

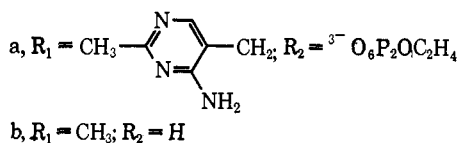
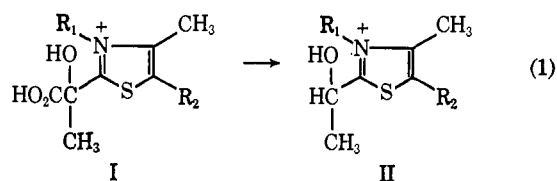
Mechanisms of Thiamine-Catalyzed Reactions. Decarboxylation of 2-(1-Carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium Chloride^{1a}

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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 (IIa). 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 10⁶. 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.² On the basis of investigations of nonenzymatic model reactions, Breslow³ 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



IIa was isolated from reaction mixtures which contained pyruvic acid and pyruvate decarboxylase holoenzyme.⁴⁻⁶ Moreover, it was shown that pyruvate decarboxylase apoenzyme catalyzes the formation of acetaldehyde from IIa.^{4,7} Results similar to these

(1) (a) This research was supported by grants from the National Science Foundation and a fellowship to J. C. from the William F. Milton Fund of Harvard University. (b) To whom all inquiries should be addressed.

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(3) R. Breslow, *Chem. Ind.* (London), 893 (1957).

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have also been obtained for pyruvate dehydrogenase, the enzyme which catalyzes the thiamine pyrophosphate-dependent oxidative decarboxylation of pyruvic acid to acetyl coenzyme A.⁸

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 (IIb) 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-Carboxy-1-hydroxyethyl)-4-methylthiazole. *n*-Butyllithium (213 ml of a standardized⁹ 1.55 *N* solution in hexane) was added with stirring to anhydrous ether (280 ml) at -75° , under an

(7) H. W. Goedde, B. Ulrich, C. Stahlmann, and H. Holzer, *Biochem. Z.*, **343**, 204 (1965).

(8) P. Scriba and H. Holzer, *ibid.*, **334**, 473 (1961); H. W. Goedde, H. Inouye, and H. Holzer, *Biochim. Biophys. Acta*, **50**, 41 (1961); L. P. Hager and L. O. Krampitz, *Fed. Proc.*, **22**, 536 (1963).

(9) H. Gilman and A. H. Haubein, *J. Am. Chem. Soc.*, **66**, 1515 (1944).

atmosphere of argon. 4-Methylthiazole¹⁰ (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 attain room temperature and was then rapidly neutralized by pouring *into* ethanolic hydrogen chloride (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 yield a fraction (17.8 g) (bath temperature 115–145°, pressure 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 g, 10.4%): mp 41.5–43°, raised to 44.5–45.5° (from *n*-pentane); λ_{\max} (EtOH) 252 nm (ϵ 4825); nmr (CCl₄) 1.29 (3 H, t, $J = 7$ Hz), 1.80 (3 H, s), 2.39 (3 H, d, $J = 1$ Hz), 4.24 (q, $J = 7$ Hz) overlying 4.32 (s) (3 H total), 6.79 (1 H, m).

Anal. Calcd for C₉H₁₃NO₃S: C, 50.23; H, 6.09; N, 6.51. Found: C, 50.21; H, 6.16; N, 6.41.

In a second preparation a higher boiling fraction was collected and investigated (bath temperature 140–170°, pressure 0.005 mm). This material, which was initially a yellow, viscous liquid at room temperature, was cooled under isopropyl ether–hexane to yield a colorless crystalline solid (1.13 g from 12.4 g of distillate): mp 69–70°, unchanged on recrystallization from *n*-pentane; nmr (CCl₄) 1.78 (3 H, s), 2.29 (3 H, d, $J = 1$ Hz), 2.45 (3 H, d, $J = 1$ Hz), 6.3 (1 H, broad s), 6.71 (1 H, m), 7.15 (1 H, m); ν_{\max} (Nujol) 1675 cm⁻¹, which is characteristic of a conjugated ketone;¹¹ λ_{\max} (CH₃CN) 240 nm (ϵ 6400 based on mol wt 268.2) and 309.5 (7920), which approximate the sum of the spectra of 2-acetyl-4-methylthiazole (λ_{\max} 233 (4170) and 300 (6510)) and 2-(1-hydroxyethyl)-4-methylthiazole (λ_{\max} 247 (3900)) in CH₃CN.¹² These data 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.

Anal. Calcd for C₁₁H₁₅N₂O₂S₂: C, 49.25; H, 4.51; N, 10.45; S, 23.91. Found: C, 49.23; H, 4.63; N, 10.07; S, 23.78.

2-(1-Carbethoxy-1-hydroxyethyl)-3,4-dimethylthiazolium Fluoroborate. To trimethylxonium fluoroborate¹³ (618 mg) dissolved in the minimum amount of anhydrous nitromethane was added a solution of 2-(1-carbethoxy-1-hydroxyethyl)-4-methylthiazole (899 mg), also dissolved in the minimum of nitromethane. After the ensuing exothermic reaction, the mixture was set aside for 1 hr at room temperature and then evaporated to dryness at reduced pressure. The residue was washed thoroughly with anhydrous ether, covered with a layer of *n*-pentane, cooled, and agitated until it solidified. Filtration gave the thiazolium fluoroborate (1128 mg, 85%): mp 63.5–66°, raised to 71–71.5° (63%, from acetone–ether); λ_{\max} (H₂O) 216 nm (ϵ 3080), 260.5 (6390); nmr (D₂O) 1.20 (3 H, t, $J = 7$ Hz), 2.01 (3 H, s), 2.50 (3 H, d, $J = 1$ Hz), 3.95 (3 H, s), 4.29 (2 H, q, $J = 7$ Hz), 7.75 (1 H, m).

