Carbon-13 and Deuterium Isotope Effects on Oxalacetate Decarboxylation by Pyruvate Carboxylase†

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ABSTRACT: Deuterium and ^{13}C isotope effects for the enzymic decarboxylation of oxalactate showed that both deuterium- and ^{13}C-sensitive steps in the reaction are partially rate limiting. A normal α-secondary effect of 1.2 per deuterium was calculated for the reaction in which pyruvate-d_3 was the substrate, suggesting that the enolate of pyruvate was an intermediate in the reaction. The large normal α-secondary deuterium isotope effect of 1.7 when oxalacetate-d_2 was the substrate suggests that the motions of the secondary hydrogens are coupled to that of the primary hydrogen during the protonation of the enolate of pyruvate. The reduction in the magnitude of the ^{13}C isotope effect for the oxamate-dependent decarboxylation of oxalacetate from 1.0238 to 1.0155 when the reaction was performed in D_2O (primary deuterum isotope effect = 2.1) clearly indicates that the transfer of the proton and carboxyl group between biotin and pyruvate does not occur via a single concerted reaction. Mechanisms in which biotin is activated to react with CO_2 (prior to transfer of the proton on N-1) by bond formation between the sulfur and the ureido carbon, or in which the sequence of events is decarboxylation of oxalacetate, proton transfer from biotin to enolpyruvate, and carboxylation of enolbiotin, predict that the ^{13}C isotope effect in D_2O should be substantially lower than the observed value. A stepwise mechanism that does fit the data is one in which a proton is removed from biotin by a sulfhydryl group on the enzyme prior to carboxyl transfer, as long as the sulfhydryl group has an abnormally low pK. The ^{13}C fractionation factors of C-4 of oxalacetate and of the carbamate of pyrrolidine are 1.0075 and 1.0123 relative to aqueous CO_2.

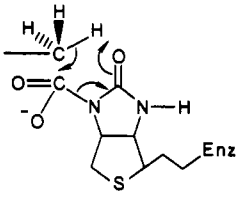
Pyruvate carboxylase (EC 6.4.4.1) catalyzes the following reactions:

$$HCO_3^- + MgATP + E\text{–biotin} \underset{}{\overset{\text{acetyl-CoA, } Mg^{2+}}{\rightleftharpoons}} E\text{–biotin–}CO_2^- + MgADP + P_i \quad (1)$$

$$E\text{–biotin–}CO_2^- + \text{pyruvate} \rightleftharpoons E\text{–biotin} + \text{oxalacetate} \quad (2)$$

The basic chemistry involved in reaction 2 is the transfer of the carboxyl group to form oxalacetate and the concomitant transfer of a proton from pyruvate to biotin. The reaction occurs with retention of configuration at carbon 3 of pyruvate (Retey & Lynen, 1965). In addition, loss of a proton to the solvent from pyruvate only occurs if either the components of the first partial reaction or oxalacetate is present (Mildvan et al., 1966). This suggests that proton removal from pyruvate only occurs if the biotin is carboxylated. Rose et al. (1976) also found that with transcarboxylase there is transfer of tritium between the substrates of the partial reactions, and they suggested that biotin acts as a proton transporter. The proton is presumably carried on N-1, which exchanges with water rather slowly at neutral pH (58 s^{-1} at pH 7.5; Fry et al., 1985). The proton could not be carried on the ureido oxygen of biotin because such a proton would exchange with solvent too rapidly (>5000 s^{-1}).

Several mechanisms have been proposed for reaction 2. Retey and Lynen (1965) proposed a concerted reaction with the ureido oxygen of biotin as the proton acceptor:



This mechanism involves unprecedented chemistry and unlikely geometry. Further, it requires enolization of biotin in the reverse direction and does not explain the data of Rose et al. (1976) on tritium transfer unless hydrogen can be directly transferred between N-1 and the ureido oxygen during enolization. From their experiments with β-fluoropropionyl coenzyme A (CoA) and propionyl-CoA carboxylase, Stubbe and Abeles (1977) and Stubbe et al. (1980) suggested that the reaction proceeds via a carbanion mechanism similar to that shown in Scheme I. No chemical precedent exists for attack of a carbanion on an ionized carboxyl group, but decarboxylation to CO_2 would permit the carboxyl transfer by reaction of this CO_2 with the enolate of either biotin or pyruvate.

Sauers et al. (1975) suggested that CO_2 trapped in the active site was an intermediate in all biotin-mediated carboxyl transfers, and the fact that reaction 2 is pH-independent (Attwood & Cleland, 1986) suggests a mechanism in which decarboxylation precedes proton transfer, with the enolate of either biotin or pyruvate directly removing a proton from the other substrate to give an enolate of it, which reacts with CO_2 to accomplish the transfer (Scheme II). In this mechanism there is no base on the enzyme involved in proton transfers.

A further mechanism is suggested by the recent discovery by Fry et al. (1985) that the N-1 proton of biotin exchanges

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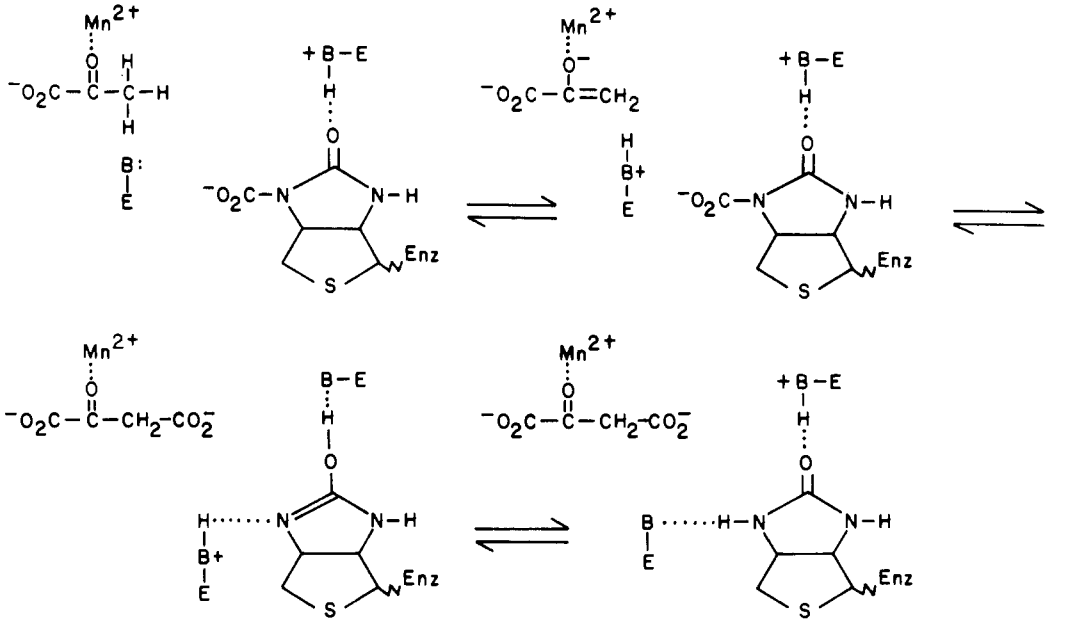
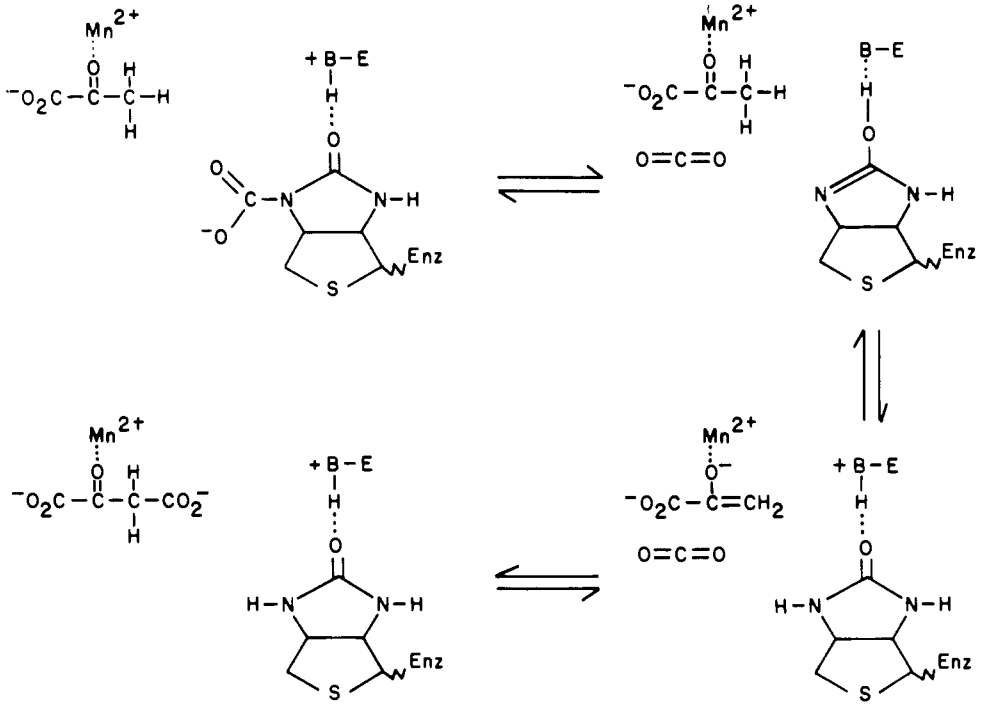
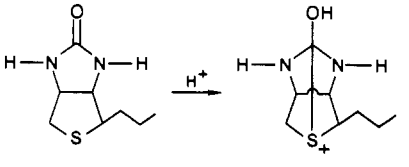
Scheme I



