from spinach and from *Rhodospirillum rubrum*.

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# Ribulose-1,5-bisphosphate Carboxylase: Primary Deuterium Kinetic Isotope Effect Using [3-$^2$H]Ribulose 1,5-Bisphosphate†

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ABSTRACT: The primary deuterium kinetic isotope effect for the reaction of [3-$^2$H]ribulose 1,5-bisphosphate with $CO_2$ in the reaction catalyzed by ribulose-1,5-bisphosphate carboxylase has been determined. By use of highly purified substrates containing less than 0.13% of the C-3 epimer xylulose 1,5-bisphosphate (this material is known to be a potent competitive inhibitor), the kinetic isotope effect has been shown to be 1.2 ± 0.2 ($V_{max}$) and 1.3 ± 0.3 ($V_{max}/K_m$). These values are clearly too small to be intrinsic isotope effects for the rate-limiting removal of the C-3 proton from ribulose 1,5-bisphosphate. The results confirm the conclusions from the tritium experiments reported in the previous two papers and emphasize the fine balance between the forward and reverse reactions of the enediol intermediate.

In the original formulation of the mechanism of action of ribulose-1,5-bisphosphate carboxylase (RuBP carboxylase),[1] Calvin (1954) proposed that the first catalytic step involves the abstraction of the proton at C-3 of RuBP to form the 2,3-enediol. It was suggested that this enediol then attacks enzyme-bound $CO_2$ to give a six-carbon species, the hydrolysis of which leads to the products, two molecules of 3-phospho-D-glycerate. [The pathway is illustrated in Scheme I of the first paper of this series (Saver & Knowles, 1981).]

From experiments with unlabeled RuBP in tritiated water (Saver & Knowles, 1982) and with [3-$^3$H]RuBP in unlabeled water (Sue & Knowles, 1982), the reality of the enolization step of Calvin's mechanism was demonstrated. It was shown that the enolization step is reversible, and this reversibility can result in the incorporation of a solvent proton onto carbon 3 of the remaining RuBP. Significant discrimination against tritium in the consumption of C-3-labeled substrate was also observed, signifying an isotope effect in the proton abstraction step that occurs after the binding of RuBP but before the first irreversible step in the reaction. It was concluded that proton abstraction from RuBP is at least partially rate determining in the early steps of the carboxylase reaction. Identification of the rate-determining step of the *overall* catalyzed reaction, however, can be made only by examining all the steps in the enzymic cycle, catalytic or other. For that purpose, the steady-state rates of 3-phosphoglycerate formation from the enzyme-catalyzed reaction of unlabeled RuBP and of [3-$^2$H]RuBP have been measured. If the proton abstraction step is rate determining in catalytic turnover, deuterium substitution in the substrate will result in an observed primary kinetic isotope effect.

Since the proposed experiment involved the precise measurement of reaction rates, it was important that the RuBP samples used differed only in the hydrogen isotope at C-3. In particular, it was critical that the substrates not be variably contaminated with xylulose 1,5-bisphosphate (XuBP), which is the product of nonenzymatic epimerization at C-3 (Paech et al., 1978) and has been reported to be a potent competitive inhibitor of RuBP carboxylase [$K_m$(RuBP) ≈ 20 μM and $K_i$(XuBP) ≈ 3 μM for the enzyme from spinach (McCurry & Tolbert, 1977)]. Samples of labeled and unlabeled RuBP were therefore prepared by enzymatic synthesis from 6-phosphogluconate immediately prior to the velocity measurements, and a method was established to determine the extent of XuBP contamination in the preparations of RuBP. We report here the synthesis of RuBP and of [3-$^2$H]RuBP that are contaminated by XuBP to less than 0.13%, and the steady-state kinetic parameters for the consumption of these substrates by the carboxylase.