Anal. Calcd for C₁₀H₁₄BF₃NO₃S: C, 37.87; H, 5.09; N, 4.42; S, 10.11. Found: C, 37.86; H, 5.18; N, 4.42; S, 10.25.

2-(1-Carbethoxy-1-hydroxyethyl)-3,4-dimethylthiazolium Chloride. The corresponding thiazolium fluoroborate (317 mg, 1.0 mmole) in 2.5 ml of water was applied to a column of Bio-Rad anion exchange resin (AG 1-X8, 50–100 mesh, chloride ion form, 3.6 ml, 5 mequiv) and eluted with water (40 ml). The eluate was evaporated under reduced pressure to give the thiazolium chloride (181 mg, 67%), mp 107–111° (highly dependent on the rate of heating; 2.8°/min in this case). The ultraviolet and nmr spectra of this compound are identical with those of the fluoroborate salt.

Anal. Calcd for C₁₀H₁₄ClNO₃S: C, 45.18; H, 6.07; N, 5.27; S, 12.06. Found: C, 44.99; H, 6.11; N, 5.23; S, 11.95.

2-(1-Carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium Chloride (CHDT Chloride). The corresponding ethyl ester (399 mg, 1.5 mmoles) was dissolved in 37% hydrochloric acid (6 ml) and maintained at 83° for 18 min. The solution was cooled and evaporated to dryness under reduced pressure. The residue was dissolved in 3 *N* ethanolic hydrochloric acid and ether was added to precipitate the product (331 mg): mp 60–63° dec, raised to 80–85° dec (35%, small colorless crystals from 1:3 mixture of 3 *N* ethanolic hydro-

chloric acid–ether); λ_{\max} (H₂O) 212 nm (ϵ 2750), 262 (6410); nmr (D₂O), 2.00 (3 H, s), 2.50 (3 H, d, $J = 1$ Hz), 3.99 (3 H, s), 7.74 (1 H, m); these signals are assigned to the methyl group of the CH₂C(OH)COOH function, the methyl group at carbon 4, the N-methyl group, and the hydrogen atom at carbon 5, respectively, by reference to the nmr spectrum of 3,4-dimethylthiazolium iodide. Hafferl, *et al.*,¹⁴ have shown for this compound that the chemical shifts of the methyl group at carbon 4, the N-methyl group, and the hydrogen atom at carbon 5 fall at 2.57 (d, $J = 1$ Hz), 4.14 (s), and 7.82 (m), respectively, in D₂O.

Anal. Calcd for C₈H₁₂ClNO₃S: C, 40.42; H, 5.09; N, 5.89; S, 13.49. Found: C, 40.36; H, 5.30; N, 5.80; S, 13.59.

2-(1-Hydroxyethyl)-3,4-dimethylthiazolium Chloride. This compound was prepared from 2-(1-hydroxyethyl)-4-methylthiazole by the procedure described earlier:¹⁵ nmr (D₂O) 1.61 (3 H, d, $J = 6.5$ Hz), 2.50 (3 H, d, $J = 1$ Hz), 3.95 (3 H, s), 5.47 (1 H, q, $J = 6.5$ Hz), 7.67 (1 H, m).

3,4-Dimethylthiazolium Chloride. A 40-ml sample of 1 *M* 3,4-dimethylthiazolium iodide¹⁶ in water was applied to a column (30 cm × 7.0 cm²) of Bio-Rad AG 1-X8, 50–100 mesh, anion exchange resin in the chloride ion form. Elution with water followed by evaporation of the eluent gave the chloride as colorless, hygroscopic crystals: mp 183–185° (raised to 184–185° from acetone–ethanol); nmr (D₂O) 2.51 (3 H, d, $J = 1 \pm 0.3$ Hz), 4.08 (3 H, s), 7.74 (1 H, m), 9.70 (less than 1 H; this signal disappeared completely in 12 min because the hydrogen at carbon 2 exchanged rapidly with solvent deuterium¹⁷).

Anal. Calcd for C₆H₈ClNS: C, 40.14; H, 5.39; Cl, 23.67; N, 9.37; S, 21.43. Found, after drying *in vacuo* over P₂O₅: C, 39.79; H, 5.64; Cl, 23.78; N, 9.52; S, 20.71.

Product Analysis. CHDT chloride (48.0 mg) in 0.01 *N* hydrochloric acid (25 ml) was maintained at 67° for 10 hr. Kinetic data (see below) indicate that complete reaction occurs in 8 hr under these conditions. The mixture was evaporated to dryness, under reduced pressure, to yield 2-(1-hydroxyethyl)-3,4-dimethylthiazolium chloride as colorless crystals (36.65 mg, 94%): mp 168–171°, mmp 167–172°: the nmr spectrum was identical with that of the authentic compound.

Reaction of 2-(1-Carbethoxy-1-hydroxyethyl)-4-methylthiazole with Iodomethane. The ester (253 mg) and iodomethane (2 ml) were heated for 6 hr at 105° in a sealed tube. An oil started to separate after 10 min and had solidified after 2 hr. The tube was cooled, opened, the contents were added to anhydrous ether (50 ml). The solid which was filtered off (109 mg) was identified as 3,4-dimethylthiazolium iodide by its melting point (116–118° (lit.¹⁶ mp 117–118°)) and nmr spectrum in D₂O.

