# Carbon-13 Kinetic Isotope Effects on Pyruvate Decarboxylation. 2. Solvent Effects in Model Systems[1]

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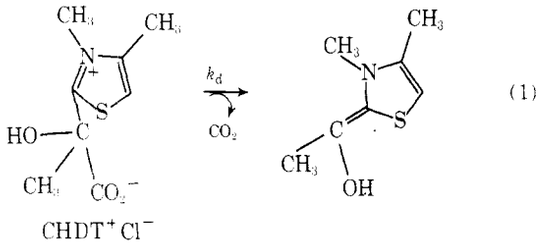
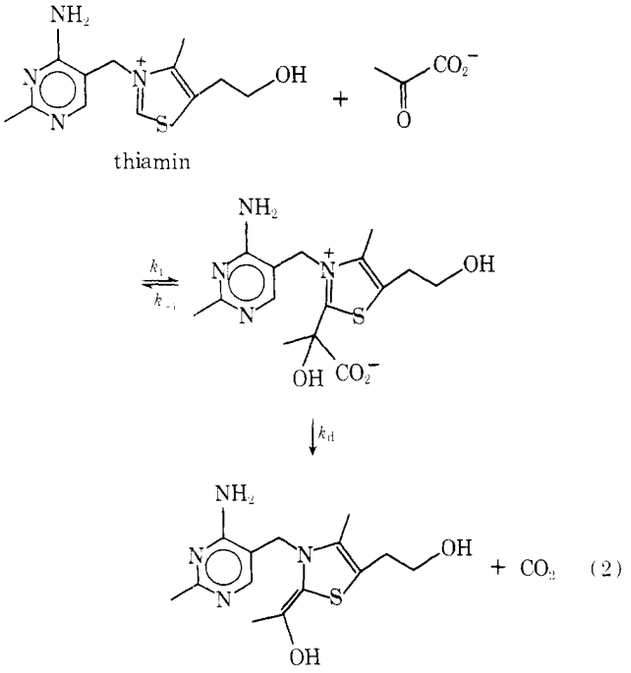
Carbon-13 kinetic isotope effects were determined for the decarboxylation of pyruvate by thiamin and for the decarboxylation of 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride (a covalent pyruvate–thiamin adduct) in water and in aqueous ethanol. The kinetic isotope effect for the decarboxylation step in the second system increased from 1.051 in $H_2O$ to ~1.058 in 50% v/v ethanol. The isotope effect in the thiamin catalyzed model reaction increased from the 0.992 inverse effect observed in $H_2O$ to 1.007 in 50% aqueous ethanol. The rate of decomposition of the covalent thiamin–pyruvate adduct to reactants relative to its rate of decarboxylation is greater in ethanol than in water. Further, the results suggest that the changes of the vibrational force constants in going from the ground state to the transition state are greater in aqueous ethanol than in water.

Carbon-13 kinetic isotope effect (KIE) studies provide a convenient, if seldom employed, technique for elucidating the rate-determining step in a multistep reaction mechanism which involves C–C bond breaking or making. We recently reported $^{13}C$ KIE values[2] for the decarboxylation of pyruvate by yeast pyruvate decarboxylase (E.C.4.1.1.1) and for two model systems: (1) 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride ($CHDT^+Cl^-$), a model KIE for the decarboxylation step,



$CHDT^+Cl^-$ (1)

and (2) thiamin catalyzed decarboxylation, a model KIE for the two steps, covalent pyruvate–coenzyme adduct formation followed by decarboxylation.



thiamin (2)

The experimental KIE values of 1.051 for reaction 1, and 0.992 for reaction 2, and the KIE of 1.002 to 1.011 observed for the holoenzyme-catalyzed reaction were interpreted to mean that in both the thiamin model and in the enzymic reaction decarboxylation is not rate limiting, i.e., $k_{-1}/k_d < 1$.

Attachment of a fluorescent dye to the active site of pyruvate decarboxylase has suggested the existence of a hydrophobic environment.[3] In addition, model studies closely resembling those in eq 1 and 2 demonstrated that the reactions proceed faster in ethanol than in water[4] and have led Lienhard's group to suggest that the enzyme may accelerate the reaction by creating a low polarity environment around the active site. Based on these suggestions, we undertook a study to demonstrate the effect of the solvent dielectric constant on the rate-limiting step in the model reaction depicted in eq 2.

