Secondary β-Deuterium Isotope Effects in Decarboxylation and Elimination Reactions of α-Lactylthiamin: Intrinsic Isotope Effects of Pyruvate Decarboxylase

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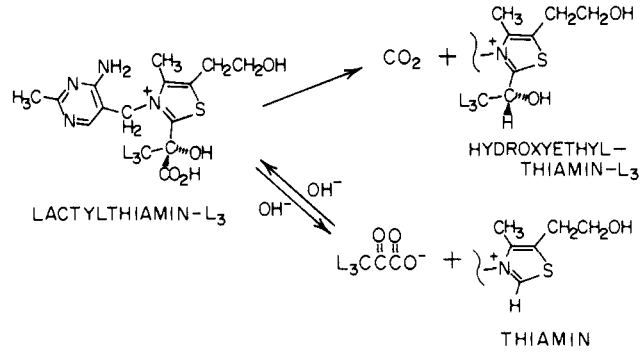
Abstract: The reactions of the adduct of pyruvate and thiamin, lactylthiamin (2-(lact-2-yl)thiamin), are accurate nonenzymic models for reactions of intermediates formed during catalysis by pyruvate decarboxylase. The enzymatic reaction generates lactylthiamin diphosphate from pyruvate and thiamin diphosphate. β-Deuterium isotope effects were determined for the nonenzymic reactions, and the results were related to isotope effects on the enzymic reaction. 2-(Lact-2-yl-β-d_3)thiamin was prepared by condensation of methyl pyruvate-d_3 with thiamin followed by hydrolysis. The isotope effect for decarboxylation of lactylthiamin in acidic solution at 25 °C (k_{H3}/k_{D3}) is 1.09 (standard deviation (SD) 0.015) in pH 3.8, 0.5 M sodium acetate: isotope effect = 1.095 (SD 0.014) in 0.001 M HCl. The reaction was also studied using 38% ethanolic aqueous sodium acetate (pH 3.8 before mixing with ethanol) since the enzymic sites are less polar than water and the reaction is significantly accelerated by the cosolvent. The isotope effect is within statistical range of that for the reaction in water, 1.105 (SD 0.016), indicating that acceleration by the solvent does not change the extent of hyperconjugative stabilization of the transition state relative to the ground state. The isotope effect for the base-catalyzed elimination of pyruvate from lactylthiamin was determined from kinetic studies by using multiwavelength analysis for reactions in pH 11 sodium carbonate solution. The isotope effect (k_{H3}/k_{D3}) is 1.12 (SD 0.01), which is slightly higher than the effect on decarboxylation. The isotope effects indicate that there is considerable interaction between the methyl protons of the lactyl group and the incipient electron-rich center which develops as either carbon dioxide or pyruvate is lost. The isotope effects are slightly larger than the effect reported for the catalytic rate in pyruvate decarboxylase with pyruvate-d_3 as a substrate and inverse to the effects for V/K terms. This suggests that the enzymic reaction is subject to rate-limiting processes which are less isotope sensitive but that the reactions of enzyme-bound lactylthiamin diphosphate probably account for most of the observed effect on k_{cat} in the enzymic reaction.

The mechanism of enzymic reactions can be best understood if one has a detailed knowledge of the properties of the chemical reactions which occur during the catalytic sequence.[1-7] The study of closely related nonenzymic reactions provides a means of deducing specific functions of the enzyme. Analysis of enzymic and nonenzymic kinetic isotope effects provides details about the relative properties of sequential transition states.[8,9] In order to apply such measurements to an enzymic system, the intrinsic barriers and isotope effects for individual steps need to be known.[10]

We have been investigating catalysis of the decarboxylation of pyruvate by enzymes which utilize thiamin diphosphate as a cofactor.[1,4,11,12] The nonenzymic catalysis of the decarboxylation of pyruvate by thiamin proceeds by an analogous pathway.[13] The central intermediate in the nonenzymic reaction is the adduct of pyruvate and thiamin, lactylthiamin.[1] The reactions of this adduct serve as a model for the enzyme-catalyzed process involving lactylthiamin diphosphate as an intermediate.

