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Carbon Kinetic Isotope Effects on Pyruvate Decarboxylation Catalyzed by Yeast Pyruvate Decarboxylase and Models[1]

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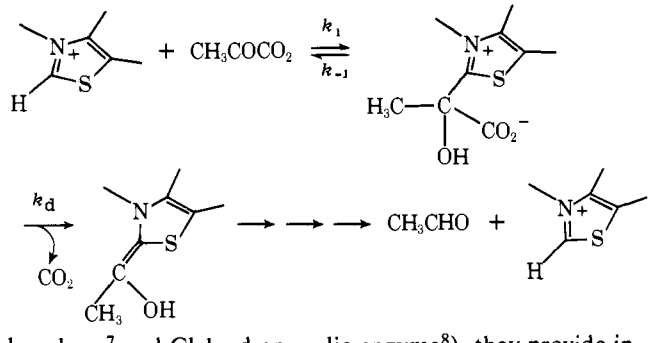
Abstract: Carbon-13 kinetic isotope effects were determined on pyruvate decarboxylation catalyzed by the enzyme yeast pyruvate decarboxylase and by thiamin, and in $CHDT^+Cl^-$ (2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride). The $CHDT^+Cl^-$ gave an effect of 1.051 corresponding to the maximum isotope effect anticipated for CO_2 loss. Thiamin-catalyzed decarboxylation gave a pH-independent inverse isotope effect of 0.992 indicating that in that model decomposition of the covalent adduct formed between thiamin and pyruvate to reactants has a higher activation energy than the subsequent decarboxylation step. The enzymatic isotope effect was found to be normal varying from 1.002 at pH 7.5 to 1.011 at pH 5.0. At pH 5.00 the isotope effect was found to be temperature independent. The results were interpreted to mean that in the pH range employed decarboxylation is faster than the decomposition of the enzyme-bound thiamin–pyruvate covalent complex. A model is presented to account for the observed pH dependence of the enzymatic kinetic isotope effect.

Yeast pyruvate decarboxylase (EC 4.1.1.1) (PDCase) catalyzes the decarboxylation of pyruvate to acetaldehyde with the assistance of thiamin pyrophosphate and Mg(II) as cofactors.[2] Numerous studies have shown that pyruvate and the coenzyme form a covalent complex. In fact, 2-(α-hydroxy)-ethylthiamin pyrophosphate (the adduct of acetaldehyde and the coenzyme) can be isolated from the enzymic reaction.[3] Scheme I accounts for the known facts.

Carbon kinetic isotope effects on the release of CO_2 can elucidate whether the decarboxylation is the slow, rate-controlling step in the series of steps culminating with CO_2 release (assuming this last step to be essentially irreversible). While few such experiments have been reported (Seltzer et al. on oxaloacetate decarboxylose,[4] O'Leary on isocitrate dehydrogenase,[5] acetoacetate decarboxylase,[6] and glutamate decarboxylase,[7] and Cleland on malic enzyme[8]), they provide information not readily accessible by any other technique.

Scheme I



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In this paper results on ^{13}C kinetic isotope effects on CO_2 release for the yeast pyruvate decarboxylase enzyme are reported. To facilitate interpretation of the enzymic results similar isotope effects were also determined for decarboxylation of $CHDT^+Cl^-$ (2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride) and for the thiamin-catalyzed pyruvate decarboxylation. The first model[9] provides the full decarboxylation isotope effect that can be anticipated. The second model is closer to the enzyme mechanism in that it incorporates a sequence of two steps: the binding of pyruvate to the coenzyme and the decarboxylation step (see Scheme I). This model enables us to decide if formation of the thiamin-pyruvate covalent adduct or its subsequent decarboxylation is rate limiting. Knowledge of the model isotope effects ultimately allows us to interpret the isotope effects observed in the enzymatic decarboxylation process.

Experimental Section

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

Enzyme Purification. The enzyme was purified from yeast slurry provided by the Anheuser-Busch Brewing Co. (Newark, N.J.) and approximately following Ullrich's procedure.[10] The precipitated wet yeast (200 g) resulting from centrifugation was mixed with a solution of 10 mL of glycerol, 0.1 g of Na_2EDTA, and 0.6 g of $(NH_4)_2SO_4$ in 70 mL of water. This slurry was stirred overnight at 0–5 °C and then centrifuged at 10 000*g*. The supernatant solution contained enzyme with a specific activity of ca. 5 units/mg.

The commercial enzyme (Sigma, St. Louis, Mo.) had a specific activity of 18 units/mg.