## Results and Discussion

The isotope effects were calculated according to Bigeleisen's formula[5] from data obtained by measuring isotope ratio $^{45}CO_2/^{44}CO_2$:

$$k^{12}/k^{13} = \frac{\log(1-f)}{\log\left(1 - f\frac{N_x}{N_{x0}}\right)} \quad (3)$$

where $N_x$ is the isotope ratio at low fractional conversion $f$ corrected for the natural abundance of $^{12}C^{16}O^{17}O$, and $N_{x0}$ is the corrected ratio at 100% reaction ($f = 1.0$).

**$CHDT^+Cl^-$.** Table I summarizes the data in water and in aqueous ethanol. Clearly, the KIE increases with increasing ethanol content. The rate of decarboxylation has also been found to increase with added ethanol.[4a] Even with the large $f$ value employed (this was necessitated by the limited quantity of substrate available for this study) error analysis[5b] indicates less than ±0.002 uncertainty in the isotope effect due to the cumulative uncertainties of $f$ and $N_x$ measurements.

Solvent effects on heavy-atom KIE measurements are rare in the literature. The solvent isotope effect on malonic acid decarboxylation was reported to be small: 1.034 in $H_2O$ at 137 °C;[6] 1.032 in dioxane at 99.1 °C;[7] and 1.032 in quinoline at 138 °C.[8] Cromartie and Swain[9] reported $k_{water}/k_{ethanol}$ chlorine isotope effects of 0.999 84 for cyclization of 2-chloroethanol and 1.000 56 for the reverse reaction. In *tert*-butyl alcohol the solvent isotope effect was 1.000 36 for the forward and 1.000 30 for the reverse reaction.

Since $CHDT^+Cl^-$ retains its dipolar ionic character in the range of ethanol–water mixtures employed here,[4a] one can write the solvent effect in terms of a single rate constant for the two isotopically labeled species:

$$\frac{(k_d/k_d^*)_{30\%\ \text{ethanol}}}{(k_d/k_d^*)_{H_2O}} = \frac{1.054}{1.051} \simeq 1.003 \quad (4)$$

and

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**Table I. $^{13}C$ Kinetic Isotope Effect on $CHDT^+Cl^-$ Decarboxylation**

| Temp, °C | Conc $CHDT^+Cl^-$, mM, in solvent | $f$[a] | $N_x \times 10^6$ [b] | $N_{x0}$[c]/$N_x$ | | $k^{12}/k^{13}$ [d] |
|---|---|---|---|---|---|---|
| 45.6 | 16.8 in $H_2O$ | 0.1914 | 10124 | 1.0459 | | 1.0511 |
| 45.6 | 16.8 in $H_2O$ | 0.1988 | 10131 | 1.0452 | | 1.0506 |
| | | | | | Av | 1.0509[f] |
| 45.6 | 15.8 in 30% ethanol[e] | 0.1897 | 10104 | 1.0480 | | 1.0534 |
| 45.6 | 15.8 in 30% ethanol | 0.2275 | 10113 | 1.0471 | | 1.0537 |
| | | | | | Av | 1.0536[f] |
| 25.6 | 15.8 in 50% ethanol[e] | 0.2004 | 10061 | 1.0525 | | 1.0583 |
| 25.6 | 15.8 in 50% ethanol | 0.2223 | 10080 | 1.0505 | | 1.0574 |
| | | | | | Av | 1.0581[f] |

[a] Fractional reaction determined from kinetic data in ref 4a. [b] Isotope ratio at fractional reaction $f$. [c] Isotope ratio at $f = 1.00$; equal to 10588 ± 9 for five separate determinations with 95% confidence limits. [d] Calculated isotope effect according to eq 3. [e] 30% ethanol–70% $H_2O$ (v/v). [f] In ref 2 we demonstrated that the enzymatic KIE at pH 5.00 is temperature independent between 10 and 37 °C.

$$\frac{(k_d/k_d^*)_{50\%\ \text{ethanol}}}{(k_d/k_d^*)_{H_2O}} = \frac{1.058}{1.051} \simeq 1.007 \qquad (5)$$