Scheme II



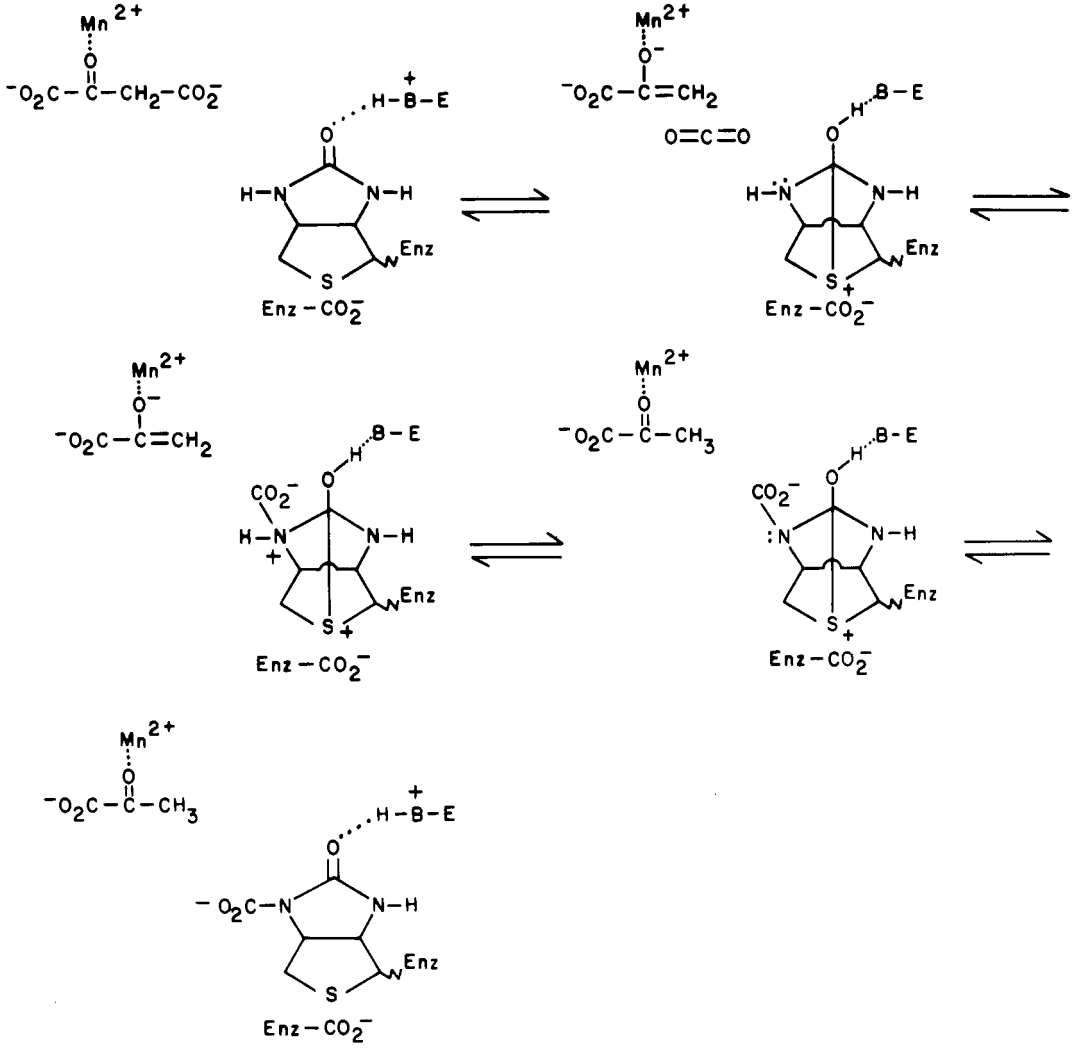
in acid by a process involving two protons. They postulate that protonation of the ureido oxygen permits sulfur attack on the ureido carbon to give a cage structure in which N-1 becomes a secondary amine and is readily protonated by a second hydrogen ion:



An enzyme could catalyze such a condensation by steric compression, protonation of the ureido oxygen by lysine, and stabilization of the sulfonium center by a nearby carboxyl group. N-1 would then be nucleophilic enough to react readily with CO_2 resulting from decarboxylation of oxalacetate. Proton transfer from the zwitterionic carbamate to the enolate of pyruvate followed by relaxation of the steric compression and cleavage of the C–S bond would complete the reaction (Scheme III).

Cheung and Walsh (1976) observed a kinetic deuterium isotope effect when deuterated pyruvate was used as a substrate for pyruvate carboxylase, and hence, proton transfer is at least partially rate limiting. If carboxyl transfer is also at least partially rate limiting, then the observed ^{13}C isotope effect would provide a means of distinguishing between the two proposed mechanisms. In the concerted mechanism, the ^{13}C-sensitive step is also deuterium sensitive, and thus with a deuterated substrate the ^{13}C isotope effect should increase or remain the same as that in the reaction with the nondeuterated substrate (Cleland, 1982). On the other hand, if the reaction

Scheme III



is stepwise, as in the mechanisms in Schemes I–III, the observed ^{13}C isotope effect should be reduced when a deuterated substrate is used because the ^{13}C-sensitive step becomes less rate limiting.

In the continuous decarboxylation of oxalacetate by pyruvate carboxylase, which is stimulated by oxamate, the only substrate required is oxalacetate (Attwood & Cleland, 1986). Since the CO_2 that results from the decarboxylation of oxalacetate can easily be collected and analyzed in an isotope ratio mass spectrometer, this system provides an opportunity to study ^{13}C isotope effects in reaction 2. In this paper we shall present the results of both deuterium and ^{13}C isotope effect experiments and use them to define a mechanism for reaction 2.