β-Deuterium isotope effects are potentially useful probes of the transition-state structure of the model system and the related enzymic reactions. Alvarez and Schowen[14] have shown that the enzymic reaction is subject to kinetic isotope effects when pyruvate-d_3 is compared with pyruvate as a substrate. However, the results in that study could not be compared to a nonenzymic process, and the intrinsic effects could only be estimated with considerable uncertainty. Thus, in order to determine the inherent sensitivity of the reaction to β-deuterium substitution, we undertook a study of the reactions of lactylthiamin and (lactyl-d_3)thiamin. The results serve as a basis for analysis of the related enzyme reactions.



Experimental Section

Materials. Pyruvic acid and Diazald (diazomethane precursor) were purchased from the Aldrich Chemical Company. Thiamin chloride hydrochloride was purchased from Novopharm Limited. Sodium pyruvate and ethyl pyruvate were obtained from the Sigma Chemical Company, and deuterium oxide (99%) was from MSD Isotopes Limited. Reagents were obtained from Fisher Scientific and BDH.

Synthesis. Pyruvic-d_3 Acid. The procedure of White was followed.[15] Freshly distilled pyruvic acid (10 g) was dissolved in deuterium oxide and the solution refluxed for 24 h. The product was isolated by continuous extraction with ether. The procedure was repeated twice to equilibrate the deuterium content of the sample and the solvent. The resulting pyruvic-d_3 acid was distilled under vacuum.

Lactylthiamin and (Lactyl-d_3)thiamin. Methyl pyruvate-d_3 was prepared by reaction of pyruvic-d_3 acid with diazomethane in ether. The integrated proton NMR spectrum of this material showed the methyl

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group of the acetyl moiety to be 93% deuterated (by comparison of the integrations of the methyl ester group and the acetyl methyl group). The material was dissolved in absolute ethanol containing sodium ethoxide and thiamin, following the previously reported procedure which uses the ethyl ester of unlabeled pyruvate.[1] (Methyl pyruvate-d_3 is transesterified to the ethyl ester under the reaction conditions.) The resulting ethyl (lactyl-d_3)thiamin was analyzed by proton NMR which shows that the acetyl methyl group contained 88% deuterium. The product undergoes no further exchange and was hydrolyzed in concentrated DCl to give (lactyl-d_3)thiamin. The absence of further exchange was evidenced by conducting the hydrolysis of ethyl lactylthiamin in deuterium oxide containing DCl (12 M). The same relative integrated intensity for the methyl group of lactylthiamin was obtained as in the case of the same material hydrolyzed in concentrated HCl.

Methods. Instruments. Proton NMR spectra were recorded on a Varian T-60 spectrometer. Chemical shifts are reported relative to an external Me_4Si reference. UV spectra were obtained with a Varian Cary 210 spectrometer. Kinetic data were collected from the spectrometer through a microprocessor-controlled interface to a Commodore 2001 (PET) computer. All reactions were conducted with samples contained in 1-cm cuvettes in the jacketed five-cell holder of the spectrometer. The temperature of the cells was maintained by circulation through the jacket of water kept within 0.1 °C of a set point by a Neslab Exacal EX-100 apparatus. Temperature of reaction solutions was determined by measuring the temperature in a blank (water) sample using a Fisher 119 meter equipped with a Yellow Springs thermistor. For isotope effect studies, samples were run simultaneously in batches using the automatic sample changing feature of the spectrometer. Solutions were transferred with calibrated mechanical pipets.

Kinetic Methods. Decarboxylation of Lactylthiamin. Samples of lactylthiamin were prepared from ethyl lactylthiamin (10 mg) by hydrolysis in concentrated HCl (0.5 mL) for at least 12 h. The HCl was removed under vacuum. The sample was dissolved in 1 mL of water and stored frozen. For kinetic studies, a 20-μL portion of the thawed solution was added to 2.9 mL of either 0.5 M or 0.1 M sodium acetate buffer (pH 3.8), 0.001 M HCl, or an ethanol/water solution, contained in a 3-mL (1 cm) stoppered quartz cuvette. Solutions were maintained at 25.2 °C. In all studies, five samples were run simultaneously in the sample changer of the spectrometer, with three samples having a deuterated methyl group in the lactyl moiety and two samples without deuterium. For studies of the effect of solvent, buffers were prepared by adding 40 mL of 95% ethanol to 60 mL of 0.1 M acetate buffer, pH 3.8. In these experiments, four samples were studied simultaneously, with two having a deuterated methyl group.