## Materials and Methods

### *Materials*

Enzymes, cofactors, and other materials were as specified in Saver & Knowles (1982) and Sue & Knowles (1982). Sodium borodeuteride was obtained from Ventron Corp. Dihydroxyacetone phosphate (as the dimethyl ketal dimonocyclohexylammonium salt) was from Sigma, and glycolaldehyde phosphate (as the diethyl acetal dimonocyclohexylammonium salt) was from Calbiochem.

*(S)-[4-$^2$H]NADH.* To a solution of dihydroxyacetone (~60 mg) in water (5 mL) was added sodium borodeuteride (140 mg), and the mixture was left for 30 min at room temperature. The solution was then treated with formic acid (500 μL) and the resulting solution concentrated by rotary evaporation. Water was added and the sample taken to dryness. The resulting precipitate was dissolved in water and the solution

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[1] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; RuBP carboxylase, ribulose-1,5-bisphosphate carboxylase; XuBP, D-xylulose 1,5-bisphosphate; $NAD^+$, nicotinamide adenine dinucleotide; NADH, reduced $NAD^+$; $NADP^+$, nicotinamide adenine dinucleotide phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl.

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passed through a column (5 mL) of Dowex-50 ($H^+$ form). The eluate was taken to dryness, and repeated evaporations of added methanol [the residue was dissolved in a few drops of water, and methanol (~5 mL) was added] were carried out to remove borate (Zill et al., 1953). An assay of the dissolved product [using glycerol-3-phosphate dehydrogenase/$NAD^+$ and glycerol kinase/ATP in 250 mM hydrazine–500 mM glycine buffer, pH 9.0, containing $MgCl_2$ (2 mM)] indicated 242 μmol of [2-$^2$H]glycerol. The mass spectrum of tris(trifluoroacetyl)glycerol shows a strong $M^+ - 113$ peak ($M^+ - CF_3COO$) at $m/z$ 267. The ratio of the ion intensities at $m/z$ 268 and $m/z$ 267 was used to determine the extent of deuterium incorporation in the [2-$^2$H]glycerol sample.

Glycerokinase (74 units) was added to a solution (61.9 mL) of 250 mM hydrazine–500 mM glycine buffer, pH 9.0, containing $MgCl_2$ (2 mM), ATP (1.83 mM), $NAD^+$ (1.45 mM), [2-$^2$H]glycerol (1.62 mM), and glycerol-3-phosphate dehydrogenase (162 units). After 5 h at room temperature, the solution was titrated to pH 8.0 with HCl, diluted to ~750 mL with water, and subjected to chromatography on a column (5.0 × 35.8 cm) of DEAE-cellulose equilibrated with 65 mM $NH_4HCO_3$, pH 8.0. The column was eluted with a linear gradient (1.4 L plus 1.4 L) of $NH_4HCO_3$ (65–250 mM). The fractions containing NADH were pooled, and the solution was concentrated to near dryness. Several evaporations of added 2-propanol were carried out to remove bicarbonate, and the sample was finally taken to dryness. The yield of isolated [4-$^2$H]NADH was ~30% on the basis of starting $NAD^+$.

D-[*4-$^2$H*]*Fructose 1,6-bisphosphate* was prepared enzymically from dihydroxyacetone phosphate and D-[1-$^2$H]glyceraldehyde 3-phosphate (the latter being made in situ from 3-phospho-D-glycerate and [4-$^2$H]NADH) by the method described earlier for the $^3$H-labeled material (Sue & Knowles, 1982). The yield was 46% on the basis of starting [4-$^2$H]-NADH. The deuterium content of [4-$^2$H]fructose 1,6-bisphosphate was assessed as follows. A solution of [4-$^2$H]-fructose 1,6-bisphosphate (~0.5 μmol) in $NH_4HCO_3$ buffer, pH 8.0 (4.35 mL), was treated with a solution of alkaline phosphatase (2 units) in 200 mM $NH_4HCO_3$ (10 μL) and incubated at room temperature for 75 min. A solution of $NaBH_4$ (4.8 mg) in water (500 μL) was then added. After 2 h, the mixture was acidified with Dowex-50 ($H^+$ form) (1 mL). The resulting suspension was sonicated briefly to remove $CO_2$ and then applied to a column (1 mL) of Dowex-50 (cyclohexylammonium form). The eluate was concentrated under reduced pressure. Several evaporations of added MeOH were performed, and the sample was finally dried in vacuo. This material was converted to the pertrifluoroacetyl derivative, which was analyzed by mass spectroscopy. The peaks at $m/z$ 506 and $m/z$ 505 in the mass spectrum derive from the labeled and unlabeled $C_4$ ion fragments and record the extent of labeling in the parent molecule regardless of whether cleavage occurs between C-1 and C-2 or between C-4 and C-5. That the deuterium label resides on C-3 or C-4 of the [4-$^2$H]fructose 1,6-bisphosphate is confirmed by the absence of isotopic enrichment in the $C_2$ ion fragment at $m/z$ 253.

