Mechanisms of Thiamine-Catalyzed Reactions. Decarboxylation of 2-(1-Carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium Chloride

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Abstract: Previous investigations have indicated that the thiamine pyrophosphate-dependent enzymatic decarboxylation of pyruvate to acetaldehyde proceeds via the decarboxylation of 2-(1-carboxy-1-hydroxyethyl)thiamine pyrophosphate (Ia) to 2-(1-hydroxyethyl)thiamine pyrophosphate (Ila). This paper reports the synthesis of an analog of Ia, 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride (CHDT chloride), and the kinetics of the decarboxylation of CHDT to 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion in water, ethanol-water mixtures, and ethanol. We conclude from the dependence upon pH of the observed first-order rate constants for the decarboxylation of CHDT in water at 67° that the reactive species is the one in which the carboxyl group is ionized. The rate of decarboxylation of this dipolar ion is markedly increased in solvents less polar than water; the half-times for decarboxylation are 24.0 hr in water at 45.6° and 3.2 min in absolute ethanol at 26.0°. Comparison of this model with the pyruvate decarboxylase reaction shows that the enzyme accelerates the decarboxylation of Ia in water by a factor of at least 10. We propose that the enzymatic catalysis is effected through binding of the thiazolium portion of Ia in a region of the enzyme less polar than water and suggest that such an enzymatic solvent effect is a major cause of catalysis in many thiamine pyrophosphate-dependent enzymatic reactions.

The decarboxylation of pyruvate to acetaldehyde by the enzyme pyruvate decarboxylase is a reaction which requires the coenzyme thiamine pyrophosphate. On the basis of investigations of nonenzymatic model reactions, Breslow proposed that the enzymic reaction proceeds by way of 2-(1-carboxy-1-hydroxyethyl)thiamine pyrophosphate (Ia), which is formed from thiamine pyrophosphate and pyruvic acid by reaction of the thiazolium ring, ionized at carbon 2, with the carbonyl group of pyruvic acid. Decarboxylation of this intermediate yields 2-(1-hydroxyethyl)thiamine pyrophosphate (IIa), which can then lose acetaldehyde and so regenerate the thiamine pyrophosphate. Subsequently, the proposed intermediate Ila was isolated from reaction mixtures which contained pyruvic acid and pyruvate decarboxylase holoenzyme. Moreover, it was shown that pyruvate decarboxylase apoenzyme catalyzes the formation of acetaldehyde from Ila. Results similar to these have also been obtained for pyruvate dehydrogenase, the enzyme which catalyzes the thiamine pyrophosphate-dependent oxidative decarboxylation of pyruvic acid to acetyl coenzyme A.

In order to understand the enzymatic decarboxylation of pyruvic acid in greater detail, we have synthesized 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium (CHDT, Ib) chloride, an analog of Ia, and determined the kinetics of its nonenzymatic decarboxylation to 2-(1-hydroxyethyl)-3,4-dimethylthiazolium (Ib) chloride. The results of this study are reported here, together with a comparison of this model reaction with the enzymatic reaction and a discussion of how catalysis might be achieved in the enzymatic reaction.

Experimental Section

General. Starting materials were reagent grade. Extracts were dried over anhydrous sodium sulfate. Melting points were determined on a hot-stage apparatus and are uncorrected. Nuclear magnetic resonance (nmr) spectra were recorded on Varian A-60 or T-60 instruments with tetramethylsilane as the internal standard when the solvent was carbon tetrachloride, and as the external standard in chloroform when the solvent was deuterium oxide. Nmr spectra are expressed as signal in parts per million downfield from tetramethylsilane (relative integrated intensity of the signal; multiplicity of the signal with s = singlet; d = doublet, t = triplet, q = quartet, and m = unresolved multiplet; coupling constant, accurate to ±0.5 Hz). Ultraviolet spectra were obtained with a Cary Model 14 or a Zeiss PMQ II spectrophotometer with 1-cm path length cells. Infrared spectra were recorded on a Perkin-Elmer Model 137 spectrometer. A Radiometer Model 25 SE pH meter was used for pH measurements. Elemental analyses were performed by the Scandinavian Microanalytical Laboratory, Herlev, Denmark.

2-(1-Carboxethoxy-1-hydroxyethyl)-4-methylthiazole. n-Butyllithium (213 ml of a standardized 1.55 N solution in hexane) was added with stirring to anhydrous ether (290 ml) at −75°, under an

atmosphere of argon. 4-Methylthiazole\(^9\) (0.33 mole) was then added dropwise during 45 min, followed by ethyl pyruvate (0.33 mole) which was added as rapidly as possible and with vigorous stirring. The mixture was allowed to stand at room temperature and was then rapidly neutralized by pouring into an ethanolic hydrochloric acid (103 ml, 3.20 N). Water (300 ml) was added, the organic layer was separated, and the aqueous layer was extracted with dichloromethane. The combined organic layers were dried, evaporated, and the residue (52.6 g) was distilled under high vacuum to 178°C (bath temperature 115–146°C; C, 0.001–0.004 mm) which contained the required product. This distillate was diluted with an equal volume of ether, cooled, and agitated until solid material was obtained. Filtration yielded colorless crystals of 2-(1-carbethoxy-1-hydroxyethyl)-4-methylthiazole \((7.36 \text{ g, 10.7%})\) mp 41.5–45.5°C (from n-propanol-ether \((	ext{EtOH}) 252 \text{ nm (e 216)} \text{ nm \text{nm}}\) ); \(2.29 \text{ (g, Q, J = 1 Hz, F)}\) 4.24, Q, J = 1 Hz), 7.15 (1 H, H, m), 7.15 (1 H, m); v,.,(NuJol) 1675 cm\(^{-1}\), which is characteristic of a conjugated ketone; \(\lambda_{\text{max}} \text{ (CH}_{3}\text{CN) 240 nm (e 6400 based on mol wt 268.2)}\) and 309.5 (7920), which suggest that this by-product is 2-(4-methylthiazole), 1-hydroxy-1-[2-(4-methylthiazole)] ethyl ketone, which would result from attack by the 2-carbanion of 4-methylthiazole on the ester group of the 2-(1-carbethoxy-1-hydroxyethyl)-4-methylthiazole anion.