Enzyme Assay and Kinetic Measurements. The enzymatic reaction could be followed by pH stat[11] (i.e., $CH_3COCO_2^- +$ enzyme + TPP $\rightarrow CH_3CHO + CO_2 + OH^-$), coupled enzyme assay (alcohol dehydrogenase, NADH),[10] and Warburg respirometry.[12]

(a) pH stat titration was employed in the determination of both low conversion and 100% reaction times of the enzymatic reaction and the low conversion reaction times of the thiamin-catalyzed decarboxylation. 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 of the 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 the titrigraph that was equipped with a plastic stirrer. With the recorder running 10 μL of enzyme (CO_2 free) was introduced via a microliter pipet. Figure 1 shows the titrigraph of the consumption of 0.01 N HCl in maintaining the preset pH 5.00. 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 500 μL of enzyme solution. f, the fractional reaction, could be calculated from the number of moles of 0.01 N HCl consumed divided by the total number of moles of pyruvate at the reaction time employed in the scaled-up reaction. As an example, at 29 min reaction time the average of three samples shows 0.774 ± 0.02 mL of 0.01 N HCl consumed and the fractional reaction f is $(0.774 \times 0.01)/(2 \times 0.2) = 0.0194$.

The 100% reaction ($f = 1.0$) was also determined by pH stat. In a typical determination (Figure 1), 100 μL of enzyme solution was added to 2 mL of 0.01 M pyruvate in a pH stat with the recorder running. After 25 min there was no more acid consumed. Pyruvate (1 mL, 0.02 M) was then added to see if enzyme activity still persisted (to ensure that the previous amount of pyruvate had been quantitatively converted to CO_2 and acetaldehyde). This was apparently the case (see Figure 1). The average of three samples for the 100% reactions was found to be 97.5 ± 2.9% of the theoretical value in 25 min. A scaled-up version of this system consisted of 20 mL of 0.01 M pyruvate and 1000 μL of enzyme from which CO_2 was collected over a period of 3 h to overnight (~12 h). The collection of CO_2 and the determination of f were performed on the same day for both low percent and 100% conversion experiment.

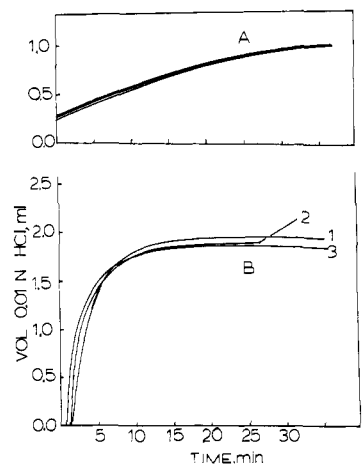


Figure 1. Titrigraph determination of f (fractional reaction) at pH 5.00 in the enzymic decarboxylation reaction. (A) Triplicate determinations of low fractional conversion (see Experimental Section for details). (B) Triplicate determinations of 100% reaction ($f = 1.0$). Curve 2 demonstrates that enzyme activity still remained as added pyruvate led to further hydroxide ion release (see Experimental Section for details).

(b) The UV method was employed to assay both commercially available and in-house purified enzyme.[10]

(c) The Warburg respirometer method[12] was employed in the determination of the fractional reaction of thiamin-catalyzed decarboxylation.

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-necked flask equipped with separatory funnel, drying tube, a syringe cap, and a stopcock) was purged three times (filled, then evacuated to below 50 μm) with high-purity CO_2-free N_2. Next, the reaction vessel was filled with CO_2-free N_2, followed by injection through the side arm of 100 mL of pyruvate solution (previously degassed by bubbling through it CO_2-free N_2 for at least 30 min).

The solution was stirred and thermostated at 30 °C for 30 min. Next 0.5 mL of pyruvate decarboxylase was injected through a rubber septum to initiate the reaction. The reaction was quenched (syringe cup) with 5 mL of concentrated H_2SO_4 at the time when ca. 200 μmol of CO_2 was evolved (as calculated from the f values determined above).

Next, the reaction vessel was 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. Next, the liquid nitrogen was replaced by dry ice and the CO_2 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, the CO_2 was also 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.[13,14] The atom fraction of ^{13}C, N_x, corrected for $^{12}C^{16}O^{17}O$, was calculated from the expression:

$$10^6 N_x = \frac{\bar{r}_{\text{sample}} 11\ 134}{\frac{1}{2}(\bar{r}_{\text{tank before}} + \bar{r}_{\text{tank after}})} - 800$$

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; 800 is the number provided by the manufacturer to compensate for the ^{17}O isotope ratio ($^{12}C^{16}O^{17}O$). $11\ 134 \times 10^{-6}$ is the average value of 1362 readings of the 45/44 mass ratio of tank CO_2 during the entire course of the present experiments.

Table I. Isotope Effect on $CHDT^+Cl^-$ Decarboxylation

Temp, °C	Concn, mM	Reaction time, min	Half-life[a] min	f[b]	N_{x0} (or N_x)[c] ($\times 10^6$)	N_{x0}/N_x	k^{12}/k^{13}
67.0	6.3 in H_2O	645	50	1.0	10 582		
67.0	6.3 in H_2O	666	50	1.0	10 583		
67.0	6.3 in H_2O	2380	50	1.0	10 591		
67.0	67.3 in 0.652 M HCl (pH ~ 0.18)	369.8	693	0.3698	10 166	1.0410	1.0521
45.6	16.8 in H_2O (pH 5.0)	438	1429	0.1914	10 124	1.0459	1.0511
45.6	16.8 in H_2O (pH 5.0)	457	1429	0.1988	10 131	1.0452	1.0506

[a] Calculated from Table II and Figure 5, ref 9a. [b] Fractional reaction. [c] N_{x0} for $f = 1.0$, N_x for $f \neq 1.00$.