Materials and Methods

Materials. D_2O (99.8 atom % D) was from Aldrich. All other materials were high-purity preparations obtained from commerical suppliers, except for chicken liver pyruvate carboxylase, which was purified as described by Goss et al. (1979) to specific activities of 18–22 units (mg of protein)$^{-1}$ (1 unit of enzymic activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of oxalacetate min^{-1} at 30 °C under saturating substrate conditions).

Deuterium Isotope Effects Using Trideuteriopyruvate. Pyruvate-d_3 was prepared as follows: 132 mg of oxalacetic acid was dissolved in 10 mL of D_2O and left for 30 min. The pH of the solution was then adjusted to 7.5 with NaOD in D_2O, and 0.5 mL of 20 mM anhydrous $MgSO_4$ in D_2O was added. The solution was sealed in a vial and left for 48 h, after which the concentration of pyruvate was determined by spectrophotometric assay using lactate dehydrogenase and NADH. A similar assay using malate dehydrogenase showed no residual oxalacetate. An internal standard of acetate was added to an aliquot of the solution, and the sample was examined by proton NMR. By integrating the areas under the proton peaks corresponding to pyruvate and acetate, the concentration of protonated pyruvate in the sample was determined, and the percent of pyruvate-d_3 present in the sample was found to be 99.4. The remainder of the reaction mixture was then lyophilized, and the resultant solid was stored desiccated at 0 °C. When solutions of pyruvate-d_3 or unlabeled pyruvate were prepared for kinetic experiments, the concentration of pyruvate was determined by enzymic assay with lactate dehydrogenase.

Kinetic assays were performed in a volume of 1 mL at 25 °C, pH 7.5, containing 2.5 mM ATP, 20 mM $NaHCO_3$, 0.036–0.22 mM pyruvate, 7 mM $MgCl_2$, 0.25 mM acetyl-CoA, 0.24 mM NADH, 11 units of malate dehydrogenase, 0.1 M *N*-[tris(hydroxymethyl)amino]glycine (Tricine), and 1.2 μg of pyruvate carboxylase. The release of oxalacetate was monitored by its conversion to malate as indicated by the decrease in absorbance of NADH at 340 nm.

Deuterium Isotope Effect on Nonenzymic Decarboxylation of Oxalacetate. Reaction mixtures contained 0.1 M Tricine, 5 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM oxalacetate in either H_2O or D_2O at 25 °C and pH 7.5. At

various times after the addition of oxalacetate to the solutions, aliquots were removed and assayed for oxalacetate by the malate dehydrogenase assay.

Deuterium Isotope Effects on Enzymic Decarboxylation of Oxalacetate. Two types of experiment were performed, ones in which the oxalacetate was deuterated and ones in which it was not. In all of the experiments the reaction volume was 3 mL, and a 10-cm path-length cuvette was used. The reactions were performed as described by Attwood and Cleland (1986) except that each reaction was started by the addition of oxalacetate, and thus, the background rates of oxalacetate decarboxylation were determined in separate assays. All reactions were carried out at 25 °C and pH 7.5, and all oxalacetate concentrations were determined by the malate dehydrogenase assay.

In the experiments using unlabeled oxalacetate, the assay components, including 0.1 M Tricine buffer, were prepared in either H_2O or D_2O, and oxalacetate was added as a concentrated solution in 0.1 M Tricine, pH 7.5, in H_2O. In the experiments using oxalacetate-d_2, the assay components were prepared in D_2O. A solution of oxalacetic acid was prepared in D_2O and left for 1 h. After this time an aliquot of the solution when examined by proton NMR showed no signal corresponding to the methylene protons of oxalacetate. The solution was then made 0.1 M in Tricine and adjusted to pH 7.5 with NaOD in D_2O. The concentration of oxalacetate was determined with the malate dehydrogenase assay.

^{13}C Isotope Effects on Oxalacetate Decarboxylation. The technique for determination of ^{13}C isotope effects was that of O'Leary (1980) in which the natural abundance of ^{13}C in the C-4 position of oxalacetate is used as a trace label to minimize errors caused by atmospheric CO_2 contamination. Reaction mixtures for the nonenzymic decarboxylation of oxalacetate contained 0.1 M Tricine and in the low conversion samples 5 mM EDTA. In the 100% conversion samples EDTA was replaced by 50 mM $MgCl_2$, which catalyzes the decarboxylation reaction. The pHs of the solutions were adjusted to pH 7.5 with saturated NaOH (or NaOD) after being degassed with N_2 for 4 h. A concentrated solution of oxalacetate in 0.1 M Tricine buffer that had been degassed was prepared, and the pH was adjusted to 7.5 with saturated NaOH (or NaOD). The reactions were then initiated by the addition of small volumes of the oxalacetate solution so that the final concentration of oxalacetate was 1 mM in final volume of 50 mL. In the low-conversion samples the concentration of oxalacetate was determined by removing an aliquot of the reaction mixture immediately after the addition of oxalacetate and assaying this fraction by the malate dehydrogenase assay. The final concentration of oxalacetate was determined in a similar fashion.

The low-conversion samples were allowed to react for 6.5–7.5 h, while the 100% conversion samples were left for 24 h. In both cases the reactions were quenched by the addition of 3 mL of concentrated H_2SO_4. Before the addition of H_2SO_4 to the low-conversion samples, the reaction vessles were chilled in ice, and the solutions were kept cold during the addition of the acid. Controls were performed to ensure that the addition of the acid did not result in further decarboxylation of oxalacetate. Such controls showed no decarboxyaltion of oxalacetate as determined by enzymic assay or CO_2 formation determined by manometric assay. In all cases the CO_2 was isolated as soon as possible after the reactions were quenched. Analysis of the CO_2 samples was carried out in a Finnegan Delta-E isotope ratio mass spectrometer. All ratios were corrected for ^{17}O according to Craig (1957).

The reaction mixtures for the enzymic decarboxylation of oxalacetate contained 0.1 M Tricine, 5 mM EDTA, and 0.25 mM acetyl-CoA and for the oxamate-dependent reactions 4 mM oxamate. These solutions were degassed for 4 h with N_2 before their pHs were adjusted to 7.5 with saturated NaOH (or NaOD). A solution of 25 mM NADH containing 0.1 mg mL^{-1} carbonic anhydrase in 0.1 M Tricine, pH 7.5, was degassed for 2–3 h with N_2. NADH was added to the reaction mixtures to a final concentration of 1 mM, along with 25 units of lactate dehydrogenase and 5 mg of pyruvate carboxylase. The experiments were then performed as described for the nonenzymic decarboxylations, and the reactions were allowed to proceed until 8–20% of the oxalacetate was decarboxylated. Reaction time was 10 min in the presence of oxamate (14–20% decarboxylation) and 70 min in the absence of oxamate (8–10% decarboxylation).

By calculation of the amount of CO_2 produced by nonenzymic decarboxylation during the course of the oxamate-independent reaction and use of the experimental value for the nonenzymic ^{13}C isotope effect, the observed isotope effect was corrected to give the true oxamate-independent isotope effect. Similarly, the observed ^{13}C isotope effect for the reaction in the presence of oxamate was corrected for both nonenzymic and oxamate-independent decarboxylation to give the isotope effect for the oxamate-dependent reaction alone.

The D_2O from Aldrich was significantly enriched in ^{18}O (and thus presumably ^{17}O) with a content of 0.28 atom % ^{18}O. In order to avoid enrichment of the CO_2 resulting from the decarboxylation of oxalacetate with ^{18}O and ^{17}O (thus necessitating a larger correction for ^{17}O and a less accurate isotope effect), the D_2O was normalized prior to use by bubbling dry CO_2 through it in the presence of 0.5 g L^{-1} carbonic anhydrase. To follow the normalization process, samples of the D_2O were removed, freed from CO_2 by sparging with CO_2-free dry N_2, and equilibrated with natural abundance CO_2 with the D_2O in large excess. This CO_2 was isolated and analyzed for its ^{18}O content. After 12-h normalization, the ^{18}O content of the D_2O had decreased from 0.28 to 0.21 atom % ^{18}O (i.e., had decreased by 90% of the excess over natural abundance). The deuterium content remained unchanged from the original 99.8 atom % D as determined by proton NMR and density measurements. Separate 100% conversion samples were run in normalized D_2O.