The conversions of lactylthiamin and (lactyl-d_3)thiamin to (hydroxyethyl)thiamin and (hydroxyethyl-d_3)thiamin were monitored by observing the small increase in absorbance at 255.5 nm for the aqueous solutions containing acetate, at 270 nm for solutions containing HCl, and at 257 nm for ethanolic solutions. The wavelengths were chosen to maximize the observed change while avoiding high absorbance due to the buffer. The data were collected automatically over a period of 400 min or five half-lives for the aqueous solutions and over 85 min for the more rapid reaction in aqueous ethanol. Ninety-nine equally time-spaced data points were collected for each sample and stored in the computer. The collected data were stored on diskettes and later analyzed by using the kinetic overrelaxation algorithm[16] in a BASIC program. For each run, a rate constant ratio for the deuterated vs. undeuterated samples was determined. For the buffer experiments, seven sets of runs were done with 0.5 M acetate and seven with 0.1 M acetate. Four sets of runs were conducted in 0.001 M HCl. The isotope effects from the sets were averaged to give the mean isotope effect. For the reactions in aqueous ethanol, a series consisted of at least three sets of runs. Seven series were conducted, and these were averaged to give a mean isotope effect.

Conversion of Lactylthiamin to Thiamin and Pyruvate. Lactylthiamin undergoes a base-catalyzed elimination reaction to release pyruvate and the thiamin ylide (which is rapidly protonated).[1] At high pH, the elimination process is more rapid than decarboxylation. The thiamin which is produced reacts with hydroxide and is converted to a ring-opened form.[17] This subsequent reaction complicates the observation of the initial process. However, by following the absorbance changes at two wavelengths, sufficient independent data are generated to separate the first reaction from the second if the ratio of changes in the extinction coefficient for the material undergoing the second reaction can be determined independently.[1] The methodology has been explained in detail, and its application will be presented below. In each run, absorbance data were collected as a function of time at a single wavelength or at two wavelengths. The information was stored and processed by using a BASIC program.

In order to analyze the data, the ratio of change in extinction coefficients at the two wavelengths under the reaction conditions, Q, was determined for the decomposition of thiamin (pH 11, 0.5 M sodium carbonate). Thiamin chloride hydrochloride (21 mg) and sodium pyruvate (7 mg) were dissolved in 2 mL of 1 M HCl. Thirty microliters of this solution was added to 2.8 mL of carbonate buffer in a 1-cm cuvette maintained at 25.2 °C in the spectrometer sample holder. Four samples were prepared, and the reaction of each was monitored at 264 and 270 nm. Data were collected every 4 min, and 65 points were obtained for each sample. The first-order rate constants were determined by using the kinetic overrelaxation algorithm.[16] The ratio of differences in absorbances at the two wavelengths at the start and end of the reaction ($A(0) - A(\infty)$) gives the value of Q.

Elimination of Pyruvate from Lactylthiamin. Samples of ethyl lactylthiamin were hydrolyzed in HCl as described for the kinetic studies of the decarboxylation reaction and concentrated. Then, a 2-mg portion of the residual solid was added to 1 mL of water. Twenty-microliter portions of this solution were added to 2.8 mL of 0.5 M sodium carbonate, pH 11 buffer. Four samples were run simultaneously, two which contained a deuterium in the lactyl methyl group and two which did not. Sixty-five data points were recorded at 1-min intervals for each sample at 264 and 270 nm. The absorbances at both wavelengths were utilized along with the value of Q to generate the function $Z(t)$ (eq 1). $Z(t)$ was

$$Z(t) = [A(270,t) - A(270,0)] - Q[A(264,t) - A(264,0)] \quad (1)$$

then processed as first-order kinetic data (with $Z(t)$ replacing absorbance). Four series of runs were conducted with each series consisting of five or more sets of runs.