Spectrophotometric Titration of CHDT Chloride. A single, freshly prepared stock solution of the thiazolium chloride (2.42 × 10⁻³ *M*) in 10⁻³ *N* HCl was used for the entire spectrophotometric titration. For each measurement a 0.50-ml aliquot of this solution was added to 4.50 ml of a solution of buffer. The stock buffer solutions were all made up to 1.00 *M* ionic strength with potassium chloride. The optical density of each solution was measured, in a 10-mm cell, at 275 nm, against a blank which was identical in every respect except for omission of the thiazolium chloride.

Kinetic Measurements. Approximately 1.5 × 10⁻⁴ *M* solutions of CHDT chloride were prepared by dissolving accurately weighed amounts of the substrate in 100 ml of buffer solution. Ionic strength was maintained throughout at 1.00 *M* by addition of potassium chloride to the aqueous buffer and lithium chloride to the ethanol–water buffers. For each kinetic run carried out in aqueous solution 5-ml aliquots of reaction mixture were placed in 14 test tubes with Teflon-lined screw-on caps. The tubes were incubated in a constant-temperature bath at 67.00 ± 0.05° for 15 min and then were withdrawn periodically, cooled in ice, and the optical density (OD) of the contents was measured at 270 nm and, in some cases, over the entire ultraviolet spectrum. Reaction tubes which were

(14) W. Hafferl, R. Lundin, and L. L. Ingraham [*Biochemistry*, 2, 1298 (1963)] obtained the nmr spectrum of 3,4-dimethylthiazolium iodide in 0.1 *N* sodium acetate buffer, pD 3.6, in D₂O and reported the chemical shifts relative to the methyl signal of the acetate buffer. We have determined that the methyl group of 0.1 *N* sodium acetate buffer, pD 3.6, in D₂O has a δ value of 2.06 relative to the external standard of tetramethylsilane in chloroform, and the chemical shifts in the text are given relative to tetramethylsilane.

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(17) P. Haake, L. P. Bausher, and W. B. Miller, *ibid.*, 91, 1113 (1969).

(10) E. L. McNelis, Thesis, Columbia University, 1960.

(11) L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," Methuen and Co., Ltd., London, 2nd ed, 1958, p 136.

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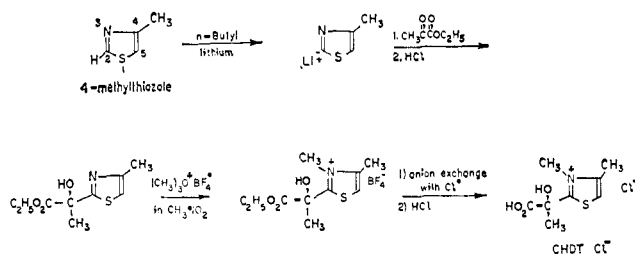


Figure 1. The synthesis of CHDT chloride.

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 $(OD_{270 \text{ nm}} \text{ at time } t - OD_{270 \text{ nm}} \text{ after complete reaction})$ vs. time by use of the equation

$$\ln \left(\frac{OD_0 - OD_\infty}{OD_t - OD_\infty} \right) = k_{\text{obsd}} t$$

An nmr method was also used to measure the rates of decarboxylation 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-carbomethoxy-1-hydroxyethyl)-4-methylthiazole with iodomethane was attempted at 105°; the only isolable product was 3,4-dimethylthiazolium iodide. A potent alkylating 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-carbomethoxy-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°)

(18) C. T. Eyles, P. Sykes, and J. E. Downes, *J. Chem. Soc.*, 4265 (1965).

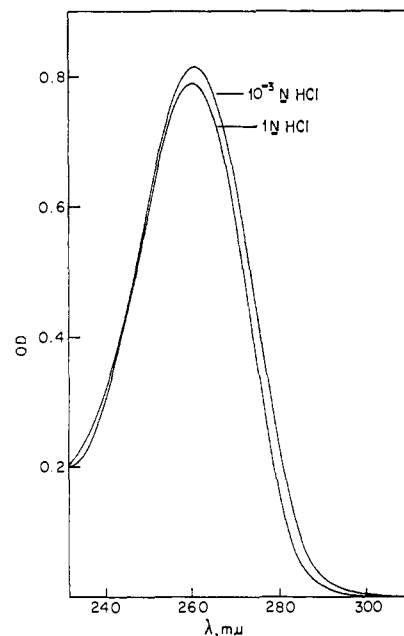


Figure 2. Ultraviolet spectra of $1.26 \times 10^{-4} M$ CHDT chloride in 1 *N* HCl and $10^{-3} N$ HCl-1 *M* KCl.

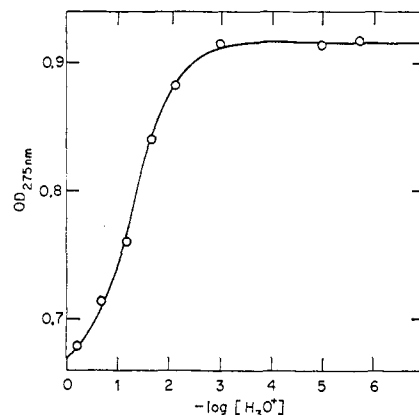


Figure 3. Spectrophotometric titration of CHDT chloride at 275 nm. The optical density of $2.42 \times 10^{-4} M$ CHDT at 20–25° is plotted as a function of the negative logarithm of the hydronium ion concentration. The buffers used in the ranges of $-\log [H_3O^+]$ were HCl, 0–2; 0.025 *M* sodium formate, 3; and 0.025 *M* sodium acetate, 5 and 6. The ionic strength was maintained with KCl at 0.9 *M*. The line through the points is calculated on the basis of an apparent *pK* of 1.31.

