Use of Multiple Isotope Effects To Study the Mechanism of 6-Phosphogluconate Dehydrogenase†

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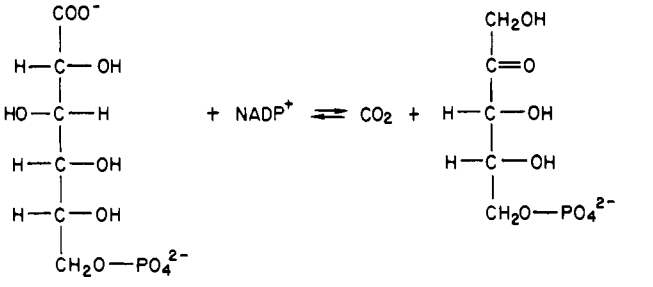
ABSTRACT: The multiple isotope effect method of Hermes et al. [Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106–5114] has been used to study the mechanism of the oxidative decarboxylation catalyzed by 6-phosphogluconate dehydrogenase from yeast. ^{13}C kinetic isotope effects of 1.0096 and 1.0081 with unlabeled or 3-deuterated 6-phosphogluconate, plus a ^{13}C equilibrium isotope effect of 0.996 and a deuterium isotope effect on V/K of 1.54, show that the chemical reaction after the substrates have bound is stepwise, with hydride transfer preceding decarboxylation. The kinetic mechanism of substrate addition is random at pH 8, since the deuterium isotope effect is the same when either NADP or 6-phosphogluconate or 6-phosphogluconate-*3-d* is varied at fixed saturating levels of the other substrate. Deuterium isotope effects on V and V/K decrease toward unity at high pH at the same time that V and V/K are decreasing, suggesting that proton removal from the 3-hydroxyl may precede dehydrogenation. Comparison of the tritium effect of 2.05 with the other measured isotope effects gives limits of 3–4 on the intrinsic deuterium and of 1.01–1.05 for the intrinsic ^{13}C isotope effect for C–C bond breakage in the forward direction and suggests that reverse hydride transfer is 1–4 times faster than decarboxylation.

Hermes et al. (1982) have shown that, by measuring the ^{13}C isotope effect with a deuterated and nondeuterated substrate, it was possible to distinguish mechanisms in which the deuterium- and ^{13}C-sensitive steps were the same or different and, in the latter case, to tell which step came first. A concerted transition state was seen for the C–H cleavage catalyzed by glucose-6-phosphate dehydrogenase, while NADP malic enzyme was shown to catalyze a stepwise mechanism with dehydrogenation preceding decarboxylation. In further applications of this method, we report in this paper that 6-phosphogluconate dehydrogenase has a stepwise mechanism like that of malic enzyme, while in the following paper we demonstrate that prephenate dehydrogenase catalyzes a concerted oxidative decarboxylation of its natural substrate.

Yeast 6-phosphogluconate dehydrogenase (EC 1.1.1.44) isolated in crystalline form by Pontremoli et al. (1961) catalyzes the oxidative decarboxylation of 6-phosphogluconate by NADP to give ribulose 5-phosphate and CO_2 (eq 1). The



(1)

reaction is readily reversible with an equilibrium constant of 79 mM for eq 1 as written (CO_2, not bicarbonate ion, is the product; Villet & Dalziel, 1969). It has long been assumed, in accord with the known mechanism for the decarboxylation of β-keto acids in solution (Bender & Breslow, 1962), that the decarboxylation occurred in a two-step process, with formation of 3-keto-6-phosphogluconate via hydride transfer to NADP preceding the C–C bond breakage. On the basis of tritium exchange experiments, Lienhard & Rose (1964) tentatively suggested an enzyme-bound enediol form of ribulose 5-phosphate as an intermediate. Although 3-keto-6-phosphogluconate has never been trapped as an intermediate product, when 2-deoxy-6-phosphogluconate was used as the substrate, 2-deoxy-3-keto-6-phosphogluconate was isolated from the reaction mixture, since decarboxylation was greatly slowed down (Rippa et al., 1973). This intermediate was reduced by NADPH to the starting material or decarboxylated to 1-deoxyribulose 5-phosphate when incubated with the yeast enzyme and NADPH.

In this paper we use deuterium isotope effects to establish a random kinetic mechanism for the yeast enzyme, use the multiple isotope effect methodology of Hermes et al. (1982) to prove that the mechanism is stepwise, and use tritium, ^{13}C, and deuterium isotope effects to estimate limits on the intrinsic deuterium, tritium, and ^{13}C isotope effects, as well as the forward commitment for 6-phosphogluconate and the internal partition ratio for the 3-keto intermediate.

Experimental Procedures

Materials. Glucose-3-*t* was purchased from New England Nuclear, and sodium borodeuteride (98 atom % D) was from Sigma. Mes,[1] Hepes, Taps, acetyl phosphate, NADP, NADPH, ribulose 5-phosphate, glucose 6-phosphate, 6-phosphogluconate, ribose 5-phosphate, α-ketoglutarate, thiamine pyrophosphate, 1,2:5,6-diisopropylidene-D-glucofuranose, transketolase, glutamate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, xylulose-5-phosphate epimerase, hexokinase, yeast alcohol dehydrogenase, carbonic anhydrase, acetate kinase, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase, and 6-phosphogluconate

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonate; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonate.