*6-Phospho*-D-[*4-$^2$H*]*gluconate* was prepared from [4-$^2$H]-fructose 1,6-bisphosphate by the method described for the $^3$H-labeled material (Sue & Knowles, 1982). The yield was 90%, based on starting fructose 1,6-bisphosphate.

[*3-$^2$H*]*Ribulose 1,5-bisphosphate* was prepared from 6-phospho[4-$^2$H]gluconate according to the procedure described for the preparation of unlabeled RuBP (Saver & Knowles, 1982). The deuterium content of [3-$^2$H]RuBP was determined by mass spectral examination of the corresponding pentakis-(trifluoroacetyl)pentitols, derived in the following manner. An aqueous solution of RuBP (0.1 μmol, sodium salt) was treated with a solution of $NaBH_4$ (10 mg) in water (100 μL). Excess reagent was destroyed with 1 drop of acetic acid. The sample was concentrated under reduced pressure, and further evaporations of added water were performed to remove traces of acetic acid. The mixture of pentitol 1,5-bisphosphates was dissolved in 200 mM $NH_4HCO_3$ (500 μL) and the solution treated with alkaline phosphatase (4.1 units) for 30 min. The solution was then treated with Dowex-50 ($H^+$ form) (0.5 mL) and the suspension sonicated and applied to a column (0.5 mL) of Dowex-50 ($NH_4^+$ form). The eluted sample was subjected to several evaporations of added water and methanol to remove borate and then derivatized with trifluoroacetic anhydride for mass spectral analysis. In the mass spectrum of the resulting pentakis(trifluoroacetyl)pentitols, the $M^+ - 113$ peaks ($M^+ - CF_3COO$) at $m/z$ 520 and $m/z$ 519 were used to monitor the extent of labeling. The location of the isotope at C-3 of the $C_5$ unit was confirmed by the presence of isotopic enrichment in the $C_3$ ion fragment ($m/z$ 380 and $m/z$ 379) and the absence of enrichment in the $C_2$ ion fragment at $m/z$ 253.

[*3-$^2$H*]*RuBP* was prepared twice, and the mass spectroscopic analyses of the deuterium contents of the [2-$^2$H]glycerol, the [4-$^2$H]fructose 1,6-bisphosphate intermediate, and the final product [3-$^2$H]RuBP are listed in Table I.

Table I: Deuterium Content of the Trifluoroacetylated Polyols Derived from Compounds in the Synthetic Route to [3-$^2$H]RuBP

| labeled compd | mass spectral ($m/z$) ratio compared | deuterium content[a] (%) prepn A | deuterium content[a] (%) prepn B |
|---|---|---|---|
| [2-$^2$H]glycerol | 268:267 | 97.8 ± 0.8 | 95 ± 1 |
| [4-$^2$H]fructose bisphosphate[b] | 506:505 | 94 ± 1 | 90 ± 3 |
| [3-$^2$H]RuBP[c] | 520:519 | 92.3 ± 0.8 | 89 ± 2 |

[a] Expressed as the percentage above natural abundance. The errors are derived from the standard deviations in the measured mass spectral ratios. [b] Analyzed as the hexitol mixture after dephosphorylation and borohydride reduction. [c] Analyzed as the pentitol mixture after borohydride reduction and dephosphorylation.

