Ribulose-1,5-bisphosphate Carboxylase: Enzyme-Catalyzed Appearance of Solvent Tritium at Carbon 3 of Ribulose 1,5-Bisphosphate Reisolated after Partial Reaction†

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ABSTRACT: When ribulose 1,5-bisphosphate is allowed to react with carbon dioxide in tritiated water in the carboxylation reaction catalyzed by ribulose-1,5-bisphosphate carboxylase from *Rhodospirillum rubrum*, the ribulose 1,5-bisphosphate reisolated after partial reaction is found to be labeled. The specific radioactivity of the remaining substrate pool rises during the course of the reaction. Experiments in deuterium oxide show that the isotopic label resides on carbon 3. Earlier failures to detect this exchange process probably derive from the use of enzyme that was, in the absence of carbon dioxide, inactive. The present results provide direct evidence for the intermediacy of the enediol between C-2 and C-3 of ribulose 1,5-bisphosphate and show that the enolization step is at least partially rate limiting in the overall carboxylase reaction. The specific radioactivity of the product 3-phospho-D-glycerate remains constant throughout the course of the reaction at about one-sixth that of the solvent. This strengthens the argument against the involvement of "sticky" protons in the reaction.

Ribulose-1,5-bisphosphate carboxylase catalyzes the condensation of D-ribulose 1,5-bisphosphate (RuBP)[1] with CO_2 to yield two molecules of 3-phospho-D-glycerate. The enzyme mediates the principal reaction involved in the fixation of CO_2 by photosynthetic organisms (McFadden, 1973). Mechanistically, the original pathway proposed by Calvin (1954) and outlined in Scheme I has, with minor additions, successfully accommodated the results of subsequent mechanistic investigations. The enzyme has been shown to require an "activating" CO_2 (distinct from the substrate CO_2) and magnesium ion for full catalytic activity (Lorimer et al., 1976; Laing & Christeller, 1976; Miziorko, 1979; Lorimer, 1979), and it has also been demonstrated that the enzyme will catalyze an oxygenase reaction in which dioxygen reacts with RuBP to produce one molecule of 3-phospho-D-glycerate and one molecule of phosphoglycolate (Andrews et al., 1971; Andrews & Lorimer, 1978; Lorimer et al., 1977).

In the Calvin mechanism for the carboxylase reaction, the RuBP is enolized by the abstraction of a proton from C-3 to generate an enediol (or enediolate) intermediate that then attacks CO_2. The resulting six-carbon intermediate, 2-carboxy-3-ketopentitol 1,5-bisphosphate, is then hydrolyzed to yield two molecules of 3-phospho-D-glycerate. This outline of events is supported by the facts that (i) carbon dioxide is the substrate [as distinct from bicarbonate (Cooper et al., 1969)] and becomes attached to C-2 of RuBP, (ii) carbon–carbon bond cleavage occurs between C-2 and C-3 of the RuBP (Müllhofer & Rose, 1965), (iii) the C-3 hydrogen of RuBP is lost to the medium (Fiedler et al., 1967), (iv) the hydrogen that becomes attached to C-2 of the "upper" phosphoglycerate molecule (Scheme I) is derived from the solvent (Fiedler et al., 1967), (v) the "lower" phosphoglycerate molecule acquires one of its carboxyl group oxygen atoms from water, and (vi) both the C-2 and the C-3 oxygen atoms of RuBP are retained in the products of the carboxylase-catalyzed reaction (Lorimer, 1978; Sue & Knowles, 1978). The participation of the six-carbon intermediate has been supported by inhibition studies with substrate analogues (Wishnick et al., 1970; Siegel & Lane, 1973; Pierce et al., 1980) and by the detection of a labeled material (deriving from $^{14}CO_2$) with electrophoretic properties expected for such a species (Sjödin & Vestermark, 1973).