Table II. Thiamin-Catalyzed Decarboxylation Isotope Effects, 30 °C

pH	[Pyruvate], M	[Thiamin], M	f[a]	$N_x \times 10^6$	N_{x0}/N_x[b]	k^{12}/k^{13}	Average[c]
6.50	0.16	0.02	0.0103	10 656	0.9933	0.9933	
6.50	0.16	0.02	0.0103	10 694	0.9898	0.9898	
6.50	0.16	0.02	0.0104	10 642	0.9946	0.9946	
6.50	0.16	0.02	0.0104	10 671	0.9919	0.9919	0.9924 ± 0.0028
7.00	0.182	0.0091	0.0113	10 653	0.9936	0.9936	
7.00	0.182	0.0091	0.0113	10 688	0.9904	0.9904	0.9920 ± 0.0068
7.50	0.182	0.0091	0.0120	10 702	0.9891	0.9891	
7.50	0.182	0.0091	0.0119	10 705	0.9888	0.9888	0.9890 ± 0.0007
8.00	0.182	0.0091	0.0039	10 661	0.9929	0.9929	
8.00	0.182	0.0091	0.0039	10 672	0.9918	0.9918	0.9924 ± 0.0024
8.60	0.154	0.023	0.0133	10 641	0.9947	0.9947	
8.60	0.154	0.023	0.0134	10 638	0.9950	0.9950	0.9949 ± 0.0007
						Overall av	0.9921 ± 0.0014

[a] Fractional reaction. [b] N_{x0} taken from Tables I and III. [c] Within 95% confidence limits.

An acetaldehyde-containing sample was run to prove that our procedure for CO_2 collection did not introduce acetaldehyde or acetic acid into the gas sample to be analyzed on the mass spectrometer. CO_2-free 0.5 M acetate buffer (20 mL) was introduced into a 100-mL flask (CO_2-free atmosphere). The sample was frozen and attached to the vacuum system. CO_2 from the standard tank (corresponding to approximately 200 μmol, 1 mmHg according to the vacuum gauge reading) was transferred into the flask via a vacuum line. Then the standard CO_2 collection technique was followed. Duplicate samples gave 45/44 ratios of $11\,132 \times 10^{-6}$ and $11\,137 \times 10^{-6}$, respectively. These values are well within the error limits of the 45/44 mass ratio of tank CO_2. This proves to our satisfaction that acetaldehyde is not being measured in the mass spectrometer under our experimental protocol for CO_2 collection during the decarboxylation reaction.

Results

The isotope effects were obtained according to Bigeleisen's formula:[15]

$$k^{12}/k^{13} = \frac{\log(1-f)}{\log[1-f(N_x/N_{x0})]}$$

where N_x is the isotope ratio at low fractional conversion f corrected for natural abundance $^{12}C^{16}O^{17}O$ and N_{x0} is the corrected ratio at 100% reaction ($f = 1.0$). Error analysis indicates a maximum error of 0.002 due to cumulative uncertainties in f and N_x measurements.

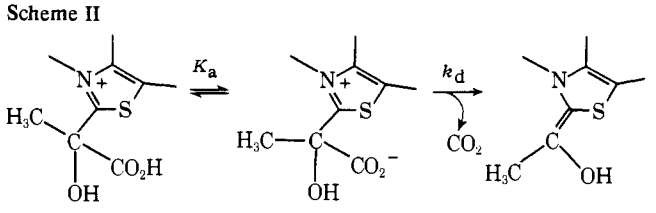
$CHDT^+Cl^-$. Table I presents the results obtained on this model system at two different pHs: 0.186 and 5.00. The isotope effect observed is 1.051 − 1.052. Employing Scheme II for the two isotopically labeled species (since Crosby et al.[9a] demonstrated that only the dipolar ion undergoes decarboxylation):

$$k^{12}/k^{13} = \frac{k_d}{k_d^*} = 1.051$$

This magnitude compares with the 1.06 value for divalent metal ion catalyzed decarboxylation of oxaloacetic acid[4] and of some other decarboxylation values cited by Dunn[16] in a recent review.

Thiamin-Catalyzed Decarboxylation. Table II presents data at pH 6.5, 7.0, 7.5, 8.0, and 8.6. N_{x0} for these results had to be taken from the $CHDT^+Cl^-$ and PDCase data since (N_{x0} values for these sets are in excellent agreement with each other) thiamin catalyzes the condensation of pyruvate to acetolactate (Scheme III); hence, the CO_2 is liberated from two sources.