D-*Xylulose 1,5-Bisphosphate.* XuBP was prepared by the aldolase-catalyzed condensation of glycolaldehyde phosphate and dihydroxyacetone phosphate (McCurry & Tolbert, 1977). The addition of triosephosphate isomerase free aldolase (~9 units) initiated the reaction in 1.47 mL of 100 mM citrate–NaOH buffer, pH 6.5, containing 2-mercaptoethanol (2 mM), dihydroxyacetone phosphate (3.65 mM), and glycolaldehyde phosphate (4.18 mM). The reaction mixture was left at 30 °C for 150 min. Acid-washed charcoal (100 mg) was then added to the solution. The charcoal was removed by centrifugation followed by filtration of the supernatant through paper. The charcoal pellet was washed twice with water. The combined filtrates were then purified by chromatography on a column (1.0 × 6.8 cm) of DEAE-cellulose equilibrated with 50 mM triethanolamine hydrochloride buffer, pH 7.0, and eluted with a linear gradient (25 mL plus 25 mL) of the same buffer (50–300 mM). The fractions were assayed for phosphate released by alkaline phosphatase, and XuBP was assayed by using aldolase and glycerol-3-phosphate dehydrogenase/NADH in 100 mM triethanolamine hydrochloride, pH 8.0. The fractions containing XuBP corresponded to a peak well separated from dihydroxyacetone phosphate and glycolaldehyde phosphate, which eluted together. XuBP (in 30% yield, based on dihydroxyacetone phosphate) was then precipitated as the barium salt.

The concentration of XuBP determined by enzymatic assay of a sample was precisely half of that of $P_i$ liberated by alkaline

phosphatase. The sample contained no fructose 1,6-bisphosphate, as assayed with fructose 1,6-bisphosphatase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase/$NADP^+$. For gas chromatographic and mass spectrometric analysis, XuBP was derivatized as follows. An aqueous solution of XuBP (0.5 μmol) was treated with $NaBH_4$ (0.62 mg). After 1 h at room temperature, the sample was incubated with alkaline phosphatase (2.1 units in 10 μL of 200 mM $NH_4HCO_3$). After 2 h, acetic acid (100 μL) was added, and the sample was evaporated to dryness. After repeated evaporations of added $H_2O$–MeOH, an aqueous solution of the remaining residue was passed through a column of Dowex-50 ($H^+$ form) (0.5 mL) over Dowex-50 ($NH_4^+$ form) (0.5 mL). The eluted sample was subjected to several more evaporations of added $H_2O$–MeOH, dried in vacuo, and finally derivatized by reaction with trifluoroacetic anhydride. The resulting pentakis(trifluoroacetyl)pentitols were analyzed by gas chromatography and by mass spectrometry. The mass spectrum showed characteristic peaks at $m/z$ 519, 505, 379, and 253.

*Methods*

*Assay for XuBP.* The assay mixture contained XuBP (~0.004 μmol) in 100 mM triethanolamine hydrochloride buffer, pH 8.0 (1.13 mL), containing NADH (0.02–0.06 mM), $MgCl_2$ (20 mM), $NaHCO_3$ (50 mM), EDTA (4 mM), and dithiothreitol (1 mM). Glycerol-3-phosphate dehydrogenase (~10 units) was added, and when the absorbance at 340 nm had stabilized, aldolase (~40 units) was added. The amount of XuBP was calculated from the "burst" of absorbance that occurs on the addition of aldolase. When the assay is applied to a freshly prepared solution of RuBP (2.6–7.0 μmol), any contaminating XuBP still causes a burst in the absorbance at 340 nm, though there is a slow rate of change of the absorbance after the burst. [Presumably, RuBP is very slowly cleaved by aldolase. The relative rates of cleavage, $V_{max}$ values of RuBP, XuBP, and fructose 1,6-bisphosphate, have been reported to be <0.005, 0.3, and 1.0, respectively. The $K_m$ values for these three substrates are all very similar (Gray & Barker, 1970; Mehler & Cusic, 1967).] Extrapolation of the linear parts of the absorption vs. time curve allows the burst size to be calculated. When this method is used, XuBP contamination of RuBP samples can be quantitated at levels of 0.10 ± 0.02%.