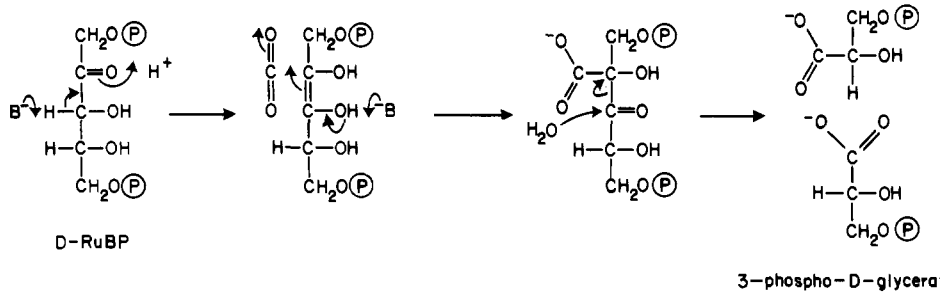
The existence of an enediol or enediolate intermediate deriving from RuBP in the carboxylase reaction requires the enzyme-catalyzed abstraction of a proton from C-3. If this proton (now attached to the enzymic base, B) (Scheme II) can exchange rapidly with the solvent, it is expected that the enolization catalyzed by RuBP carboxylase will result in the exchange of protons between C-3 of RuBP and the solvent (Scheme II). Such exchange is well precedented in the reactions catalyzed by aldolase and by triosephosphate isomerase, for example (Rose, 1962), and would, in the case of the carboxylase, further validate the mechanism outlined in Scheme I. Early attempts to demonstrate this exchange met with failure, however: when RuBP was incubated with the carboxylase in the absence of the cosubstrate CO_2, very little tritium from tritiated water washed into the substrate (Hurwitz et al., 1956; Simon et al., 1964). Conversely, when specifically labeled [3-^{3}H]RuBP was incubated with the enzyme in the absence of CO_2, very little isotope was washed out into the unlabeled solvent (Fiedler et al., 1967). Various explanations could account for these results: (i) the tritium kinetic isotope effect could be very large; (ii) the abstracted proton might be sequestered by the enzyme and be unavailable for exchange with solvent; or (iii) the enzyme could be unable to carry out any catalytic steps in the absence of activating CO_2 and magnesium ion. So that the most likely cause of failure, (iii), could be circumvented, enzyme that has been preactivated by incubation with CO_2 and magnesium must be used, and this, of course, means that the reaction mixture contains all the components required for the overall reaction of RuBP. When [3-^{3}H]RuBP was used as substrate in the carboxylase reaction, more than 98% of the tritium label appeared in the medium, and less than 0.1% was found in the product phosphoglycerate

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received March 3, 1982.* This work was supported by the National Science Foundation and the National Institutes of Health.

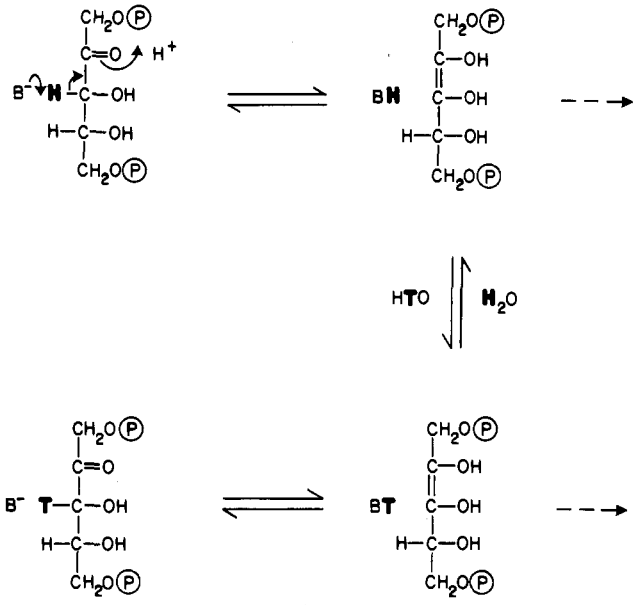
[1] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; RuBP carboxylase, ribulose-1,5-bisphosphate carboxylase; XuBP, D-xylulose 1,5-bisphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; $NADP^+$, nicotinamide adenine dinucleotide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

0006-2960/82/0421-5398$01.25/0

Scheme I: Calvin's Mechanism for the Reaction Catalyzed by RuBP Carboxylase



Scheme II: Carboxylase-Catalyzed Exchange of Protons between RuBP and Solvent[a]



[a] B is an enzymic base.

(Fiedler et al., 1967). The C-3 proton of RuBP is not, therefore, delivered to the product in the last step. On the basis that, in the above reaction, the rate of fixation of CO_2 was 5 times faster than the rate of appearance of tritium in the water, it was suggested that the removal of the C-3 proton was rate limiting (Fiedler et al., 1967). In the absence of any knowledge of the occurrence of washout of label from the [3-^{3}H]RuBP remaining after partial reaction, however, no quantitative estimate of the discrimination against tritiated substrate can be made.

To evaluate the importance of the putative enolization step in the carboxylase reaction, we report here the results of exchange–conversion experiments, in which we have measured the appearance of tritium at C-3 of the RuBP that remains after partial enzyme-catalyzed reaction in tritiated water.