Table I. First-Order Rate Constants and Isotope Effects for the Decarboxylation of Lactylthiamin and (Lactyl-d_3)thiamin at 25 °C[a]

reaction medium	$10^4 k_H$, s^{-1}	$10^4 k_D$, s^{-1}	k_H/k_D
pH 3.8, 0.5 M NaOAc	1.478	1.414	1.045
	1.543	1.448	1.065
	1.575	1.488	1.058
	1.586	1.509	1.058
	1.571	1.460	1.076
	1.595	1.453	1.097
	1.572	1.461	1.075
	1.420	1.363	1.042
pH 3.8, 0.1 M NaOAc	1.244	1.164	1.068
	1.375	1.280	1.074
	1.395	1.323	1.054
	1.480	1.370	1.080
	1.290	1.233	1.046
	1.280	1.210	1.058
	1.331	1.252	1.063
	1.410	1.314	1.073
0.001 M HCl	1.204	1.138	1.055
	1.283	1.200	1.069
	1.314	1.225	1.072
	1.217	1.112	1.069
			1.067[b] mean

[a] Temperature variation between runs: ±0.2 °C. [b] Standard deviation: 0.014.

Results

Decarboxylation of Lactylthiamin. The decarboxylation reaction was studied at pH 3.0 and 3.8 (under these conditions the pyrimidine is protonated and the carboxyl is unprotonated). The reaction was followed at 255.5 nm where the change is most readily observed but is nonetheless very small. For example, the total change in absorbance is 0.0165 OD when the initial absorbance is 0.82. The reaction in HCl, pH 3, was followed at 270 nm with a change in absorbance of 0.069 OD when the initial absorbance is 1.44 OD.

The individual runs were fitted to the integrated first-order rate law with correlation coefficients of at least 0.999 999 and standard deviations for the data of less than 2%. The rate constants for decarboxylation of lactylthiamin and (lactyl-d_3)thiamin at pH 3 and 3.8 are presented in Table I. The observed isotope effect (k_H/k_D) in pH 3.8, 0.5 M sodium acetate solution is 1.063 (SD 0.018) and 1.067 (SD 0.009) at the same pH in 0.1 M buffer. In 0.001 M hydrochloric acid, the isotope effect is 1.072 (SD

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Table II. First-Order Rate Constants for the Decarboxylation of Lactylthiamin and (Lactyl-d_3)thiamin in 38% Ethanolic, Aqueous, pH 3.8 Sodium Acetate at 25 °C[a]

no. of sets	10^3k_H, s^{-1}	10^3k_D, s^{-1}	k_H/k_D
4	1.293	1.184	1.092
4	1.295	1.194	1.085
4	1.248	1.137	1.097
2	1.230	1.143	1.076
3	1.271	1.196	1.063
2	1.330	1.203	1.105
3	1.168	1.081	1.080
3	1.154	1.090	1.058
			1.082 mean

[a] Temperature variation between runs: ±0.2 °C. [b] Standard deviation: 0.015.

0.016). Since the results under the three conditions are within range of each other, the average isotope effect of 1.067 (SD 0.014) will be used for the pH range 3–3.8.

Since the deuterated material contains 88% D and 12% H, in the lactyl methyl group, the isotope effect can be corrected to that of 100% deuterated material by application of the rule of the geometric mean which states that the effect of each deuterium on $\Delta G^{\ddagger}$ is independent (eq 2). Since the isotopes are randomly

$$(k_H/k_{D3})^{1/3} = (k_H/k_{D2})^{1/2} = k_H/k_D = x \quad (2)$$

distributed, the actual distribution can be calculated by standard probability formulations. Since the sample is 88% deuterium, in a randomized sample of 25 units, 22 will be deuterium and 3 will be hydrogen. The relative weights for the various distributions are determined by standard combinatorial functions:[18]

$$3H,0D = {}^3C_3 = 1 \quad (3)$$

$$2H,1D = ({}^3C_2)({}^{22}C_1) = 66 \quad (4)$$

$$1H,2D = ({}^3C_1)({}^{22}C_2) = 693 \quad (5)$$

$$0H,3D = {}^{22}C_3 = 1540 \quad (6)$$

where

$${}^xC_y = x!/[(x-y)!y!]$$

This gives a normalized set of ratios of 0.002:0.028:0.030:0.67 for the four cases. By application of the rule of the geometric mean[19]

$$0.67(k_H/k_{D3}) + 0.30(k_H/k_{D2}) + 0.028(k_H/k_D) = (k_H/k_D)_{obsd} =$$
$$0.67(k_H/k_D)^3 + 0.30(k_H/k_D)^2 + 0.028(k_H/k_D) = 1.067 \quad (7)$$

