰ at 24 C. Deleens *et al.*, in several experiments with spinach and maize carboxylases, found varying isotope fractions with varying enzyme preparations and plant sources; Δ dissolved CO_2 values ranged from −40 to −80 ‰ (8). Even different preparations of the same plant varied as much as 40 ‰ with respect to isotope fractionation. Recently, Christeller *et al.* (5) have reported a series of isotope fractionation experiments with carboxylase purified from *Glycine max* (soybean). They found a constant Δ dissolved CO_2 of −25.7 to −30.4 ‰ which was not affected by temperature or $NaHCO_3$ concentration.

Several plant enzymes of varying purification states have been used in the above experiments. Moreover, these experiments have been conducted with carboxylases having similar quaternary structure, amino acid composition, and catalytic properties (12). Presumably, reaction mechanisms and hence isotopic fractionations should not vary as greatly as the literature suggests. Although carbon isotope fractionations ranged from −5.5 ‰ for tomato enzyme (20) to −80 ‰ for spinach enzyme (8), experimental techniques also varied which may account for part of the disparate Δ dissolved CO_2 values recorded by these investigators.

Spinach leaf RuBP carboxylase is a large protein having a mol wt of 557,000 and is composed of eight large subunits, mol wt 55,800, and eight small subunits, mol wt 12,000 (21, 22). RuBP carboxylases have been isolated from procaryotic systems which vary in mol wt, structural composition, and regulatory properties. For example, when isolated and purified from the purple, nonsulfur, bacterium *Rhodospirillum rubrum*, the carboxylase has a mol wt of 114,000, is composed of only two large subunits, and is not regulated by the effector 6-P-gluconate (12, 27). Furthermore, two structurally and catalytically distinct RuBP carboxylases have been isolated from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* (9). Peak I carboxylase is structurally and catalytically similar to the enzyme from higher plants. Peak II carboxylase has a mol wt of 360,000 and is composed of only six large subunits. It is also apparent that a diversity of structure may exist in the blue-green algae (28, 29), similar to the bacteria (9, 27); *i.e. Hydrogenomonas eutropha*, a chemosynthetic bacterium, has a high mol wt carboxylase similar in structure to that of the spinach enzyme (12).

In this paper we have examined carbon isotope fractionation by RuBP carboxylases from spinach, *R. rubrum*, *R. sphaeroides*, *Agmenellum quadruplicatum*, and *H. eutropha*, for several reasons. First, we have produced a reproducible fractionation value, Δ dissolved CO_2, by carefully controlling reaction conditions and purification procedures of both the enzyme and the product phosphoglyceric acid. Second, certain reaction conditions were purposely modified to test their effects on the fractionation: $NaHCO_3$ concentration, O_2 level, temperature, buffer, substrate, and metal cofactors. In addition, several purification states of each enzyme were used in order to determine the effect on the Δ dissolved CO_2. Moreover, in an attempt to see if enzymes of different mol wt, purity, quaternary structure, and catalytic properties affect the subsequent fractionation, we have varied the enzyme source.

MATERIALS AND METHODS

Spinach Enzyme Preparations. Three types of spinach RuBP carboxylase were used in experiments: A, homogeneous carboxylase purified by the procedure of Paulsen and Lane (21); B,

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[2] Department of Microbiology.

[3] Abbreviations: PGA: 3-phosphoglyceric acid; RuBP: ribulose 1,5-bisphosphate; Δ dissolved CO_2 = $\delta^{13}C$ (CO_2 fixed) − $\delta^{13}C$ dissolved CO_2; R5P: ribose 5-phosphate.

partially purified carboxylase purchased from the Sigma Chemical Co.; and C, carboxylase from freshly lysed chloroplasts prepared by the method of Bahr and Jensen (2), slightly modified by the omission of sodium isoascorbate in solutions A and B, and by the use of nine layers of cheesecloth to remove cell debris. Spinach for preparations type A and C was obtained from a local market. Specific activity of enzyme preparation B was between 0.1 and 0.3 μmol of CO_2 fixed min^{-1} mg $protein^{-1}$. The specific activity of enzyme preparation A was 1.4 μmol of CO_2 fixed min^{-1} mg $protein^{-1}$ (Lowry). Specific activities of preparations C are reported in Table III. All preparations were assayed by coupling RuBP carboxylase activity to the spectrophotometric assay for PGA (see below) or as previously described (9).

Growth of *A. quadruplicatum* Strain PR-6. Cells were grown at 39 C in a glass, water-jacketed growth chamber (Special Cat. No. 9450-5002 Bellco Glass Inc.) with a working volume of about 300 ml. The culture chamber is an adaptation of the original design of Myers and Clark (16), an apparatus for continuous culture of *Chlorella*. The chamber was illuminated by a bank of four 15-w deluxe cool white fluorescent bulbs 9 cm from the algal chamber, and a 13-w cool white fluorescent bulb positioned in the center of the chamber. The culture medium, ASP-2, contained the regular components (32) with the following constituents increased in concentration: 5 g of $NaNO_3$/l, 0.4 g of PO_4/l, and 16 μg of B_{12}/l. The pH was adjusted to 7.8 with HCl before autoclaving. The chamber was gassed with 1% CO_2 mixed with air when growing cells for algal enzyme type A. For cells used in preparing algal enzyme type B, a tank of 1.25% CO_2 in air was used. The cell yield from the chamber was approximately 1 g dry wt/day.

Purification Procedures for RuBP Carboxylase from *A. quadruplicatum* Strain PR-6. Two methods were used to isolate carboxylase from PR-6. In the first preparation A, the cell paste, approximately 125 g wet wt, was suspended in 2 volumes of 20 mM Tris-HCl (pH 7.9), 1 mM EDTA, and 5 mM 2-mercaptoethanol (TEM buffer) and disrupted in a chilled French pressure cell at 1250 p.s.i. The cell extract was then prepared as previously described (28). RuBP carboxylase was purified by batchwise elution of protein from DEAE-cellulose (Fig. 1). Fractions 0.25 M NaCl through 0.4 M NaCl contained the peak of carboxylase activity and were pooled. To the pooled fractions, solid ammonium sulfate was added, and the solution slowly brought to 75% saturation. The precipitate was suspended in a minimum amount of 10% (v/v) glycerol in buffer. This fraction had a specific activity of 0.24 μmol of CO_2 fixed/mg of protein · min and was used as algal enzyme type A (see Table IV).