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dehydrogenase were from Sigma. The latter was a crystalline suspension in $(NH_4)_2SO_4$ that was diluted 10-fold with CO_2-free buffer prior to use for ^{13}C isotope effect studies. Ribulose 5-phosphate was also prepared by the method of Pontremoli & Mangiarotti (1962). D-Glucose-3-*d* was synthesized from 1,2:5,6-di-*O*-isopropylidene-D-glucofuranose by the procedure of Koch & Perlin (1970) by oxidation to 1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuran-3-ulose, reduction with sodium borodeuteride, tosylation, and conversion of 1,2:5,6-di-*O*-isopropylidene-3-*O*-tosyl-α-D-allofuranose-3-*d* into 3-*O*-benzoyl-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose-3-*d*, which was deblocked by standard techniques.

6-Phosphogluconate-*3-d* was prepared from D-glucose-*3-d* by using hexokinase and glucose-6-phosphate dehydrogenase with acetyl phosphate, acetate kinase, acetaldehyde, and alcohol dehydrogenase to recycle the nucleotides. The reaction mixtures contained in 50-mL total volume 5 mmol of D-glucose-*3-d*, 1 mM ATP, 1 mM NAD, 2 mM $MgCl_2$, 15 mmol of acetyl phosphate, 7.5 mmol of acetaldehyde, 85 units of acetate kinase, 90 units of yeast alcohol dehydrogenase, 90 units of hexokinase, and 200 units of *L. mesenteroides* glucose-6-phosphate dehydrogenase. The pH of the reaction was maintained at 8–8.5 with KOH. Glucose was consumed after 6 h as determined by a coupled enzymatic assay for ADP formation in 1-mL total volume containing 0.1 M Hepes, pH 8, 2.5 mM ATP, 5 mM $MgCl_2$, 1 mM NAD, and 7.5 units each of hexokinase and glucose-6-phosphate dehydrogenase. Reactions were stopped by lowering the pH to 5 with Dowex 50-H^+, which also removed Mg^{2+}. The solution was filtered, treated with 0.5 g of acid-washed activated charcoal, and stirred for 1 h at 25 °C to remove most of the nucleotides. Charcoal was removed by filtration through Celite. Enzymes were removed by ultrafiltration by using an Amicon Series 80 apparatus with a PM-30 semipermeable membrane filter (excludes M_r >30000). The pH was lowered to 3 with HCl, and the filtrate was concentrated by rotary evaporation at 35 °C to remove acetaldehyde and acetic acid. The syrup was diluted to the desired concentration and the pH adjusted to 7.6 with 1 M KOH. Steps in the synthesis and specific deuteration at C-3 of glucose were monitored by ^1H NMR. Unlabeled 6-phosphogluconate was prepared in a similar manner from D-glucose.

6-Phosphogluconate-*3-t* was synthesized from 250 μCi of D-glucose-*3-t* in 0.75 mL containing 0.2 M Hepes, pH 8.5, 0.2 mM ATP, 0.2 mM NADP, 1 mM $MgCl_2$, and 13 units each of hexokinase and glucose-6-phosphate dehydrogenase. After an end point was reached (no further absorbance increase at 340 nm was observed), the solution was allowed to stand for an additional hour to convert the lactone to 6-phosphogluconate-*3-t*. The pH was lowered to 1 with HCl to denature the enzymes and destroy NADPH (the acid-degradation product of NADPH is eluted before NADPH from the MP-1 column later used to isolate NADPH for the measurement of isotope effects and thus does not need to be removed from the stock 6-phosphogluconate solutions). The solution was diluted to 200 mM with unlabeled 6-phosphogluconate in 1 mL and the pH adjusted to 8.

Nomenclature. The nomenclature used is that of Northrop (1977) in which isotope effects on kinetic or thermodynamic parameters are defined by leading superscripts. Thus, T, 13, D, or α-D refers to tritium, ^{13}C, primary deuterium, or α-secondary deuterium isotope effects, respectively. Where necessary, following subscripts are used. For example, $^{13}(V/K_{6\text{-P-gluconate-}3\text{-}d})$ is the ^{13}C isotope effect on V/K with 6-phosphogluconate-*3-d* as substrate. For further discussion of nomenclature, see Cook & Cleland (1981a).

Initial Velocity Studies. All kinetic studies were performed by monitoring absorbance changes with either a Cary 118 spectrophotometer or a Beckman DU monochromator equipped with a Gilford OD converter and a 10-mV recorder. The assay temperatures were maintained at ±0.1 °C of the stated values with thermospacers and a circulating water bath. pH values were measured with a Radiometer 26 pH meter equipped with a combined microelectrode standardized to ±0.01 pH unit. 6-Phosphogluconate dehydrogenase activity was measured spectrophotometrically at 25 °C by following formation of NADPH from NADP at 340 nm in 2 mL total volume, 1 cm path length cuvettes. Assays were typically conducted in 0.1 M Hepes buffer, pH 8.0, containing 1 mM NADP, variable amounts of 6-phosphogluconate, 0.2 mM dithiothreitol, 2 mM $Mg(OAc)_2$, 25 mM NH_4Cl, and 25 mM α-ketoglutarate (the same conditions used for determination of the ^{13}C isotope effects).

Deuterium isotope effects on initial velocities are obtained at saturating nucleotide concentrations (1 mM) by comparing deuterated and nondeuterated substrates. To check for an ordered or random kinetic mechanism, the isotope effects were also examined at saturating levels of deuterated and unlabeled substrates (2 mM) with NADP as the variable substrate.

The pH dependence of the deuterium isotope effects with 6-phosphogluconate dehydrogenase was determined by varying the concentrations of 6-phosphogluconate and 6-phosphogluconate-*3-d* at saturating levels of NADP (1 mM at pH >6 and 3 mM at pH 5 and 6). The 0.1 M buffers used (K-Mes at pH 5.05 and 5.94, K-Hepes at pH 7.34 and 8.0, and K-Taps at pH 9.1) contained 2 mM $Mg(OAc)_2$, 25 mM α-ketoglutarate, 25 mM NH_4Cl, and 0.5 mM dithiothreitol.