The isotope effect k_H/k_D is 1.028 (SD 0.004) per deuterium or 1.09 (SD 0.015) for the compound with three deuteriums in the methyl group for the reaction at pH 3.8 and 1.095 (SD 0.014) in 0.001 M HCl. The difference in the isotope effects under the two sets of conditions is not statistically significant.

Isotope Effect in Alcohol/Water. Considerable evidence has been presented that indicates that the catalytic decarboxylation process of thiamin diphosphate dependent enzymes occurs in an environment of reduced polarity.[20,21] It is important to know if the isotope effect changes if the reaction occurs in a solvent less polar than water. The decarboxylation reaction was studied for a solution made from 60 parts pH 3.8, 0.1 M sodium acetate buffer and 40 parts 95% ethanol (mixed solution is 38% ethanol by volume). Under these conditions, the decarboxylation reaction is about 10 times faster than in the absence of ethanol. The observed first-order rate constants and isotope effects for decarboxylation of lactylthiamin and (lactyl-d_3)thiamin are presented in Table II.

Table III. Difference in Initial and Final Extinction Absorbances for the Decomposition of Ring-Opened Thiamin at Two Wavelengths and the Ratio (Q) of These Changes at the Two Wavelengths for a Series of Runs

abs, 270 nm	abs, 264 nm	Q
0.4424	0.5642	0.7841
0.4120	0.5260	0.7832
0.4504	0.5737	0.7851
0.4543	0.5776	0.7865
0.4794	0.6107	0.7850
0.4548	0.5785	0.7861
0.4015	0.5132	0.7823
0.4182	0.5328	0.7849
0.3696	0.4693	0.7875
0.4486	0.5717	0.7846
0.4594	0.5850	0.7853
0.4358	0.5545	0.7859
0.4497	0.5720	0.7861
0.4552	0.5789	0.7863
0.4737	0.6040	0.7842
0.4538	0.5789	0.7839
0.4613	0.5889	0.7833
0.4477	0.5694	0.7862
		0.7850 mean

[a] Standard deviation: 0.003.

The observed isotope effect from eight series of runs is 1.082 (SD 0.015). Correction of the observed isotope effect for the 88% extent of deuteration of the methyl group as described in the preceding section gives a ratio k_H/k_{D3} of 1.105 (SD 0.016), and the isotope effect per deuterium is 1.034 (SD 0.0004). These values are essentially the same as those observed for the reaction in water.

Isotope Effect on Elimination of Pyruvate from Lactylthiamin. In solutions more basic than approximately pH 9.5, lactylthiamin is preferentially converted to pyruvate and thiamin. In basic solution, thiamin undergoes a reaction with 2 equiv of hydroxide to undergo a ring-opening process.[16] The ring-opened thiamin undergoes a further slow reaction which is irreversible. The presence of these further reactions makes it necessary to study the reaction by methods that compensate for this. The "two-wavelength method" described in the Experimental Section, which has been employed previously to study this reaction, was used.[1] For the two step reaction in eq 8, the rate constant for the first

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \quad (8)$$

step is the slope of a plot of ln $(Z_\infty - Z_t)$ vs. time, where the function Z is defined in eq 1. The two-wavelength method requires determination of the ratio Q which is the differences of extinction coefficients of B and C at two wavelengths.