Although not homogeneous, such a preparation was substantially free of phycocyanin and other major contaminants. For the purposes of this investigation, relatively large amounts of enzyme were required; the subsequent purification of the enzyme through

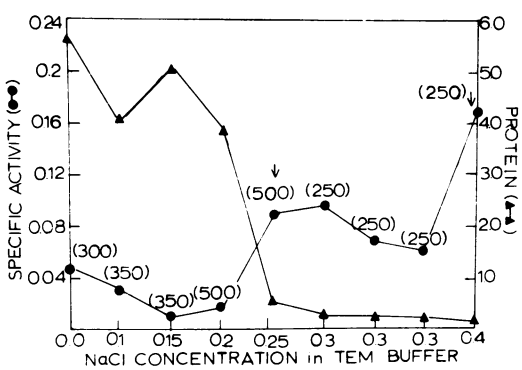

FIG. 1. Purification of RuBP carboxylase from PR-6 by DEAE-cellulose. Specific activity (μmol of CO_2 fixed/min/mg of protein) is indicated by ●——●. Protein (mg/ml) is indicated by ▲——▲. Arrows indicate the range of fractions which were pooled. Number in parentheses over specific activity (●) indicates the volume of the NaCl-buffer wash (ml).

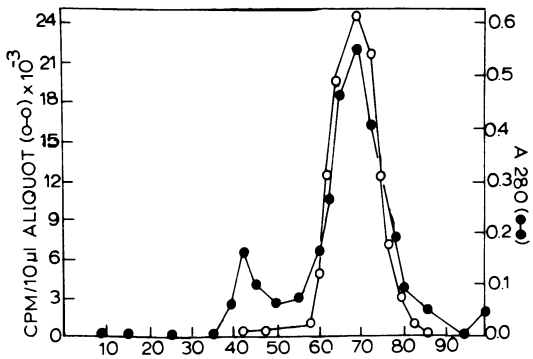

FIG. 2. Purification of RuBP carboxylase from *R. rubrum* by a Sephadex G-200 column. Counts/min/fraction aliquot are indicated by O——O. Protein (mg/ml) is indicated by ●——●.

sucrose gradients (28) was judged to be unwise due to problems of dilution, stability, and low yield at this stage.

Cells of strain PR-6, 6 g wet wt, were suspended in 2 volumes of a buffer containing 20 mM Tris-H_2SO_4 (pH 8), 1 mM EDTA, 10 mM $MgCl_2$, 50 mM $NaHCO_3$, and 5 mM 2-mercaptoethanol (TEMMB buffer). This cell suspension in a beaker in an ice bath was disrupted by sonication with a Branson model S-125 sonicator in four 30-sec bursts. After each 30-sec sonic treatment, the probe was removed and immersed in ice water until cooled. Broken cells were removed by centrifugation at 15,000*g* for 15 min at 0 to 4 C. The supernatant was then used in carbon isotope experiments (B), (see Table IV) or further purified by an acid precipitation method (28). The PR-6 carboxylase was precipitated from the above supernatant with 1 N acetic acid at about pH 6 and redissolved in TEMMB. This RuBP carboxylase was used for experiments (B) after further purification by ammonium sulfate precipitation.

Growth of *R. rubrum*. A culture of *R. rubrum* standard strain was kindly supplied by J. Lascelles. The culture was maintained in the synthetic malate medium of Ormerod *et al.* (18), with 0.2% ammonium sulfate as the nitrogen source and with 0.1% $NaHCO_3$. The butyrate medium was the same but with 0.6% butyrate. Inocula for a 3-liter low form flask (Corning No. 4422) used for growing butyrate cells was about 250 ml of exponential phase malate cells grown in a prescription bottle. Cultures were placed 30 cm beneath banks of 60-w incandescent bulbs and grown at 28 C. All cultures were harvested at the midexponential phase of growth by centrifugation at 6,000*g* for 15 min. Cells were washed once with distilled H_2O and frozen at −70 C until needed.

Purification of RuBP Carboxylase from *R. rubrum*. Cells (about 60 g wet wt) grown on butyrate and stored at −70 C were thawed and suspended at 0 to 4 C in a buffer containing 20 mM Tris-H_2SO_4 (pH 8). The cell suspension was disrupted by sonication in three 20-sec bursts in the presence of glass beads (0.17–0.18 mm). The sonic probe was immersed in an ice bath between treatments. All purification procedures were carried out at 0 to 4 C and basically followed the protocol previously described (27). Peak fractions from the DEAE-cellulose column were pooled and concentrated by ultradiffusion techniques (Amicon filtering). The concentrated solution contained 7 mg of protein/ml. Thirty-two mg of this fraction were then loaded onto a Sephadex G-200 column (2.5 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 7.5) and 2 mM EDTA. The flow rate was 0.6 ml/min and 3-ml fractions were collected. The A_{280} was read for each fraction, and all fractions containing protein were assayed for carboxylase activity (Fig. 2). At this point, the peak fractions 68 through 76 were considered to be 90 to 95% homogeneous by polyacrylamide disc gel electrophoresis. The fractions were pooled and concentrated by ultradiffusion to a volume of 5 ml, 1.4 mg of protein/ml. The specific activity of this preparation was 1.8 μmol of CO_2 fixed min^{-1} mg of protein.

Preparation of *H. eutropha* RuBP Carboxylase. Lyophilized

cells of *H. eutropha*, grown autotrophically, were a generous gift from R. Repaske, NIH, and were stored at −20 C until used. The cells were thawed in TEMB buffer pH 8 (minus 2-mercaptoethanol) at a ratio of 8:1, and incubated with 25 mg of hen egg white lysozyme at room temperature for 15 min. The cells were then disrupted by sonication in four 30-sec bursts. Cell debris and unbroken cells were removed by centrifugation at 20,000*g* for 15 min at 0 to 4 C. The supernatant was further clarified by centrifugation at 48,000*g* for 70 min, and was dialyzed overnight in TEMB buffer. The supernatant was then made to 45% saturation with ammonium sulfate, and the precipitated protein was removed by centrifugation at 20,000*g* for 15 min. The pellet, which contained the RuBP carboxylase activity, was redissolved in 5 ml of TEMB buffer. The ammonium ion was removed by passing the protein solution through a Sephadex G-25 column (1 × 20 cm). Fractions containing protein were then absorbed onto DEAE-cellulose which had previously been washed and equilibrated with TEMB buffer. Protein was eluted batchwise with increasing concentrations of NaCl in TEMB buffer. The carboxylase eluted in the 0.13 M NaCl and 0.15 M NaCl buffer washes. The 0.15 M NaCl wash was brought to 50% saturation with ammonium sulfate, and the precipitated protein spun down at 20,000g for 15 min. The pellet was redissolved in TEMB and dialyzed overnight. The protein solution was then carefully decanted from debris and denatured protein. The protein solution had a concentration of 0.38 mg of protein/ml as determined by *A* at 280 and 260 nm. The solution was then concentrated by ultradiffusion using Pellicon PSED membranes (Millipore). The concentrate, 0.73 mg of protein/ml, had an *A* 280/260 ratio of 1.31, and a specific activity of 0.6 μmol of CO_2 fixed/min · mg of protein. This enzyme preparation was used in isotope experiments 2 and 3 while the ammonium sulfate precipitate of the crude extract was used in experiment 1 (see Table V).