Analytical Data. CHDT chloride \((48.0 \text{ mg})\) in 0.01 N hydrochloric acid \((25 \text{ ml})\) was maintained at 67°C for 10 hr. Kinetic Product Analysis. CHDT chloride \((48.0 \text{ mg})\) in 0.01 N hydrochloric acid \((25 \text{ ml})\) was maintained at 67°C for 10 hr. Kinetic 

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Chloride. This compound was prepared from 2-(1-hydroxyethyl)-4-methylthiazole by the procedure described earlier:\(14\) nmr \((\text{D}_{2} \text{O}) 2.51 (3 \text{ H, J = 1 ppm from acetone-}

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(14) W. Hafferl, R. Lundin, and L. L. Ingraham [Biochemistry, 2, 1299 (1963)] obtained the nmr spectrum of 3,4-dimethylthiazolium iodide in 0.1 N sodium acetate buffer, \(pD 3.6, \text{ D}_{2} \text{O and reported the chemical shifts of the methyl groups of 0.1 N sodium acetate buffer,}\)

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(20) F. G. White and L. L. Ingraham, ibid., 84, 3109 (1962).
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weighed before and after the period at 67° were found to have lost
less than 1% of their solvent by evaporation. For decarboxylations
in ethanol-water buffers at 45.6°, the procedure used to measure
the rate constants was identical with that used in water;
however, the method was modified slightly for measurement of
rates in aqueous ethanol at 25.95°. In the latter cases the reactions
were carried out in 10-mm cells in the thermostated cell block of a
Zeiss PMQ II spectrophotometer. The observed first-order rate
constants were obtained from semilogarithmic plots of (ODm
at time t - ODm at complete reaction) vs. time by use of the
equation
\[
\ln \left( \frac{OD_0 - OD_m}{OD_1 - OD_m} \right) = k_{\text{obs}} t
\]

An nmr method was also used to measure the rates of decar-
boxylation in four aqueous buffers. The reactions were carried out
in stoppered nmr tubes and were monitored on a Varian A-60
spectrometer, fitted with a VA-6040 variable-temperature controller,
by following the disappearance of the singlet at δ 2.00 due to the
methyl group α to the carboxyl group of the reactant and/or the
appearance of the signal at δ 1.161 due to the corresponding methyl
group of the 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion. The
integrated intensities of these signals were measured relative to the
sum of the integrals of the 4-methyl group (three hydrogen atoms),
which served as an internal standard. Observed first-order rate
constants were taken as the slopes of semilogarithmic plots of the
fraction of CHDT remaining, based on the integrations of the nmr
signals, against time.

Results

Synthesis of CHDT Chloride. The synthesis of this thiazolium chloride is outlined in Figure 1. Details of the
synthesis together with the characterization of this compound and of the intermediates are given in the
Experimental Section.

Prior to this work Eyles, et al.,18 had attempted to synthesize the very similar compound, 2-(1-carboxy-1-
hydroxyethyl)-3-benzylthiazolium bromide. They were unsuccessful; the reaction of the intermediate, 2-(1-
carbomethoxy-1-hydroxyethyl)thiazole, with benzyl bromide yielded 3-benzylthiazolium bromide. We
observed the same reaction, loss of the side chain from the 2 position when the quaternization of
2-(1-carboxethoxy-1-hydroxyethyl)-4-methylthiazole with iodomethane was attempted at 105°; the only isolable
product was 3,4-dimethylthiazolium iodide. A potent alkylation agent, such as the trimethyloxonium ion,
appears to be required for alkylation of the thiazole under conditions sufficiently mild to prevent loss of the
side chain at carbon 2. The variable melting point of 2-(1-carbethoxy-1-hydroxyethyl)-3,4-dimethylthiazolium
chloride is undoubtedly due to thermal elimination of the side chain from the 2 position because if heating
is continued, the melt resolidifies with evolution of gas at approximately 135° (bp of ethyl pyruvate, 144°)


and remelts at 176-180° (mp of 3,4-dimethylthiazolium chloride, 184-185°).