Scheme II



$$\frac{d(CO_2)/dt}{d(CO_2^*)/dt} = \frac{k_d(S)}{k_d^*(S^*)} = \frac{k^{12}}{k^{13}}\frac{(S)}{(S^*)}$$

Scheme III

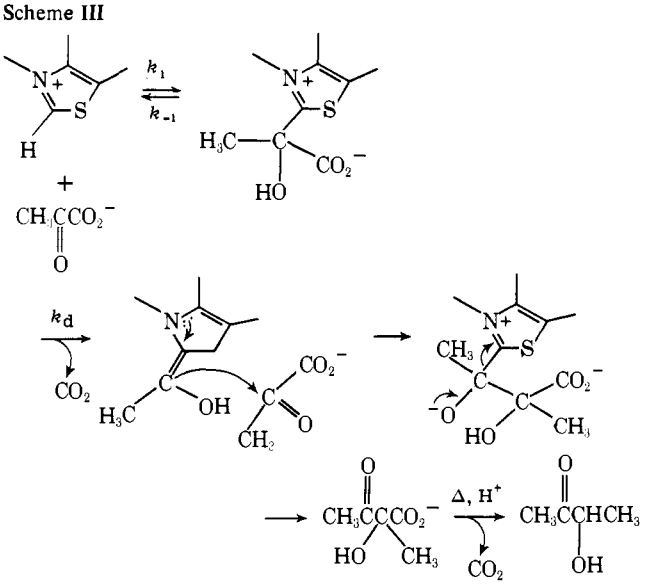


Table III. Isotope Ratios and Kinetic Isotope Effects at 30 °C for the Holopyruvate Decarboxylase Catalyzed Reaction

pH	[Buffer], M	[Pyruvate], M	f	$N_x \times 10^6$	k^{12}/k^{13}
5.00	0.5 acetate	0.192	0.0424	10 469	1.0113
5.00	0.5 acetate	0.095	0.154	10 471	1.0119
5.00	0.1 acetate	0.095	0.0787	10 476	1.0108
5.00	0.5 acetate	0.195	0.0052	10 480	1.0101
5.00				Av	1.0110 ± 0.0011
5.50	0.5 acetate	0.096	0.0235	10 517	1.0066
5.50	0.5 acetate	0.096	0.0240	10 521	1.0062
5.50	0.5 acetate	0.096	0.0286	10 511	1.0071
5.50	0.5 acetate	0.096	0.0285	10 513	1.0069
5.50				Av	1.0067 ± 0.0005
6.00	0.5 citrate	0.0199	0.142	10 508	1.0079
6.00	0.5 citrate	0.0398	0.0265	10 513	1.0070
6.00	0.5 citrate	0.0992	0.0412	10 524	1.0059
6.00	0.5 citrate	0.0992	0.0412	10 530	1.0053
6.00				Av	1.0065 ± 0.0016
6.50	0.5 phosphate	0.0198	0.0850	10 550	1.0035
6.50	0.5 phosphate	0.0198	0.0870	10 549	1.0036
6.50	0.5 phosphate	0.195	0.0310	10 521	1.0062
6.50	0.5 phosphate	0.195	0.0293	10 525	1.0058
6.50				Av	1.0048 ± 0.0020
7.0	0.5 phosphate	0.0191	0.105	10 552	1.0033
7.0	0.5 phosphate	0.0191	0.0402	10 568	1.0016
7.0	0.5 phosphate	0.191	0.0410	10 546	1.0038
7.0	0.5 phosphate	0.191	0.0412	10 553	1.0032
7.0				Av	1.0030 ± 0.0013
7.5	0.5 phosphate	0.091	0.0637	10 548	1.0036
7.5	0.5 phosphate	0.192	0.0103	10 572	1.0012
7.5	0.5 phosphate	0.192	0.0103	10 565	1.0018
7.5				Av	1.0022 ± 0.0023
6.00[a]	0.5 citrate	0.0095	1.0	N_{x0} = 10585 ± 5; av of 16 runs	

[a] All values are given in Table IV.

Since formation of acetolactate (and subsequently of acetoin) is very much slower than is the release of the first CO_2 (unpublished from this laboratory) low conversion (1% or less) to CO_2 corresponds to the first pyruvate decarboxylation. N_{x0} from the other experiments ($CHDT^+Cl^-$ and PDCase) should be applicable. A total of 12 determinations demonstrates that the isotope effect is inverse and essentially pH independent in the pH range employed.