Preparation of *R. sphaeroides* Peak I and Peak II Carboxylases. The RuBP carboxylases were purified from heterotrophically grown cells (9) and were a gift from J. Gibson, University of Texas at Austin.

General Reaction Conditions. In all experiments 3-ml Reacti-Vials (Pierce Chemical Co.) with Teflon-coated rubber caps were used with a final reaction volume of 2.5 ml/vial. Experiments using preparation A of PR-6 enzyme employed three vials; spinach preparations A and B, PR-6 preparation B, and *R. rubrum* experiments used two vials. Experiments with *H. eutropha* carboxylase used four or five vials.

Experiments using spinach type C preparations employed three to four vials as indicated in Table III. In all experiments, vials containing buffer, $MgCl_2$, and DTT were bubbled with tank N_2 or O_2 for 10 min at room temperature before the addition of $NaHCO_3$. In all experiments except those using spinach preparation C, RuBP carboxylase was added with the $NaHCO_3$, and the vials incubated at 30 C for 10 min before initiating the reaction with RuBP. In spinach experiments C, RuBP or ATP and R5P were added simultaneously with the bicarbonate and the reaction initiated with chloroplast suspension. All experiments were performed with laboratory lights off and at 30 ± 0.1 C except the one experiment indicated in Table II, or those indicated in Table IV.

Assay and Purification of PGA. PGA was assayed via the coupled enzymes, P-glyceric acid phosphokinase and 3-P-glyceraldehyde dehydrogenase, according to the method of Andrews *et al.* (1). When PGA was present in the assay mixture NADH oxidation was followed on a Cary model 118 C spectrophotometer at 340 nm (conservative detection limit 0.01 μmol of PGA ml^{-1}).

After boiling the reaction mix (5 min) and removing the denatured protein by centrifugation, the supernatant was then absorbed on a Dowex AG 1-X8 (Cl^-) column (15 × 1.4 cm) and washed with approximately 10 ml of distilled H_2O. PGA was eluted between 30 and 50 ml with 0.2 M NaCl. PGA was then barium-precipitated by a modification of the method of Neuberg and Lustig (17). The modifications included the following: adjusting the pH of the PGA-NaCl solution with 1 N HCl to pH 4 to 5 prior to precipitation, and gassing the PGA-NaCl solution with N_2 gas for 10 min before adding freshly weighed solid $BaCl_2$ (0.1 g). Crystals generally formed overnight with refrigeration. PGA crystals were separated by centrifugation and dried in a vacuum oven over P_2O_5 at 60 C. The purification procedure for PGA was checked for isotopic fractionation (see Table I).

Chemicals. Spinach RuBP carboxylase (Lot No. 104C-7900), disodium D-ribose-5-P, D(−)3-P-glyceric acid sodium salt, the coupled enzymes from yeast, P-glyceric acid phosphokinase and 3-P-glyceraldehyde dehydrogenase, and the DEAE-cellulose used in purifying *R. rubrum* and *H. eutropha* carboxylase were obtained from Sigma Chemical Co.

The PGA assayed as 60 ± 5% free acid via the above enzymes. The tetrasodium salt of D-RuBP was purchased from Calbiochem (Lot No. 220030). This Lot No. was used in all experiments. The RuBP was assayed via Sigma RuBP carboxylase and the coupled enzymic PGA assay above, to be 45 ± 5% pure. Cellex-D, DEAE-cellulose, used to isolate PR-6 carboxylase, was obtained from Bio-Rad Biological Laboratories. A single sample of $NaHCO_3$ was used in all experiments.

Reaction Conditions for Spinach RuBP Carboxylase Experiments Using Different Metal Cofactors. With Mn^{2+} as cofactor, homogeneous preparation A was used. The enzyme was dialyzed overnight in 50 mM phosphate buffer (pH 7.6) with 0.1 mM EDTA and 10 mM 2-mercaptoethanol. With Ni^{2+} as cofactor, preparation B from Sigma was used in three experiments. The enzyme was dialyzed in 50 mM Tris-HCl (pH 7.6) and 50 mM $NaHCO_3$ for 24 hr to ensure the removal of any Mg^{2+} ions. For isotope fractionation experiments, the reaction mix did not contain DTT or 2-mercaptoethanol as the presence of reduced sulfhydryl groups caused a dark brown precipitate to form with Ni^{2+}.

In one experiment using Ni^{2+} as cofactor and in the two experiments using Ni^{2+}/Mg^{2+} (1:1), preparation A was again used. The enzyme was inactive without DTT with all metal cofactors including Mg^{2+}, so in order to assay the enzyme or run isotope fractionation experiments, the RuBP carboxylase was preincubated for 1 hr in 10 mM DTT. After the preincubation, the protein solution was passed through a Sephadex G-25 column with 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 50 mM $NaHCO_3$. This procedure removed DTT, yet the carboxylase remained activated. This preparation was used immediately in isotope fractionation experiments.

Combustion of PGA. Samples of CO_2 gas for isotope ratio analysis were prepared from the Ba-PGA crystals by combustion in a vacuum line. Dried Ba-PGA crystals were placed on a glass fiber filter (Gelman type A, 24 mm) coated with copper oxide to aid combustion. To prevent contamination of the CO_2 gas prepared from PGA, 100 μl of 50% (v/v) H_3PO_4 were added to the combustion boat containing the filter and Ba-PGA before combustion. Any atmospheric CO_2 or barium carbonate was thus removed. The CO_2 from recovered PGA was from 1 to 2 ml.

The PGA was combusted for 10 min under O_2 at temperature of 800 C. Gasses were circulated via a Toepler pump. Water was removed with dry ice and ethanol traps. The combustion line was precise to ±0.2 ‰ as checked by occasional combustion of laboratory reference oil, AER.