*Measurements of Isotope Effect.* Freshly prepared samples of labeled and unlabeled RuBP were used as substrates in the determination of the $V_{max}$ and $K_m$. Each pair of determinations was run on the same day. RuBP concentrations were varied between 0.025 and 0.12 mM, the reactions being run at 30.0 °C under $N_2$. The final concentrations in the reaction mixture (0.5 mL) were 100 mM triethanolamine hydrochloride buffer, pH 8.0, 20 mM $MgCl_2$, 50 mM $NaHCO_3$, 4 mM EDTA, 1 mM dithioerythritol, 0.25 mM NADH, 10 mM ATP, ~8 units of phosphoglycerate kinase, ~18 units of glyceraldehyde-3-phosphate dehydrogenase, ~7 units of triosephosphate isomerase, ~6 units of glycerolphosphate dehydrogenase, and 0.0016 unit of RuBP carboxylase. The buffer solution containing $MgCl_2$, EDTA, and dithioerythritol was flushed with $N_2$ before use, and the NADH, ATP, RuBP, and coupling enzymes were added in a small volume. Bicarbonate was then added, and the reaction was initiated with activated carboxylase. The reference cuvette contained all components except the carboxylase.

*Measurement of the Loss of Deuterium from [3-$^2$H]RuBP during an Initial Velocity Experiment.* The carboxylase reactions were run either with or without the coupling enzymes used in the velocity measurements. The reaction in the presence of coupling enzymes was run in a cuvette under nitrogen as if a velocity measurement was being made, except that the volume of the reaction mixture was 2.5 mL, and it contained 0.14 μmol of [3-$^2$H]RuBP. The reaction was initiated with activated carboxylase. After ~2.5 min, the solution was added to $NaBH_4$ (11 mg in 100 μL of water). After acidification with acetic acid (3 drops), the sample was taken to dryness under reduced pressure. Two evaporations of added $H_2O$ and five evaporations of added MeOH were then performed. The dried sample was dissolved in water (2 mL), and the solution was treated twice with acid-washed charcoal (200 mg) and centrifuged for 2 min. The remaining charcoal pellet was washed 3 times with water. The combined filtrates were diluted and applied to a column (1.0 × 8.0 cm) of DEAE-cellulose equilibrated with 65 mM $NH_4HCO_3$, pH 8.0. The column was eluted with a linear gradient (30 mL plus 30 mL) of $NH_4HCO_3$ (65–200 mM). The fractions were assayed for phosphate released by alkaline phosphatase, and those corresponding to the pentitol bisphosphates were pooled, and the mixture was treated with alkaline phosphatase (~10 units in 50 μL of 200 mM $NH_4HCO_3$). After incubation for 30 min, the sample was treated with Dowex-50 ($H^+$ form) (3 mL) and sonicated to remove $CO_2$. The suspension was applied to a column (3 mL) of Dowex-50 ($NH_4^+$ form) and washed with water. The eluate was taken to dryness, and borate was removed by repeated evaporations of added MeOH. The residue was dried in vacuo and then treated with trifluoroacetic anhydride for mass spectral analysis.