PDCase-Catalyzed Decarboxylation. Table III presents all isotope ratios and kinetic isotope effects observed in the enzyme reaction at 30 °C at pH 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. Table IV presents all N_{x0} values. There is a slight but real increase in kinetic isotope effect going from neutral to slightly acidic media: k^{12}/k^{13} is 1.0030 ± 0.0013 at pH 7.0, 1.0065 ± 0.0016 at pH 6.0, and 1.0110 ± 0.0011 at pH 5.0. The large number of experimental determinations obtained at pH 5.0 at different temperatures (Table V) enhances our confidence in the pH dependence claimed. The kinetic isotope effect at pH 5.00 was shown to be temperature independent between 10 and 37 °C, the average of 23 values being 1.010. This implies that there is no change in the rate-limiting step in this temperature range.

As this work was being rewritten into a full paper we became aware of very similar work published by O'Leary.[17] That author reported a kinetic isotope effect of 1.0083 ± 0.0003 at 25 °C, pH 6.8. While the results of this study and that of O'Leary differ somewhat in that pH range, the small differences could be due to differences in instrumentation, enzyme purification, fractional reaction employed, N_{x0}, etc. In any case, the principal conclusions (see below) are unaltered by the differences in magnitudes between the two studies.

Table IV. Corrected Isotope Ratio N_{x0} of Pyruvate Decarboxylation Catalyzed by Holoenzyme at pH 6.00 and 30 °C in 0.5 M Citrate Buffer

[Pyruvate], M	Reaction time, min	$N_{x0} \times 10^6$
0.0095	210	10 582
0.0095	210	10 589
0.0095	210	10 578
0.0095	900	10 579
0.0095	316	10 572
0.0095	317	10 577
0.0091	720	10 580
0.0091	720	10 581
0.0087	1180	10 589
0.0087	1180	10 602
0.0087	2880	10 587
0.0087	2880	10 593
0.0087	1205	10 605
0.0083	1205	10 583
0.0091	1449	10 575
0.0087	924	10 585
	Av	10 585 ± 5

Discussion

Whereas the decarboxylation of $CHDT^+Cl^-$ may be viewed essentially as a single-step mechanism, the thiamin- and enzyme-catalyzed decarboxylations necessitate additional steps which involve the formation and dissociation of the adduct according to the first two steps in Scheme I, as abbreviated below.

Here T may be either free or enzyme-bound thiamin and S is free or enzyme-bound pyruvic acid. The asterisk denotes the ^{13}C label. The decarboxylation step is assumed to be irreversible. The dry N_2 atmosphere employed and the low CO_2 pressure generated as well as all relevant literature to date make it unlikely that reversibility obtains. Steady-state con-

Table V. Temperature Dependence of Kinetic Isotope Effects in Pyruvate Decarboxylase Catalyzed Reaction at pH 5.00 in 0.1 M Total Acetate Buffer

Temp, °C	[Pyruvate], M	f[a]	$N_x \times 10^6$	N_{x0}/N_x[b]	k^{12}/k^{13}	Av[c]
10	0.190	0.0111	10 481	1.0099	1.0100	
10	0.190	0.0112	10 487	1.0093	1.0094	
10	0.182	0.0153	10 484	1.0096	1.0097	
10	0.182	0.0151	10 487	1.0093	1.0094	1.0094 ± 0.0004
15	0.195	0.0145	10 482	1.0098	1.0099	
15	0.195	0.0106	10 475	1.0105	1.0106	
15	0.195	0.0107	10 474	1.0106	1.0107	1.0104 ± 0.0008
20	0.194	0.0290	10 482	1.0098	1.0099	
20	0.194	0.0293	10 480	1.0100	1.0102	
20	0.194	0.0230	10 488	1.0092	1.0093	
20	0.194	0.0229	10 483	1.0097	1.0098	1.0098 ± 0.0005
25	0.198	0.0194	10 479	1.0101	1.0102	
25	0.198	0.0195	10 480	1.0100	1.0101	
25	0.198	0.0186	10 474	1.0106	1.0107	
25	0.198	0.0181	10 470	1.0110	1.0111	1.0105 ± 0.0006
37	0.191	0.0097	10 484	1.0096	1.0096	
37	0.191	0.0099	10 480	1.0100	1.0100	
37	0.191	0.0092	10 475	1.0105	1.0105	
37	0.191	0.0096	10 487	1.0093	1.0093	1.0099 ± 0.0007

[a] Fractional reaction. [b] N_{x0} from Tables III and IV. [c] 95% confidence limit.

$$T + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} TS \xrightarrow{k_d} \text{product} + CO_2$$

$$T + S^* \underset{k_1^*}{\overset{k_{-1}^*}{\rightleftharpoons}} TS^* \xrightarrow{k_d^*} \text{product} + C^*O_2$$

ditions on the TS and TS* covalent complexes lead to the following rate expression for the isotope effect (eq 1).

$$\frac{k^{12}}{k^{13}} = \frac{k_1\left(1 + \frac{k_{-1}^*}{k_d^*}\right)}{k_1^*\left(1 + \frac{k_{-1}}{k_d}\right)} \qquad (1)$$

Equation 1 is similar to that used by other investigators.[4,16] Note, however, that the rate constants controlling the formation and dissociation of the adduct are assumed to be isotope dependent. This isotope effect had been neglected by earlier investigators.