Acid Dissociation Constant of CHDT. The ultraviolet spectrum of CHDT in 1 M hydrochloric acid has
\( \lambda_{\text{max}} \) at 260 nm (Figure 2). This spectrum is characteristic of that expected for a thiazolium ion; for example,
2-(1-hydroxyethyl)-3,4-dimethylthiazolium chloride has \( \lambda_{\text{max}} \) 255 nm.24 The spectrum of CHDT in 10-3 M
hydrochloric acid-1 M potassium chloride is similar to, but not identical with, that of the compound in 1 M HCl:
\( \lambda_{\text{max}} \) is shifted to 262 nm and the extinction coefficient is slightly larger. It seemed very likely that the basis
for this small difference in the spectra was the ionization of the carboxyl group of CHDT, and the results of a
spectrophotometric titration of CHDT at 275 nm (Figure 3) support this interpretation.
In a spectrophotometric titration the optical densities of solutions with the same total concentration of CHDT should vary with the hydronium ion concentration, \( (H_3O^+) \), in accord with the equation

\[
\frac{O_{D_B} - O_D}{(H_3O^+)} = \frac{O_{D_A} - O_{D_B}}{K_a'}
\]

where \( K_a' \) is the apparent acid dissociation constant and \( O_{D_A}, O_{D_B}, \) and \( O_D \) are the optical densities of the acid, base, and partially ionized forms, respectively.\(^{(19)}\)

A plot of \((O_{D_B} - O_D)/(H_3O^+)\) against \( O_D \) using the data in Figure 3 was linear and yielded a value of 1.31 for \( pK_a' \).

There are insufficient data in the literature to permit an exact estimation of what \( pK_a \) value should be expected for the carboxyl group of CHDT. The \( pK_a \) value of the carboxyl group of isoserine, \( \text{H}_3\text{N}^+\text{CH}_2-\text{CH(OH)CO}_2\text{H} \), which slightly resembles CHDT, is 2.8.\(^{(20)}\)

Decarboxylation of CHDT in Aqueous Buffers. We obtained the complete ultraviolet spectrum of reaction mixtures in which the decarboxylation of CHDT had proceeded for various time periods at 67.0° and at pH values of 1.00, 3.00, 5.32, and 7.28. At each pH value the spectrum of the product was identical with that of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium chloride, with \( \lambda_{\text{max}} \) at 255 nm. Moreover, with the exception of the run at pH 7.28 (see below), the yields of the decarboxylation product calculated from the initial concentration of CHDT, the final optical density at 255 m\( \mu \), and the extinction coefficient of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion at this wavelength (\( \epsilon 5400 \)) were quantitative. The series of spectra at each pH value (except pH 7.28, see below) exhibited an isosbestic point near 253 nm. The occurrence of the isosbestic points demonstrates that there is no significant accumulation of an ultraviolet-absorbing intermediate during the course of the decarboxylation. Figure 4 illustrates these results with the data obtained at pH 3.

Two other reactions which might have been expected to occur are the elimination of 3,4-dimethylthiazolium ion from CHDT and from 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion. However, the ultraviolet spectra of an equimolar mixture of 3,4-dimethylthiazolium ion and either pyruvic acid or acetaldehyde in 0.5 and 10\(^{-2} \) N hydrochloric acid, adjusted to 1 M ionic strength with potassium chloride, exhibit \( \lambda_{\text{max}} \) at 249 nm (\( \epsilon 3500-3800 \)) and are therefore substantially different from that of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium chloride. Consequently, on the basis of the product spectra, as well as on the basis of direct isolation of the product (see Experimental Section) and the nmr spectra of the product (see below), we conclude that these two reactions did not occur to any significant extent.

Under all the conditions which we used the decarboxylation was first order for at least two half-times. The dependence of the observed first-order rate constants for decarboxylation (\( k_{\text{obsd}} \)) upon the acidity of the medium (shown in Figure 5) indicates that the species of CHDT reactive in decarboxylation is that in which the carboxyl group is ionized. In this case, the rate law for decarboxylation is

\[
-k_{\text{obsd}}(\text{CHDT})_{\text{total}} = k(\text{CHDT}^\pm) = \frac{kK_a'}{K_a' + (H_3O^+)}(\text{CHDT})_{\text{total}} = k_{\text{obsd}}(\text{CHDT})_{\text{total}}
\]

so that

\[
\frac{1}{k_{\text{obsd}}} = \frac{(H_3O^+)}{kK_a'} + \frac{1}{k}
\]

where \( k \) is the rate constant for decarboxylation of CHDT\(^\pm \), the species in which the carboxyl group is ionized (a zwitterion); \( K_a' \) is the apparent acid dissociation constant of the carboxyl group; and \( (\text{CHDT})_{\text{total}} \) is the total concentration of CHDT. When the data in Figure 5 were plotted according to eq 4, we obtained...
a straight line which yielded values of \(1.45 \times 10^{-3}\) min\(^{-1}\) and \(5.0 \times 10^{-2}\) \(M\) for \(k\) and \(K_a'\) at 67° and 1 \(M\) ionic strength. The solid curve in Figure 5, which was calculated from eq 3 with these values for \(k\) and \(K_a'\), shows good agreement with the experimental points, except at the two highest pH values. Further verification of the rate law is the finding that the value of \(K_a'\) determined by the spectrophotometric titration of CHDT (4.9 \(\times 10^{-2}\) \(M\)) is the same as that given by the kinetics. It is unnecessary to make a correction for the fact that the value of \(pK_a'\) from the spectrophotometric titration refers to 20–25°, since the dissociation constants of carboxylic acids are nearly independent of temperature. For example, the \(pK\) of the carboxyl group of glycine is 2.36 at 20° and 2.32 at 55°, 21

When the rate measurements were made, we observed that for runs made at pH values above 6.3 the optical density continued to fall slowly long after decarboxylation should have been complete, and dropped considerably below the calculated final value. The rate constants in these cases were calculated by using the final optical density expected in the absence of other reactions. This treatment probably explains the small positive deviations of the rate constants at pH 6.8 and 7.3 from the plateau value in Figure 5. A control experiment was carried out by maintaining a 1.72 \(\times 10^{-4}\) \(M\) solution of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium chloride in 0.025 \(M\) potassium phosphate buffer, pH 7.3, at 67°. No shift in \(\lambda_{max}\) from 255 nm occurred, but the optical density at 255 nm dropped from 0.93 to 0 over a period of 8 days. This slow disappearance of the thiazolium compound is probably due to base-catalyzed opening of the ring22 and subsequent hydrolytic reactions of the product.