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 *N* hydrochloric acid has λ_{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 λ_{max} 255 nm.¹⁵ The spectrum of CHDT in $10^{-3} N$ hydrochloric acid-1 *M* potassium chloride is similar to, but not identical with, that of the compound in 1 *N* HCl: λ_{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.

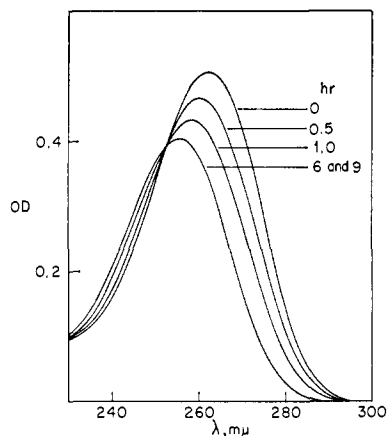


Figure 4. Spectral time course of the decarboxylation of 8×10^{-5} M CHDT chloride in 10^{-3} N HCl, at 67.0° and 1 M ionic strength.

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

$$\frac{\text{OD}_B - \text{OD}}{(\text{H}_3\text{O}^+)} = \frac{\text{OD}}{K_a'} - \frac{\text{OD}_A}{K_a'} \quad (2)$$

where K_a' is the apparent acid dissociation constant and OD_A , OD_B , and OD are the optical densities of the acid, base, and partially ionized forms, respectively.¹⁹ A plot of $(\text{OD}_B - \text{OD})/(\text{H}_3\text{O}^+)$ against OD 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, $\text{H}_3\text{N}^+\text{CH}_2\text{-CH}(\text{OH})\text{CO}_2\text{H}$, 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 λ_{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 $\text{m}\mu$, and the extinction coefficient of 2-(1-hydroxyethyl)-3,4-dimethylthiazolium ion at this wavelength (ϵ 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-di-

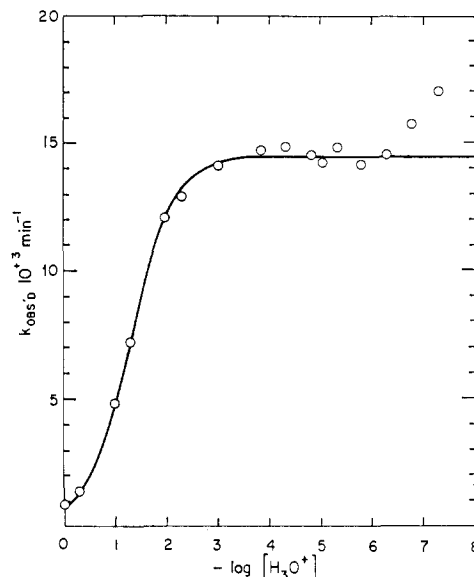


Figure 5. The dependence of the observed first-order rate constants for the decarboxylation of CHDT chloride upon the hydronium ion concentration of the reaction mixture, at 67.0° and 1 M ionic strength. The buffers used in the various $-\log [\text{H}_3\text{O}^+]$ ranges were 0-2.3, hydrochloric acid; 3, 0.025 M sodium formate; 3.8-5.3, 0.025 M sodium acetate; 5.7-7.3, 0.025 M sodium phosphate. The solid curve was calculated on the assumption that only the zwitterionic species of CHDT undergoes decarboxylation (see the text).

methylthiazolium 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^{-3} N hydrochloric acid, adjusted to 1 M ionic strength with potassium chloride, exhibit λ_{max} at 249 nm (ϵ 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_{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

$$\frac{-d(\text{CHDT})_{\text{total}}}{dt} = k(\text{CHDT}^\pm) = \frac{kK_a'}{K_a' + (\text{H}_3\text{O}^+)}(\text{CHDT})_{\text{total}} = k_{\text{obsd}}(\text{CHDT})_{\text{total}} \quad (3)$$

so that

$$\frac{1}{k_{\text{obsd}}} = \frac{(\text{H}_3\text{O}^+)}{kK_a'} + \frac{1}{k} \quad (4)$$

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

(19) V. Gold, "pH Measurements," Methuen and Co., Ltd., London, 1956, Chapter VIII.

(20) O. H. Emerson, P. L. Kirk, and C. L. A. Schmidt, *J. Biol. Chem.*, 92, 449 (1931).

a straight line which yielded values of $14.5 \times 10^{-3} \text{ min}^{-1}$ 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 $20\text{--}25^\circ$, 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 λ_{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 50° : $\Delta F^\ddagger = +26.0 \text{ kcal/mole}$; $\Delta H^\ddagger = +31.2 \text{ kcal/mole}$; $\Delta S^\ddagger = +16 \text{ cal/deg mole}$.

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 δ 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-dimethylthiazolo-

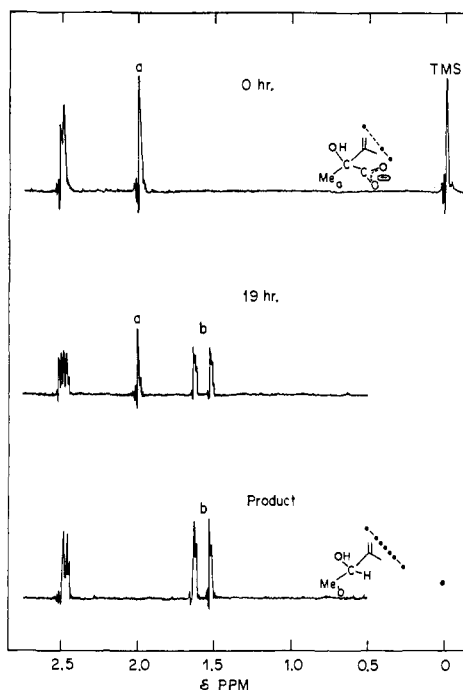


Figure 6. Nmr spectra taken during the decarboxylation of $0.17 M$ CHDT in $1.15 N$ HCl at $67.0 \pm 0.5^\circ$.