Equation 1 is now used to decide whether the dissociation of the adduct TS or the decarboxylation determines the rate of the reaction under two limiting conditions. (1) If the dissociation of the covalent adduct becomes rate limiting, i.e., $k_{-1}/k_d \ll 1$, eq 1 yields $k^{12}/k^{13} \simeq k_1/k_1^*$, which represents a secondary kinetic isotope effect due to the binding step. Such effects have been estimated between 0.983 (inverse) and 1.012 (normal) by Bigeleisen and Wolfsberg.[18] (2) On the other hand, if the rate is limited by the decarboxylation step, i.e., $k_{-1}/k_d \gg 1$, the observed isotope effect becomes $k^{12}/k^{13} \simeq k_1k_dk_{-1}^*/k_1^*k_d^*k_{-1}$. Invoking theoretical estimates for secondary isotope effects[18] and the results of the present investigation from $CHDT^+Cl^-$ for k_d/k_d^* (=1.05), the isotope effect k^{12}/k^{13} is estimated to be between 1.03 and 1.06.[19]

Thiamin-Catalyzed Reaction. The observed value of 0.992 ± 0.002 for the thiamin model clearly dictates the first limit; i.e., the dissociation of the pyruvate-thiamin covalent adduct to reactants is subject to a larger activation energy than the decarboxylation step.

It is of relevance to mention that in the amine-catalyzed decarboxylation of acetoacetic acid, formation of Schiff base and decarboxylation were found to proceed at similar rates (employing a different experimental approach).[20] Thus, in these examples of nucleophilic catalysis decarboxylation is not strictly rate limiting even in the absence of enzyme. In fact, these results demonstrate that in nonenzymic decarboxylations CO_2 loss need not be rate limiting and that k_1 and k_{-1} may have significant isotope effects associated with them contrary to what is usually assumed.

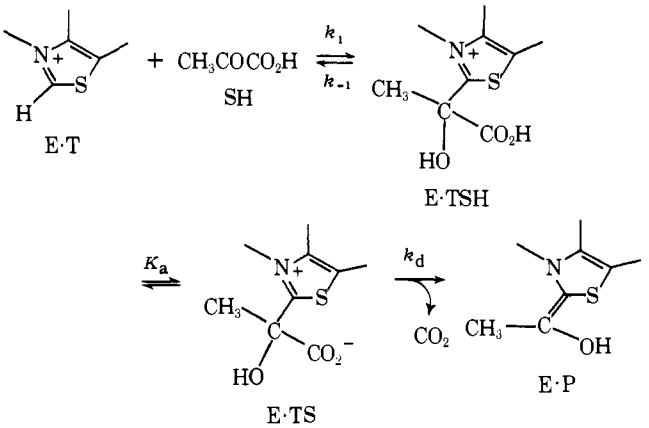
It should be pointed out that the observed isotope effect, k^{12}/k^{13}, represents an upper limit to k_1/k_1^*. Clearly, the isotope effect on the decarboxylation, k_d/k_d^*, is greater than the possible maximum of the secondary isotope effect, k_{-1}/k_{-1}^*, or $k_dk_{-1}^*/k_d^*k_{-1} > 1$. Therefore, according to eq 1, $k_1/k_1^* \leq k^{12}/k^{13}$ (=0.992). Thus, the isotope effect on the adduct formation rate constant is definitely inverse and substantially different from unity.

Holoenzyme-Catalyzed Reaction. The immediate mechanistic conclusion which may be reached from the enzyme study is similar to that obtained from the thiamin model, since the observed isotope effects (1.002–1.010) are definitely below the range estimated for the case where decarboxylation becomes rate limiting (1.03–1.06; see limit 2 above).

It is of interest to carry out a more detailed analysis of the enzymatic decarboxylation by selecting a scheme that accounts for the observed pH dependence of the isotope effect. This behavior can be interpreted to follow an apparent pK of 5–5.5.

Scheme IV presents a possible mechanism that accounts for

Scheme IV



the observed pH dependence, where E · T is enzyme-bound coenzyme and SH is un-ionized pyruvic acid.

In abbreviated form for the two isotopically labeled species:

$$E{\cdot}T + SH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}TSH \underset{H^+}{\overset{K_a}{\rightleftharpoons}} E{\cdot}TS \xrightarrow{k_d} EP + CO_2 \quad (2)$$

$$E{\cdot}T + S^*H \underset{k_{-1}^*}{\overset{k_1^*}{\rightleftharpoons}} E{\cdot}TS^*H \underset{H^+}{\overset{K_a^*}{\rightleftharpoons}} E{\cdot}TS^* \xrightarrow{k_d^*} EP + {}^*CO_2 \quad (3)$$

In view of the proposed existence of a hydrophobic active site in the enzyme[9] this scheme appears particularly attractive since un-ionized pyruvic acid would be preferred to pyruvate in such a medium.[21]