Mass Spectrometry. Carbon isotope measurements were made using a 60-degree sector field, 15.24-cm radius of curvature, isotope ratio mass spectrometer (Nuclide Corp. Model RMS-60) as described by McKinney *et al.* (13).

Relative isotope compositions are reported 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 standard used for comparison in this paper is the Peedee belemnite standard (7). The fractionation value of Δ dissolved CO_2 is equal to the $\delta^{13}C$ (CO_2 fixed) minus the $\delta^{13}C$ dissolved CO_2

where: $\delta^{13}C$ (CO_2 fixed) = 6($\delta^{13}C$ PGA) − 5($\delta^{13}C$ RuBP) (see ref. 20). $\delta^{13}C$ (dissolved CO_2) is derived from the measured $NaHCO_3$ value using the data of Mook *et al.* (15) for the $NaHCO_3$-CO_2 carbon isotope equilibrium.

RESULTS

The PGA purification procedure did not affect the isotope ratio of standard PGA (Table I). Experiments 1 and 2 checked for possible ion exchange column fractionation and column bleed. In experiment 3 in which all reaction components were present, including the major organic contaminant Tris buffer at a known $\delta^{13}C$ (−37 ‰), the PGA differed only +0.11 ‰, which is within the precision limits of the combustion process and mass spectral analysis.

Carbon Isotope Fractionation by Spinach RuBP Carboxylase. Table II shows that with highly purified RuBP carboxylase preparation A and partially purified preparation B, the Δ dissolved CO_2 averaged −36.4 ‰ for 12 experiments. It can be seen that fractionation is independent of enzyme purity. Also within the range of saturating $NaHCO_3$ concentrations tested (21), 45 to 195 mM, the fractionation values did not vary appreciably.

RuBP carboxylase also has an oxygenase activity in which RuBP is oxygenated to form P-glycolate (1). This reaction is favored by high O_2 tensions, high pH, and low levels of $NaHCO_3$. P-glycolate formation, which would theoretically decrease the Δ dissolved CO_2 value, did not occur at our experimental levels of $NaHCO_3$, as evidenced by the experiment in which the reaction mix was gassed with 100% O_2 which did not decrease the maximum fractionation value. Furthermore, although we are not making an issue of the temperature effect at this time, the one experiment at 39 C did not show the large effect which Whelan *et al.* (33) reported. Christeller *et al.* (5), in more extensive work on this point, also failed to find a temperature effect.

The results of experiments utilizing freshly lysed spinach chloroplasts are presented in Table III. The experiments show that the Δ dissolved CO_2 was independent of the initial substrate added. This is important because as mentioned RuBP was 45 ± 5% pure. Impurities include ribulose-5-P (Ru5P), inorganic PO_4, H_2O, and possibly some Tris buffer. RuBP is extremely difficult to purify to 100% purity, as it is highly unstable, and may convert to Ru5P. The calculation of Δ dissolved CO_2 involves the $\delta^{13}C$ of the RuBP, which obviously may be affected by carbon impurities. In contrast ribose-5-P is stable and reportedly 99% pure (Sigma Chemical Co.). R5P was converted to Ru5P via endogenous P-riboseisomerase, and Ru5P to RuBP using ATP and endogenous P-ribu-

Table I. Isotopic control on PGA purification.

Experiment No.	$\delta^{13}C$ PGA[1] after 1st Dowex column o/oo	$\delta^{13}C$ PGA after 2nd Dowex column o/oo
1[2]	-17.53	-18.29
2[2]	-18.10	-18.23
3[3]	-18.46	

[1] $\delta^{13}C$ of sodium PGA was -18.35 o/oo of a sample taken directly from bottle.

[2] Twenty-three mg sodium PGA (60% free acid) was adsorbed and eluted from Dowex column as in Materials and Methods. Fractions containing PGA were pooled. One-half of the mixture was adsorbed and eluted from a second Dowex column. PGA was further purified and analyzed as in Materials and Methods.

[3] PGA plus total reaction mix (see Table II) minus RuBP were incubated at 30 C for 6 hr. PGA was purified and analyzed as in Materials and Methods.

Table II. Carbon isotope fractionation by homogeneous (A) and partially purified (B) spinach ribulose-1,5-bisphosphate carboxylase.

Experiments were performed in 150 mM tris (pH 7.6), 6.4 mM $MgCl_2$, 1.4 mM DTT, and $NaHCO_3$ as indicated. Six mg of the enzyme preparations were routinely used. Temperature was 30±0.1 C except for the one experiment indicated.

Spinach enzyme preparation	RuBP added, mg	($NaHCO_3$) mM	PGA[1] formed (μ mol)	$\delta^{13}C$ PGA o/oo	$\delta^{13}C$ CO_2 fixed[2] o/oo	Δ Dissolved CO_2[3] o/oo
A	20.5	120	38	-23.0	-50.5	-38.1
A	20.5	120	46	-22.7	-48.7	-36.3
A	16.0	120	32	-22.4	-46.9	-34.5
A	16.0	120	33	-22.9	-49.9	-37.5
						Av. = -36.6
B	-	157	22	-21.8	-43.3	-30.9
B	22.0	157	45	-22.8	-49.3	-36.9
B	25.0	195	52	-23.0	-50.5	-38.1
B (O_2)[4]	20.5	195	49	-22.9	-49.9	-37.5
B	20.0	45	40	-22.2	-45.7	-33.3
B	20.0	90	43	-22.8	-49.3	-36.9
B (39C)[5]	17.0	195	47	-22.8	-49.3	-38.3
B	23.0	195	45	-23.0	-50.5	-38.1
						Av. = -36.3

[1] Number given is PGA formed after reaction went to completion, assayed as NADH oxidation via the coupled enzymes, phosphoglyceric acid phosphokinase and 3-phosphoglyceraldehyde dehydrogenase. PGA was recovered from reaction mix on Dowex AG-1 X 8 (Cl^-) column.

[2] 5/6 ($\delta^{13}C$ RuBP) + 1/6 ($\delta^{13}C$ (CO_2 fixed)) = $\delta^{13}C$ PGA. $\delta^{13}C$ RuBP was -17.5 o/oo (see ref 20).

[3] Δ Dissolved CO_2 = $\delta^{13}C$ of (CO_2 fixed) - $\delta^{13}C$ of dissolved CO_2. The $\delta^{13}C$ of the dissolved CO_2 was calculated as -12.4 o/oo. The $\delta^{13}C$ of the $NaHCO_3$ used was -4.0 o/oo.

[4] In this experimentthe reaction mix was bubbled with O_2.

[5] Experimental temperature was 39 C.