The carboxylase-catalyzed reaction of [3-$^2$H]RuBP *without* accompanying coupling enzymes was accomplished as follows. Nitrogen-flushed buffer [100 mM triethanolamine hydrochloride, pH 8.0 (728 μL) at 30 °C, containing $MgCl_2$ (20 mM), EDTA (4 mM), and dithioerythritol (1 mM)] was introduced by syringe into a nitrogen-flushed flask containing $NaHCO_3$ (4.2 mg). [3-$^2$H]RuBP (0.28 μmol) in water (250 μL) was then added. A portion (480 μL) was removed as a control and transferred to a sealed, nitrogen-flushed vial. In the remaining portion, the reaction was initiated by addition of a buffered solution (16 μL) of activated carboxylase (0.0016 unit); the control sample was supplemented with the same volume of water. After 3 min, the reaction sample was added to an aqueous solution (100 μL) of $NaBH_4$ (10 mg). The control sample was similarly treated. Both samples were then taken to dryness, and several evaporations were performed, two of added water and five of added MeOH. The samples were then purified by chromatography and worked up for mass spectrometry as described above.

## Results

*Determination of $V_{max}$ and $K_m$ for Unlabeled RuBP and for [3-$^2$H]RuBP.* To ensure that the measurement of reaction rates would monitor only the consequence of deuterium substitution at carbon 3 of RuBP, it was important that the protonated and deuterated samples were handled in the same manner prior to the kinetic analyses. The preparations of unlabeled and 4-$^2$H-labeled 6-phosphogluconates from the respective fructose 1,6-bisphosphates, and the preparations of unlabeled and labeled RuBP from the respective 6-phosphogluconates, were therefore done identically in parallel.

The kinetic assays were run under nitrogen and were initiated by the addition of a solution of activated carboxylase to a buffered solution containing saturating levels of $CO_2$ and either unlabeled RuBP or [3-$^2$H]RuBP. The time course of the reactions was followed spectrophotometrically (Racker, 1962) by coupling the carboxylase-catalyzed formation of 3-phosphoglycerate with the reactions catalyzed by 3-

Table II: Kinetic Parameters for the Carboxylase-Catalyzed Reaction of Unlabeled and 3-$^2$H-Labeled RuBP

| prepn | RuBP samples | deuterium content (%) | $V_{max}$ [a] | $K_m$ [a] ($\mu$M) | $^HV_{max}/^DV_{max}$ obsd | $^HV_{max}/^DV_{max}$ cor [b] | $^H(V_{max}/K_m)/^D(V_{max}/K_m)$ obsd | $^H(V_{max}/K_m)/^D(V_{max}/K_m)$ cor [b] |
|---|---|---|---|---|---|---|---|---|
| A | protio | | 5.4 ± 0.3 | 33 ± 5 | 1.1 ± 0.1 | 1.2 ± 0.2 | 1.2 ± 0.2 | 1.2 ± 0.2 |
| | deuterio | 92.3 | 4.8 ± 0.08 | 34 ± 1 | | | | |
| B | protio | | 10.5 ± 0.5 | 33 ± 4 | 1.2 ± 0.2 | 1.2 ± 0.2 | 1.3 ± 0.2 | 1.4 ± 0.2 |
| | deuterio | 89.0 | 8.8 ± 0.4 | 36 ± 3 | | | | |
| | | | | | | av: 1.2 ± 0.2 | | av: 1.3 ± 0.3 |

[a] Determined from plots of $v$ vs. $v/[s]$. The errors are the standard errors from least-squares analysis of these plots. The units of $V_{max}$ are micromolar per minute. [b] Corrected for the incomplete isotopic labeling of the deuterated sample.

phosphoglycerate kinase/ATP, glyceraldehyde-3-phosphate dehydrogenase/NADH, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase/NADH. Four moles of NADH is therefore oxidized for every mole of RuBP consumed in the carboxylase reaction. The observed initial velocities were measured from the linear part of the progress curves (time span of 1–2 min) that followed a short lag phase (0.25–1 min). A sufficient excess of the coupling enzymes and cofactors was used to ensure that the observed reaction rates were proportional to the concentration of the carboxylase. It was shown that the rates were unaffected by the addition of carbonic anhydrase to the assay mixture; that is, the rate of bicarbonate dehydration is not rate limiting. The results of the $V_{max}$ and $K_m$ determinations for two different preparations of RuBP and of its 3-$^2$H-labeled analogue are listed in Table II.