The method is applicable to the breakdown of lactylthiamin which involves the three steps in eq 9. The rate constants have

$$\text{lactylthiamin} \xrightarrow{k_1} \text{thiamin} + \text{pyruvate} \xrightarrow{k_2} \text{ring-opened thiamin} \xrightarrow{k_3} \text{product} \quad (9)$$

previously been determined.[1] At pH 11.0, 25.0 °C, the values (in the order 1, 2, 3) are 1.6×10^{-3}, 1×10^{-2}, and 3×10^{-4} s^{-1}. Since the second step is fast compared to the first and third steps, thiamin accumulates only at low steady-state levels and the observed products are the next two. Therefore, the system can be treated for the purpose of analysis as having two steps, and Q is the ratio of differences of extinction coefficients of ring-opened thiamin and the decomposition product. In order to establish the initial steady state, thiamin and pyruvate are added along with lactylthiamin. After a few minutes, the system is well-behaved. The values determined for Q from the proportionality constants

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Table IV. First-Order Rate Constants for the Elimination of Pyruvate from Lactylthiamin and (Lactyl-d_3)thiamin (pH 11, 0.5 M Sodium Carbonate) at 25 °C[a]

no. of sets[b]	10^3k_H, s^{-1}	10^3k_D, s^{-1}	k_H/k_D
6	1.685	1.537	1.096
7	1.430	1.317	1.086
5	1.505	1.355	1.110
5	1.236	1.131	1.093
			1.10[c] mean

[a] Temperature variation between runs: ±0.2 °C. [b] Each set consisted of two samples with deuterium in the lactyl methyl group and two without. Temperature between sets may vary by ±0.2 °C. [c] Standard deviation: 0.01.

in 18 runs are presented in Table III.

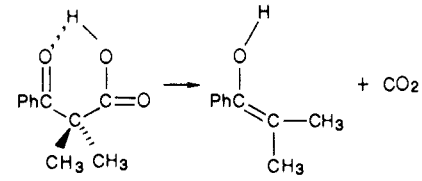
The elimination reaction was studied at pH 11.0 where the rate of expulsion of pyruvate from lactylthiamin is 30 times the rate of decarboxylation. Since the elimination reaction (k_1) is base catalyzed, the pH of the reaction must be carefully controlled, and all hydrochloric acid (used to prepare the substrate from its ester) was carefully removed from the samples by lyophilization before addition to the pH 11 sodium carbonate buffer solution.

A plot of Z vs. time gives an apparent first-order rate constant as its slope. The data give correlation coefficients of not less than 0.9999 and standard deviations of less than 3%. Table IV summarizes rates and isotope effects for the four series of runs on the breakdown of lactylthiamin and (lactyl-d_3)thiamin to thiamin and pyruvate. The observed averaged effect is 1.10 (SD 0.01) for 88% (lactyl-d_3)thiamin. By use of the correction procedure described in the section on the decarboxylation reaction, the β-deuterium kinetic isotope is 1.12 (SD 0.01) for the fully deuterated methyl group or 1.038 (SD 0.003) per deuterium.

Discussion

Hyperconjugation as a stabilizing factor in transition states has been cited as the principal source of kinetic β-deuterium isotope effects.[22] In the formation of carbonium ions and other electron-deficient species, hyperconjugation stabilizes transition states by allowing incipient positive charge to be delocalized to protons.[23] Negative hyperconjugation, in which negative charges are stabilized, has been invoked for reactions involving carbanion formation by proton removal[24,25] and in decarboxylation.[26] In the present study, the loss of CO_2 generates excess electron density in the residual portion of the reacting molecule and the transition state is expected to be stabilized by negative hyperconjugation. The magnitude of the isotope effect can be an indicator of transition-state structure if the system is calibrated.

The magnitude of the β-deuterium isotope effect on decarboxylation of lactylthiamin is indicative of considerable interaction between the excess electron density and the methyl group in the transition state. As a basis for comparison, we studied the β-deuterium isotope effect on the decarboxylation of 2,2-dimethylbenzoylacetic acid.[26] The isotope effect for the undissociated form of the carboxylic acid is 1.01 per deuterium. The decarboxylation of the less reactive conjugate base has an isotope effect of 1.02 per deuterium. For lactylthiamin, the isotope effect is 1.03 per deuterium. The increasing isotope effect is consistent with increasing hyperconjugative stabilization of transition states. The small isotope effect for 2,2-dimethylbenzoylacetic acid is consistent with the special stabilization offered by the cyclic transition state involving internal proton transfer to generate the product enol.[27]



The conjugate base of 2,2-dimethylbenzoylacetic acid cannot react via this cyclic mechanism, and the larger isotope effect is consistent with its lower reactivity. A more detailed comparison with lactylthiamin is not appropriate since in that case the reactant is zwitterionic while the transition state is less polar. The presence of a hydroxyl group at the β-position of lactylthiamin also changes the nature of the transition state, possibly directing hyperconjugative stabilization to the methyl group.