Table III. Carbon isotope fractionation by ribulose-1,5-bisphosphate carboxylase from freshly lysed spinach chloroplasts using RuBP or R5P and ATP as substrates.

Experiments were performed in 30 mM HEPES buffer (pH 7.8), 24 mM $MgCl_2$, 5 mM DTT, 96 mM $NaHCO_3$, 3.3 mM RuBP (free acid) or 3.3 mM ATP plus 5.7 mM [illegible] and 0.3 to 0.4 ml of chloroplast suspension. All experiments were gassed with N_2; incubation temperature was 30 C.

Substrate	mg Chl/Total vol (ml)	Specific activity μ mol CO_2 fixed mg Chl^{-1} hr^{-1}	PGA[1] formed (μ mol)	$\delta^{13}C$ PGA o/oo	$\delta^{13}C$ substrate o/oo	$\delta^{13}C$ CO_2 fixed[2] o/oo	Δ Dissolved CO_2[3] o/oo
RuBP	0.312/7.5	54	50	-23.1	-17.5	-51.1	-38.7
Ribose-5-P	0.312/7.5	68	50	-19.7	-14.4	-46.2	-33.8
Ribose-5-P	0.18/10.0	22	49	-20.7	-14.4	-52.2	-39.8
Ribose-5-P	0.18/10.0	22	45	-19.9	-14.4	-47.4	-35.0
							Av. -36.8

[1] Number given is PGA found after reaction went to completion. PGA recovered from a Dowex AG-1X8 (Cl^-) column eluted with a linear NaCl gradient from 0.15 to 0.4 M NaCl, after removing ATP and ADP with charcoal. PGA assayed as in Table I.

[2] 5/6 ($\delta^{13}C$ RuBP or R5P) + 1/6 ($\delta^{13}C$ (CO_2 fixed)) = $\delta^{13}C$ PGA.

[3] See Table II.

Table IV. Carbon isotope fractionation by ribulose-1,5-bisphosphate carboxylase from Agmenellum quadruplicatum strain PR-6

Experiments were performed in 150 mM tris (pH 7.6) or 150 mM HEPES (pH 6.7), 6.4 mM $MgCl_2$, 1.4 mM DTT, and 95 mM $NaHCO_3$. Twenty to 24 mg of Na_4RuBP was routinely used. The amount of enzyme added in experiments using algal enzyme type "A" was 0.6 mg; experiments using algal enzyme type "B" used either 10 mg of high-speed supernatant enzyme or 2 mg of further purified enzyme (see Materials and Methods).

Algal enzyme type	Buffer & final pH	Temp. (C)	PGA[1] formed (μ mol)	$\delta^{13}C$ PGA o/oo	$\delta^{13}C$ (CO_2 fixed)[2] o/oo	Δ Dissolved[3] CO_2 o/oo
A	Tris /7.8	39	42	-22.1	-45.1	-33.6
A	Tris /7.8	30	45	-21.4	-40.9	-28.5
A	Tris /7.8	30	48	-21.7	-42.7	-30.3
A	HEPES/7.2	30	38	-21.3	-40.3	-27.9
A	HEPES/7.2	30	36	-22.6	-48.1	-35.7
A	Tris /7.8	35	48	-22.4	-46.9	-35.0
A	Tris /7.8	35	37	-21.9	-43.9	-32.0
						Av. -31.9
B[4]	Tris /7.8	30	54	-21.3	-40.3	-27.9
B[4]	Tris /7.8	30	31	-22.6	-48.1	-35.7
B[5]	Tris /7.8	30	56	-22.4	-46.9	-34.5
B[5]	Tris /7.8	30	56	-22.1	-45.1	-32.7
						Av. -32.7

[1,2,3] See Table II.

[4] Enzyme preparation was the crude, high speed supernatant.

[5] Enzyme preparation was the acid-precipitated fraction further purified by ammonium sulfate.

lokinase. Δ dissolved CO_2 values calculated from R5P and RuBP experiments were similar, the average value of −36.8 ‰ corresponds almost exactly with those of enzyme preparations A and B. In addition, all extraneous material present in preparations B and C had no effect on fractionation.

Carbon Isotope Fractionation by RuBP Carboxylase from *A. quadruplicatum* Strain PR-6. Results of these experiments are shown in Table IV. Two preparations of cells and enzymes were used. Algal enzyme type A was purified by batchwise elution of enzyme from DEAE-cellulose with NaCl buffer (Fig. 1). The average isotope fractionation value was −31.9 ‰, with a spread of 7.8 ‰ in Δ dissolved CO_2 values. This number corresponds quite closely with the average obtained from spinach carboxylase of 36.6 ‰, yet is measurably different, about 13%. Experiments A were run at three different temperatures, 30, 35, 39 C, again to check the effect of temperature on the isotope fractionation which has been reported in the literature (23, 33). Carbon isotope fractionation did not decrease with increasing temperature in these experiments. The average Δ dissolved CO_2 value at 30 C was −30.6 ‰; at 35 C, −33.5 ‰; and for one experiment at 39 C, −33.6 ‰. The buffer was changed in two experiments to test if buffer material was contaminating the recovered PGA. Tris had a $\delta^{13}C_{PDB}$ of −37 ‰, whereas HEPES buffer had a $\delta^{13}C_{PDB}$ of −20.2 ‰. If the presence of buffer made a strong contribution to the PGA which was combusted, this would be reflected in the $\delta^{13}C$ value of PGA. Such was not the case.

Carbon Isotope Fractionation by *R. rubrum* and *H. eutropha*. The enzyme from *R. rubrum* used in this series of experiments was rigorously purified to about 95% homogeneity (Fig. 2), except for the one experiment indicated in Table V which used less homogeneous enzyme. The temperature, 30 C, and reaction conditions were kept constant. Of four experiments, the average Δ dissolved CO_2 value was −32.1 ‰ with a range of 4.2 ‰.

Two different preparations of carboxylase were used in the *H. eutropha* fractionation experiments (Table V). Experiment 1 utilized a crude ammonium sulfate preparation, whereas experiments 2 and 3 utilized a highly purified, high specific activity (0.6 μmol CO_2 fixed/mg of protein min) enzyme. The range of fractionation values is greater, 6 ‰, than that seen in *R. rubrum* experiments, but the average value clearly indicated a more negative average Δ dissolved CO_2, −38.7 ‰.

Carbon Isotope Fractionation by RuBP Carboxylase from *R.*

Table V. Carbon isotope fractionation by Rhodospirillum rubrum, Hydrogenomonas eutropha, and Rhodopseudomonas sphaeroides RuBP carboxylases.