Since the investigation of kinetic isotope effects naturally requires the presence of the isotope in the compound undergoing reaction, it was necessary to determine whether significant amounts of deuterium had been washed out from the substrate [3-$^2$H]RuBP during the time of the initial velocity experiments described above. [3-$^2$H]RuBP was therefore allowed to react with RuBP carboxylase (with or without coupling enzymes present), and the reaction was quenched after a short time by addition to sodium borohydride. After purification, the derived pentitol bisphosphates were subjected to mass spectroscopic analysis.

From the results shown in Table III, it is evident that the amount of deuterium lost from [3-$^2$H]RuBP during the early part of the carboxylase reaction is such that the measured reaction rate for the 3-$^2$H-labeled compound would have been increased by less than 10%. This difference is certainly within the limits of experimental error in the determination of $^HV_{max}/^DV_{max}$ and $^H(V_{max}/K_m)/^D(V_{max}/K_m)$.

Table III: Deuterium Loss from [3-$^2$H]RuBP during the Time Span of an Initial Velocity Experiment

| [3-$^2$H]RuBP sample | deuterium content [a] (% of total) | loss of deuterium (% of original) |
|---|---|---|
| starting material | 87 ± 1 | |
| after carboxylase-catalyzed reaction [b] | 78 ± 1 | 10.3 |
| starting material | 88.5 ± 0.9 | |
| after carboxylase-catalyzed reaction [c] | 81 ± 1 | 8.5 |
| after incubation [c] in absence of carboxylase | 88 ± 1 | 0.6 |

[a] The errors are standard deviations of repeated scans of the mass spectra. [b] In the presence of coupling enzymes. For conditions, see the text. The reaction was quenched after 2.5 min, when the reaction was ~23% complete (more carboxylase having been added than normally present in a velocity measurement). [c] In the absence of coupling enzymes. For conditions, see the text. The reaction was quenched after 3 min, when the reaction was ~3% complete (the normal amount of carboxylase having been added).

## Discussion

Before discussion of the implications of the very small primary kinetic isotope effect for the deprotonation at C-3 of RuBP in the carboxylase reaction, the validity of the observation must be established.

There are three main reasons why a more normal kinetic isotope effect might have been missed: (i) the reaction of the protonated sample might have been selectively inhibited; (ii) the deuterated sample might have reacted more rapidly than expected; and (iii) some process other than the carboxylase-catalyzed reaction was limiting the rate of the observed reaction.

First, it is unlikely that the protonated substrate would contain an inhibitor that was not also in the deuterated sample, since precautions had been taken to prepare the unlabeled RuBP and [3-$^2$H]RuBP concurrently and in precisely the same manner, despite the ready availability of the former. Even so, it was in principle possible that, in each of the independent determinations, the unlabeled RuBP was more contaminated than the labeled sample, by the C-3 epimer, XuBP. XuBP is a powerful inhibitor of the carboxylase, so a method was devised to assess the XuBP content of substrate RuBP samples. This assay showed that the XuBP contamination of the unlabeled RuBP was maximally 0.13%. From the known $K_i$ and $K_m$ values for XuBP and RuBP, respectively, this level of XuBP would cause a fractional inhibition of the observed reaction rate of approximately 0.5%. Since the substrates were prepared simultaneously by identical methods, and two complete preparations were conducted, the likelihood of a consistent impurity in one of them is negligible.

Second, the [3-$^2$H]RuBP sample might have reacted faster than expected because washout of the deuterium label may have generated unlabeled substrate during the course of the assay. This possibility was disproved by experiments that demonstrated that the loss of deuterium early in the carboxylase reaction (in the time span of an initial velocity measurement) is very small and cannot contribute significantly to the measured rate of the carboxylase-catalyzed reaction.