If C–C stretching is the principal contribution to the KIE, these results would imply that the changes in the C–C stretching force constant in going from the reactant to the transition state are greater in aqueous ethanol than in water.[10]

**Thiamin-Catalyzed Decarboxylation.** Table II summarizes the data in 50% (v/v) ethanol. The most striking feature of the results is that the KIE changes from an inverse (0.992) to a normal (1.007) value on transferring the decarboxylation from water to aqueous ethanol. We have shown[2] that the rate expression for contrasting the $^{12}C$ and $^{13}C^*$ isotopic reaction rates is:

$$k^{12}/k^{13} = k_1/k_1^* \frac{(1 + [k_{-1}^*/k_d^*])}{(1 + [k_{-1}/k_d])} \qquad (6)$$

It has been found previously that the ratio $k_{-1}/k_d$ is small, i.e., the rate of decomposition of the thiamin–pyruvate adduct into reactants is slower than the rate of decarboxylation.[2] The observed ratio of $k^{12}/k^{13}$ is largely determined by the secondary isotope effect on $k_1$ and $k_{-1}$. However, the observed change in $k^{12}/k^{13}$ cannot be explained solely on the basis of a solvent isotope effect on $k_1$ and $k_{-1}$. For the decarboxylation step of the $CHDT^+Cl^-$ system the change is 1.007. A transfer between solvents would probably induce smaller changes in the secondary isotope effects $k_1/k_1^*$ and $k_{-1}/k_{-1}^*$ respectively than in $k_d/k_d^*$. In order to account for the observed change of $k^{12}/k^{13}$ by a factor of 1.015 (0.992 to 1.007), according to eq 6, an increase of the ratio of $k_{-1}/k_d$ is required, i.e., $(k_{-1}/k_d)_{50\%\ \text{ethanol}} > (k_{-1}/k_d)_{H_2O}$. We therefore conclude that the rate of decomposition of the thiamin–pyruvate adduct into reactants relative to the rate of its decarboxylation is enhanced in aqueous ethanol as compared with water. Our conclusions are in satisfactory qualitative agreement with earlier kinetic measurements. It was found that $k_{d,\text{ethanol}}/k_{d,H_2O}$ is ca. 9000 for $CHDT^+Cl^-$ [4a] and that $k_{-1}$ for the lyate ion catalyzed decomposition of the thiazolium–ethyl pyruvate covalent adduct to reactants is ca. $4 \times 10^4$ faster in ethanol than in water.[4b]

**Table II. $^{13}C$ Kinetic Isotope Effects on Pyruvate Decarboxylation Catalyzed by Thiamin in 50% Ethanol (v/v) Buffered by 0.05 M $NH_4^+Cl^-$ with $(NH_4^+)/(NH_3) = 2$**

| Pyruvate, M | Thiamin, M | $f$[a] | $N_x$[b] | $N_{x0}$[c]/$N_x$ | $k^{12}/k^{13}$ [d] |
|---|---|---|---|---|---|
| 0.1818 | 0.0455 | 0.0119 | 10534 | 1.0048 | 1.0048 |
| 0.0909 | 0.0455 | 0.0075 | 10510 | 1.0071 | 1.0071 |
| 0.0909 | 0.0455 | 0.0075 | 10505 | 1.0076 | 1.0076 |
| 0.0909 | 0.0455 | 0.0075 | 10528 | 1.0054 | 1.0054 |
| 0.0909 | 0.0455 | 0.0075 | 10495 | 1.0086 | 1.0086 |
| 0.0909 | 0.0455 | 0.0075 | 10490 | 1.0090 | 1.0090 |
| 0.0909 | 0.0455 | 0.0075 | 10504 | 1.0077 | 1.0077 |
| 0.0909 | 0.0455 | 0.0075 | 10499 | 1.0082 | 1.0082 |
| | | | | Av | 1.0073 ± 0.0012 |
| In $H_2O$[e] | | | | | 0.9921 ± 0.0014 |

[a] Fractional reaction. [b] Isotope ratio at the fractional reaction $f$. [c] Isotope ratio at $f = 1.0$; the average value of the $CHDT^+Cl^-$ $N_{x0}$ (see Table I, footnote c) and the holoenzyme $N_{x0}$ (Table IV, ref 2, the average of 16 determinations) as these two values are within experimental error identical. [d] Calculated isotope effect according to eq 3. [e] From ref 2, Table II; the average of 12 determinations in the pH range of 6.5–8.6.