We observe that the rate of decarboxylation of lactylthiamin is increased by a factor of 10 in 38% aqueous ethanol compared to the reaction in water, as is expected from the work of Lienhard and co-workers.[28] Since the transition state for decarboxylation is less polar than the reactant, transfer to a less polar reaction medium decreases the reaction barrier. The decreased barrier to reaction should decrease demand for stabilization of the transition state. Any effect on transition-state structure would be potentially reflected in a changed isotope effect. Similarly, the trend would extend to the transition state of the enzymic reaction. We find that to the accuracy of our measurements, the isotope effect is invariant with changing solvent. This can be extrapolated to predict that the enzymic step in which enzyme-bound lactylthiamin diphosphate undergoes decarboxylation reaction will also show an intrinsic isotope effect on V_{max} of 1.10 for pyruvic-d_3 acid. The formation of lactylthiamin diphosphate and elimination of acetaldehyde from (hydroxyethyl)thiamin diphosphate should be subject to isotope effects on the order of that for the elimination of pyruvate from lactylthiamin (1.12).

Determination of the isotope effect on k_1 would permit a complete model for the enzymic reaction comparison to be obtained, but this rate constant cannot be measured directly since the intermediate is produced under steady-state conditions. Determination of the isotope effect on the equilibrium constant (k_1/k_{-1}) would also yield this value, but the reported method for determination of the equilibrium constant is not sufficiently precise to yield an isotope effect.[1] Of course, one cannot conclude that the isotope effect on k_1 will be the same as on k_{-1}.

Alvarez and Schowen report secondary β-deuterium isotope effects in the reaction of pyruvate and pyruvate-d_3 catalyzed by pyruvate decarboxylase.[14] The isotope effect on V_{max} is 1.085 (SD 0.006). For this enzyme, formation and decarboxylation of enzyme-bound lactylthiamin diphosphate are each partially rate determining. The observed isotope effect on V_{max} is approximately the same as that which we observe for decarboxylation of lactylthiamin in water (1.067) and somewhat smaller than that for the reaction in ethanol/water (1.10). The latter value probably is best for comparison to the enzymic reaction since the active site is hydrophobic. It is considerably smaller than the isotope effect on elimination (1.12). These results suggest that for the enzymic reaction, steps which are less isotope sensitive possibly are partially rate determining, given the hydrophobic nature of the active site. Such steps may include substrate binding, product release, and conformational changes of the protein. However, the steps associated with reactions of lactylthiamin diphosphate will be the major factors in determining k_{cat} since the enzymic effect is significant and only the elimination of acetaldehyde from (hydroxyethyl)thiamin is a process which could also contribute to the isotope effect. The magnitude of the observed isotope effect on V_{max} is consistent with significant transition-state stabilization in the enzymic reaction occurring through negative hyperconju-

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gation from the lactyl methyl group of the coenzyme adduct.

Jordan, O'Leary, Deniro, and their co-workers have investigated ^{13}C isotope effects (for the carboxyl group of pyruvate) in pyruvate decarboxylase.[8,9] These groups have concluded that decarboxylation is partially rate determining (for V_{max}) in the enzymic reaction. The intrinsic ^{13}C effect for decarboxylation as measured by Jordan in a model system is roughly a measure of the extent of rehybridization in the transition state along the coordinate corresponding to the breaking of the C1–C2 bond of pyruvate and is not complicated by the rate at which the product is released.[29] The isotope effect increases by about 5% in going from the reaction in water to 30% aqueous ethanol, indicative of a slightly more extensive change of structure in arriving at the transition state in the presence of the cosolvent. Since the reaction is faster in the cosolvent, this result indicates that the transtion state becomes more productlike in the cosolvent. This is a reasonable conclusion since it is assumed that the reaction involves conversion of a polar molecule to a less polar intermediate.[1,28] In our study, the β-deuterium isotope effect provides information which is complementary to that from the ^{13}C isotope effect. Although the decarboxylation is faster and the transition state is more productlike, there is little change in the amount of stabilization provided by the β-substituent. The type of stabilization offered by the β-substituents is localized and does not compete with that offered by the solvent. Thus, the two types of isotope effects are consistent with a transition state which is more advanced in the more reactive system but which has a fixed amount of hyperconjugative interaction.