The values of \(k_{obsd}\) for the decarboxylation of CHDT in \(10^{-3}\) \(N\) hydrochloric acid and at 1 \(M\) ionic strength were determined spectrophotometrically at five temperatures over the range from 45 to 67°. Since the carboxyl group is about 98% ionized at this concentration of hydronium ion, these rate constants are, within a few per cent, those for the decarboxylation of the zwitterion species of CHDT. The activation parameters for the reaction were calculated from the linear Arrhenius plot according to the equations of transition state theory23 and have the following values at 25°:

\[
\Delta H^\ddagger = +31.2 \text{ kcal/mole; } \Delta S^\ddagger = +16 \text{ cal/deg mole.}
\]

As a check upon the spectrophotometric method, the decarboxylation of CHDT was also examined by means of nmr spectroscopy. Figure 6 illustrates the results with data from the reaction in 1.15 \(N\) HCl at 67°. Upon decarboxylation in water, the singlet due to the side chain methyl group of CHDT is replaced by an upfield doublet; this signal is split because of the adjacent hydrogen atom in the product. Also, the doublet at \(\delta 2.50\) from the methyl group at carbon 4 is shifted very slightly upfield in the product (Figure 6). When the decarboxylation was carried out in deuterium oxide, this region of the spectrum was the same except for the fact that the signal due to the side chain methyl group of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion appeared as a singlet rather than a doublet because the carboxyl group had been replaced by a deuterium atom rather than a hydrogen atom. Table I presents the rate constants for the decarboxylation of CHDT obtained from the nmr measurements. These constants are in reasonable agreement with those obtained by the spectrophotometric method (see text above and Figure 5). The rate constants obtained in acetate buffer in a pH range where the carboxyl group of CHDT is completely ionized show that there is little or no solvent deuterium isotope effect upon the rate of decarboxylation. Consequently, the solvent isotope effect of 2.3 observed in 1.15 \(N\) acid must be the result of an isotope effect of 2.3 upon \(K_a'\) (see eq 3). This conclusion is in agreement with the fact that the ionization constants of carboxylic acids in \(H_2O\) have been found to be 2.5–3.2 times larger than the values in \(D_2O\). 24

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(22) G. D. Maier and D. E. Metzler [ibid., 79, 4386 (1957)] describe this reaction with thiamine.
Decarboxylation of CHDT in Ethanol–Water Mixtures and Dimethyl Sulfoxide. Complete spectra, taken at various times during the reaction of CHDT in 90% (v/v) ethanol–water buffered with acetate buffer, showed an isosbestic point near 254 nm and demonstrated the quantitative formation of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion. The kinetics of decarboxylation in all the ethanol–water mixtures were first order; Table II gives the values of \( k_{\text{obsd}} \). Note that the values of \( k_{\text{obsd}} \) in 50 and 90% (v/v) ethanol–water are not changed by varying the buffer ratio tenfold. These findings show that CHDT was completely ionized in the ethanol–water acetate buffers which were used and consequently that all the values of \( k_{\text{obsd}} \) in Table II are values for the decarboxylation of the enamine has purposely been written with the hydroxyl group and nitrogen cis to one another, since an examination of Ealing Corp. CPK models shows that in the other geometrical isomer there is considerable steric interaction between the N-methyl group and the vinylic methyl group.

A lower estimate of the rate constant for the protonation of the enamine by H\(_2\)O can be made in the following way. Mieyal, et al., have determined by nmr spectroscopy that the \( \alpha \)-hydrogen atom of 2-(1-hydroxyethyl)thiamine (see IIa) undergoes exchange with the deuterium atoms of D\(_2\)O and that the first-order rate constant for the exchange at pD 8.4 and 50° is 0.14 hr\(^{-1}\). Under these conditions the predominant pathway for exchange is almost certainly the abstraction of the \( \alpha \)-hydrogen atom by deuteroxide ion followed by deuteration of the enamine by D\(_2\)O (the second reaction in eq 5, B\(^-\) = OD\(^-\)). Since no enamine was detected by the nmr spectroscopy even when the exchange was 80% complete, the position of equilibrium between the thiazolium salt and the neutral enamine must lie far in the direction of the thiazolium salt at pD 8.4. Consequently, the rate-determining step in the exchange reaction is abstraction of the hydrogen atom by deuteroxide ion, and the pseudo-first-order rate constant for deuteration of the enamine by D\(_2\)O must be at least five times greater than the observed first-order rate constant of 0.14 hr\(^{-1}\). This minimal estimate of 0.7 hr\(^{-1}\) is itself ten times larger than the first-order rate constant for the decarboxylation of the CHDT zwitterion at 50° (0.072 hr\(^{-1}\)). Moreover, at the acidic pH values at which the decarboxylation of CHDT was carried out, we expect protonation of the enamine by the dipolar ion species in dimethyl sulfoxide may be even larger. The rate acceleration relative to water as the solvent is at least 10\(^4\). Jurch and Ramey have reported that 4-pyridylacetic acid hydrochloride decarboxylates in dimethyl sulfoxide with a half-time of 4.1 hr at 30°, but is stable in D\(_2\)O for 24 hr at 90°.