R. rubrum experiments were performed in 150 mM tris (pH 7.6), 6.4 mM $MgCl_2$, 4 mM DTT, and 95 mM $NaHCO_3$. Seventeen to 20 mg Na_4RuBP was used, temperature was 30 C. R. sphaeroides experiments were performed in 150 mM tris-HCl (pH 7.6), 6.4 mM $MgCl_2$, 2.8 mM DTT, and 95 mM $NaHCO_3$; 0.4 mg of enzyme was used in each experiment. Twenty to 24 mg of Na_4RuBP was routinely used. Temperature was 30 C, and final assay pH was 7.8.

Enzyme Source and Experiment	RuBP Case/experiment (mg)	PGA[1] formed (μmol)	$\delta^{13}C$ PGA o/oo	$\delta^{13}C$ (CO_2 fixed)[2] o/oo	Δ dissolved CO_2[3] o/oo
R. rubrum					
1	0.42	50	-21.7	-42.7	-30.3
2[4]	0.50	56	-22.4	-46.9	-34.5
3	0.63	43	-21.7	-42.7	-30.3
4	0.63	29	-22.2	-45.7	-33.3
				Av.	-32.1
H. eutropha					
1	2.0	33	-22.7	-48.7	-36.3
2	1.8	63	-22.9	-49.9	-37.5
3	1.8	44	-23.7	-54.7	-42.3
				Av.	-38.7
R. sphaeroides					
Peak I					
A	0.4	54	-22.0	-44.7	-32.3
B	0.4	46	-21.7	-42.6	-30.2
C	0.4	45	-21.9	-43.0	-31.6
				Av.	-31.4
Peak II					
A	0.4	44	-21.4	-40.9	-28.5
B	0.4	50	-21.7	-42.7	-30.3
C	0.4	50	-21.0	-38.5	-26.1
				Av.	-28.3

[1,2,3] See Table II.

[4] Enzyme preparation was taken from Sephadex G-200 fractions which were not homogeneous.

sphaeroides. Two structurally and catalytically distinct RuBP carboxylases have been isolated from *R. sphaeroides* (9). Separate enzyme preparations which, although not homogeneous, contained only one of these carboxylases, were used in isotope fractionation experiments (Table V). Peak I, the large 550,000 mol wt carboxylase, gave fractionation values from −30.2 to −32.3 ‰ with an average of −31.4 ‰ (three determinations). Peak II, the enzyme without the small subunits, fractionated stable carbon isotopes slightly differently, with an average Δ dissolved CO_2 of −28.3 ‰. Both sets of experiments were assayed at pH 7.8, although the peak II enzyme is more active at pH 7.2 (9). It is difficult to ascertain whether these average fractionations are truly different, as the number of experiments was small.

Carbon Isotope Fractionation by Spinach RuBP Carboxylase and the Effect of Metal Cofactors. A fractionation of −36.5 ‰ with respect to dissolved CO_2 for 16 experiments by spinach carboxylase activated by Mg^{2+} was unchanged by enzyme purity, $NaHCO_3$ concentration, substrate, temperature, or presence or absence of other proteins.

With Mn^{2+} as the metal cofactor, the average fractionation from two experiments was −41.7 ‰, a difference of −5.1 ‰ from Mg^{2+}-activated spinach experiments (Table VI). The specific activity of the Mn^{2+}-activated enzyme was approximately 20% of the Mg^{2+}-activated enzyme. With Ni^{2+} as metal cofactor, the fractionation value became less negative, by +6.8 ‰ to an average of −29.8 ‰. The specific activity of the Ni^{2+}-activated enzyme was approximately 80% of the Mg^{2+}-activated enzyme. When both Mg^{2+} and Ni^{2+} (1:1 ratio) were used to activate the enzyme, from two experiments the average was −36.3 ‰ which corresponds quite closely with the enzymic fractionation experiments activated with only Mg^{2+}.

DISCUSSION

The over-all rationale of the experiments presented here included several additional considerations. The reactions were always allowed to run to completion in terms of the RuBP. If this were not so, theoretically, the fractionation value obtained would be a mixture of the $^{13}CO_2/^{12}CO_2$ fractionation and the [^{13}C]-RuBP/[^{12}C]RuBP fractionation, and therefore would be greater than the $^{13}CO_2/^{12}CO_2$ fractionation measured. It is not clear whether this was the case in all of the experiments reported in the literature. RuBP utilization was monitored via PGA production using the coupled PGA assay enzymes and via RuBP carboxylase rates. At least twice the estimated completion time was allowed before stopping the reaction. Second, the reagents used were from the same source, *i.e.* one sample of $NaHCO_3$ at a known $\delta^{13}C$ (−4.0 ‰) was always used. The assay pH (initial and final value, 7.8), N_2 gassing, and high levels of $NaHCO_3$ were purposely chosen to minimize RuBP oxygenase activity.

Another consideration of all of these experiments is that for one particular RuBP carboxylase, the Δ dissolved CO_2 value was not significantly altered by more conventional reaction modifications. First, enzyme purity had no effect in spinach, PR-6, *R. rubrum,* and *H. eutropha* experiments. Moreover, even spinach chloroplast extracts, which contained other cellular components, introduced no changes in Δ dissolved CO_2 values. Second, the method of purification did not affect Δ dissolved CO_2 values in spinach and PR-6. Third, different reaction temperatures in the spinach and PR-6 experiments had no effect on the Δ dissolved CO_2 corroborating Christeller *et al.* (5) and the whole plant data of Troughton *et al.* (31).

The experiments with Mg^{2+}, Ni^{2+}, and Mn^{2+}, using spinach carboxylase, are the only examples where for one enzyme, the Δ dissolved CO_2 values differed significantly. The role of the metal cofactor in the RuBP carboxylase reaction is unclear (12). Mg^{2+} has been shown to activate the enzyme during preincubation presumably by creating a stable ternary complex with the enzyme and HCO_3^- ions (2). It has been suggested that the metal cofactor is present at the active RuBP carboxylase site close to the binding sites of CO_2 and the intermediate, carboxy ribitol bisphosphate (14). If the metal cofactor is changed, the stable ternary complex could be changed. Moreover, changing the metal cofactor would

Table VI. Carbon isotope fractionation by spinach RuBP carboxylase and the effect of metal cofactors.