lium 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

Table I. Rate Constants for the Decarboxylation of CHDT at 67.0° ,^a Obtained by Nmr Measurements

[CHDT + Cl ⁻], M	Buffer	$10^3 k_{\text{obsd}}$, ^b min ⁻¹
0.175	1.15 N HCl-H ₂ O	0.61
0.155	1.15 N DCl-D ₂ O ^c	0.27
0.225	0.32 M NaOAc; 0.18 M HOAc; H ₂ O ^{d,e}	23
0.225	0.32 M NaOAc; 0.18 M DOAc; D ₂ O ^{e,d,f,g}	21

^a $\pm 0.5^\circ$. ^b The first-order plots of data obtained for 0–50% reaction in $1.15 N$ acid and for 0–75% reaction in the acetic acid buffers were linear, except for the case of the reaction in acetate buffer in H₂O, and yielded these rate constants. The plot for the exception showed some scatter, and the constant is not more accurate than $\pm 30\%$ of the value given. ^c 99.87 atom % deuterium. ^d $1.0 M$ ionic strength with KCl. ^e pH at 25° , 4.83. ^f Prepared from acetic anhydride and D₂O. ^g pH meter reading at 25° , 4.93.

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 H₂O have been found to be 2.5–3.2 times larger than the values in D₂O.²⁴

(24) R. P. Bell and A. T. Kuhn, *Trans. Faraday Soc.*, **59**, 1789 (1963).

(21) E. J. King, *J. Am. Chem. Soc.*, **73**, 155 (1951).

(22) G. D. Maier and D. E. Metzler [*ibid.*, **79**, 4386 (1957)] describe th's reaction with thiamine.

(23) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed, John Wiley & Sons, Inc., New York, N. Y., 1961, p 98.

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_{obsd} . Note that the values of k_{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_{obsd} in Table II are values for the decarboxylation of the

Table II. Kinetic Data for the Decarboxylation of CHDT in Ethanol-Water Mixtures

EtOH (v/v)	T, °C ±0.05°	Buffer ratio ^a	Ionic strength, ^b M	k_{obsd} , min ⁻¹
0	45.60	2.38	1.00	4.85×10^{-4}
10	45.60	2.38	1.00	8.67×10^{-4}
20	45.60	2.38	1.00	1.91×10^{-3}
30	45.60	2.38	1.00	5.13×10^{-3}
40	45.60	2.38	1.00	1.26×10^{-2}
50	45.60	0.75	1.00	2.67×10^{-2}
50	45.60	2.38	1.00	2.68×10^{-2}
50	45.60	7.55	1.00	2.61×10^{-2}
50	25.95	2.38	1.00	1.86×10^{-2}
60	25.95	2.38	1.00	3.99×10^{-3}
70	25.95	2.38	1.00	8.38×10^{-3}
80	25.95	2.38	1.00	1.78×10^{-3}
90	25.95	7.68	1.00	3.74×10^{-2}
90	25.95	2.30	1.00	3.58×10^{-2}
90	25.95	0.77	1.00	3.64×10^{-2}
100	25.95	2.42	1.00 ^c	0.180
100	25.95	2.40	0.750 ^c	0.259
100	25.95	2.40	0.500 ^c	0.424
100	25.95	2.40	0.250 ^c	0.808
100	25.95	2.40	0.025	2.18

^a The ratio [sodium acetate]/[acetic acid]; all reaction mixtures contained 0.025 M sodium acetate. ^b Adjusted with lithium chloride. ^c A small amount of sodium chloride that precipitated from these buffers was filtered off before use.

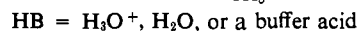
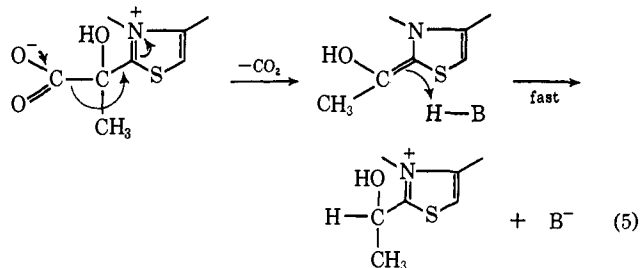
zwitterionic species (rate constant, k , in eq 3). The first-order rate constant for the decarboxylation of CHDT[±] in water at 26° and 1 M ionic strength can be estimated from the Arrhenius plot; its value is 2×10^{-5} min⁻¹. Consequently, decarboxylation occurs 9000 times more rapidly in absolute ethanol 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_{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

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 D₂O for 24 hr at 90°.²⁶

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



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 H₂O can be made in the following way. Mieyal, *et al.*, have determined by nmr spectroscopy that the α -hydrogen atom of 2-(1-hydroxyethyl)thiamine (see IIa) undergoes exchange with the deuterium atoms of D₂O and that the first-order rate constant for the exchange at pD 8.4 and 50° is 0.14 hr⁻¹.²⁷ Under these conditions the predominant pathway for exchange is almost certainly the abstraction of the α -hydrogen atom by deuteroxide ion followed by deuteration of the enamine by D₂O (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 D₂O 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

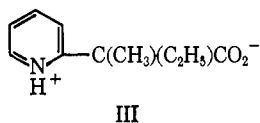
(25) The dissociation constants of carboxylic acids in dimethyl sulfoxide vary from 10⁻⁸ (acetic acid) to 20 (picric acid) times the values in water: I. M. Kolthoff, M. K. Chantooni, Jr., and S. Bhowmik, *J. Am. Chem. Soc.*, **90**, 23 (1968).