Determination of Substrate Concentrations. V/K isotope effects determined by comparing the slopes of double-reciprocal plots are not sensitive to the presence of inhibitors in the deuterated and unlabeled substrates but are only as precise as the relative concentrations of the substrates known. 6-Phosphogluconate, 6-phosphogluconate-*3-d*, and 6-phosphogluconate-*3*-t concentrations were determined enzymatically by using 5–10 units of yeast 6-phosphogluconate dehydrogenase and 1 mM NADP in K-Hepes buffer, pH 8.0 at 25 °C, containing 1 mM dithiothreitol. NADP solutions were calibrated enzymatically in 2-mL total volume containing 0.1 M Hepes, pH 8, 5 mM glucose 6-phosphate, and 2.4 units of *L. mesenteroides* glucose-6-phosphate dehydrogenase. The reaction mixture for determining NADPH concentrations contained 0.1 M Hepes, pH 8, 4 units of glutamate dehydrogenase, 5 mM α-ketoglutarate, and 5 mM NH_4Cl. The assay for ribulose 5-phosphate was conducted in 1-mL total volume with 0.1 M Hepes, pH 7.5, 3 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate, 4 mM arsenate, 0.5 mM NAD, 0.3 mM ribose 5-phosphate, 1 mM dithiothreitol, 3.2 units of glyceraldehyde-3-phosphate dehydrogenase, and 0.3 unit of transketolase. An aliquot of ribulose 5-phosphate was added and the absorbance at 340 nm recorded to indicate the amount of xylulose 5-phosphate present. After 0.2 unit of xylulose-5-phosphate epimerase was added and allowed to reach an end point, the net change in A_{340} measured the ribulose 5-phosphate concentration (Cooper et al., 1958).

^{13}C Kinetic Isotope Effects. The ^{13}C isotope effects on the 6-phosphogluconate dehydrogenase reaction were determined by the method of O'Leary (1980) with the natural abundance of ^{13}C in the substrate as the label. Reaction mixtures contained in 15-mL total volume 0.1 M Hepes, 24 mM 6-phosphogluconate or 6-phosphogluconate-3-*d*, 1 mM NADP,

0.2 mM dithiothreitol, 2 mM $Mg(OAc)_2$, and 30 mM α-ketoglutarate (to reoxidize NADPH with NH_4Cl and glutamate dehydrogenase), with the pH adjusted to 8 with KOH. The assay solutions minus the enzymes and NH_4Cl were degassed for at least 6 h with CO_2-free N_2 before addition of 70 units of glutamate dehydrogenase in 0.05 mL and of 30 mM NH_4Cl (added as solid). The reactions were then initiated by addition of a sufficient quantity of 6-phosphogluconate dehydrogenase (3 units in 0.11 mL) to give 15–25% reaction of the 15-mL sample in 75 min at 25 °C. The reactions were quenched by acidification with 0.5 mL of concentrated H_2SO_4, and the CO_2 was isolated on a high-vacuum line. Aliquots were removed before initiation and after quenching the reaction and analyzed for 6-phosphogluconate to establish the extent of reaction. The 100% conversion samples were treated identically except that a 5-mL volume of the 24 mM substrate mixture was diluted to 15 mL and was incubated overnight with 13.5 units (0.5 mL) of 6-phosphogluconate dehydrogenase and 350 units of glutamate dehydrogenase (0.25 mL). 6-Phosphogluconate assays indicated that greater than 99.7% reaction had occurred in all cases.

The ratios of $^{13}C/^{12}C$ in the CO_2 from both low-conversion and complete-conversion samples were measured with a Nuclide Associates RMS 6-60 isotope ratio mass spectrometer equipped with a dual-inlet system. The low- and complete-conversion samples from a particular experiment were always analyzed on the same day to minimize any day to day variations in the mass spectrometer.

^{13}C Equilibrium Isotope Effect for 6-Phosphogluconate Dehydrogenase Reaction. A 15-mL mixture of 20 mM ribulose 5-phosphate, 30 mM NADPH, and 500 mM $KHCO_3$ was allowed to reach chemical and isotopic equilibrium in the presence of 15 units of 6-phosphogluconate dehydrogenase at pH 8.0, 25 °C, in a sealed flask with a 70 -mL gas volume. The reaction mixtures also contained 0.1 M Hepes, 0.2 mM dithiothreitol, 2 mM $Mg(OAc)_2$, and 1 mg of carbonic anhydrase and were sparged with CO_2-free N_2 gas prior to addition of 6-phosphogluconate dehydrogenase in 0.3 mL. After 28 h the CO_2 atmosphere and liquid HCO_3^- phases were sampled, and all remaining CO_2 was removed by acidification with H_2SO_4 and purging with CO_2-free N_2 gas. The 6-phosphogluconate was quantitatively decarboxylated by the coupled enzymatic procedure used for the 100% samples in the kinetic experiments [the pH was adjusted to 8, and 15 mM α-ketoglutarate and NH_4Cl (added as solids), 70 units (in 0.1 mL) of glutamate dehydrogenase to recycle NADP, 1 mM dithiothreitol, and 15 units (in 0.3 mL) of 6-phosphogluconate dehydrogenase were added and allowed to react overnight]. Isotope ratio measurements on the three CO_2 samples following purification provided the isotope effect on the 6-phosphogluconate $\rightleftharpoons$ CO_2(g) + ribulose 5-phosphate and 6-phosphogluconate $\rightleftharpoons$ HCO_3^- + ribulose 5-phosphate equilibria directly. Since dissolved CO_2 is the true substrate (Villet & Dalziel, 1969), these numbers were corrected for the known isotope exchange equilibria between the dissolved CO_2 and CO_2(g) ($^{13}K_{eq}$ = 1.0011) or dissolved CO_2 and HCO_3^- ($^{13}K_{eq}$ = 1.009) (Mook et al., 1974).