Third, the possibility must be faced that some reaction other than the carboxylase-catalyzed consumption of RuBP was limiting the rate of the change being measured. Control experiments were performed to demonstrate the adequacy of the coupled enzyme system by showing that the measured reaction rate is proportional to the carboxylase concentration at levels higher than those used in the velocity measurements. This leaves only the possibility that the concentration of cosubstrate $CO_2$ could have limited the reaction rate. In all the experiments described (Saver & Knowles, 1982; Sue & Knowles, 1982; present work), bicarbonate was present at 50 mM. This, under the conditions used, corresponds to a $CO_2$ concentration of 1.1 mM, which is in excess of the known $K_m$ for $CO_2$ of 0.3

mM (Christeller & Laing, 1978). Finally, that the *rate* of $CO_2$ production was not slow was proved by the insensitivity of the measured reaction rate to the presence of carbonic anhydrase.

The control experiments described above establish the validity of the rate determinations, and it is concluded that the observed isotope effects on $V_{max}$ and $V_{max}/K_m$ are indeed near unity. The experimental values of 1.2 ± 0.2 and 1.3 ± 0.3, respectively, are too small to constitute an *intrinsic* primary kinetic isotope effect for the rate-limiting removal of the C-3 proton from RuBP. [For the rate-determining cleavage of a C–H bond $\alpha$ to a carbonyl group, primary deuterium isotope effects in nonenzymic systems are about 5 (March, 1977), and in enzyme-catalyzed reactions, values of 3 or more have been observed [e.g., see Leadlay et al. (1976)].] Some transition state in the carboxylase-catalyzed reaction must therefore be overshadowing that for the cleavage of the C-3–H bond, and proton abstraction from RuBP cannot be cleanly rate limiting during the steady-state turnover of substrate molecules by the enzyme.

*Rate-Limiting Step.* The results presented in the preceding two papers have demonstrated that ribulosebisphosphate carboxylase proceeds via the enolization of RuBP and that the transition state for enolization is only partly rate limiting in the overall reaction. Thus, while discrimination against tritiated substrate is observed (Sue & Knowles, 1982), the proton abstraction step is not cleanly rate limiting since exchange of solvent tritium into remaining substrate is also significant (Saver & Knowles, 1982). The results obtained from the tritium experiments provide a maximum value of 0.44 for the mixed fractionation factor for the binding and enolization steps ($\Phi_{1,2}$), which is equivalent to an intrinsic kinetic tritium isotope effect ($\Phi_S/\Phi_{1,2}$) of *at least* 2.6. The *apparent overall* tritium isotope effect calculated from the data of Sue & Knowles (1982) is 1.25 ± 0.08. The incursion of other transition states (that of the condensation and, possibly, that for the isotope exchange process) evidently decreases the kinetic significance of the enolization step in the overall reaction. In the present paper, the validity of this conclusion is confirmed, since the overall kinetic deuterium isotope effect on $V_{max}/K_m$ is 1.3 ± 0.3. The error limits of these isotope effects, and their absolute magnitude, are such as to preclude any more detailed analysis. [Indeed, if eq 4.6 of Albery & Knowles (1976) is used to predict the deuterium isotope effect on $V_{max}/K_m$, using the range of parameters listed in Table III of the previous paper (Sue & Knowles, 1982), values near 1.8 are obtained. However, the accuracy of the results, the precise applicability of the Swain–Schaad relationship (Swain et al., 1958) to mixed fractionation factors, and uncertainty about fractionation in the exchange steps make such an exercise of questionable validity.]

It is clear that two or more transition states in the overall reaction catalyzed by the carboxylase are of detectable kinetic significance: the enolization step in which the C-3 proton of RuBP is abstracted, a subsequent isotopically insensitive step that is presumably the condensation of the enediol with $CO_2$, and, possibly, the exchange step in which protons from the medium replace the proton(s) on the conjugate acid of the enzyme base. As is being observed with increasing frequency for many different enzyme-catalyzed reactions, no single elementary step is cleanly rate limiting, the partitioning of enzyme-bound intermediates being rather finely balanced between the forward and reverse reactions.

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