Determination of ^{13}C Fractionation Factor of Oxalacetate. The ^{13}C isotope effect on the equilibrium between bicarbonate and oxalacetate was determined from the natural abundance of ^{13}C in these compounds. The following reaction reached equilibrium in less than 5 min:

$$HCO_3^- + MgATP + \text{pyruvate} \underset{}{\overset{Mg^{2+},\ \text{acetyl-CoA}}{\rightleftharpoons}} MgADP + P_i + \text{oxalacetate}$$

The equilibrium concentrations of pyruvate and oxalacetate were determined by enzymic assay to give the equilibrium constant:

$$K_{eq} = \frac{[HCO_3^-][MgATP][\text{pyruvate}]}{[P_i][MgADP][\text{oxalacetate}]} = 0.1$$

Under these conditions, the enzyme retained 89% of its activity after 7 h.

Reaction solutions were degassed for 4 h with N_2 before the pH was adjusted to 7.5 with saturated NaOH. Pyruvate, oxalacetate, and bicarbonate were added later as solutions in degassed 0.1 M Tricine, pH 7.5. Reaction mixtures contained 10 mM ATP, 2 mM ADP, 2 mM sodium bicarbonate, 50 mM K_2HPO_4, 1 mM pyruvate, 2 mM oxalacetate, 14 mM $MgCl_2$,

and 0.25 mM acetyl-CoA in 25 mL of 100 mM Tricine, and the reaction was started by addition of 0.9 mg of pyruvate carboxylase. In two flasks, enzyme, pyruvate, oxalacetate, bicarbonate, and acetyl-CoA were omitted.

A solution containing 20 mM NADH and 0.1 mg mL^{-1} carbonic anhydrase in 0.1 M Tricine, pH 7.5, was degassed for 3 h with N_2. This solution was then filtered at 4 °C in an Amicon concentrator with N_2 pressure and a PM10 filter to remove the enzyme, and the filtrate was collected under N_2. The contents of flasks not containing enzyme were filtered in a similar way and collected under N_2 in flasks containing 5 mL of the degassed 20 mM NADH solution, 100 units of malate dehydrogenase, 0.5 mL of 0.1 M sodium bicarbonate, and 0.5 mL of a solution containing 50 mM pyruvate and 100 mM oxalacetate. The flasks were sealed at room temperature for 3 h, after which no oxalacetate was found, showing complete conversion to malate. The contents of the two reaction mixtures containing pyruvate carboxylase were filtered in the same way after 300 or 400 min and the filtrates incubated with NADH and malate dehydrogenase as described above.

The contents of all four flasks were acidified to pH 3.3 with 0.05 mL of concentrated H_2SO_4. The CO_2 from the bicarbonate in solution was isolated and analyzed as described for the kinetic ^{13}C isotope effects. Each flask was then adjusted to pH 7.2 with 0.08 mL saturated NaOH. A 50 mM solution of NADP was degassed at low pH, adjusted to pH 7.2, and made 21 units mL^{-1} in malic enzyme. A total of 2.5 mL of this solution was added to each flask, and they were incubated for 4 h to decarboxylate the malate. The solutions were acidified with 1 mL of concentrated H_2SO_4, and CO_2 was isolated in the usual fashion. The ratio of the $^{13}C/^{12}C$ ratios for bicarbonate and oxalacetate in the equilibrium reaction mixtures gave the equilibrium isotope effect. The flasks not containing pyruvate carboxylase gave the mass ratios in the bicarbonate and oxalacetate at the start of the reaction.

Determination of ^{13}C Fractionation Factor for N,N-Tetramethylenecarbamate (Pyrrolidine Carbamate). Dioxane was dried by being refluxed overnight with sodium and was distilled just before use from fresh sodium and benzophenone. Pyrrolidine carbamate was formed by sparging 2 mL of 2.0 M pyrrolidine in dioxane with CO_2 for 10 min. Proton NMR spectra showed that carbamate formation is complete in 5 min under these conditions. After the solution was sparged, the needle was withdrawn, and the gas phase above the solution (25 mL) was flushed with CO_2 for 10 min. The vessel was then sealed and shaken gently for 3 days at room temperature. A reaction vessel that did not contain pyrrolidine was treated identically to determine the amount and isotopic content of CO_2 dissolved in dioxane.

Aliquots were transferred from the reaction vessels to CO_2 isolation vessels that contained 0.1 mL of 1 M H_2SO_4 and had been flushed with nitrogen. A syringe equipped with a stopcock was used to remove 1-mL aliquots from the gas phase above the solutions, while 0.05-mL aliquots were removed from the pyrrolidine-containing solution and 0.2-mL aliquots from the blank. The CO_2 was isolated and its $^{13}C/^{12}C$ ratio determined as described above.

The observed ratio of ^{13}C contents in the carbamate solution and its gas phase will equal $F_p x + F_b y$, where F_p is the true ratio for carbamate and CO_2 gas (that is, the desired fractionation factor), F_b is the corresponding ratio for dissolved CO_2 determined in the blank (the vapor pressure isotope effect), and x and y are the proportions of CO_2 in the solution in the form of carbamate and dissolved CO_2 (the latter assumed equal to the value in the blank).

Data Analysis. Data from the deuterium isotope effect experiments where the effect was on V/K alone were fitted to eq 3 by the least-squares method with a program written by Duggleby (1981), where F_i is the fractional deuteration of the substrate and VKI is the isotope effect minus 1 on V/K. The $^{13}(V/K)$ or ^{13}k isotope effects were computed from eq 4,

$$v = VA/[K(1 + F_i VKI) + A] \quad (3)$$

$$^{13}(V/K) \text{ or } ^{13}k = \frac{\log (1 - f)}{\log (1 - fR_f/R_0)} \quad (4)$$

where R_0 and R_f are $^{13}C/^{12}C$ isotope ratios for the initial substrate (as measured in product at $f = 1.0$) and product at fractional conversion f. Replicates from the same experiment were averaged to give mean $^{13}(V/K)$ values.

RESULTS

Deuterium Isotope Effects on Pyruvate Carboxylation. An isotope effect on 2.8 on V/K was observed when pyruvate-d_3 and unlabeled pyruvate were used as substrates, but no effect was observed on V.

Deuterium Isotope Effects on Enzymic Decarboxylation of Oxalacetate. The primary D_2O solvent isotope effect when unlabeled oxalacetate was the substrate in either H_2O or D_2O was 2.1 on V/K and 1.0 on V. The overall solvent D_2O isotope effect, which also includes α-secondary deuterium isotope effects from oxalacetate-d_2, was obtained by comparing the reaction in H_2O using unlabeled oxalacetate with that in D_2O using oxalacetate-d_2. The effect on V/K was 3.3, and that on V was 1.0. The α-secondary deuterium isotope effect from oxalacetate-d_2 alone was obtained by comparing the reactions in D_2O, where either unlabeled or dideuterated oxalacetate was the substrate. The effect was 1.7 on V/K and 1.0 on V. If we assume that the isotope effect on V/K for monodeuteration is the square root of the value for oxalacetate-d_2, we obtain 1.3 for this value.