Finally, if the active site of holopyruvate decarboxylase indeed resembles alcohol in its microenvironment rather than water, these results suggest that part of the observed enzymic KIE (1.002 to 1.011 depending on pH) is due to the apolar environment. In the absence of environmental factors the observed KIE would be even smaller or inverse.

## Experimental Section

**Reagents.** Sodium pyruvate and thiamin hydrochloride were purchased from Sigma and were used without further purification. Inorganic reagents were of highest purity available from Fisher Scientific. Buffers were prepared from sodium acetate and acetic acid (pHs 5.00 and 5.50), sodium citrate and citric acid (pH 6.00), monobasic and dibasic phosphate (pHs 6.50, 7.00, and 7.50), and sodium borate (pHs 8.00 and 8.60). $CHDT^+Cl^-$ was kindly provided by Dr. G. E. Lienhard of Dartmouth Medical School.

pH-stat titration was employed in the determination of the low conversion reaction times of the thiamin-catalyzed decarboxylation. The decarboxylated product (eq 2) is quickly protonated under the conditions employed (pH <8.6, $pK_a = 17$[4a]) and $OH^-$ production can be used to monitor the reaction rate.[11] A Radiometer (Copenhagen) pH meter Model 26 equipped with titrator 11, autoburet ABU 12, stirring system, and recorder REA (300) was used. The chart speed was set at 1 min/cm or 30 s/cm. The titration speed was set at 5, 10, or 20 depending on the rate of acid consumption.

In a typical determination at low fractional conversion 2.00 mL of 0.2 M pyruvate (adjusted to pH 5.00 and flushed for 30 min with $CO_2$-free high-purity $N_2$) was pipetted into a plastic beaker on a titrigraph that was equipped with a plastic stirrer. With the recorder running, thiamin solution ($CO_2$ free) was introduced via a microliter pipet. Three samples were run and recorded. On the same day a scaled up reaction was run to collect $CO_2$ employing 100 mL of 0.2 M pyruvate and scaled up thiamin. The fraction, $f$, in the scaled up reaction could be calculated from the number of moles of 0.01 N HCl consumed divided by the total number of moles of pyruvate. The Warburg respirometer was also employed in the determination of the fractional reaction of thiamin-catalyzed decarboxylation. The kinetic information from ref 4a was employed to estimate the fractional reaction of $CHDT^+Cl^-$.

**Reaction under $CO_2$-free $N_2$ and Collection of $CO_2$.** The entire procedure was performed on a high-vacuum line. First the reaction vessel (a three-neck flask equipped with separator funnel, drying tube, and a syringe cap) was purged three times (filled then evacuated to below 50 μm) with high-purity $CO_2$-free $N_2$. The reaction vessel was

filled with $CO_2$-free $N_2$ and 100 mL of pyruvate solution (previously degassed by bubbling through it $CO_2$-free $N_2$ for at least 30 min) was injected through the sidearm. The solution was stirred and thermostatted at 30 °C for 30 minutes. Next 0.5 mL of thiamin (or $CHDT^+Cl^-$ sans pyruvate) was injected through a rubber septum to initiate the reaction. The reaction was quenched (syringe cap) with 5 mL of concentrated $H_2SO_4$ after ca. 200 μmol of $CO_2$ had evolved (as calculated from the $f$ values determined above).

The reaction vessel was then attached to the vacuum line at a different point and frozen with liquid $N_2$ and the nitrogen in the vessel was removed by the vacuum pump until no further significant pressure decrease in vacuum gauge reading could be observed. The flask was then warmed slightly and refrozen in a dry ice–acetone bath and the $CO_2$ was distilled into a U tube which was cooled in liquid $N_2$. The liquid nitrogen was replaced by dry ice and the $CO_2$ was passed to the Toepler pump bulb. The dry ice–acetone was removed from the U tube and the condensed gases were pumped until the vacuum gauge read below 50 μm. Then the gas was transferred to a sample tube for mass spectrometric measurement. In the reaction catalyzed by thiamin, $CO_2$ was purified by passage through $H_2SO_4$.