Materials and Methods

Materials

RuBP carboxylase was isolated from *Rhodospirillum rubrum* as described previously (Sue & Knowles, 1978). The enzyme was >95% homogeneous on polyacrylamide disc gel electrophoresis and had a specific catalytic activity of 2.94 units/mg. A unit of enzyme activity is defined as that amount which catalyzed the reaction of 1 μmol of RuBP per min at 30 °C under the conditions of assay reported by Racker (1962), assuming a value for $A^{0.1\%}_{280nm}$ of 0.974 (Tabita & McFadden, 1974). It should be noted that a more recent determination gives an $A^{0.1\%}_{280nm}$ of 1.20 (Stringer et al., 1981). Phosphoribulokinase (as a partially purified powder, from spinach) was from Sigma. Solutions were prepared freshly by dissolution of the powder in 100 mM triethanolamine hydrochloride buffer, pH 8.0, containing $MgCl_2$ (20 mM), EDTA (1 mM), and 2-mercaptoethanol (1 mM). The solution was clarified by brief centrifugation. Aldolase (rabbit muscle), alkaline phosphatase (bovine intestine), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), α-glycerophosphate dehydrogenase (rabbit muscle), phosphoglucose isomerase (yeast), glucose-6-phosphate dehydrogenase (yeast), 3-phosphoglycerate kinase (yeast), 6-phosphogluconate dehydrogenase (yeast), and glycerol kinase (*Candida mycoderma*) were obtained as crystalline suspensions in ammonium sulfate, from Sigma. D-Fructose 1,6-bisphosphatase (rabbit muscle, suspension in ammonium sulfate) and carbonic anhydrase (bovine erythrocytes, dialyzed and lyophilized) were also from Sigma. Triosephosphate isomerase (crystalline, from chicken muscle) was a gift from J. Belasco. Where relevant, enzymes were pretreated with bromohydroxyacetone phosphate to remove triosephosphate isomerase activity (de la Mare et al., 1972) and then dialyzed against 100 mM triethanolamine hydrochloride buffer, pH 8.0, containing 2-mercaptoethanol (5 mM). ATP, NADH, NAD^+, $NADP^+$, and 6-phosphogluconate were obtained from Sigma.

Trifluoroacetic anhydride was obtained from Aldrich. Dowex-50W (100–200 mesh, 4% cross-linked) was from Sigma. Diethylaminoethylcellulose (DE 52) was from Whatman.

Buffer solutions were prepared from the highest grade commercial materials and distilled–deionized–distilled water.

D-*Ribulose 1,5-Bisphosphate.* In the procedures described below, columns of DEAE-cellulose were run at 4 °C, and columns of Dowex-50 were run at room temperature. For our first experiments, RuBP was purchased from Sigma as the barium salt or was prepared from D-ribose 5-phosphate by using the method of Horecker et al. (1958) and purified by chromatography on DEAE-cellulose.

These methods were superseded by the synthesis of RuBP from 6-phosphogluconate, developed by J. M. Sue for the preparation of the samples of specifically labeled RuBP (Sue & Knowles, 1982a,b). This synthesis, if carried out no more than a day in advance, yielded RuBP that contained at most 0.15% of its C-3 epimer, XuBP. The addition of 6-phosphogluconate dehydrogenase (2.3 units) initiated the reaction in an incubation mixture (1.42 mL) containing 100 mM triethanolamine hydrochloride buffer, pH 8.0, $MgCl_2$ (20 mM), EDTA (4 mM), 2-mercaptoethanol (1 mM), $NADP^+$ (5.2 mM), ATP (14.9 mM), 6-phosphogluconate (3.2 mM), and phosphoribulokinase (9.2 units). After 1 h at room temperature, the reaction was terminated by centrifugation of the mixture through an ultrafiltration membrane cone at 3000 rpm for 20 min. The cone was rinsed with 10 drops of water, and to the combined ultrafiltrates were added NADH (0.05 μmol), glycerol-3-phosphate dehydrogenase (~60 units), and aldolase (~3 units). After 40 min at room temperature, acid-washed charcoal (113 mg) was added, and the mixture was centrifuged for 2 min. The supernatant was treated again with charcoal