Metal and Experiment No.	PGA formed (μmol)	$\delta^{13}C$ PGA o/oo	$\delta^{13}C$ CO_2 fixed o/oo	Δ Dissolved CO_2 o/oo
Mg^{2+} [1] Ave. of 16 experiments	42	-22.7	-49.0	-36.6
Mn^{2+} [2]	38	-23.8	-55.3	-42.9
Mn^{2+}	48	-23.4	-52.9	-40.5
			Av.	-41.7
Ni^{2+} [3]	43	-20.9	-37.9	-25.5
Ni^{2+}	50	-21.3	-40.3	-27.9
Ni^{2+}	38	-22.1	-45.1	-32.7
Ni^{2+} [4]	28	-22.2	-45.7	-33.3
			Av.	-29.8
Ni^{2+}/Mg^{2+} [5]	27	-23.3	-52.3	-39.9
Ni^{2+}/Mg^{2+}	40	-22.1	-45.1	-32.7
			Av.	-36.3

[1] Experiments were performed with 6.4 mM $MgCl_2$, using spinach preparations A, B, and C.

[2] Experiments were performed with 6.4 mM $MnCl_2$ using the homogeneous spinach preparation A.

[3] Experiments were performed with 6.4 mM $NiCl_2$ using the spinach preparation B from Sigma.

[4] The experiment was performed with 6.4 mM $NiCl_2$ using the preactivated spinach preparation A.

[5] Experiments were performed with 3.2 mM $NiCl_2$ and 3.2 mM $MgCl_2$ with preactivated spinach preparation A.

modify the topography of the active site. Modifications of this sort could conceivably alter reaction rates and perhaps even the reaction mechanism, resulting in a new kinetic isotope effect. Furthermore, if the metal cofactor were close to the binding site of CO_2, then perhaps a different isotopic equilibrium between $NaHCO_3$—dissolved CO_2—CO_2-enzyme might occur.

Comparing fractionation values from more than one RuBP carboxylase, one can see from Tables II through VI that Δ dissolved CO_2 values are essentially the same (Fig. 3). While enzymes which catalyze the same reaction according to identical mechanisms should fractionate carbon isotopes to the same extent, the point of interest is that minor changes in reaction mechanisms may be reflected in substantial fractionation shifts. The results of these experiments show that the fractionation of CO_2 *in vitro* by RuBP carboxylase averages −34.1 ‰ *versus* the $\delta^{13}C$ of the dissolved CO_2 calculated from the $\delta^{13}C$ $NaHCO_3$ used (15) for a range of enzymes and sources. RuBP carboxylase from the photosynthetic bacteria *R. rubrum* is unique in that it is a dimer of catalytic subunits, yet this enzyme gave similar Δ dissolved CO_2 values to that of the spinach carboxylase, a protein structurally and catalytically distinct. The enzyme from *A. quadruplicatum* fractionated CO_2 to the same extent as the *R. rubrum* enzyme. *R. sphaeroides* peak II enzyme, a carboxylase consisting of multiple aggregates of the large subunit and lacking the small subunits (9), gave the lowest average fractionation value, but still was similar to PR-6 and *R. rubrum*. Peak I enzyme from the same organism fractionated carbon isotopes quite similar to its own isozyme, peak II, and to the other carboxylases tested. Experimental values obtained with the *H. eutropha* enzyme were also in this −34 ‰ range. The fact that these values are similar fits with previously reported whole plant/cell carbon data which show that most C_3 plants vary from $\delta^{13}C$ of −22 to −34 ‰ (26, 30). If the Δ dissolved CO_2 values from RuBP carboxylase vary as greatly as reported in the literature, from −5.5 (20) to −80 ‰ (8), this variation along with other potential variables such as the $\delta^{13}C$ of the CO_2 source, respiration rate, photorespiration, and lipid content might create a greater deviation in $\delta^{13}C$ values for whole C_3 plants than the reported −21 to −36 ‰ (26, 30). Moreover, the fact that these enzymes which were isolated from such a wide variety of organisms, a chemosynthetic procaryote, three photosynthetic procaryotes (one a blue-green alga), and a higher plant, all show similar fractionation values indicates that the mechanism for these different carboxylases is similar.

Nevertheless, the average values for Δ dissolved CO_2 by the various enzymes do vary, up to 27% (*H. eutropha versus* peak II *R. sphaeroides*). In Figure 3, all experiments are plotted on the Δ dissolved CO_2 scale and can be compared to the fractionation

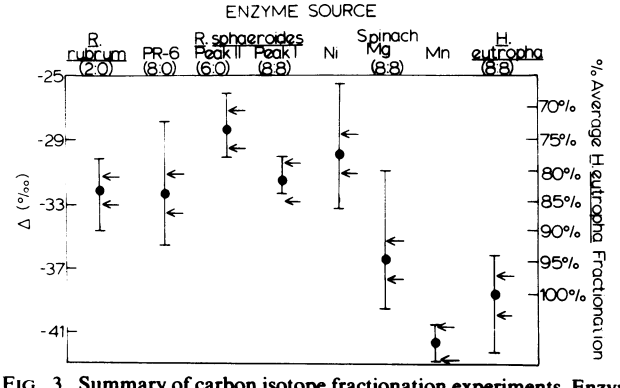

FIG. 3. Summary of carbon isotope fractionation experiments. Enzyme source and the subunit structure are located at the top of each column. Large subunit is the first number in the parentheses; small subunit is the second: (L:S). Bars indicate the total range of experimental values. Dot ● indicates the average of fractionation values. Arrows indicate the instrumental error: ± 0.2 ‰ in measuring PGA. During the calculation of Δ dissolved CO_2, the error is multiplied by 6 and equals ± 1.2 ‰.

range seen in whole C_3 plants. The average value obtained numerically from these series of experiments is presumed to be a valid and useful way of representing Δ dissolved CO_2. In Figure 3, with the average *H. eutropha* fractionation value set at 100%, one can easily compare the percentage difference in average Δ dissolved CO_2 values in relation to the average value obtained from *H. eutropha* experiments.

There are several possible reasons why the average fractionation values for the five enzyme systems studied were different. The over-all fractionation may be made up of two factors, an equilibrium isotope effect and a kinetic isotope effect. An equilibrium isotope effect in the preequilibration of CO_2 with the enzyme (2) might account for part of the variability; however, at this time there are no data to evaluate the magnitude of this contribution. A kinetic isotope effect would account for a substantial portion of the magnitude of the fractionation value observed. Kinetic isotope effect theory predicts that fractionation may vary with slight changes in the nature of the activated complex, here 2-carboxy-3-ketoribitol 1,5-bisphosphate (24). Based on the data of Table VI showing the strong shift in fractionation with different metals, we suggest that the spread for the five enzyme systems can be due to subtle differences in the activated complex.