From the fractionation factors[1] for pyruvate (0.84) and methylene groups next to a keto group (0.93) given by Cleland (1980), we can calculate the α-secondary deuterium isotope effect for pyruvate as $1.3 \times 0.84/0.93 = 1.2$. Again assuming that the α-secondary deuterium isotope effects for pyruvate are multiplicative, the primary deuterium isotope effect for deuterated pyruvate may be calculated from the observed value with pyruvate-d_3 as $2.8/(1.2)^2 = 1.9$.

In the experiments performed in D_2O (assuming that enzyme-bound biotin behaves in the same way as free biotin), the proton on N-1 of the enzymic biotin has exchanged with the solvent, since the exchange rate of this proton in free biotin is 58 s^{-1} (Fry et al., 1985). Thus in the oxalacetate decarboxylation experiments there is a primary deuterium effect on the transfer of the N-1 proton from biotin to pyruvate. Since the fractionation factor of the proton on N-1 of biotin has been measured by Dr. A. S. Mildvan (personal communication) to be 0.97, the expected primary deuterium isotope effect resulting from deuteration of the N-1 proton of biotin can be calculated as $1.9 \times 0.97/0.84 = 2.2$. Thus the calculated isotope effect for deuterated biotin is essentially the same as the primary solvent D_2O effect, suggesting that the major component of the primary solvent D_2O effect is the isotope effect due to deuteration of the N-1 proton of biotin. Other D_2O solvent isotope effects are thus small.[2]

[1] A fractionation factor is an equilibrium isotope effect for exchange of label between a compound and a reference molecule (water for deuterium or ^{18}O or CO_2 in aqueous solution for ^{13}C). When the fractionation factor is above unity, the heavy atom enriches in the compound; when it is less than unity, it enriches in the reference molecule.

Table I: Deuterium Isotope Effects on Pyruvate Carboxylation and Oxalacetate Decarboxylation[a]

reaction	type of isotope effect	$^{D}(V/K)$ or ^{D}k
pyruvate carboxylation	obsd with pyruvate-d_3	2.8
by pyruvate	calcd primary	1.9
carboxylase	calcd α-secondary (per D)	1.2
oxalacetate	obsd primary solvent D_2O	2.1
decarboxylation by	obsd α-secondary for OAA-d_2	1.7
pyruvate carboxylase	calcd α-secondary (per D)	1.3
	calcd primary for biotin-d	2.2
nonenzymatic	obsd solvent D_2O	1.4

[a] The experimental details and the methods of calculation of the isotope effects are described in the text.

Table II: ^{13}C Isotope Effects on Oxalacetate Decarboxylation[a]

reaction conditions	$^{13}(V/K)$ or ^{13}k	no. of determinations
nonenzymic in H_2O	1.0527 ± 0.0001	3
nonenzymic in D_2O	1.0507 ± 0.0016	3
enzymic (−oxamate) in H_2O	1.0323 ± 0.0002	2
enzymic (−oxamate) in D_2O	1.0252 ± 0.0005	2
enzymic (+oxamate) in H_2O	1.0238 ± 0.0005	3
enzymic (+oxamate) in D_2O	1.0155 ± 0.0007	3

[a] Experimental details and methods of calculation of the isotope effects are described in the text.

Deuterium Isotope Effect on Nonenzymic Decarboxylation of Oxalacetate. The measured effect on the observed first-order rate constant was 1.4. The values of all deuterium isotope effects are shown in Table I.

^{13}C Isotope Effects on Oxalacetate Decarboxylation. Table II shows the ^{13}C isotope effects on both the enzymic and nonenzymic decarboxylation of oxalacetate in both H_2O and D_2O. The values shown for the enzymic decarboxylations are corrected for the nonenzymic decarboxylation occurring during the course of the reaction. The oxamate-dependent values have also been corrected for oxamate-independent decarboxylation of oxalacetate during the reaction. These corrections typically resulted in reductions from the observed isotope effects of 0.3%, e.g., from 1.0272 to 1.0238.

^{13}C Fractionation Factor of Oxalacetate. After 300- or 400-min incubation of pyruvate carboxylase with its reactants under conditions where chemical equilibrium was reached in less than 5 min, the ratio of the $^{13}C/^{12}C$ mass ratios of C-4 of oxalacetate and bicarbonate was 1.00149 or 1.00145, respectively. Thus $^{13}K_{eq}$ is 1.00147. The mass ratios of the starting bicarbonate and oxalacetate showed that during attainment of isotopic equilibrium the bicarbonate became enriched in ^{13}C. Since the ^{13}C fractionation factor of bicarbonate relative to aqueous CO_2 is 1.009, that of C-4 of oxalacetate is 1.009/1.00147 = 1.0075.

^{13}C Fractionation Factor of N,N-Tetramethylenecarbamate. Dioxane sparged with CO_2 contained 105 mM dissolved CO_2 with a ^{13}C content 0.9983 times that in the gas phase in equilibrium with it. In the presence of pyrrolidine, 27% of the CO_2 in the solution was dissolved CO_2, and 73% was carbamate. The calculated fractiontion factor of the carbamate relative to CO_2 gas was 1.0112 ± 0.0007, corresponding to 1.0123 relative to aqueous CO_2 (CO_2 gas has a fractionation factor relative to aqueous CO_2 of 1.00106; Mook et al., 1974). Note the depletion of ^{13}C content in dissolved CO_2 is actually larger in dioxane (0.17%) than in water (0.11%).

Discussion

Deuterium Isotope Effects. As found previously by Cheung and Walsh (1976), when pyruvate-d_3 is the substrate, there is a sizeable isotope effect on V/K but none on V. The deuterium isotope effects on the decarboxylation of oxalacetate by pyruvate carboxylase are also on V/K but not on V. This shows that the rate-limiting part of the reaction is the decarboxylation of the enzyme-carboxybiotin complex and that any deuterium-sensitive steps within this part of the reaction are not rate limiting. Presumably, the conformation changes that lead to decarboxylation of carboxybiotin limit V; these must be insensitive to D_2O.

The fact that the α-secondary deuterium effects on V/K are normal both for pyruvate carboxylation and for oxalacetate decarboxylation suggests that the enolate of pyruvate is an intermediate in the reaction. The fractionation factor for phosphoenolpyruvate, which is the closest analogue to the enolate of pyruvate for which a value has been obtained, is 0.78 (Cleland, 1980). Since the fractionation factors for oxalacetate and pyruvate are 0.93 and 0.84, respectively, the equilibrium isotope effects for the formation of the enolate of pyruvate from pyruvate and oxalacetate are 1.1 and 1.2, respectively. The observed isotope effects for single deuterium substitution of 1.2 and 1.3 exceed these values, however, suggesting that (1) the interconversion of pyruvate and its enolate is a major rate-limiting step for V/K (the primary deuterium isotope effects of 1.9–2.1 argue the same thing) and (2) the bending motions of the secondary hydrogens in going from a tetrahedral to a trigonal structure are coupled to the movement of the primary hydrogen and become part of the reaction coordinate motion (this causes the observed kinetic secondary isotope effect to exceed the equilibrium isotope effect). Similar large α-secondary deuterium isotope effects have been seen for a number of dehydrogenases where hydride transfer is coupled to the bending motions of the hydrogen at C-4 of the nicotinamide ring of NAD (Cook et al., 1981; Hermes et al., 1984; Hermes & Cleland, 1984).

The relatively small solvent D_2O effect of 1.4 on the nonenzymic decarboxylation of oxalacetate is intriguing, since the rate-limiting step is clearly enolpyruvate formation on the basis of the large ^{13}C isotope effect of 1.053. This large ^{13}C value reflects (1) carbon motion in the transition state as carbon of mass 12 becomes collinear with two oxygens of combined mass 32 and (2) a transition state that is probably fairly late. The fractionation factor of the methylene hydrogens of oxalacetate has clearly not reached that of enolpyruvate (the equilibrium isotope effect is 1.4 for oxalacetate-d_2), unless there is no primary deuterium isotope effect at all from the proton that must be transferred from buffer or solvent to form the enol. The ^{13}C isotope effect measured in D_2O was slightly lower than that in H_2O, although this apparent difference may not be significant.