and centrifuged. The charcoal pellet was washed 3 times with water, and the combined supernatants were filtered through paper. The filtrate was loaded onto a column (1.0 × 8.0 cm) of DEAE-cellulose equilibrated with 70 mM triethanolamine hydrochloride buffer, pH 7.0. After elution of the column with a linear gradient (30 mL plus 30 mL) of triethanolamine hydrochloride (70–300 mM), pH 7.0, RuBP was precipitated from the appropriate fractions (pooled volume, ~10 mL) by addition of a saturated solution of $BaCl_2$ (1 mL) and ethanol (5 mL) at room temperature. After being stirred for 30 min, the mixture was centrifuged at 3000 rpm for 10 min. The precipitate was washed twice with aqueous ethanol (100 and 200 μL, 80% v/v) and dried under a nitrogen stream and then in vacuo for 1 h. This material was stored at −70 °C. The barium salt of RuBP (0.5–2 mg) was converted to the sodium salt by treatment with Dowex-50 (H^+ form) (20 μL of moist resin plus one drop of water), followed by filtration of the suspension through a column of Dowex-50 (Na^+ form) (200 μL of moist resin). At least 95% of the RuBP eluted in the first 600 μL of filtrate. Solutions of RuBP sodium salt were kept at 4 °C until needed.

The yield of RuBP from 6-phosphogluconate depends on the purity and the amount of the enzyme phosphoribulokinase used in the synthesis and is between 40 and 90% before chromatography. The amount of RuBP reaches a maximum during the course of the dehydrogenase–kinase reaction. The later fall in the RuBP concentration appears to derive from phosphatase activity present in commercial preparations of phosphoribulokinase. The recovery of RuBP from the chromatography and precipitation steps was between 74 and 90%. The amount of RuBP assayable by using the carboxylase was, for the peak fractions, within 10% of the amount estimated by phosphate assay of the phosphate released from the derived pentitol bisphosphates by alkaline phosphatase.

Methods

Assays. Assays of carboxylase activity were carried out as follows. The enzyme was first activated by dialysis overnight at 4 °C against 100 mM triethanolamine hydrochloride buffer, pH 8.0, containing $MgCl_2$ (20 mM), $NaHCO_3$ (50 mM), EDTA (4 mM), and dithiothreitol (1 mM). The coupling enzymes (3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase) were also dialyzed against the activation buffer. The reaction was initiated by the addition of activated carboxylase to the activation buffer (1 mL) containing NADH (0.25 mM), ATP (10 mM), RuBP (0.3 mM), and the coupling enzymes, at 30 °C. The proportionality between the rate of the reaction and the amount of carboxylase was checked routinely.

Kinetic experiments were done as follows. A solution of 100 mM triethanolamine hydrochloride, pH 8.0, containing $MgCl_2$ (20 mM), EDTA (4 mM), and dithioerythritol (1 mM) was flushed with nitrogen for 1–3 h. The reaction mixture contained, in an optical cuvette, the following: (i) buffer solution (35 μL) containing NADH (3.57 mM) and ATP (142.9 mM), (ii) an unbuffered solution of RuBP sodium salt (75 μL), and (iii) a buffered solution of the coupling enzymes (20 μL). The cuvette was capped with a rubber septum and flushed briefly with nitrogen. The next additions, made under nitrogen, were (iv) buffer solution to bring the total volume to 460 μL and (v) buffer solution (30 μL) containing $NaHCO_3$ (833 mM). The reaction mixture, at 30 °C, was initiated with activated carboxylase (10 μL of a solution containing 0.16 unit/mL), added by syringe, and the absorbance was monitored at 340 nm. The velocity of the reaction in progress was unaffected by the addition of carbonic anhydrase (>160 units) or of a second portion of coupling enzymes.

The amount of contaminating XuBP in RuBP samples was measured as described by Sue & Knowles (1982b).

The phosphate content of a sample was determined by incubation of the sample with alkaline phosphatase (~0.2–4.0 units in 200 mM NH_4HCO_3) followed by measurement of the liberated phosphate according to Ames (1966).

Radioactivity. Radioactive samples of ≤100 μL were dissolved in scintillation fluid (10 mL) composed of toluene (2460 mL), ethanol (1120 mL), naphthalene (210 g), and Liquifluor (160 mL). Counting was done in glass vials, using a Beckman LS-233 liquid scintillation system. Counting errors were ≤2%.

Mass Spectrometry. Mass spectra were taken on an AEI MS9 mass spectrometer. Quantitative determinations of ion intensities were obtained by slow and repeated scanning of the relevant peaks. Trifluoroacetyl esters of polyols were analyzed at a source temperature of 60–110 °C.

Gas chromatography was performed on a Varian Aerograph Model 1440 single-column flame-ionization detector gas chromatograph, with nitrogen as the carrier gas. The trifluoroacetyl esters of pentitols were analyzed on a column (6 ft) of OV-11 (10%) on Supelcoport 100/120 mesh (Supelco, Inc., Bellefonte, PA), at a column temperature of 150 °C and an injector temperature of 200 °C.