**Discussion**

**Mechanism of Decarboxylation.** The kinetic study of the decarboxylation of CHDT clearly shows that the zwitterion is the species which decarboxylates. The most likely mechanism for the reaction is decarboxylation to yield as the initial product the planar neutral enamine, which is protonated in a subsequent rapid reaction (eq 5). In this mechanism the structure of the

\[
\begin{align*}
\text{HO-} & \quad \text{CH}_3 \\
\text{HO-} & \quad \text{CH}_3
\end{align*}
\]

\[\text{HB} = \text{H}_2\text{O}^+, \text{H}_2\text{O}, \text{or a buffer acid}\]

\[\text{HB} = \text{H}_2\text{O}^+, \text{H}_2\text{O}, \text{or a buffer acid}\]

zwitterionic species (rate constant, \( k \), in eq 3). The first-order rate constant for the decarboxylation of CHDT\(^*\) in water at 26° and 1 M ionic strength can be estimated from the Arrhenius plot; its value is 2 \( \times 10^{-2} \) min\(^{-1}\). Consequently, decarboxylation occurs 9000 times more rapidly in absolute ethanol with 1 M lithium chloride than in water. Furthermore, reduction of the ionic strength in absolute ethanol from 1.0 to 0.025 M causes a 12-fold increase in the rate.

An attempt was made to follow the decarboxylation of CHDT in dimethyl sulfoxide-\(d_6\) by nmr spectroscopy. However, during the preparation of a 0.2 M solution of CHDT chloride in dimethyl sulfoxide-\(d_6\) at room temperature there was immediate evolution of gas; and the nmr spectrum, which was taken within 3 min of dissolution, was identical with that of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion. Consequently, \( k_{\text{obsd}} \) under these conditions must be greater than 0.7 min\(^{-1}\). Since probably only a small fraction of the carboxylic acid was ionized in dimethyl sulfoxide,\(^{25}\) the specific rate constant for decarboxylation of


hydronium ion, rather than by water, to be the predominant reaction. Consequently, at an acid pH value the first-order rate constant for protonation might be considerably greater than the minimal estimate of 0.7 hr⁻¹. This analysis shows, in agreement with our results, that the spectrophotometric detection of the enamine intermediate during decarboxylation should not be possible.

The mechanism of decarboxylation of CHDT closely resembles that proposed for the decarboxylation of 2-methyl-2-(2-pyridyl)butyric acid (III). The fact that this compound decarboxylated more rapidly in neutral aqueous solution than in strong acid or base suggested that the zwitterion was the reactive species, and the intermediacy of a planar enamine was clearly shown by the finding that decarboxylation of optically active III yielding racemic 2-s-butylpyridine.

The enormous increases that occur in the rates of decarboxylation of the CHDT dipolar ion upon change of the solvent from water to ethanol and to dimethyl sulfoxide show that the transition state has a structure in which the charge separation is considerably less than in the reactant zwitterion. A further analysis of these solvent effects in terms of the solvent effect upon the solvation of the reactant and of the transition state requires values for the solvent activity coefficients of CHDT± in ethanol and dimethyl sulfoxide with reference to water, which have not been determined. However, on the basis of solvent activity coefficients that have been reported for acetate ion, tetraalkylammonium ions, and uncharged polar organic compounds, it seems likely that the carboxylate group of the reactant is less strongly solvated in ethanol and much less strongly solvated in dimethyl sulfoxide than in water, that the thiazolium portion may actually be more strongly solvated in ethanol and dimethyl sulfoxide, and that the transition state is solvated more strongly in the nonaqueous solvents than in water.

Comparison with Pyruvate Decarboxylase. The evidence presented in the beginning of this article indicates that 2-(1-carboxy-1-hydroxyethyl)thiamine pyrophosphate (Ia) is the intermediate which undergoes decarboxylation in the pyruvate decarboxylase reaction. In addition to that evidence, Holzer and Beaucamp have reported that small amounts of radioactive Ia can be isolated from reaction mixtures containing pyruvate-¹⁴C or -²¹⁴C and pyruvate decarboxylase at pH 6 by paper chromatography of the methanolic extracts obtained upon treating the reaction mixtures with methanol at 55-60°C. However, since the half-time for decarboxylation of CHDT± is only 3 min in ethanol at 26°C, the compound which Holzer and Beaucamp isolated may not have been Ia. The finding that carbon dioxide, rather than bicarbonate, is the initial product in enzymatic thiamine pyrophosphate-dependent decarboxylations of α-keto acids is in agreement with the mechanism expected on the basis of our model.

In order to make a valid comparison with pyruvate decarboxylase, it is necessary to estimate the non-enzymic rates of decarboxylation of Ia (species with the carboxyl group ionized) on the basis of the rates of decarboxylation of CHDT±. Ia would be expected to decarboxylate somewhat more rapidly than CHDT± because the (4-amino-2-methyl-5-pyrimidinyl)methyl group, especially in its protonated form (pK_a ~ 5.5), is more electron withdrawing than a methyl group. However, two lines of reasoning suggest that this inductive effect does not make the rate of decarboxylation of Ia more than about 50 times that of CHDT±. First, a rate constant for the decarboxylation of Ia can be estimated from the rate constants for the decarboxylation of acetocetate anion in water

\[
k = 21 \times 10^{-6} \text{ min}^{-1} \text{ at } 25^\circ \text{C},
\]

for the abstraction of each α-hydrogen atom from acetone by hydroxide ion

\[
k = 1.7 M^{-1} \text{ min}^{-1} \text{ at } 25^\circ \text{C},
\]