Tritium Isotope Effects. $^{T}(V/K)$ for the reaction catalyzed by 6-phosphogluconate dehydrogenase was determined at saturating nucleotide levels by the internal competition method (Cleland, 1982). Reaction mixtures contained in 1 mL 0.1 M Hepes, pH 8, 25 mM NADP, 2 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 20 mM 6-phosphogluconate-*3-t* (1.25 μCi of $^3H/\mu$mol). The reaction was either run to completion (to calculate the specific activity of NADPH when all the 6-phosphogluconate-*3-t* had been consumed) or stopped at 20, 35, or 50% conversion by rapid addition of 45% KOH to bring the pH to 12. After removal of denatured protein, tritiated NADPH was separated from unreacted 6-phosphogluconate-*3-t* and NADP on 1.5 × 10 cm AG-MP-1 anion-exchange columns (Bio-Rad) equilibrated with 0.175 M LiCl, pH 10.0 at 25 °C, by washing with 150 mL of the same buffer and by elution with 150 mL of 0.4 M LiCl, pH 10 (Viola et al., 1979). The extent of reaction was determined by performing end-point assays for 6-phosphogluconate before initiation of the reaction with 6-phosphogluconate dehydrogenase and after stopping the reaction with KOH. For each reaction, 4-mL fractions containing tritiated NADPH and showing a constant specific activity were used to give the NADPH specific activity in cpm per micromole. The concentration of NADPH was determined from A_{340} by using 6.2 mM^{-1} cm^{-1} as the extinction coefficient. A Beckman LS 100C scintillation counter was used to count 1-mL aliquots of each fraction.

Data Analysis. In determinations of deuterium isotope effects, reciprocal initial velocities were plotted against reciprocal substrate concentrations, and the data were fitted by the least-square method with the computer programs of Cleland (1979) to eq 2–4, which assume isotope effects on both

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (2)$$

$$v = VA/[K(1 + F_iE_{V/K}) + A] \quad (3)$$

$$v = VA/[(K + A)(1 + F_iE_V)] \quad (4)$$

V and V/K, isotope effects on V/K only, or equal effects on both, respectively. F_i is the fraction of deuterium label in the substrate, and E_V and $E_{V/K}$ are the isotope effects minus 1 for V and V/K, respectively. Individual saturation curves were fitted to eq 5, and initial velocity patterns were fitted to eq

$$v = VA/(K + A) \quad (5)$$

6. Data were also fitted to the forms of these equations where

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (6)$$

the logarithm was taken of both sides (this changes the assumed error distribution from one with constant errors in initial velocities to one with proportional errors). Data for the pH dependence of the deuterium isotope effect were fitted to eq 7, where K_1 is the dissociation constant for a group on the

$$\log y = \log (C/1 + K_1/H) \quad (7)$$

enzyme, y is $^{D}(V/K_{6\text{-P-gluconate}}) - 1$, and C is the pH independent value of y. ^{13}C and tritium isotope effects on V/K were determined with eq 8, where R_f is the $^{13}C/^{12}C$ ratio (or the

$$^{13,T}(V/K) = \ln (1 - f)/\ln [1 - f(R_f/R_0)] \quad (8)$$

specific activity of tritiated NADPH) at fractional reaction f and R_0 is the $^{13}C/^{12}C$ ratio (or specific activity of tritiated NADPH) at f = 1.0 (that is, after complete reaction).

Results

Deuterium Isotope Effects. The kinetic parameters for 6-phosphogluconate dehydrogenase obtained from initial velocity studies at several pH values are summarized in Table I. When 6-phosphogluconate and 6-phosphogluconate-*3-d* were varied at saturating NADP concentrations at pH 8, isotope effects were observed on both V and $(V/K_{6\text{-P-gluconate}})$ that were nearly the same (1.55 ± 0.02 and 1.61 ± 0.04 in two trials with data fitted to the log form of eq 4). Deuterium isotope effects with enzyme from Boehringer-Mannheim were nearly the same as those with the enzyme from Sigma (1.55 ± 0.04). All subsequent studies were performed with the

Table I: Kinetic Parameters for 6-Phosphogluconate Dehydrogenase Reaction vs. pH

pH	V^a	K_{NADP} (mM)	$K_{i\,NADP}$ (mM)	V/K_{NADP}	$K_{6\text{-P-gluconate}}$ (mM)	$K_{i\,6\text{-P-gluconate}}$ (mM)	$V/K_{6\text{-P-gluconate}}$
5.93[b]	8.9 ± 0.5	0.033 ± 0.005	(0.001 ± 0.003)	270 ± 40	0.068 ± 0.007	(0.002 ± 0.006)	131 ± 15
8.0[c]	34.9 ± 0.6	0.025 ± 0.001	nd[d]	1400 ± 50	0.153 ± 0.004	nd[d]	228 ± 7
9.03[e]	1.9 ± 0.2	0.031 ± 0.009	0.023 ± 0.006	61 ± 16	3.13 ± 0.45	2.4 ± 1.0	0.6 ± 0.1

[a] Velocities are expressed as μmol of NADPH min^{-1} (mg of enzyme)$^{-1}$ (45 units/mg). [b] 0.1 M Mes; fitted to the log form of eq 6. [c] 0.1 M Hepes; fitted to eq 4 at saturating NADP or 6-phosphogluconate. [d] nd, not determined. [e] 0.1 M Taps; fitted to the log form of eq 6.