Derivatization. Trifluoroacetyl esters of polyols were prepared by addition of equal volumes of ethyl acetate and trifluoroacetic anhydride to freeze-dried samples followed by heating to 70–90 °C for 5 min. The mass spectrum of a pertrifluoroacetylated alcohol shows a peak at $M^+ - 113$, which arises from the loss of a trifluoroacetyl group from the molecular ion.

Reaction of RuBP in Tritiated Water. The approximate extents of reaction were estimated from a concomitant spectrophotometric assay at 370 nm in a 1-mm cuvette. The extinction coefficient for NADH at 370 nm is 2500 M^{-1} cm^{-1}.

For the exchange vs. conversion experiment, the following components were contained in a mixture (196 μL) prepared under nitrogen: RuBP (0.4 μmol of the free acid), NaOH (1.6 μmol), and nitrogen-flushed carboxylase assay buffer (without coupling enzymes) containing $NaHCO_3$ (50 mM). Tritiated water (4 μL, of specific radioactivity 5 Ci/mL) was injected into the reaction mixture, and after the solution was mixed, two portions (10 μL) were withdrawn for the determination of the specific radioactivity of the solution. The mixture was then incubated at 30 °C, and the reaction was initiated by the addition of activated carboxylase (0.015 unit). At appropriate times, portions (200 μL) were withdrawn and added to solid $NaBH_4$ (~6 mg). After 30 min, excess borohydride was destroyed by the addition of acetic acid (five drops).

Each quenched sample was freeze-dried by bulb to bulb distillation in vacuo. This procedure was followed by two more lyophilizations of added H_2O (~1 mL). The sample was then dissolved in water (10 mL) and applied to a column (0.7 × 8.5 cm) of DEAE-cellulose equilibrated with 50 mM NH_4HCO_3, pH 8.0. The column was washed with this buffer until no appreciable radioactivity eluted from the column. The radioactive compounds were then recovered by elution from the column with a linear gradient (25 mL plus 25 mL) of NH_4HCO_3 (50–200 mM).

Results

Position of Solvent-Derived Isotope in RuBP. The proton of RuBP that is most likely to exchange with the medium during the carboxylase reaction is the one at C-3, since this is the proton that is removed in the overall catalytic process (Fiedler et al., 1967). Since, however, a small amount of nonspecific labeling may occur at other positions of RuBP [this

Table I: Deuterium Labeling As Assessed by the *m/z* 253 and the *m/z* 379 Peaks in the Mass Spectrum of the Pentakis(trifluoroacetyl)pentitols

source of sample	*m/z* 254:253 ratio [a] (C_2 species)	*m/z* 380:379 ratio [a] (C_3 species)
enzyme reaction [b]	0.11 (±0.01)	0.38 (±0.01)
controls [c]		
i	0.12 (±0.02)	0.12 (±0.01)
ii	0.12 (±0.01)	0.15 (±0.02)
	0.07 [d]	0.10 [d]

[a] Averaged from 6-10 scans of the mass spectrum. The values in parentheses are standard deviations. [b] Incubated 23.5 min in 100 mM triethanolamine hydrochloride buffer, pH 8.0, 30 °C. The extent of reaction was ~60%. [c] Incubation in the absence of enzyme. Quenched by borohydride either immediately (i) or after 24 min at 30 °C (ii). [d] Predicted from the natural abundance of isotopes.

is clear from the fact that some labeling of RuBP was observed from an incubation of inactive carboxylase in tritiated water in the absence of CO_2 (Simon et al., 1964)], it was necessary to show that the carboxylase-catalyzed incorporation of label occurs only at C-3 of remaining RuBP. For this purpose, RuBP was incubated with active carboxylase in ^2H_2O, and the label distribution in reisolated RuBP was assessed by mass spectrometric analysis. The carboxylase-catalyzed reaction was allowed to proceed in ^2H_2O and was quenched after partial reaction by the addition of sodium borohydride. The reduction of the remaining RuBP to the two pentitol bisphosphates (epimers at C-2) ensured that the labile protons, those α to the carbonyl group of RuBP, would not be lost in the subsequent workup. After purification by ion-exchange chromatography, the pentitol bisphosphate sample was treated with alkaline phosphatase, and the resulting mixture of pentitols was trifluoroacetylated and then examined by mass spectrometry to determine the extent and position of deuterium labeling.