It should be noted that a mechanism that postulates the protonation of E · TS as a side chain according to (abbreviations as in Scheme IV):

$$\begin{array}{ccccc} E{\cdot}T + S & \rightleftharpoons & E{\cdot}TS & \longrightarrow & EP \\ & & H^+ \downarrow\uparrow K_a & & CO_2 \\ & & E{\cdot}TSH & & \end{array} \quad (4)$$

(S is ionized pyruvate being bound to enzyme-bound thiamin pyrophosphate) results in an isotope effect that is essentially independent of pH. A possible pH dependence of the kinetic isotope effect may arise via the protolytic equilibrium of ETSH. However, this acid dissociation equilibrium isotope effect (K_a/K_a^*), as shown below, is considerably smaller than the magnitude of the pH dependence observed.

Another possible mechanism that results in a pH-dependent isotope effect involves the decarboxylation of E · TSH^+ (abbreviations as in Scheme IV) according to eq 5:

$$E{\cdot}T + S \rightleftharpoons E{\cdot}TS \underset{K_a}{\overset{H^+}{\rightleftharpoons}} E{\cdot}TSH \xrightarrow[CO_2]{} EP \quad (5)$$

This expression is ruled out by the pH dependence here found and is also unlikely in light of the model studies[9] which indicated that in $CHDT^+Cl^-$ the substrate analogous to E· TS decarboxylated only.

Steady-state assumptions on E·TS and E·TSH in eq 2 and 3 lead to the following rate expression for the observed isotope effect (eq 6).

$$\frac{k^{12}}{k^{13}} = \frac{k_1}{k_1^*} \frac{1 + \dfrac{k_{-1}^*(H^+)}{k_d^* K_a^*}}{1 + \dfrac{k_{-1}}{k_d}\dfrac{(H^+)}{K_a}} \quad (6)$$

For the following discussion it is useful to estimate the magnitude of K_a/K_a^*. This estimate was carried out by calculating the ^{13}C equilibrium isotope effect on the ionization of formic acid based on harmonic force fields of formic acid and formate ion[22] employing Wilson's F-G-matrix method.[23] A computer program was used which was developed by Schachtschneider and Snyder[24] and modified by Wolfsberg and Stern.[25] From the calculated reduced isotopic partition function ratios s/s^*f[26] for formic acid and formate ion at 300 K (HCOOH, s/s^*f = 1.175 383; $HCOO^-$, s/s^*f = 1.172 770) the ^{13}C isotope effect on the ionization of formic acid is obtained as K_a/K_a^* = 1.0022. The calculated values indicate a change of only 0.0005 over the temperature range of 270-370 K.

This value is in reasonable agreement with the ^{13}C isotope effect on the ionization of benzoic acid which was determined as K_a/K_a^* = 1.0014 ± 0.001.[27]

The two limits of eq 6 are similar to those for eq 1 discussed above: (1) $(k_{-1}/k_d)[(H^+)/K_a] \ll 1$, or:

$$k^{12}/k^{13} = k_1/k_1^* \quad (7)$$

a limit reached at high pH for a pK_a near 5 and if $k_{-1} \leq k_d$. This limiting isotope effect was estimated between 0.983 and 1.012.[18] (2) If the acidity-dependent term dominates in eq 6:

$$\frac{k^{12}}{k^{13}} = \frac{k_1}{k_1^*}\frac{k_1^*}{k_{-1}}\frac{k_d}{k_d^*}\frac{K_a}{K_a^*} \quad (8)$$

As $K_a/K_a^* \simeq 1.002$, k^{12}/k^{13} lies between 1.03 and 1.06 (see second limit on eq 1 discussed above).

The observed isotope effect is clearly close to the first limit (eq 7). In addition, eq 6 may now be used to estimate from the observed data points at pH 5, 6, and 7.0 the ratio k_{-1}/k_d which compares the rate of dissociation of enzyme-bound thiamin-substrate covalent adduct with the rate of decarboxylation. For this estimate of k_{-1}/k_d it is important to realize that, according to eq 6, for small values of $(k_{-1}/k_d)[(H^+)/K_a]$ the isotope effect k^{12}/k^{13} varies approximately linearly with the hydrogen ion concentration.

The third column in Table VI represents the best linear fit to the observed data at pH 5.0, 6.0, and 7.0 quoted in the second column. To estimate k_{-1}/k_d in conjunction with eq 6, values for the isotope effects k_d/k_d^* and k_{-1}/k_{-1}^* also have to be known. The $CHDT^+Cl^-$ study furnishes the values of k_d/k_d^* of 1.051. The isotope effect k_{-1}/k_{-1}^* again is estimated within the range expected for secondary isotope effects;[18] i.e., $0.983 \leq k_{-1}/k_{-1}^* \leq 1.012$. k_1/k_1^* is just the high pH limit of the linear fit, and according to eq 6 it has a value of 1.0043.