Table II: pH Dependence of Deuterium Isotope Effects for 6-Phosphogluconate Dehydrogenase at 25 °C

pH	$^D(V/K_{6\text{-P-gluconate}})^a$	pH	$^D(V/K_{6\text{-P-gluconate}})^a$
5.05	2.54 ± 0.06	8.0	1.54 ± 0.02
5.94	2.11 ± 0.07	9.10	1.37 ± 0.02
7.34	1.93 ± 0.05		

[a] Data were fitted to eq 4 (which assumes equal isotope effects on V and V/K), except at pH 5, where the isotope effect on V was not significantly different from unity and the data were fitted to eq 3.

Sigma enzyme. When NADP was varied at saturating 6-phosphogluconate and 6-phosphogluconate-*3-d* at pH 8, the same deuterium isotope effects were observed on V and (V/K_{NADP}), and these values were only slightly higher than the values obtained at saturating NADP and variable 6-phosphogluconate or 6-phosphogluconate-3-*d* (1.77 ± 0.04). Table II tabulates the deuterium isotope effects on V/K as a function of pH when 6-phosphogluconate or 6-phosphogluconate-*3-d* are varied at saturating NADP. The V/K isotope effects are largest at pH 5.05 and approach unity as the pH is raised to 9.1. When the data for $^D(V/K_{6\text{-P-gluconate}})$ from fits of the data to eq 4 were fitted to eq 7, a pK of 8.8 ± 0.2 was obtained.

Tritium Isotope Effects. With 6-phosphogluconate-*3-t*, the specific activities of NADPH for reactions run to 20.2 and 34.7% completion at pH 8 were (1.27 ± 0.03) × 10^4 and (1.30 ± 0.11) × 10^4 cpm/μmol, compared to a specific activity of (2.38 ± 0.22) × 10^4 cpm/μmol for a reaction run to 100% completion. A reaction run to 49.8% completion had a specific activity of (1.24 ± 0.14) × 10^4 cpm/μmol compared to a specific activity of (2.26 ± 0.09) × 10^4 cpm/μmol for the 100% reaction. The tritium isotope effect on V/K calculated from eq 8 gave an average value of 2.05 ± 0.12 for the three experiments, which agrees with the value of 2.04 obtained by Palm et al. (1968) at pH 9.0, 21 °C, with the yeast enzyme.

^{13}C Kinetic and Equilibrium Isotope Effects. The $^{13}(V/K_{6\text{-P-gluconate}})$ and $^{13}(V/K_{6\text{-P-gluconate-}3\text{-}d})$ kinetic isotope effects were determined from comparison of $^{13}C/^{12}C$ in CO_2 from partial reactions and $^{13}C/^{12}C$ ratios in complete-conversion reactions. These ratios are tabulated, together with the ratios needed for determination of $^{13}K_{eq}$ for the reaction 6-phosphogluconate ⇌ ribulose 5-phosphate + CO_2, in Table III. The isotope effects determined either by use of eq 8 [$^{13}(V/K)$ kinetic isotope effects] or directly from the ratios ($^{13}K_{eq}$ equilibrium isotope effect) are given below each set of experimental data.

Discussion

Mechanistic Deductions from Deuterium Isotope Effects. The kinetic parameters determined from the initial velocity studies (Table I) agree well with those previously reported for the enzyme from *Candida utilis* (Pontremoli et al., 1961). Previous kinetic studies on 6-phosphogluconate dehydrogenase from sheep liver (Villet & Dalziel, 1972) or yeast (Pontremoli et al., 1962) demonstrated that the mechanism was sequential but could not determine whether it was ordered or random. Since the deuterium isotope effects for yeast 6-phosphogluconate dehydrogenase are essentially the same regardless of which substrate is varied in the presence of saturating levels of the other, the yeast enzyme has a random mechanism in this direction. [If the mechanism involved compulsory binding of NADP first, $^D(V/K)$ would have been 1.0 when NADP was varied at saturating levels of 6-phosphogluconate or 6-phosphogluconate-*3-d* (Cook & Cleland, 1981a).]

$V/K_{6\text{-P-gluconate}}$ decreased below a pK of 6.2 and above a pK of 7, while V/K_{NADP} decreased below a pK of 7.4 and above a pK of 8.4 (Rippaw et al., 1972). The pH profile for V_{max} showed pK values of 6.8 and 8.4. From equilibrium dialysis experiments as a function of pH, the dissociation constant for 6-phosphogluconate increased above a pK of 7.3 (Rippa et al., 1972), suggesting that the pK of 7 seen in the $V/K_{6\text{-P-gluconate}}$ profile is due to a group on the enzyme involved in the binding of 6-phosphogluconate. As shown in Table II, the deuterium isotope effect on $V/K_{6\text{-P-gluconate}}$ decreases above a pK of 8.8, at the same time that V and $V/K_{6\text{-P-gluconate}}$ or V/K_{NADP} is reported to decrease by Rippa et al. (1972). This unusual

Table III: ^{13}C Isotope Effects at pH 8.0, 25 °C, for 6-Phosphogluconate Dehydrogenase

substrate	isotope ratios (×10^5)[a] low conversion	100% conversion	% reaction	$^{13}(V/K)$	$^{13}K_{eq}$[g]
6-phosphogluconate	1193.2	1203.1	0.205	1.0093	
	1192.3	1202.9	0.203	1.0100	
				1.0096 ± 0.0006[b]	
6-phosphogluconate-*3-d*	1204.0	1212.9	0.149	1.0080	
	1204.1	1213.0	0.196	1.0082	
				1.0081 ± 0.0002[b]	
6-P-gluconate	1186.6[c]	1191.5,[d] 1204.4[f]	0.993[e]		
	1187.6[c]	1192.0,[d] 1203.7[f]	0.993[e]		
					0.997 ± 0.001,[d] 0.995 ± 0.001[f]