The mass spectrum of the pentakis(trifluoroacetyl)pentitols (Chizhov et al., 1969) enables one to distinguish between a deuterium atom at C-3 and labels at all other carbon positions. The parent ion fragments in the mass spectrometer to produce a three-carbon species (at *m/z* 379) that necessarily contains the C-3 carbon, and this fragment displays an increased mass if a proton at *any* carbon center has exchanged with solvent ^2H_2O. By determining the extent to which the two-carbon ion fragment (at *m/z* 253) which derives from C-1 + C-2 and C-4 + C-5 is also deuterated, it is possible to identify the fractional labeling due to deuterium at the C-3 center alone. From the results in Table I, it is clear that the deuterium enrichment found in the C_3 ion fragment is not due to labeling at C-1, C-2, C-4, or C-5 of the parent pentakis(trifluoroacetyl)pentitols, since there is no enrichment in the C_2 ion fragment. Consequently, the position of deuterium incorporation in RuBP catalyzed by the carboxylase is essentially limited to the C-3 position of RuBP.

The ratios of *m/z* 254:253 and *m/z* 380:379 measured for the control samples (deriving from the incubation of RuBP in deuterated water in the absence of enzyme) are both somewhat higher than the ratios predicted from the natural abundance of isotopes (Table I). The discrepancies are small and do not affect the conclusion that the enzyme catalyzes the exchange of hydrogen isotope between the solvent and C-3 of the remaining RuBP.

Exchange vs. Conversion Catalyzed by RuBP Carboxylase. The carboxylase reaction was performed under nitrogen in tritiated buffer with initial substrate concentrations of 2 mM RuBP and 50 mM $NaHCO_3$. At different times, samples were removed and added to sodium borohydride. Concurrently, a control incubation containing the coupling enzymes and cofactors required for a spectrophotometric assay was run to determine the time required for appropriate extents of reaction. Tritiated water was removed from the quenched samples by bulb to bulb distillation in vacuo, and the remaining material was subjected to ion-exchange chromatography on DEAE-cellulose. Fractions containing the pentitol bisphosphates and 3-phospho-D-glycerate were assayed for phosphate content and radioactivity, from which the specific radioactivities of each compound were calculated. The extent of reaction was estimated from the relative amounts of pentitol bisphosphates and 3-phosphoglycerate, based on the known total and specific radioactivities.

The results from these experiments are summarized in Table II. The specific radioactivity of the product 3-phospho-D-glycerate is independent of the extent of reaction and has an

Table II: Tritium Content of Product 3-Phospho-D-glycerate and of Remaining Ribulose Bisphosphate as a Function of the Extent of the Carboxylase Reaction [a]

sample	specific radioactivity of solvent [b] (cpm/μmol × 10^{-5})	specific radioactivity [c] (cpm/μmol × 10^{-5}) remaining bisphosphate	specific radioactivity [c] (cpm/μmol × 10^{-5}) product phosphoglycerate	extent of reaction [d] (%)	tritium content (specific radioactivity as % of solvent) of remaining bisphosphate	tritium content (specific radioactivity as % of solvent) of product phosphoglycerate
1	7.0 ± 0.1	0.911 ± 0.007	0.55 ± 0.01	21	13.0 ± 0.4	7.9 ± 0.4
		1.91 ± 0.02	0.55 ± 0.01	38	27 ± 1	7.9 ± 0.4
		3.26 ± 0.03	0.54 ± 0.01	56	47 ± 2	7.7 ± 0.4
		5.17 ± 0.06	0.56 ± 0.01	70	74 ± 3	8.0 ± 0.4
		7.37 ± 0.05	0.54 ± 0.01	82	105 ± 3	7.8 ± 0.3
		8.41 ± 0.06	0.54 ± 0.02	86	120 ± 4	7.7 ± 0.5
2	7.7 ± 0.1	2.43 ± 0.03	0.64 ± 0.01	39	32 ± 1	8.3 ± 0.2
		9.0 ± 0.6	0.59 ± 0.02	83	117 ± 8	7.7 ± 0.5
		17 ± 1	0.59 ± 0.02	98	225 ± 14	7.7 ± 0.5
control [e]	7.3 ± 0.1	0.095 ± 0.002		*f*	1.30 ± 0.06	
		0.301 ± 0.008		*g*	4.1 ± 0.2	

[a] In 100 mM triethanolamine hydrochloride buffer, pH 8.0, 30 °C. [b] Duplicate portions (10 μL) of the reaction mixture were removed for scintillation counting before the addition of carboxylase to initiate the reaction. [c] Average value determined for the appropriate fractions from the ion-exchange column. [d] Estimated from the total amounts of bisphosphate and phosphoglycerate, which was determined from the known total and specific radioactivities. Enzyme sample 1 was quenched at 38 min for 86% conversion and enzyme sample 2 at 28 min for 98% conversion. [e] In the absence of enzyme. [f] Quenched after 55 min. [g] Quenched after 180 min.