The fractionation of whole plants/microorganisms is calculated by the following equation: $\Delta = \delta^{13}C$ plant carbon − $\delta^{13}C$ atmospheric CO_2, wherein atmospheric CO_2 has been assigned an average value of −7 ‰ (7, 10). If *in vitro* fractionation by RuBP carboxylase is compared with fractionation seen in whole C_3 higher plants/microorganisms (Fig. 4), the enzymic fractionation is consistently greater than that in whole plants with the exception of tomato carboxylase experiments in which it was less (20) and *one* experiment with sorghum carboxylase at 37 C (33). Regardless of whether the enzyme source was a procaryote or a eucaryote, C_3 or C_4, fractionation was greater *in vitro* by RuBP carboxylase than the whole cell fractionation. The simple model that carbon isotope fractionation by RuBP carboxylase should be close to that seen in whole C_3 plants is based on the assumption that RuBP carboxylase is solely responsible for fixing all of the plant's carbon. Kelly *et al.* (11) suggest that the existence of other autocatalytic pathways for CO_2 fixation is possible. We cautiously suggest that this may be part of the explanation for the rather large differences in *in vivo* and *in vitro* carbon isotope fractionation.

Moreover, it is possible that the CO_2 level in the atmosphere is at nonsaturating levels for RuBP carboxylase *in vivo* (12). This may cause a decrease in Δ dissolved CO_2. The chloroplast can be treated as either a closed or an open system. If a closed system, CO_2 fixation would occur until all CO_2 (both $^{12}CO_2$ and $^{13}CO_2$) molecules had been used up. This would result in no fractionation. If the chloroplast were a completely open system, the CO_2 being fixed (mostly $^{12}CO_2$) would not affect the total amount of CO_2 left in the pool. Theoretically, $^{12}CO_2$ would be in ample supply, and $^{13}CO_2$ would be able to diffuse away from the chloroplast: both processes resulting in maximum isotope fractionation. This hypothesis has been tested in open and closed growth chambers with a whole C_3 plant (4). In a closed system, the $\delta^{13}C$ of the whole plant carbon was 0.0 ‰, whereas in an open system, the $\delta^{13}C$ of the whole plant was −21.5 ‰. The carbon isotope fractionation experiments with RuBP carboxylase approach these "open" conditions and therefore should represent maximum fractionation values.

In summary, we have measured the Δ dissolved CO_2 for five RuBP carboxylase enzymes which differ in mol wt, structural composition, and regulatory properties. All values are similar to an average −34.1 ‰, yet are measurably different as can be seen by comparing average Δ dissolved CO_2 values (Fig. 3). Enzyme preparation, purity, or reaction temperature had little effect on the fractionation by one enzyme. Changing the metal cofactor with spinach carboxylase had a distinct effect on the average Δ dissolved CO_2 value. With Ni^{2+} as cofactor, the average fractionation value was −29.9 ‰, and with Mn^{2+} as cofactor, the average

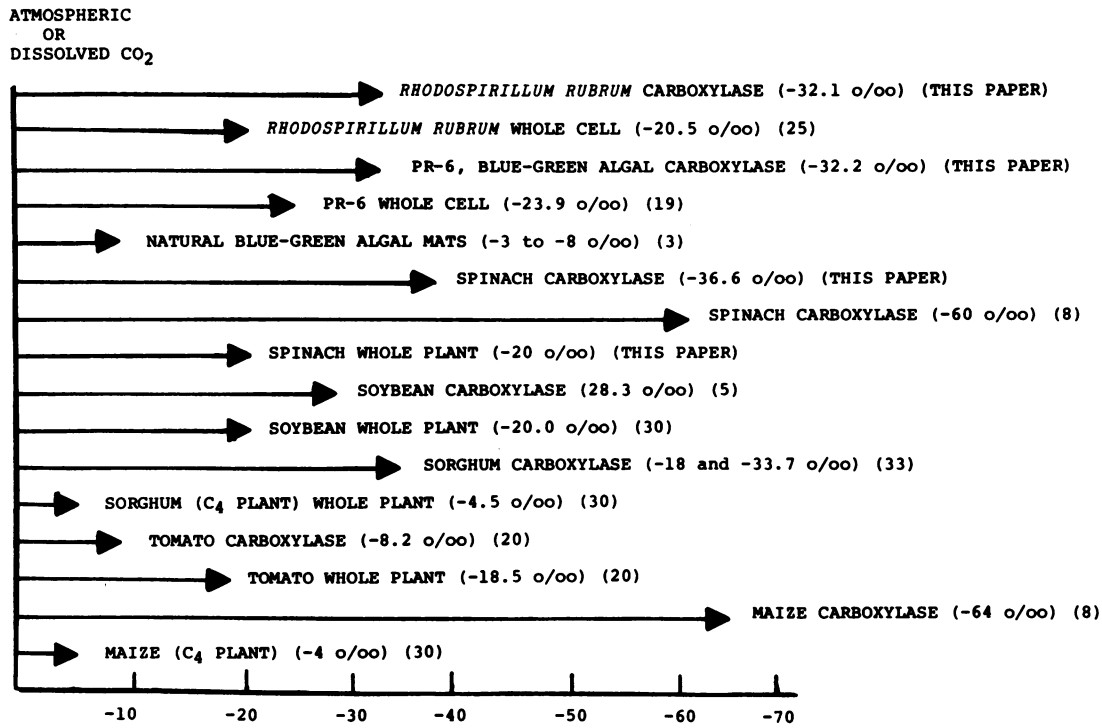

FIG. 4. Carbon isotope fractionation values (Δ) for whole plants/microorganisms compared to fractionation values (Δ dissolved CO_2) by their RuBP carboxylases. All fractionations are with respect to dissolved CO_2 or atmospheric CO_2. Length of arrows indicates fractionation value.

fractionation value was −41.9 ‰. Since the metal cofactor has been postulated to be present at the active RuBP carboxylase site, the study of carbon isotope fractionation values may be useful in studying the binding of CO_2 and the topography of the active site. On the basis of the Δ dissolved CO_2 value, it is tempting to predict that Ni^{2+} may be involved with carboxylation *in vivo* as its fractionation value is more similar to the fractionation seen in whole C_3 plants, but the experiments with equimolar concentrations of Mg^{2+} and Ni^{2+} seem to indicate that Mg^{2+} has a higher affinity for carboxylase and is probably the cofactor used by RuBP carboxylase *in vivo*.

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