[a] CO_2 was isolated as described by O'Leary (1980) and analyzed on a Nuclide Associates isotope ratio mass spectrometer. Ratios were adjusted for ^{17}O contribution to m/e 45 by subtracting 74 from decade settings for m/e 45/44, which were corrected to a tank standard of 1260. [b] Average. [c] The mass ratios under low conversion are from 6-phosphogluconate at chemical and isotopic equilibrium. [d] The mass ratios are for CO_2 sampled from the gas phase at equilibrium, with $^{13}K_{eq}$ corrected for the isotopic exchange equilibrium for $CO_2(g) \rightleftharpoons CO_2(aq)$. [e] Proportion of [$CO_2$ + HCO_3^-] of the sum of this and [6-phosphogluconate] at equilibrium. [f] The mass ratios are for CO_2 recovered by acidification of a sample of the HCO_3^- at equilibrium with $^{13}K_{eq}$ corrected for the $CO_2(aq) \rightleftharpoons HCO_3^-(aq)$ isotopic exchange equilibrium. [g] Average $^{13}K_{eq}$ for 6-phosphogluconate ⇌ CO_2 + ribulose 5-phosphate = 0.996 ± 0.001.

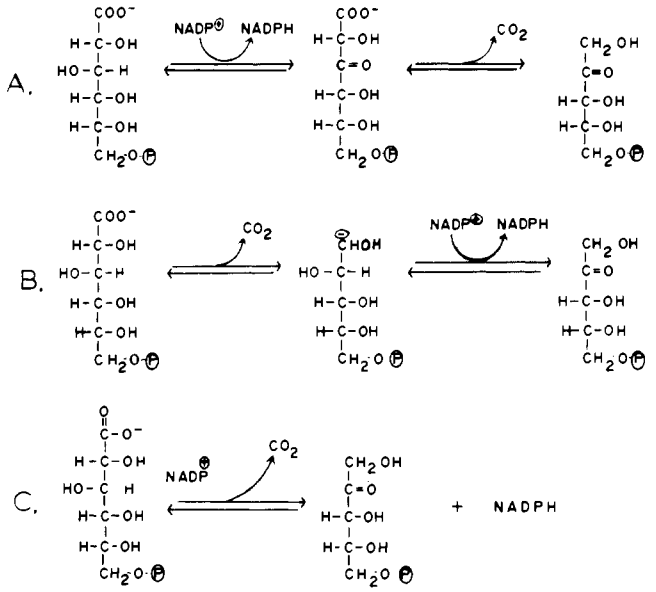

FIGURE 1: Mechanisms for 6-phosphogluconate dehydrogenase: (A) stepwise mechanism with C–H cleavage preceding C–C cleavage; (B) stepwise mechanism with C–C cleavage preceding C–H cleavage; (C) concerted C–H and C–C cleavages. An enediol or enediolate would probably be the actual first product of decarboxylation in (A) or (C), with protonation to give the ketone occurring prior to product release.

behavior suggests that pH-sensitive and isotope-sensitive steps are different, as with the alcohol dehydrogenases (Cook & Cleland, 1981c). The results are consistent with a mechanism where the 3 hydroxyl group of 6-phosphogluconate is deprotonated by a group on the enzyme, followed by oxidation of the alkoxide intermediate to give enzyme-bound 3-keto-6-phosphogluconate, which then decarboxylates, but further work will be needed to confirm such a mechanism. At low pH, $^{D}(V/K)$ appears to increase as V and V/K decrease, suggesting that 6-phosphogluconate has a finite external commitment at neutral pH (Cook & Cleland, 1981b).

Chemical Mechanism of 6-Phosphogluconate Dehydrogenase. For the decarboxylation catalyzed by 6-phosphogluconate dehydrogenase, several studies are consistent with formation of 3-keto-6-phosphogluconate via hydride transfer to NADP prior to C–C bond breakage. Thus, Lienhard & Rose (1964) proposed an enzyme-bound enol form of ribulose 5-phosphate as an intermediate in the oxidative decarboxylation of 6-phosphogluconate on the basis of tritium exchange experiments. Rippa et al. (1973) were able to isolate 3-keto-2-deoxy-6-phosphogluconate, which could be reduced by NADPH or decarboxylated, during the oxidative decarboxylation of 2-deoxy-6-phosphogluconate. By analogy, 3-keto-6-phosphogluconate should be an enzyme-bound intermediate that partitions between hydride transfer and decarboxylation steps as shown in Figure 1A. This mechanism is in accord with the known mechanisms for the decarboxylations of other β-keto acids in solution (Bender & Breslow, 1962). Despite these arguments, several alternate mechanisms must be considered. Figure 1B shows the least likely mechanism, that is, C–C bond cleavage preceeding C–H bond breakage, in which case decarboxylation of 6-phosphogluconate provides the driving force for hydride transfer. The last alternative (Figure 1C) shows a concerted oxidative decarboxylation with the proper orientation between NADP and 6-phosphogluconate promoted by the enzyme.

The stepwise and concerted mechanisms are easily distinguished by determining the ^{13}C isotope effect on V/K with both deuterated and unlabeled substrates and the deuterium isotope effect on V/K. In a concerted mechanism, deuteration of the substrate slows the bond-breaking step and makes it more rate limiting, so that the observed ^{13}C isotope effect is increased. In a stepwise mechanism, deuterating the substrate makes the ^{13}C-sensitive step less rate limiting (since the C–H bond-cleavage step is slowed down), and the observed ^{13}C isotope effect is decreased (Hermes et al., 1982).[2] The results in Table III show that the mechanism is stepwise.