**Mass Spectrometric Analysis.** The isotope ratio ($^{13}CO_2/^{12}CO_2$) was determined on a Consolidated-Nier Model 21-201 isotope ratio mass spectrometer.[13] The atom fraction of $C^{13}$, $N_x$, corrected for $C^{12}O^{16}O^{17}$, was calculated from the expression

$$10^6 N_x = \frac{\bar{r}_{\text{sample}} 11134}{1/2(\bar{r}_{\text{tank before}} + \bar{r}_{\text{tank after}})} - 800$$

where $\bar{r}_{\text{sample}}$ is the average ratio of six readings of $CO_2$ sample and $\bar{r}_{\text{tank before}}$ and $\bar{r}_{\text{tank after}}$ are the average ratios of six readings of tank $CO_2$ (Matheson Research Purity) measured before and after the sample measurement, respectively. The number 800 was provided by the manufacturer to compensate for the $O^{17}$ isotope ratio. $(C^{12}O^{16}O^{17})\cdot(11134 \pm 5) \times 10^{-6}$ is the average value of the 1362 readings of the 45/44 mass ratio of tank $CO_2$ during the entire course of the present experiments.

$N_{x0}$ for the thiamin-catalyzed reaction was the average value of the $N_{x0}^2$ determined for $CHDT^+Cl^-$ and of the $N_{x0}$ determined for the holoenzyme-catalyzed reaction[2] as these two are in close accord. This had to be done since the thiamin-catalyzed reaction leads to acetolactate (and thence acetoin) so that the $CO_2$ is liberated from two sources. However, at low conversion (even with a 100% error in the estimate of $f$ in the range of $f \simeq 0.01$) the source of $CO_2$ is exclusively pyruvate (rather than acetolactate) since the subsequent steps are much slower.

**Registry No.**—$CHDT^+Cl^-$, 29510-46-1; sodium pyruvate, 113-24-6; thiamin hydrochloride, 67-03-8.

## References and Notes

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# Oxidation of Olefins with Peroxouranium Oxide ($UO_4 \cdot 4H_2O$)[1]

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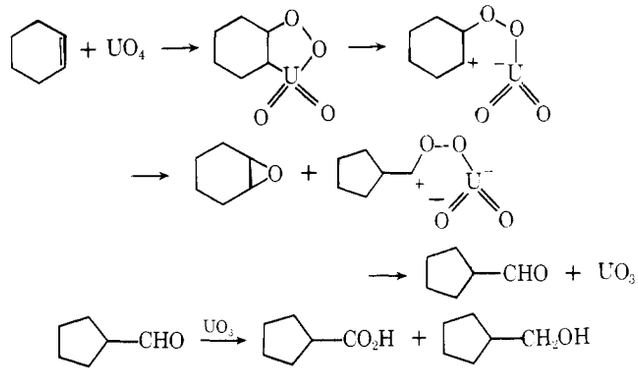
Peroxouranium oxide was found to be an effective oxidizing agent for alkenes. The oxidations are suggested to proceed through an oxyuranylation path with resulting carbocationic rearrangement of the intermediates. The used reagent may be recovered and regenerated effectively.

Although many dioxygen–metal compounds are known,[2] little is known about their reactions. The preparation of peroxouranium oxide from uranyl nitrate and hydrogen peroxide has been known for nearly a century.[3] The structure has been established by Gordon and others[4] as being a true peroxo complex, $UO_2(O_2)\cdot 4H_2O$.[2a] The aqueous chemistry of peroxouranium(VI) has been found to be complicated. Peroxouranium oxide tetrahydrate forms peruranates of varying composition with aqueous hydrogen peroxide, culminating in the formation of the most stable $UO_8^{2-}$ species.[2b] So far the oxidizing ability of $UO_4$ in organic systems was not explored.

## Results and Discussion

Peroxouranium oxide tetrahydrate was found to be an effective oxidizing reagent for hydrocarbons, particularly olefins. Data are summarized in Table I. Ring-contracted, ring-expanded, and epoxidized products were obtained. The reaction is viewed as proceeding via the complexation of the olefin by the coordinatively unsaturated uranium. The increasing electron density at the metal results in a lengthening of the metal–dioxygen bond until a metal–carbon bond is formed, while the developing charge-deficient carbon forms a bond to the displaced oxygen. The cyclic intermediate de-

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