and for the enamionization of 2-(1-hydroxyethyl)thiamine by deuteroxide ion (see above, k ~ 5 \times 10^6 M^{-1} \text{ min}^{-1} \text{ at } 50^\circ \text{C}), if one assumes that the ratio of the rate constant for decarboxylation to that for proton abstraction is the same in both cases. The value of this rate constant for the decarboxylation of Ia (pyrimidine group not protonated) in water at 50°C is 6 \times 10^{-3} \text{ min}^{-1}, which is 50 times larger than the value of 1.2 \times 10^{-3} \text{ min}^{-1} found for CHDT±. Second, the magnitude of the inductive effect can be estimated by comparing the rates of ionization of the hydrogen atom on carbon 2 of thiamine pyrophosphate and 3,4-dimethylthiazolium ion (eq 6). This reaction

should also be facilitated by inductive electron withdrawal into the substituent bonded to the quaternary nitrogen atom. According to Ulirich and Mannsbeck, the half-time for the exchange with deuterium of the hydrogen atom on carbon 2 of 0.2 M thiamine pyrophosphate in D₂O is 4.5 min at 38°C and pD 5.0, a pD value at which approximately half of the thiamine pyrophosphate is protonated on the pyrimidine ring. Haake, et al., have reported that the half-time for the corresponding reaction of 3,4-dimethylthiazolium ion in D₂O is 96 min at 33°C and pD 5.0. Consequently, the inductive effect in this reaction is about a factor of 15.

quently, pyruvate decarboxylase accelerates the de-

The factor may well be considerably greater than this

enzymic reaction could occur through interaction of

phosphate is isolated from mixtures of the holoenzyme

Ia at 30° is 2 \times 10^{-8} \text{ min}^{-1}. The turnover number of

highly purified pyruvate decarboxylase is 2340

moles of pyruvate per minute per mole of bound thia-

mine pyrophosphate at 30° and pH 6.0. Consequently, pyruvate decarboxylase accelerates the decarboxylation of Ia by a factor of at least 10^{6}-10^{9}. The factor may well be considerably greater than this value because the rate-determining step in the enzymic reaction is probably not the decarboxylation of bound Ia. The fact that 2-(1-hydroxyethyl)thiamine pyrophosphate at 30° and pH 6.0,37 consequently, pyruvate decarboxylase accelerates the decarboxylation of Ia by a factor of at least 10^{6}-10^{9}. The factor may well be considerably greater than this value because the rate-determining step in the enzymic reaction is probably not the decarboxylation of bound Ia. The fact that 2-(1-hydroxyethyl)thiamine pyrophosphate is isolated from mixtures of the holoenzyme and pyruvate suggests that the release of acetaldehyde from this bound intermediate is rate determining.

Catalytic Mechanism. The increase in the rate of decarboxylation of CHDT\textsuperscript{=} in ethanol and dimethyl sulfoxide is almost as large as the factor which we have estimated for enzymic catalysis. Thus, catalysis in the enzymic reaction could occur through interaction of the thiazolium ring of thiamine pyrophosphate with a region of the enzyme less polar than water so that either the carboxylate anion and the thiazolium cation of Ia would be more weakly solvated than in water and/or the transition state for decarboxylation and the enamine would be more strongly solvated than in water. The fact that thiamine pyrophosphate binds strongly to pyruvate decarboxylase apoenzyme (in the presence of magnesium ions) is not in conflict with this hypothesis: although the interaction between the thiazolium ring and the enzyme might be unfavorable, this could be more than compensated by strong associations of the pyrimidine group, at one end of the molecule, and of the pyrophosphate–magnesium group, at the other end of the molecule, with the protein.

The following information from the literature is in agreement with the suggestion that the active site of pyruvate decarboxylase is hydrophobic.

(1) 2-p-Toluidinylnapthalene-6-sulfonate is a dye that fluoresces strongly only in a lipophilic environment.


Its structure somewhat resembles that of thiamine pyrophosphate, except for the positive charge of the thiazolium nucleus. Ullrich has recently reported that this dye binds to apodecarboxylase, that it fluoresces upon binding, that half the bound dye is displaced from the enzyme by thiamine pyrophosphate and magnesium, and that the other half is displaced by pyruvate.38

(2) Wittorf and Gubler have found that the fluorescence spectrum of thiochrome pyrophosphate (IV) changes upon binding to apodecarboxylase in the same way that it changes upon transfer to solvents of decreasing dielectric constant.39 Also, these authors state that tetrahydrothiamine pyrophosphate (V) is a potent inhibitor of the binding of thiamine pyrophosphate to apodecarboxylase. The reason may be that the binding of this compound lacks the unfavorable interaction which occurs between the protein and the positively charged thiazolium ring of thiamine pyrophosphate.

The hypothesis of an enzymic solvent effect as the basis for the enzymic catalysis of the decarboxylation of Ia requires a consideration of the effects that an environment less polar than water would have upon the other steps of the pyruvate decarboxylase reaction. Figure 7 presents a mechanism for the entire reaction that is consistent with previous model and enzymic studies. The first step has purposely been written as a direct proton transfer between the carboxylate anion and carbon 2 of the thiazolium ring rather than proton transfer \textit{via} other acid–base groups of the enzyme in order to emphasize the hypothesis of an enzymatic solvent effect. The carbonyl acid and the ylide are less polar than the carboxylate anion and the thiazolium ring; thus, a hydrophobic environment should accelerate the proton transfer to the carboxylate anion and shift the equilibrium in the direction of the ylide, relative to the case in water. Since the pK_a of pyruvic acid is 2.5 and the pK_a of the ionization of the hydrogen at carbon 2 of the thiazolium ring has been estimated to be between 14 and 20,10,37 the fractions of un-ionized pyruvic acid and of the ylide are very small in neutral aqueous solution.