^{13}C Isotope Effects. The magnitude of the ^{13}C isotope effect observed on the oxamate-dependent, enzymic decarboxylation of oxalacetate indicates that the carboxyl group transfer step is at least partially rate limiting. The fact that the size of the effect was reduced when the reaction was run in D_2O clearly indicates that there are separate carboxyl-transfer and proton-transfer steps in the overall reaction. If the reaction were concerted, one would expect the observed ^{13}C isotope effect to stay the same or increase upon deuteration (Cleland, 1982).

[2] The above calculations are only approximate since they ignore the presence of commitments and the possibility that the intrinsic isotope effects are not strictly multiplicative because of the coupled motion of primary and secondary hydrogens in the transition state and the presence of tunneling (Hermes & Cleland, 1984). For small isotope effects, however, the errors caused by small commitments will not be large. We shall examine the secondary isotope effects in more detail for individual mechanisms below.

This stepwise type of mechanism is in agreement with the results of similar ^{13}C isotope effect experiments on transcarboxylase reported by O'Keefe and Knowles (1986).

The larger ^{13}C isotope effect observed for the oxamate-independent decarboxylation of oxalacetate than for the oxamate-dependent reaction could be taken to indicate that the decarboxylation step in this reaction is more rate limiting than that in the oxamate-dependent reaction. However, the situation is complicated by the fact that the oxamate-independent reaction is the sum of a biotin-dependent decarboxylation and a biotin-independent decarboxylation (Attwood & Cleland, 1986). It can be assumed that the biotin-dependent reaction is similar to the oxamate-dependent decarboxylation of oxalacetate and hence would have a similar ^{13}C isotope effect (Attwood & Cleland, 1986). The higher ^{13}C isotope effect observed in the overall oxamate-independent reaction must therefore be derived from the biotin-independent decarboxylation reaction, which may be a simple amine-catalyzed decarboxylation. The reduction in the magnitude of the ^{13}C isotope effect for the oxamate-independent decarboxylation of oxalacetate in D_2O may be caused by effects on both the biotin-dependent and biotin-independent reactions.

Mechanism of Carboxyl Transfer. From our results we may eliminate from consideration the concerted mechanism of Retey and Lynen (1965). However, before going on to consider possible reaction mechanisms that will fit our data, we should examine a facet of the reaction that neither of the two mechanisms so far described in this paper takes into account. This concerns the lability of the carboxybiotin moiety at the site of reaction 2. Easterbrook-Smith et al. (1976) proposed that a hydrolytic leak occurs at the site of reaction 2 at low pyruvate concentrations such that more ATP is hydrolyzed than oxalacetate is formed in the forward reaction of pyruvate carboxylase. In addition, Goodall et al. (1981) found that oxamate was able to induce the decarboxylation of the isolated enzyme–carboxybiotin complex. It would appear that oxamate mimics the effect of pyruvate in that it acts as a signal for the movement of carboxybiotin to the site of reaction 2 (Goodall et al., 1981). Other studies on the isolated enzyme–carboxybiotin complex also indicate that the movement of carboxybiotin to the site of reaction 2 increases its lability (Attwood et al., 1984). Thus, some characteristic of this enzymic subsite must enhance the tendency of carboxybiotin to be decarboxylated.

Goodall et al. (1983) attempted to address this problem by proposing a mechanism in which a proton is extracted from N-3 of carboxybiotin by a base in the site of reaction 2. This enhances the nucleophilicity of the ureido oxygen, which can then remove a proton from pyruvate in a reaction similar to that of Retey and Lynen (1965). However, our results rule out such a cyclic concerted mechanism.

We have considered three chemical mechanisms for reaction 2 in detail. The first (Scheme I) involves enolization of biotin by proton removal by a base on the enzyme, followed by carboxyl transfer and protonation of the enolate of pyruvate.[3] Thus, the ^{13}C-sensitive step or steps involving carboxyl transfer are flanked by steps that involve a primary deuterium isotope effect. If we assume no external commitments exist,[4] the key part of the mechanism will be

$$\text{E–OAA–BioNH} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{EH–OAA–enolBioN:} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{EH–enolPyr–BioNCO}_2 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \text{E–Pyr–BioNCO}_2 \quad (5)$$

where OAA is oxalacetate, BioNH is biotin, Pyr is pyruvate, and EH and E are enzyme with the catalytic base protonated or unprotonated. The steps represented by k_5 and k_6 would probably have to involve decarboxylation to enzyme-bound CO_2 as in intermediate, but the end result would be the same. The equation for the secondary deuterium isotope effect in this mechanism will be

$$^{\text{sec-D}}(V/K) = 1.7 = \frac{^{\text{sec-D}}k_5 + a + {}^{\text{sec-D}}K_{\text{eq5}}{}^{\text{sec-D}}k_7 b}{1 + a + b} \quad (6)$$

where $a = k_5/k_4$, $b = k_6/k_7$, $^{\text{sec-D}}K_{\text{eq5}}$ is 1.45 (the equilibrium isotope effect for going from oxalacetate to enolpyruvate), and $^{\text{sec-D}}k_5$ is probably about 1.2 (it will make little difference if we assume a different value between 1.0 and 1.45). Since the observed value is 1.7, the last term in the numerator must be the important one, and b is likely to be at least unity and a less than 0.2. If $b = 1.1$ and $a = 0.2$, we get 1.57 for $^{\text{sec-D}}k_7$ (or 1.25 per deuterium). This large normal secondary deuterium isotope effect (the equilibrium isotope effect is 0.86) demonstrates coupled motion between the primary and secondary hydrogens in the transition state, and is similar in size to that seen with formate dehydrogenase (Hermes et al., 1984).

Equations for the ^{13}C isotope effect in water and in D_2O for this mechanism are

$$^{13}(V/K)_{H_2O} = 1.0238 = \frac{^{13}k_5 + a + {}^{13}K_{\text{eq}}b}{1 + a + b} \quad (7)$$

$$^{13}(V/K)_{D_2O} = 1.0155 = \frac{^{13}k_5 + a^{\text{D}}k_4/^{\text{sec-D}}k_5 + {}^{13}K_{\text{eq}}b^{\text{D}}k_7{}^{\text{sec-D}}k_7/^{\text{sec-D}}k_6}{1 + a^{\text{D}}k_4/^{\text{sec-D}}k_5 + b^{\text{D}}k_7{}^{\text{sec-D}}k_7/^{\text{sec-D}}k_6} \quad (8)$$

where $^{\text{D}}k_4$ and $^{\text{D}}k_7$ are the primary deuterium isotope effects on those steps, $^{\text{sec-D}}k_5 = 1.2$, $^{\text{sec-D}}k_6 = 0.83$, and $^{\text{sec-D}}k_7$ is probably around 1.5 (see above). The primary solvent isotope effect with unlabeled oxalacetate is given by

$$^{\text{D}}(V/K) = 2.1 = \frac{^{\text{D}}K_{\text{eq3}}(1 + a^{\text{D}}k_4 + b^{\text{D}}k_7)}{1 + a + b} \quad (9)$$