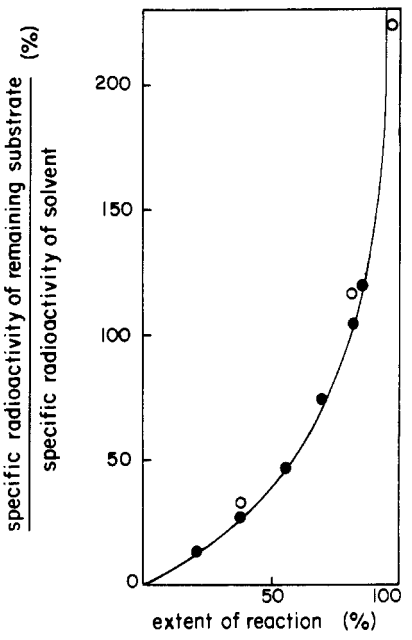


FIGURE 1: Tritium content of the remaining ribulose 1,5-bisphosphate as a function of the extent of the carboxylase-catalyzed reaction in tritiated water. Data from Table II.

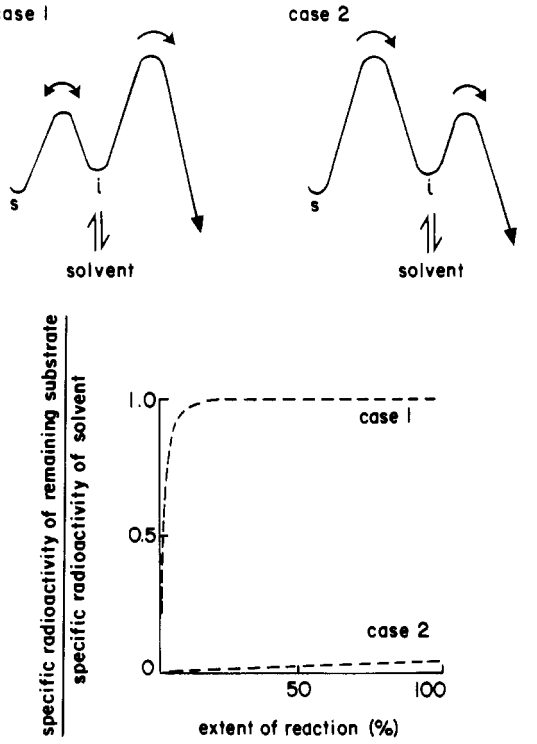
average value of 7.8% (±0.2%) that of the solvent. In contrast, the tritium content of the remaining RuBP is clearly time dependent (see Figure 1). The substrate RuBP, which enters the reaction unlabeled, incorporates tritium during the incubation with enzyme, and its specific radioactivity (as judged from the tritium content of the derived pentitol bisphosphates) rises as the carboxylase reaction approaches completion. Interestingly, the specific radioactivity of remaining RuBP at very high fractional conversion actually exceeds that of the solvent in which the reaction is run. (In a control experiment performed in the absence of enzyme, the extent of tritium labeling in the reisolated RuBP was negligible. Moreover, the rise in the specific radioactivity of the derived pentitol bisphosphates cannot come from an enzyme-catalyzed epimerization to a tritiated pentulose bisphosphate that is not a substrate for the carboxylase. Any such epimerization would *decrease* the observed rise in the specific radioactivity.)

Discussion

The experiments described in this paper were designed to establish the existence of the putative enediol in the Calvin mechanism for RuBP carboxylase (Scheme I) by observing the incorporation of solvent tritium into the C-3 position of RuBP remaining after partial reaction. Further, it was hoped that the time course of any such incorporation would define the relative rates of the early steps in the carboxylase-catalyzed reaction.