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Conclusion

It is clear that β-deuterium isotope effects are significant in the decarboxylation and elimination reactions of lactylthiamin and are directly comparable to the values obtained with pyruvate decarboxylase from yeast. The magnitude of the isotope effect is consistent with considerable stabilization by negative hyperconjugation. This stabilization is unaffected by the factors which accelerate the reaction in a nonpolar solvent. Therefore, the value we measure can be used confidently for the intrinsic isotope effect on the decarboxylation step in enzymic reactions. Since lactylthiamin diphosphate is a central intermediate in other enzymes (pyruvate oxidase, pyruvate dehydrogenase, acetolactate synthetase), the effects on V_{max} by pyruvic-d_3 acid will provide information on differences in the electronic demand of the transition states. For example, the coupling of oxidation to decarboxylation should drastically reduce the need for negative hyperconjugation by the adjacent methyl group. Further enzymic and nonenzymic studies will extend the utility of the methodology that our study has established.

Acknowledgment. The Natural Sciences and Engineering Research Council of Canada provided support through an Operating Grant (R.K.) and a Fellowship (M.B.). Professor R. L. Schowen and Dr. F. J. Alvarez kindly provided us with a preprint of their article (ref 14).

Communications to the Editor

Acetylenic Esters. Preparation and Characterization of Hitherto Unknown Alkynyl Carboxylate, RC≡COCOR′, and Alkynyl Phosphate, RC≡COPO(OR′)$_2$, Esters

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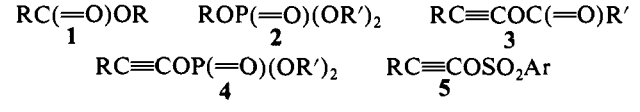
Received July 15, 1986

Carboxylate **1** as well as phosphate **2** esters are important chemical[2] and biochemical[3] functionalities. Likewise, acetylenes are well-known and useful molecules.[4] Despite the ubiquitous nature and importance of esters[2] and the diversity of functionalized acetylenes,[4] alkynyl carboxylate **3** and alkynyl phosphate **4** esters are hitherto unknown. Recently we reported[5] the first synthesis of the related alkynyl sulfonate esters **5** and in this paper we wish to disclose our preliminary results for the preparation and characterization of **3** and **4**.



The synthesis of representative alkynyl carboxylate and phosphate esters is outlined in Scheme I. Anion exchange, under strictly anhydrous conditions,[6] of known[5,7] alkynylphenyliodonium tosylates **6** with benzoate and diethyl phosphate ions gives the respective, new, iodonium salts **7** and **9**. Because of the considerable nucleophilicity of benzoate anions, the iodonium carboxylate salts **7** could not be isolated, with decomposition to the desired carboxylate esters **8** and iodobenzene being complete during anion exchange. In contrast, the iodonium phosphate salt **9a** could be isolated as a stable, crystalline salt in 80% yield, while **9b** could not be obtained pure. However, in solution, e.g., in CH_2Cl_2 at room temperature, these iodonium phosphate salts, **9**, smoothly and quantitatively convert to the desired phosphate ester **10** and the expected iodobenzene in 12–36 h.

We believe that the conversion of iodonium salts **7** and **9** to their respective esters **8** and **10**, with concomitant loss of C_6H_5I, is a result of a "nucleophilic acetylenic displacement" via an addition–elimination process as shown in Scheme II. Similar processes, namely, nucleophilic vinylic substitutions[8] via addition–elimination pathway, are well-known but are less common in acetylene chemistry.[9] This reaction is clearly dependent upon

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