where $^{\text{D}}K_{\text{eq3}}$ is determined by the fractionation factor of the base on the enzyme. If this base has a fractionation factor near unity, as would be the case for carboxyl or imidazole (or most other) groups, eq 6–9 do not have a solution with positive values of a and b. However, if we (1) assume reasonable values for $^{13}k_5$, (2) solve for b in eq 7 (assuming a is small as deduced above from the secondary isotope effect), and (3) solve for $^{\text{D}}k_7$ in eq 8, we can solve for $^{\text{D}}K_{\text{eq3}}$ in eq 9. If $^{13}K_{\text{eq}}$ is unity, $^{13}k_5$ is 1.05, $b = 1.1$, and a is small, we get 1.62 for $^{\text{D}}k_7$ and 1.58 for $^{\text{D}}K_{\text{eq3}}$, corresponding to a fractionation factor of the enzymic base of 0.61, since that of the N-1 hydrogen on biotin in 0.97. Values of $^{13}K_5$ down to 1.03 result in smaller commitments (b down to 0.26), larger values of $^{\text{D}}k_7$ (up to 3), and a slightly higher fractionation factor for the enzymic base (up to 0.65). If $^{13}K_{\text{eq}}$ is not unity, but something greater, the fractionation factor of the enzymic base can be higher (up to 0.85 for a $^{13}K_{\text{eq}}$ value of 1.0115, with $^{13}k_5 = 1.05$, $b = 2.13$, $a = 0$, and $^{\text{D}}k_7 = 2.24$).

Thus, this mechanism is capable of fitting the experimental data, but only if the enzymic base has a low fractionation

[3] In Schemes I–III we have shown Mn^{2+} forming an inner sphere complex with oxalacetate and pyruvate. The interaction may, however, be a second sphere one as suggested by Fung et al. (1974).

[4] Cheung and Walsh (1976) concluded that oxalacetate dissociated much faster than it underwent reaction, and Mildvan and Scrutton (1967) measured a rate constant for pyruvate release from the enzyme that considerably exceeded its rate of reaction.

factor. The only group with such a low value is the SH group of cysteine, which has a fractionation factor of ~0.5. But for a sulfhydryl group to be the base, it must be *ionized* in the neutral pH range and thus have a pK below 5. This is possible only when the SH group is in a tight ion pair with a positively charged group such as lysine. (The pK of the catalytic SH group on papain is ~4 in the presence of protonated histidine; Shaked et al., 1980). Thus for this mechanism to be a viable possibility, there would have to be a Lys–Cys ion pair in the active site that was separated by the binding of biotin so that the Lys polarized the ureido oxygen to induce enolization and the Cys acted as the base to accept the N-1 proton. Hudson et al. (1975) have reported that bromopyruvate inactivates sheep pyruvate carboxylase in a specific fashion (a single sulfhydryl group modified, and a much slower reaction rate with bromoacetate or iodoacetate) and that the inactivation was prevented by oxalacetate and pyruvate but not by MgATP and bicarbonate. The specificity of this reaction is reinforced by the studies of Palacian and Neet (1972) with the chicken enzyme; these authors showed that reaction of sulfhydryl groups with cystine or *N*-ethylmaleimide was a nonspecific reaction not prevented by oxalacetate. A number of sulfhydryl groups could be modified without affecting activity, but not surprisingly, complete reaction led to total inactivation. The possibility of a sulfhydryl group in the active site bears further study.

The second mechanism we will consider involves decarboxylation of oxalacetate as the first step, followed by proton transfer between biotin and enolpyruvate to generate pyruvate and enolized biotin, which then reacts with CO_2 (Scheme II). Unlike mechanism 5, this mechanism involves two ^{13}C-sensitive steps flanking a single step that will show a primary deuterium isotope effect. Again assuming that there are no external commitments

$$\text{E–OAA–BioNH} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E–enolPyr–CO}_2\text{–BioNH} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{E–Pyr–CO}_2\text{–enolBioN:} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \text{E–Pyr–BioNCO}_2 \quad (10)$$

The equation for the secondary deuterium isotope effect in water is

$$^{\text{sec-D}}(V/K) = 1.7 = \frac{^{\text{sec-D}}K_{eq3}{}^{\text{sec-D}}k_5 + {}^{\text{sec-D}}k_3a + {}^{\text{sec-D}}K_{eq3}{}^{\text{sec-D}}K_{eq5}b}{1 + a + b} \quad (11)$$

where $a = k_5/k_4$, $b = k_6/k_7$, $^{\text{sec-D}}K_{eq3} = 1.45$, $^{\text{sec-D}}K_{eq5} = 0.86$, and $^{\text{sec-D}}k_3$ is likely to be ~1.2. The minimum value of $^{\text{sec-D}}k_5$ is 1.17 if a and b are both small, again showing the coupled motions of primary and secondary hydrogens during the protonation of enolpyruvate.

The equations for the ^{13}C isotope effects in this mechanism are

$$^{13}(V/K)_{H_2O} = 1.0238 = \frac{^{13}K_{eq3} + {}^{13}k_3a + {}^{13}K_{eq3}{}^{13}k_7b}{1 + a + b} \quad (12)$$

$$^{13}(V/K)_{D_2O} = 1.0155 = [{}^{13}K_{eq3} + ({}^{13}k_3a^{\text{sec-D}}k_4 + {}^{13}K_{eq3}{}^{13}k_7b^{D}K_{eq5}{}^{\text{sec-D}}K_{eq5})/({}^{D}k_5{}^{\text{sec-D}}k_5)]/[1 + (a^{\text{sec-D}}k_4 + b^{D}K_{eq5}{}^{\text{sec-D}}K_{eq5})/({}^{D}k_5{}^{\text{sec-D}}k_5)] \quad (13)$$

where $^{13}K_{eq3} = 1.0065$ (assuming the measured fractionation factor of 1.0075 for oxalacetate and a value of 1.001 for CO_2; this is the gas-phase value since CO_2 should not be hydrated in the active site), $^{D}K_{eq5}{}^{\text{sec-D}}K_{eq5} = 0.99$, and $^{\text{sec-D}}k_4$ should be ~0.83.

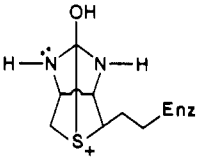
The primary deuterium isotope effect will be given by

$$^{D}(V/K) = 2.1 = \frac{^{D}k_5 + a + {}^{D}K_{eq}b}{1 + a + b} \quad (14)$$

where $^{D}K_{eq} = 1.15$.

We have been unable to find any solutions to eq 11–14 with positive values of a and b. The problem is that $^{13}(V/K)$ in D_2O ought to be closer to unity than it is (the maximum value we have been able to calculate, assuming the other experimental values, is 1.013). Thus we conclude that this mechanism, attractive though it is on chemical grounds and in view of the pH independence of the reaction, is not a viable one for reaction 2 if the experimental isotope effects for correct.

The third mechanism we have considered involves the activation of biotin by formation of a bond between the sulfur and the ureido carbon:



In this structure N-1 becomes a secondary amine and can readily attack CO_2 resulting from decarboxylation of oxalacetate to give a carbamate. This mechanism is chemically plausible if one grants the ability of the enzyme to compress biotin into this structure (it is not particularly strained, since N-1 and N-3 become tetrahedral and the C–S bonds are long enough to relieve the strain of fusing four five-membered rings in this fashion). This mechanism can be outlined as

$$\text{E–OAA–BioNH} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E–enolPyr–CO}_2\text{–BioNH:} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{E–enolPyr–BioNHCO}_2 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \text{E–Pyr–BioNCO}_2 \quad (15)$$