In the second step of the mechanism it is advantageous to have the ylide attack pyruvic acid rather than pyruvate anion because the greater electron-withdrawing effect of the COOH group should make the keto function of the acid more reactive. Also, the carbonylic acid may catalyze the condensation through the transfer of its proton to the keto oxygen before or simultaneously with the attack of the ylide. Figure 7 presents the alternative formulation in which the proton

Figure 7. A mechanism for the pyruvate decarboxylase reaction.

transfer occurs after the condensation. Since the product of the condensation is a dipolar ion, the rate of the condensation reaction may be decelerated in a hydrophobic environment relative to the rate in water. However, the reaction of carbanions with carbonyl compounds in water is known to be very rapid. For example, the second-order rate constant for the reaction of cyanide with acetaldehyde in water at 25° is $5 \times 10^4 \, M^{-1} \text{min}^{-1}$, and cyanide ion is probably a much weaker nucleophile in water than the ylide ($pK_a$ of HCN is 9.4). Thus, it seems likely that the condensation would still occur rapidly enough in a medium less polar than water to allow the enzymatic rate.

The tautomerization of the enamine, resulting from decarboxylation, to the dipolar ion is presented as a proton transfer reaction from the adjacent hydroxyl group (Figure 7); it may occur directly or with the participation of another base. Schellenberger has summarized evidence which indicates that thiamine pyrophosphate is bound to pyruvate decarboxylase in a conformation that places the 4′-amino group of the thiazolium ring; thus, the $pK_a$ for ionization of the hydroxyl group of 2-(1-hydroxyethyl)thiamine pyrophosphate in water is probably about 12. The $pK_a$ for dissociation of the $\alpha$-hydrogen atom of 2-(1-hydroxyethyl)thiamine pyrophosphate to form the enamine has not been determined. A crude estimate of its value ($pK_a = 17$) is obtained by assuming that the rate constant ($k_{OH^-}$ for abstraction of this hydrogen atom by deuteroxide ion (see above, ref 27) is related to the $pK_a$ by the same linear free energy relationship that relates the rate constants for the corresponding reaction of a series of ketones to the $pK_a$’s for ionization of these carbon acids. These $pK_a$ values of 12 and 17 show that in water the proton transfer from the hydroxyl group to the $\alpha$ carbon if favored thermodynamically. The crude estimate that $pK_a$ equals 17 also allows an estimate of the rate constant ($k_{H2O}$) for protonation of the enamine at carbon by water, since $k_{H2O} = k_{OH^-}(K_w/K_a)(155.5)$, where $K_w$ is the ion product of water. The value of $k_{H2O}$ is roughly $5 \times 10^4 \, M^{-1} \text{min}^{-1}$ at 50°.

In less polar surroundings the position of equilibrium for this tautomerization would be shifted toward the enamine and the rate of formation of the dipolar ion from the enamine would be slower. However, the large rate constant for protonation of the enamine by water suggests that the tautomerization reaction would still occur rapidly enough in a less polar medium.

The final step in the mechanism for pyruvate decarboxylase is elimination of the ylide from the alcoholate anion of 2-(1-hydroxyethyl)thiamine pyrophosphate (Figure 7). Since 2-(1-hydroxyethyl)thiamine pyrophosphate is stable in deuterium oxide at pH 8.6 for 2 hr at $60^\circ$, the release of acetaldehyde from this compound is a reaction that must be strongly catalyzed by pyruvate decarboxylase. This reaction should occur more rapidly in a hydrophobic medium for two reasons. First, in water at neutral pH the concentration of the dipolar ion shown in Figure 7, which is presumably the reactive species, will be very small, since the $pK_a$ for dissociation of the proton from the hydroxyl group is about 12. The concentration of the dipolar ion would be larger on the enzyme in aqueous solution at neutral pH because the dipolar ion is more stable than a unit positive charge in a region of lower dielectric constant. Second, the dipolar ion will eliminate more rapidly in the nonpolar environment than in water, since there is less charge separation in the transition state than in the reactant. An indication of the magnitude of the catalysis that can occur in this reaction is given by recent results on the loss of ethyl pyruvate from 2-(1-carbethoxy-1-hydroxyethyl)-3,4-dimethylthiazolium ion (eq 7). The second-order rate constant ($v = k_{[\text{thiazolium}^+] \text{[enamine]}}$) for abstraction of this hydrogen atom by deuteroxide ion (see above, ref 27) is related to the $pK_a$ by the same linear free energy relationship that relates the rate constants for the corresponding reaction of a series of ketones to the $pK_a$’s for ionization of these carbon acids. These $pK_a$ values of 12 and 17 show that in water the proton transfer from the hydroxyl group to the $\alpha$ carbon is favored thermodynamically. The crude estimate that $pK_a$ equals 17 also allows an estimate of the rate constant ($k_{H2O}$) for protonation of the enamine at carbon by water, since $k_{H2O} = k_{OH^-}(K_w/K_a)(155.5)$, where $K_w$ is the ion product of water. The value of $k_{H2O}$ is roughly $5 \times 10^4 \, M^{-1} \text{min}^{-1}$ at 50°. In less polar surroundings the position of equilibrium for this tautomerization would be shifted toward the enamine and the rate of formation of the dipolar ion from the enamine would be slower. However, the large rate constant for protonation of the enamine by water suggests that the tautomerization reaction would still occur rapidly enough in a less polar medium.