(26) G. R. Jurch, Jr., and K. C. Ramey, *Chem. Commun.*, 1211 (1968).

(27) J. J. Mieyal, R. G. Votaw, L. O. Krampitz, and H. Z. Sable, *Biochim. Biophys. Acta*, **141**, 205 (1967).

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).²⁸ The fact that this compound decarboxylated more rapidly in



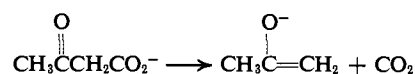
neutral aqueous solution than in strong acid or base suggested that the zwitterion was the reactive species, and the intermediacy of a planar enamine was clearly shown by the finding that decarboxylation of optically active III yielded racemic 2-*s*-butylpyridine.

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,²⁹ 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,³⁰ 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.

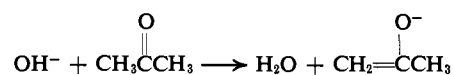
Comparison with Pyruvate Decarboxylase. The evidence presented in the beginning of this article indicates that 2-(1-carboxy-1-hydroxyethyl)thiamine pyrophosphate (Ia) is the intermediate which undergoes decarboxylation in the pyruvate decarboxylase reaction. In addition to that evidence, Holzer and Beaucamp⁵ have reported that small amounts of radioactive Ia can be isolated from reaction mixtures containing pyruvate-1-¹⁴C or -2-¹⁴C and pyruvate decarboxylase at pH 6 by paper chromatography of the methanolic extracts obtained upon treating the reaction mixtures with methanol at 55–60°. However, since the half-time for decarboxylation of CHDT[±] is only 3 min in ethanol at 26°, 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

acids³¹ is in agreement with the mechanism expected on the basis of our model.

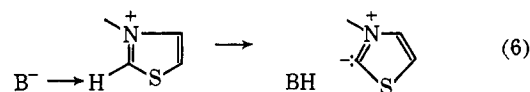
In order to make a valid comparison with pyruvate decarboxylase, it is necessary to estimate the non-enzymic rates of decarboxylation of Ia (species with the carboxyl group ionized) on the basis of the rates of decarboxylation of CHDT[±]. Ia would be expected to decarboxylate somewhat more rapidly than CHDT[±] because the (4-amino-2-methyl-5-pyrimidinyl)methyl group, especially in its protonated form ($pK_a \sim 5^{32}$), is more electron withdrawing than a methyl group.³³ 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 acetoacetate anion in water



$k = 21 \times 10^{-6} \text{ min}^{-1}$ at 25°;³⁴ for the abstraction of each α -hydrogen atom from acetone by hydroxide ion



$k = 1.7 M^{-1} \text{ min}^{-1}$ at 25°;³⁵ and for the enaminization of 2-(1-hydroxyethyl)thiamine by deuteroxide ion (see above, $k \sim 5 \times 10^3 M^{-1} \text{ min}^{-1}$ at 50°),²⁷ 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° is $6 \times 10^{-2} \text{ min}^{-1}$, 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 at carbon 2 of thiamine pyrophosphate and 3,4-dimethylthiazolium ion (eq 6). This reaction



$B^- = \text{D}_2\text{O}, \text{OD}^-$, and other bases in the reaction mixture

should also be facilitated by inductive electron withdrawal into the substituent bonded to the quaternary nitrogen atom. According to Ullrich and Mannschreck, the half-time for the exchange with deuterium of the hydrogen atom on carbon 2 of 0.2 *M* thiamine pyrophosphate in D_2O is 4.5 min at 38° and pD 5.0, a pD value at which approximately half of the thiamine pyrophosphate is protonated on the pyrimidine ring.³⁶ Haake, *et al.*, have reported that the half-time for the corresponding reaction of 3,4-dimethylthiazolium ion in D_2O is 96 min at 33° and pD 5.0.¹⁷ Consequently, the inductive effect in this reaction is about a factor of 15.

(31) R. L. Hall, B. Vennessland, and F. J. Kezdy, *J. Biol. Chem.*, **244**, 3991 (1969).

(32) D. E. Metzler in "The Enzymes," P. D. Boyer, H. Lardy, K. Myrback, Ed., Vol. 2, Part A, 1960, p 295.

(33) R. G. Yount and D. Metzler, *J. Biol. Chem.*, **234**, 738 (1959).

(34) G. Ljunggren, Dissertation, Lund, 1925; quoted by K. J. Pedersen, *J. Am. Chem. Soc.*, **51**, 2098 (1929).

(35) R. P. Bell and H. C. Longuet-Higgins, *J. Chem. Soc.*, 636 (1946).

(36) J. Ullrich and A. Mannschreck, *Biochim. Biophys. Acta*, **115**, 46 (1966).

(28) W. von E. Doering and V. Z. Pasternak, *J. Am. Chem. Soc.*, **72**, 143 (1950).

(29) A. J. Parker, *Adv. Phys. Org. Chem.*, **5**, 173 (1967).

(30) R. Alexander, E. C. F. Ko, A. J. Parker, and T. J. Broxton, *J. Am. Chem. Soc.*, **90**, 5049 (1968).