Table IV: Forward Commitments and Internal Partition Ratios Calculated for 6-Phosphogluconate Dehydrogenase[a]

assumed $^{D}k_5$	calcd values[b]		
	$^{13}k_7$	k_6/k_7	$c_{6\text{-P-gluconate}}$
3.1	1.110	0.35	2.7
3.2	1.046	0.91	2.5
3.3	1.030	1.5	2.25
3.5	1.019	2.8	1.74
3.7	1.014	4.3	1.12
3.9	1.012	6.0	0.41

[a] These limits are based on experimental isotope effects from this work: $^{D}(V/K_{6\text{-P-gluconate}}) = 1.54$, $^{13}(V/K_{6\text{-P-gluconate}}) = 1.0096$, and $^{T}(V/K_{6\text{-P-gluconate}}) = 2.05$, and the assumption that the reverse commitment for CO_2 is zero. [b] The rate constants refer to the mechanism in Figure 1A described by eq 12. $c_{6\text{-P-gluconate}} = (k_5/k_4)(1 + k_3/k_2)$. k_6/k_7 = (rate of reverse hydride transfer)/(rate of decarboxylation). The calculations used eq 21–24 of Hermes et al. (1982).

The determination of $^{13}(V/K)_H$, $^{13}(V/K)_D$, $^{13}K_{eq}$, and $^{D}K_{eq}$ also allows a distinction to be made between the stepwise mechanisms in parts A and B of Figure 1. Hermes et al. (1982) showed that when C–C bond breakage follows C–H cleavage, eq 9 holds, while when C–C breakage precedes C–H

$$\frac{^{13}(V/K)_H - 1}{^{13}(V/K)_D - 1} = \frac{^{D}(V/K)}{^{D}K_{eq}} \quad (9)$$

cleavage, eq 10 applies. Substituting the values for $^{13}(V/K)_H$,

$$\frac{^{13}(V/K)_H - ^{13}K_{eq}}{^{13}(V/K)_D - ^{13}K_{eq}} = ^{D}(V/K) \quad (10)$$

$^{13}(V/K)_D$, and $^{13}K_{eq}$[3] from Table IV, $^{D}(V/K) = 1.54$, and $^{D}K_{eq}$

[2] The general expression for a ^{13}C isotope effect on V/K is

$$^{13}(V/K)_H = \frac{^{13}k + c_f + c_r{}^{13}K_{eq}}{1 + c_f + c_r}$$

where ^{13}k is the isotope effect on the forward rate constant for the isotope-sensitive step (the intrinsic isotope effect) and $^{13}K_{eq}$ is the equilibrium isotope effect in the forward direction. The constants c_f and c_r are the forward and reverse commitments, respectively. A commitment for a given reactant is the ratio of the rate constant for the unlabeled molecule for the isotope-sensitive step and the net rate constant for release of that same reactant into solution from the intermediate that faces the isotope-sensitive step. If the deuterium-sensitive step precedes the ^{13}C-sensitive step (Figure 1A), a deuterated substrate will increase c_f, and the $^{13}(V/K)$ value will be decreased toward unity. If the deuterium-sensitive step follows the ^{13}C-sensitive one (Figure 1B), a deuterated substrate will increase c_r and decrease the $^{13}(V/K)$ value toward the $^{13}K_{eq}$ value. If, however, the deuterium- and ^{13}C-sensitive steps are the same (Figure 1C), the deuterated substrate will decrease c_f and c_r by the size of the intrinsic deuterium isotope effect, and the $^{13}(V/K)$ value will be increased.

[3] The difference between the ^{13}C equilibrium constant for decarboxylation of 6-phosphogluconate ($^{13}K_{eq} = 0.996$) and the $^{13}K_{eq}$ value of 1.0027 for the reaction catalyzed by isocitrate dehydrogenase (O'Leary & Yapp, 1978) may reflect the inductive effect of the hydroxyl on the α-carbon of 6-phosphogluconate, as opposed to having another carbon adjacent to the one lost during decarboxylation, as in isocitrate. A value of 0.999 was used for C-4 of malate by Hermes et al. (1982), on the basis of the $^{13}K_{eq}$ for isocitrate dehydrogenase and the fact that substitution of H for C on a carbon attached to the isotopic one appears to decrease the ^{13}C fractionation factor by 1.004 [see footnote r of Cleland (1980)]. Measurement of $^{13}K_{eq}$ for the reaction catalyzed by malic enzyme should help confirm these trends.

= 1.18 (Cook et al., 1980) into eq 9 and 10 gives from eq 9 1.19 ± 0.08 ≃ 1.31 ± 0.02 and from eq 10 1.12 ± 0.08 ≠ 1.54 ± 0.02. Since eq 9 is reasonably fitted by the data and eq 10 is not, a two-step mechanism with hydride transfer preceding decarboxylation is confirmed.