Equation 6 predicts from the fitted data a value of k_{-1}/k_d which varies between 0.10 (for $k_{-1}/k_{-1}^* = 0.983$) and 0.20 (for $k_{-1}/k_{-1}^* = 1.012$) for a pK_a of 5. Since the activity-pH profile indicates the presence of the pK_as certainly not smaller than 5,[11,28-30] the maximum of k_{-1}/k_d expected within this scheme is 0.20. This value reaffirms that the rate of dissociation of the enzyme-bound thiamin-substrate complex is definitely slower than the rate of decarboxylation.

The apparent pK followed by the pH dependence of the enzymatic kinetic isotope effect closely resembles the apparent pKs found in the activity-pH profile of the enzyme.[11,28-30] Whether carboxylic acid ionization is really reflected by this behavior or not cannot be determined with certainty at the present. It is an attractive possibility, however, were the hydrophobic active site to raise the pK_a of ionization from 1.31[9a] to 5-5.5.

It is significant to point out that the isotope effect found in $CHDT^+Cl^-$ (1.051) has never been approached in magnitude in any enzymic decarboxylation.[4-8] The knowledge of appropriate models is essential for a meaningful interpretation of enzymic results.

Finally, we must emphasize that our results only reflect on the relative rates of covalent enzyme-bound thiamin-pyruvate adduct dissociation and decarboxylation steps. Clearly, CO_2 loss is not rate limiting overall. Whether acetaldehyde loss is rate limiting, as suggested by the ability to isolate (−)-2-(α-hydroxy)ethylthiamin pyrophosphate from the enzyme reaction mixture,[2] remains to be demonstrated unequivocally.

Table VI. Observed and Best Linear Fit of the Isotope Effects on the Enzymatic Decarboxylation

pH	$(k^{12}/k^{13})_{obsd}$	$(k^{12}/k^{13})^a$
5	1.0110 ± 0.0011	1.0111
6	1.0065 ± 0.0016	1.0050
7	1.0030 ± 0.0013	1.0044

[a] Best linear fit to the observed data.

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Dependence of Cycloamylose–Substrate Binding on Charge

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Abstract: The direction of sodium benzoate and benzoic acid penetration of the cyclohexaamylose cavity in aqueous solution is determined by a ^1H NMR study of the respective cycloamylose complexes. Chemical-shift changes in the host and guest molecules, as well as a strong intermolecular nuclear Overhauser effect, suggest that benzoic acid has a strong orientational preference for binding in the cycloamylose cavity, while sodium benzoate binding is somewhat more random. Both benzoic acid and sodium benzoate are shown to form 1:1 AB complexes with cyclohexaamylose, and the benzoic acid is shown to generate conformational changes in the cyclic oligosaccharide's glucose rings. The dependence of cycloamylose–substrate binding on charge is discussed in terms of the energy required to move a charged species from a medium of high dielectric to a medium of low dielectric. This "insertion energy" is approximated from free energy of solution studies.

In recent years, the cycloamyloses have received a great deal of attention as enzyme active-site models.[1-3] However, little of this attention has been focused on the forces responsible for cycloamylose–substrate binding. Although there have been a number of suggestions as to the nature of the complexation driving forces (release of high-energy cavity water,[3] release of ring strain,[4] and London dispersion forces[3]) there is relatively little experimental evidence available in support of any one of these concepts. Furthermore, any explanation of the binding forces must now take into consideration Breslow's remarkable discovery that substrates bind in the cycloamylose cavity in nonaqueous solvents.[5] Although we have been unable to find any support from our solution studies for either the strain energy or high-energy water concepts, we have accumulated some evidence in favor of the London dispersion forces arguments.

One fact is undeniable: both the charge on the substrate and its direction of penetration are of great importance in regulating the stability of the cycloamylose–substrate complexes formed.[6,7] The relationship between these factors was not a clear one; e.g., sodium *p*-nitrophenolate binds 13 times more tightly in the cycloamylose cavity than the neutral phenol, while just the opposite is true of benzoic acid and its anion with the carboxylate anion binding 82 times more loosely.[6,8] It seemed likely that an understanding of this apparent anomaly would help clarify the relationship between the direction of substrate penetration and charge on substrate binding.

In this paper, we report on the direction in which benzoic acid and sodium benzoate penetrate the cyclohexaamylose cavity and compare these results with our earlier findings on the sodium *p*-nitrophenolate and *p*-nitrophenol complexes. This comparison suggests the importance of "insertion energy" in substrate binding, i.e., the energy required to move the carboxylate anion from water, a medium of high dielectric, to the cycloamylose cavity, a medium of low dielectric. This is further verified by free energy of solution studies of the guest molecules in solvents whose properties approximate those of the cyclohexaamylose cavity.

Experimental Section

Materials. The cyclohexaamylose, benzoic acid, sodium benzoate, and deuterium oxide, 99.8%, were obtained from Aldrich Chemical

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