The interactions between thiamine pyrophosphate and the pyruvate decarboxylase protein will only become precisely known through X-ray crystallography. Sable, et al., have found that thiamine and indole compounds form a weak complex in aqueous solution and, on this basis, have suggested that such an interaction may occur between thiamine pyrophosphate and the tryptophanyl residues of apoenzymes. However, the rate of ionization of the hydrogen at carbon 2 of thiamine is slower for the complex than for thiamine itself, probably because there is charge transfer from indole to the thiazolium ring. Thus, the interaction between indole and thiamine which occurs in aqueous solution

(42) The $pK_a$ for the corresponding ionization of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion is 11.4 at 25° and 0.5 M ionic strength.
does not appear to be of a type which can account for enzymic catalysis.

Conclusion
Since most enzymic reactions in which thiamine pyrophosphate is a cofactor are mechanistically similar to the pyruvate decarboxylase reaction, our results and the above discussion are relevant to many thiamine pyrophosphate-dependent enzymic reactions.


Studies on Polypeptides. XLIV. Potent Synthetic S-Peptide Antagonists
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Abstract: The concept that chemical modification of the "active" amino acid in a biologically active peptide may provide a rational approach to the discovery of competitive antagonists was tested with the S-peptide-S-protein system. A series of histidine modified analogs of S-peptide was prepared and tested for ability to compete with natural S-peptide for S-protein. 

1-(pyrazolyl)alanine, 1,3-dicarboxymethylhistidine, N-formylornithine, and ornithine proved to be potent competitive antagonists of S-peptide. The d-sulfoxides of 1-(pyrazolyl)-alanine and of 3-carboxymethylhistidine were considerably less effective inhibitors than the corresponding peptides containing methionine. 1-Carboxymethylhistidine 3-peptide was shown to possess the ability to activate S-protein with formation of active enzyme but only at high molar peptide to protein ratios. 1,3-Dicarboxymethylhistidine 3-peptide, was inactive. The replacement of arginine by ornithine in 1-(pyrazolyl)-alanine and 3-carboxymethylhistidine 3-peptide-14 significantly weakened the ability to antagonize S-peptide. The corresponding N-formylornithine derivatives were as effective as the peptides containing ornithine. Unequivocal synthetic routes to the abovementioned peptides are described.

Structure-function studies with synthetic S-peptide analogs and fragments have led us to conclude that histidine is the catalytically active amino acid residue in enzymes.

2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation. Mr. Visser's participation in this investigation was made possible through support from the Pittsburgh Plate Glass Foundation program in International Education.
4) The amino acid residues are of the L configuration. Abbreviations used are: 1-CMHis = 1-carboxymethylhistidine; 3-CMHis = 3-carboxymethylhistidine; 1,3-DiCMHis = 1,3-dicarboxymethylhistidine; Pyr(3)ala = 1-(pyrazolyl)-3-alanine; FOM = N-formylornithine.
5) thine; NArg = nitroarginine; Met = methionine d-sulfoxide; B-M-W = 1-butanol-methanol-water, 1:1:1; B-M-1N AcOH = 1-butanol-water-1N acetic acid, 1:1:1, etc.; P-M-W = 2-propanol-methanol-water, 1:1:1; P-M-2% AcOH = 2-propanol-methanol-2% acetic acid, 1:1:1, etc; Boc = t-butoxycarbonyl; t-Bu = t-butyl ester; Z = benzoxycarbonyl; TEA = triethylamine; TFA = trifluoroacetic acid; DME = dimethylformamide; DCC = dicyclohexycarbodiimide; AG 1-X2 = anion-exchange resin (Bio-Rad); tlc = thin layer chromatography; AP-M = aminopeptidase M (G. Pfeiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determan, and G. Braunitzer, Biochem., 340, 552 (1966)); (5) F. M. Richards, Proc. Natl. Acad. Sci. U. S., 44, 162 (1958); RNase S, subtilisin-modified ribonuclease A; S-peptide, the peptide obtained from RNase S; S-protein, the protein component obtained from RNase S; RNase S', the reconstituted enzyme obtained by mixing equimolar proportions of S-protein and S-peptide. According to M. S. Doscher and C. H. W. Hirs, Biochemistry, 6, 304 (1967), natural S-peptide is a mixture of at least three components, very likely S-peptide and the rest of the molecule functions as a vehicle to bring this histidine into the correct stereochemical position in the active site of the ribonuclease S molecule. In addition to its role in catalysis, the histidine residue may also contribute to binding. We are investigating analogs of S-peptide since this compound is equivalent to natural S-peptide as concerns activation of S-protein.
6) We reasoned that if these interpretations were correct and if histidine 12 did not contribute significantly to binding it should be possible to discover competitive inhibitors to S-peptide via histidine substitutions in S-peptide.
7) To test this prediction experimentally we synthesized Pyr(3)ala 12 S-peptide-14 (XIV) (Scheme I) and explored its ability to compete with S-peptide for S-protein with RNA as the substrate. This analog was selected because the molecular dimensions of Pyr(3)ala are very similar if not identical with those of histidine. The two amino acids differ markedly as concerns the acid-base properties of the ring portions of their molecules.
8) Carboxymethylation has provided significant information pertaining to the active site of pancreatic ribonuclease A. The enzyme is rapidly inactivated.

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