Intrinsic Isotope Effects, Forward Commitment, and Internal Partition Ratios Calculated for 6-Phosphogluconate Dehydrogenase. Determination of $^{13}(V/K)_H$, $^{D}(V/K)$, and $^{T}(V/K)$ also allows the calculation of limits on the intrinsic ^{13}C and deuterium isotope effects, the forward commitment for 6-phosphogluconate, and the internal partition ratio for the rate of reverse hydride transfer and the rate of decarboxylation. When the deuterium-sensitive step precedes the ^{13}C-sensitive step, the following general mechanism applies

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA' \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EX \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EPQ' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EPQ \xrightarrow{k_{11}} EQ \xrightarrow{k_{13}} E \quad (11)$$

where k_5 is the deuterium-sensitive step, k_7 is the ^{13}C-sensitive step, and k_3, k_4, k_9, and k_{10} are for non-isotope-dependent conformation changes. By assuming that CO_2 does not have a reverse commitment for the decarboxylation step [that is, $(k_8/k_9)(1 + k_{10}/k_{11})$ is much less than unity], Hermes et al. (1982) derived equations for determining the intrinsic isotope effect on decarboxylation, $^{13}k_7$, the forward commitment of 6-phosphogluconate for hydride transfer, $(k_5/k_4)(1 + k_3/k_2)$, and the partition ratio of the 3-keto-6-phosphogluconate intermediate, k_6/k_7, as a function of assumed values of the intrinsic deuterium isotope effect, $^{D}k_5$. The results of these calculations are shown in Table IV (values of $^{D}k_5$ outside the range tabulated gave negative values of either k_6/k_7 or $c_{6\text{-P-gluconate}}$).[4]

On the basis of multiple isotope effect studies similar to those in this work, NADP- and NAD-dependent malic enzymes and isocitrate dehydrogenase all catalyze reversible stepwise reactions in which a β-keto acid intermediate is formed prior to decarboxylation (Hermes et al., 1982; Hermes, 1983; Grissom & Cleland, 1983), while prephenate dehydrogenase has a concerted mechanism (Hermes et al., 1984). In the latter case, the nature of the substrate apparently dictates the type of mechanism since there is a strong driving force toward formation of the aromatic product. On the basis of the experience gained in these studies, we conclude that the use of multiple isotope effects is the method of choice for determining the mechanisms of enzymes catalyzing oxidative decarboxylations.

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Registry No. NADP, 53-59-8; EC 1.1.1.44, 9073-95-4; 6-P-gluconic acid, 921-62-0; 6-P-gluconic-*3-d* acid, 92642-71-2; D-glucose-*3-d*, 92760-46-8; deuterium, 7782-39-0.

[4] The values shown in Table IV are dependent on the accuracy of the tritium isotope effect on V/K. When a value of 2.17, corresponding to the upper limit of the standard deviation in $^{T}(V/K)$, was used, $^{D}k_5$ varied from 4.4 to 5.8, $^{13}k_7$ from 1.074 to 1.012, $c_{6\text{-P-gluconate}}$ from 1.2 to 4.7, and k_6/k_7 from 1.4 to 10.1. If the lower limit for $^{T}(V/K)$ was used (1.93), $^{D}k_5$ was unreasonably small (<3.0).

References

Bender, M. L., & Breslow, R. (1962) *Compr. Biochem. 2*, 1–218.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–108.
Cleland, W. W. (1980) *Methods Enzymol. 64*, 104–125.
Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385–428.
Cook, P. F., & Cleland, W. W. (1981a) *Biochemistry 20*, 1790–1796.
Cook, P. F., & Cleland, W. W. (1981b) *Biochemistry 20*, 1797–1805.
Cook, P. F., & Cleland, W. W. (1981c) *Biochemistry 20*, 1805–1816.
Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4853–4854.
Cooper, J., Srere, P. A., Tabachnick, M., & Racker, E. (1958) *Arch. Biochem. Biophys. 74*, 306–314.
Grissom, C. B., & Cleland, W. W. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2178.
Hermes, J. D. (1983) Ph.D. Dissertation, University of Wisconsin, Madison, WI.
Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106–5114.
Hermes, J. D., Tipton, P. A., Fisher, M. A., O'Leary, M. H., Morrison, J. F., & Cleland, W. W. (1984) *Biochemistry* (following paper in this issue).
Koch, H. J., & Perlin, A. S. (1970) *Carbohydr. Res. 15*, 403–410.
Lienhard, G. E., & Rose, I. A. (1964) *Biochemistry 3*, 190–195.
Mook, W. G., Bommerson, J. C., & Staverman, W. H. (1974) *Earth Planet. Sci. Lett. 22*, 169–176.
Northrop, D. B. (1975) *Biochemistry 14*, 2644–2651.
Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 122–152, University Park Press, Baltimore, MD.
O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83–103.
O'Leary, M. H., & Yapp, C. J. (1978) *Biochem. Biophys. Res. Commun. 80*, 155–160.
Palm, D., Rambeck, W., & Simon, H. (1968) *Z. Naturforsch., B: Anorg. Chem., Org. Chem. 23B*, 881–882.
Pontremoli, S., & Mangiarotti, G. (1962) *J. Biol. Chem. 237*, 643–645.
Pontremoli, S., DeFlora, A., Grazi, E., Mangiarotti, G., Bonsignore, A., & Horecker, B. L. (1961) *J. Biol. Chem. 236*, 2975–2980.
Pontremoli, S., Grazi, E., DeFlora, A., & Mangiarotti, G. (1962) *Arch. Sci. Biol. (Bologna) 46*, 83–91.
Rendina, A. R., Hermes, J. D., & Cleland, W. W. (1983) *Biochemistry 22*, 6A–7A.
Rippa, M., Signorini, M., & Pontremoli, S. (1972) *Arch. Biochem. Biophys. 150*, 503–510.
Rippa, M., Signorini, M., & Dallocchio, F. (1973) *J. Biol. Chem. 248*, 4920–4925.
Schimerlik, M. I., Grimshaw, C. E., & Cleland, W. W. (1977) *Biochemistry 16*, 571–576.
Swain, C. G., Stivers, E. C., Reuwer, J. F., Jr., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885–5893.
Villet, R. H., & Dalziel, K. (1969) *Biochem. J. 115*, 633–638.
Villet, R. H., & Dalziel, K. (1972) *Eur. J. Biochem. 27*, 251–258.
Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334–340.