As illustrated in Scheme II, the proposed enediol intermediate can partition between two fates: it can isomerize back to the substrate, adding a proton that may be solvent derived (i.e., "exchange"), or it can proceed forward to form product (i.e., "conversion"). The preference for one pathway or the other will depend on the relative free-energy barriers, as shown for two limiting cases in Scheme III. If the first energy barrier (the formation of the enediol) is much lower than the second (the subsequent irreversible reaction of the enediol), as in case 1, then equilibrium is rapidly established between the intermediate i and the substrate s before the conversion of the

Scheme III: Limiting Cases for the Incorporation of Isotope into Substrate (s) by Proton Exchange between an Intermediate (i) and Solvent



intermediate to product. If the reaction is performed in tritiated water, the isotopic content of the substrate reisolated after short reaction time will approximate that of the solvent, *provided that proton exchange between solvent and the participating enzymic base is fast*. For this case, the dependence of the specific radioactivity of the remaining substrate on the extent of the reaction will be as illustrated in Scheme III, case 1. If, however, the first energy barrier is higher than the second, as in case 2, the intermediate i will almost always collapse to product and only rarely revert to substrate. Since the remaining substrate can incorporate isotope only when the enediol intermediate partitions back, having picked up solvent tritium, the tritium content of the remaining substrate will not rise much during the course of the reaction. This is illustrated in Scheme III, case 2. If the partitioning of the intermediate is more evenly balanced than either case 1 or case 2, then the plot of specific radioactivity of remaining substrate vs. extent of reaction may allow the partition ratio for exchange vs. conversion to be assessed quantitatively.

The exchange–conversion curve observed for the carboxylase reaction (Figure 1) is intermediate between the two extremes depicted in Scheme III. The fact that tritium is incorporated into the substrate RuBP at all indicates that there *is* some equilibration in the early steps before the intermediate is converted to product. The initial gradient of the experimental line in Figure 1 is about 0.5, which suggests that conversion of the enediol to product is about twice as easy as exchange back to substrate. Thus, the two free-energy barriers shown in Scheme III are rather similar in height. It should be noted that since exchange is detected only by the return of the intermediate over an energy barrier that involves *tritium* transfer (from solvent to substrate), the experimental results presented here do not apply exactly to the energetics of the reaction of unlabeled material in H_2O.

The nonlinearity of the exchange–conversion curve and the high specific radioactivity of the remaining substrate toward

the end of the reaction are features that we have also observed in the exchange–conversion study of triosephosphate isomerase (Maister et al., 1976). In that work, the shape of the curve was ascribed qualitatively and quantitatively to two factors. First, the partition ratio for the enediol intermediate is defined by the relative heights of the free-energy barriers on either side of it (see Scheme III) and describes the proportion of molecules that return to substrate compared to those that proceed on to product. The partitioning of the intermediate determines the initial slope of the curve, this slope being a measure of the rate that unlabeled substrate molecules become labeled. After the reaction has proceeded for some time, a second factor becomes increasingly important. Once a tritium label is incorporated into the substrate, the intrinsic isotope effect in the proton abstraction step results in a discrimination against a tritium-labeled species. Thus, from a mixture of protonated and tritiated molecules, unlabeled molecules are selected preferentially for reaction with enzyme. In effect, the tritiated molecules are constantly being "left behind". The specific radioactivity of the remaining substrate therefore increases not only because of the exchange reaction but also because of the constant selection against the labeled substrate species. In the final stages of reaction, what little substrate remains is predominantly tritiated and has an even higher specific radioactivity than the solvent.

In terms of providing information about the carboxylase mechanism, the results presented here establish the following points.

(*a*) *RuBP carboxylase catalyzes the formation of an intermediate that is capable of proton exchange with the solvent, this exchange being specific for C-3 of RuBP.* This fact is consistent with the assumption of an enzyme-catalyzed enolization of RuBP. Although it had previously been shown that proton *abstraction* takes place in the overall catalytic reaction, the occurrence of proton *exchange* catalyzed by active enzyme has not previously been demonstrated. It is reasonable to expect that this proton transfer process is an integral part of the carboxylation mechanism.

(*b*) *Enolization must be at least partially rate limiting in the overall carboxylase reaction.* The rise in the isotopic content of the RuBP with increasing extents of reaction shows that there is significant discrimination against C-3-tritiated RuBP in the enzyme-catalyzed reaction. It therefore follows that enolization precedes an irreversible step and must be at least partially rate limiting.

(*c*) *The hydrogen that becomes attached to C-2 of the product 3-phosphoglycerate is derived from the solvent and not from RuBP.* Although the specific radioactivity of the substrate pool rises progressively during the course of the reaction, there is no corresponding increase in the specific radioactivity of the product 3-phosphoglycerate. The tritium bound to phosphoglycerate is derived from the solvent, and that protonation step shows an isotope effect for tritium transfer: the half of the molecules of phosphoglycerate that bears a solvent-derived proton (tritium) has a specific radioactivity that is about 16% that of the solvent. This 6-fold discrimination qualitatively confirms the earlier results of Fiedler et al. (1967) in their studies on the spinach enzyme.

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