The equation for the secondary isotope effect in this mechanism is

$$^{\text{sec-D}}(V/K) = 1.7 = \frac{^{\text{sec-D}}K_{eq3}(1 + {}^{\text{sec-D}}k_4a + {}^{\text{sec-D}}k_7b)}{1 + a + b} \quad (16)$$

where $a = k_5/k_4$, $b = k_6/k_7$, $^{\text{sec-D}}K_{eq3} = 1.45$, and $^{\text{sec-D}}k_4 = \sim 0.83$. The minimum value of $^{\text{sec-D}}k_7$ is 1.17 when a is small and b is large, and the value will be 1.69 (1.3 per deuterium) if $a = b = 1$. Again, the size of this value indicates coupling of primary and secondary hydrogen motions in the transition state for enolpyruvate protonation. The ^{13}C isotope effects are given by

$$^{13}(V/K)_{H_2O} = 1.0238 = \frac{^{13}K_{eq3}{}^{13}k_5 + {}^{13}k_3a + {}^{13}K_{eq3}{}^{13}K_{eq5}b}{1 + a + b} \quad (17)$$

$$^{13}(V/K)_{D_2O} = 1.0155 = [{}^{13}K_{eq3}{}^{13}k_5 + {}^{13}k_3a^{\text{sec-D}}k_4/{}^{\text{sec-D}}k_5 + {}^{13}K_{eq3}{}^{13}K_{eq5}b^{\text{sec-D}}k_7{}^{D}k_7/{}^{\text{sec-D}}k_6]/[1 + a^{\text{sec-D}}k_4/{}^{\text{sec-D}}k_5 + b^{\text{sec-D}}k_7{}^{D}k_7/{}^{\text{sec-D}}k_6] \quad (18)$$

where $^{13}K_{eq3} = 1.0065$, $^{\text{sec-D}}k_4 = \sim 0.83$, $^{\text{sec-D}}k_5 = \sim 0.9$, and $^{\text{sec-D}}k_6 = \sim 1.1$ (we assume that the fractionation factor of the N-1 hydrogen in the zwitterionic carbamate is ~1.2). Note that both secondary and primary deuterium isotope effects occur on the last step (k_7), while only secondary deuterium isotope effects are seen on the first two steps.

The primary D_2O solvent isotope effect is given by

$$^{D}(V/K) = 2.1 = \frac{^{D}k_5 + a + {}^{D}K_{eq5}{}^{D}k_7b}{1 + a + b} \quad (19)$$

where ${}^{D}k_5$ is ~0.9 and ${}^{D}K_{eq5}$ is ~0.81.

We have searched for solutions to eq 16–19 by (1) assuming values of a and b, (2) solving eq 16 and 19 for ${}^{sec\text{-}D}k_7$ and ${}^{D}k_7$, (3) solving eq 17 for ${}^{13}k_3$ and/or ${}^{13}k_5$ (usually assumed equal) in terms of ${}^{13}K_{eq5}$, and (4) substituting these values into eq 18 and solving for ${}^{13}K_{eq5}$. For values of b over unity and $a \leq b$ that do not require an unrealistically large value of ${}^{sec\text{-}D}k_7$ (more than 1.7), the primary deuterium isotope effect (${}^{D}k_7$) ranged from 3.3 to 5.4, the primary ^{13}C isotope effect varied from 1.026 to 1.048, and ${}^{13}K_{eq5}$ was always ~1.005. Thus the data fit this mechanism only if the ^{13}C fractionation factor of the zwitterionic carbamate intermediate is 0.5% less than that of CO_2. We do not know what the fractionation factor of a zwitterionic carbamate would be, but when the nitrogen is not protonated, the value is 0.8% *higher* than that of CO_2, rather than lower. Thus unless protonation of the carbamate nitrogen lowers the ^{13}C fractionation factor by over a full percent, which seems unlikely, this mechanism is not consistent with the isotope effect data.

Thus in summary, the isotope effects reported here clearly rule out a concerted chemical mechanism. Mechanisms in which ^{13}C-sensitive steps flank a central deuterium-sensitive one or in which biotin is activated by bond formation between sulfur and the ureido carbon to make N-1 into a secondary amine also appear inconsistent with the measured isotope effects. The experimental data are consistent with a mechanism where deuterium-sensitive transfers of hydrogen to an enzyme base flank a central ^{13}C-sensitive transfer of the carboxyl group (probably via CO_2), as long as the enzymic base is a sulfhydryl group in an ion pair with a positively charged group such as lysine. Further work is clearly needed to tell whether these deductions are valid or not.

Registry No. OAA, 328-42-7; D_2, 7782-39-0; ^{13}C, 14762-74-4; pyruvate carboxylase, 9014-19-1; *N,N*-tetramethylenecarbamate, 104876-17-7.

References

Attwood, P. V., & Cleland, W. W. (1986) *Biochemistry* (preceding paper in this issue).

Attwood, P. V., Wallace, J. C., & Keech, D. B. (1984) *Biochem. J. 219*, 243.

Cheung, Y.-F., & Walsh, C. (1976) *Biochemistry 15*, 3749.

Cleland, W. W. (1980) *Methods Enzymol. 64*, 104.

Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385.

Cook, P. F., Oppenheimer, N. J., & Cleland, W. W. (1981) *Biochemistry 20*, 1817.

Craig, H. (1957) *Geochim. Cosmochim. Acta 12*, 133.

Duggleby, R. G. (1981) *Anal. Biochem. 110*, 9.

Easterbrook-Smith, S. B., Hudson, P. J., Goss, N. H., Keech, D. B., & Wallace, J. C. (1976) *Arch. Biochem. Biophys. 176*, 709.

Fry, D. C., Fox, T. L., Lane, M. D., & Mildvan, A. S. (1985) *J. Am. Chem. Soc. 107*, 7659.

Fung, C.-H., Mildvan, A. S., & Leigh, J. S. (1974) *Biochemistry 13*, 1160.

Goodall, G. J., Baldwin, G. S., Wallace, J. C., & Keech, D. B. (1981) *Biochem. J. 199*, 603.

Goodall, G. J., Prager, R., Wallace, J. C., & Keech, D. B. (1983) *FEBS Lett. 163*, 6.

Goss, N. H., Dyer, P. Y., Keech, D. B., & Wallace, J. C. (1979) *J. Biol. Chem. 254*, 1734.

Hermes, J. D., & Cleland, W. W. (1984) *J. Am. Chem. Soc. 106*, 7263.

Hermes, J. D., Morrical, S. W., O'Leary, M. H., & Cleland, W. W. (1984) *Biochemistry 23*, 5479.

Hudson, P. J., Keech, D. B., & Wallace, J. C. (1975) *Biochem. Biophys. Res. Commun. 65*, 231.

Mildvan, A. S., & Scrutton, M. C. (1967) *Biochemistry 6*, 2978.

Mildvan, A. S., Scrutton, M. C., & Utter, M. F. (1966) *J. Biol. Chem. 241*, 3488.

Mook, W. G., Bommerson, J. C., & Staverman, W. H. (1974) *Earth Planet Sci. Lett. 22*, 169.

O'Keefe, S. J., & Knowles, J. R. (1986) *J. Am. Chem. Soc. 108*, 328.

O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83.

Palacian, E., & Neet, K. E. (1972) *Biochim. Biophys. Acta 276*, 297.

Retey, J., & Lynen, F. (1965) *Biochem. Z. 342*, 256.

Rose, I. A., O'Connell, E. L., & Solomon, F. (1976) *J. Biol. Chem. 251*, 902.

Sauers, C. K., Jencks, W. P., & Groh, S. (1975) *J. Am. Chem. Soc. 97*, 5546.

Schowen, K. B. J. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) p 225, Plenum, New York.

Shaked, Z., Szajewski, R. P., & Whitesides, F. M. (1980) *Biochemistry 19*, 4156.

Stubbe, J., & Abeles, R. H. (1977) *J. Biol. Chem. 252*, 8338.

Stubbe, J., Fish, S., & Abeles, R. H. (1980) *J. Biol. Chem. 255*, 236.

Wood, A. (1964) *Trans. Faraday Soc. 60*, 1263.