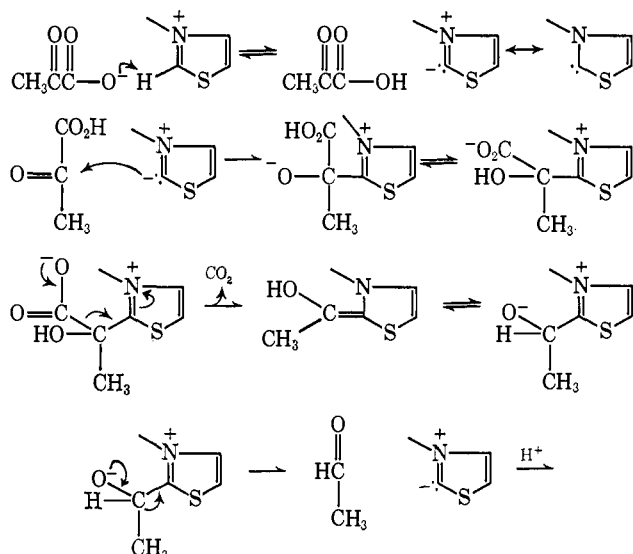


Figure 7. A mechanism for the pyruvate decarboxylase reaction.

Since the rate constant for the decarboxylation of CHDT^\pm at 30° in water, calculated from the Arrhenius plot, is $4 \times 10^{-5} \text{ min}^{-1}$, a reasonable estimate of the rate constant for the nonenzymatic decarboxylation of Ia at 30° is $2 \times 10^{-3} \text{ min}^{-1}$. The turnover number of highly purified yeast pyruvate decarboxylase is 2340 moles of pyruvate per minute per mole of bound thiamine pyrophosphate at 30° and pH 6.0.³⁷ Consequently, pyruvate decarboxylase accelerates the decarboxylation of Ia by a factor of at least 10^5 – 10^6 . 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 pyruvate⁴⁻⁶ suggests that the release of acetaldehyde from this bound intermediate is rate determining.

Catalytic Mechanism. The increase in the rate of decarboxylation of CHDT^\pm 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 ions²) 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.

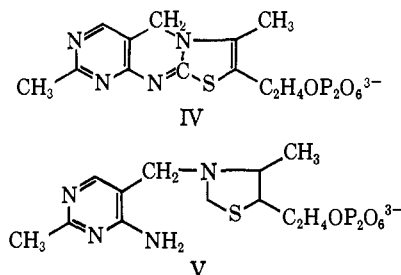
The following information from the literature is in agreement with the suggestion that the active site of pyruvate decarboxylase is hydrophobic.

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

(37) J. Ullrich, J. H. Wittorf, and C. J. Gubler, *Biochim. Biophys. Acta*, 113, 595 (1966).

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 *via* other acid–base groups of the enzyme in order to emphasize the hypothesis of an enzymic solvent effect. The carboxylic 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 $\text{p}K_a$ of pyruvic acid is 2.5 and the $\text{p}K_a$ for the ionization of the hydrogen at carbon 2 of the thiazolium ring has been estimated to be between 14 and 20,^{10,17} 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 carboxylic 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

(38) J. Ullrich, *Angew. Chem. Intern. Ed. Engl.*, 8, 82 (1969).

(39) J. H. Wittorf and C. J. Gubler, Abstracts, 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1968, Biol. 141.

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 $5 \times 10^4 M^{-1} \text{ min}^{-1}$,⁴⁰ 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 enzymic 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 to carbon (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 pyrimidine close to carbon 2 of the thiazolium ring;⁴¹ if another base participates, it may be this $-NH_2$ group. The pK_a for ionization of the hydroxyl group of 2-(1-hydroxyethyl)thiamine pyrophosphate in water is probably about 12.⁴² The pK_a for dissociation of the α -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.⁴³ These pK_a values of 12 and 17 show that in water the proton transfer from the hydroxyl group to the α carbon is favored thermodynamically. The crude estimate that pK_a equals 17 also allows an estimate of the rate constant (k_{H_2O}) for protonation of the enamine at carbon by water, since $k_{H_2O} = k_{OH^-}(K_w/K_a)$ (1/55.5), where K_w is the ion product of water. The value of k_{H_2O} is roughly $5 \times 10^5 M^{-1} \text{ min}^{-1}$ 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 pD 8.6 for 2 hr at 60°,^{27,44} 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

(40) W. J. Svirbely and J. F. Roth, *J. Am. Chem. Soc.*, **75**, 3106 (1953).

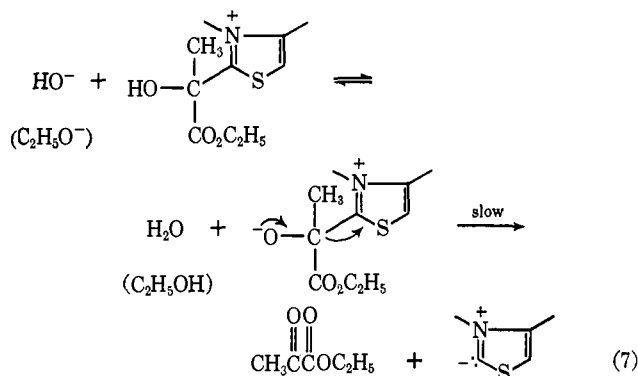
(41) A. Schellenberger, *Angew. Chem. Intern. Ed. Engl.*, **6**, 1024 (1967).

(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: G. E. Lienhard, unpublished results.

(43) R. P. Bell, *Trans. Faraday Soc.*, **39**, 253 (1943).

(44) J. Ullrich and A. Mannschreck, *Eur. J. Biochem.*, **1**, 110 (1967).

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 12.⁴² 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 due to a solvent effect is given by our recent results on the loss of ethyl pyruvate from 2-(1-carboethoxy-1-hydroxyethyl)-3,4-dimethylthiazolium ion (eq 7).⁴⁵ The second-order rate constant ($v = k_2[\text{thiazolium}^+]$



[HO^-] or [$\text{C}_2\text{H}_5\text{O}^-$] for this reaction is 4×10^4 larger in ethanol ($k_2 = 2.6 \times 10^8 M^{-1} \text{ min}^{-1}$) than in water ($k_2 = 0.62 \times 10^4 M^{-1} \text{ min}^{-1}$) at 44.7°.

In summary, these considerations indicate that those steps in the mechanism for pyruvate decarboxylase which proceed very slowly in neutral aqueous solution are markedly accelerated in a less polar solvent, whereas those steps which proceed very rapidly in water are slowed in a less polar solvent. Thus, the solvent effect causes catalysis where catalysis is needed; and consequently, the hypothesis of catalysis by an enzymatic solvent effect appears applicable to the entire pyruvate decarboxylase reaction.

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.⁴⁶ 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.^{46a} Thus, the interaction between indole and thiamine which occurs in aqueous solution

(45) J. Crosby and G. E. Lienhard, in preparation.

(46) (a) J. E. Biaglow, J. J. Míeyal, J. Suchy, and H. Z. Sable, *J. Biol. Chem.*, **244**, 4054 (1969); (b) J. J. Míeyal, J. Suchy, J. E. Biaglow, and H. Z. Sable, *ibid.*, **244**, 4063 (1969).

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,⁴⁷ our results and the above discussion are relevant to many thiamine pyrophosphate-dependent enzymic reactions.

(47) L. O. Krampitz, *Ann. Rev. Biochem.*, **38**, 213 (1969).

Studies on Polypeptides. XLIV. Potent Synthetic S-Peptide Antagonists¹⁻⁴

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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-peptide₁₋₁₄ and of N^δ-formylornithine¹⁰ and ornithine¹⁰ S-peptides₁₋₁₄ was prepared and tested for ability to compete with natural S-peptide for S-protein. β-(Pyrazolyl-3)-alanine¹², 3-carboxymethylhistidine¹², N^δ-formylornithine¹⁰ β-(pyrazolyl-3)-alanine¹², ornithine¹⁰ β-(pyrazolyl-3)-alanine¹², N^δ-formylornithine¹⁰ 3-carboxymethylhistidine¹², and ornithine¹⁰ 3-carboxymethylhistidine¹² S-peptides₁₋₁₄ proved to be potent competitive antagonists of S-peptide. The *d*-sulfoxides of β-(pyrazolyl-3)-alanine¹² and of 3-carboxymethylhistidine¹² S-peptides₁₋₁₄ were considerably less effective inhibitors than the corresponding peptides containing methionine. 1-Carboxymethylhistidine¹² S-peptide₁₋₁₄ 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-Dicarboxymethylhistidine¹² S-peptide₁₋₁₄ was inactive. The replacement of arginine by ornithine in β-(pyrazolyl-3)-alanine¹² and 3-carboxymethylhistidine¹² S-peptide₁₋₁₄ significantly weakened the ability to antagonize S-peptide. The corresponding N^δ-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-peptide⁵ analogs and fragments⁶ have led us to conclude that histidine is the catalytically active amino acid residue in

(1) See K. Hofmann, J. P. Visser, and F. M. Finn, *J. Amer. Chem. Soc.*, **91**, 4883 (1969), for paper XLIII in this series.

(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.

(3) Preliminary communications of some of the results presented in this study have appeared (a) F. M. Finn and K. Hofmann, *J. Amer. Chem. Soc.*, **89**, 5298 (1967); (b) F. M. Finn, J. P. Visser, and K. Hofmann in "Peptides 1968," E. Bricas, Ed., North Holland Publishing Company, Amsterdam, 1968, p 330.

(4) The amino acid residues are of the L configuration. Abbreviations used are: 1-CMHs = 1-carboxymethylhistidine; 3-CMHs = 3-carboxymethylhistidine; 1,3-DiCMHs = 1,3-dicarboxymethylhistidine; Pyr(3)ala = β-(pyrazolyl-3)-alanine; FOrn = N^δ-formylorni-

thine; NArg = nitroarginine; Met = methionine *d*-sulfoxide; B-M-W = 1-butanol-methanol-water, 1:1:1; B-M-1 N AcOH = 1-butanol-water-1 N 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; O-*t*-Bu = *t*-butyl ester; Z = benzyloxycarbonyl; TEA = triethylamine; TFA = trifluoroacetic acid; DMF = dimethylformamide; DCC = N,N'-dicyclohexylcarbodiimide; AG 1-X2 = anion-exchange resin (Bio-Rad); tlc = thin layer chromatography; AP-M = aminopeptidase M [G. Pfeleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determan, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)].

(5) F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, **44**, 162 (1958); RNase S, subtilisin-modified beef 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-pep-

S-peptide and that 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-peptide₁₋₁₄ since this compound is equivalent to natural S-peptide as concerns activation of S-protein.^{6c}

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-peptide₁₋₂₀ via histidine substitutions in S-peptide₁₋₁₄.

To test this prediction experimentally we synthesized Pyr(3)ala¹² S-peptide₁₋₁₄ (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.⁷

Carboxymethylation has provided significant information pertaining to the active site of pancreatic ribonuclease A.⁸ The enzyme is rapidly inactivated

tide₁₋₂₀, S-peptide₁₋₂₁, and S-peptide₁₋₂₂. For clarity natural S-peptide will be designated "S-peptide".

(6) (a) K. Hofmann, F. Finn, W. Haas, M. J. Smithers, Y. Wolman, and N. Yanaihara, *J. Amer. Chem. Soc.*, **85**, 833 (1963); (b) F. M. Finn and K. Hofmann, *ibid.*, **87**, 645 (1965); (c) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *ibid.*, **88**, 3633 (1966).

(7) K. Hofmann and H. Bohn, *ibid.*, **88**, 5914 (1966).