Synthesis and Properties of 2-Acetylthiamin Pyrophosphate: An Enzymatic Reaction Intermediate†

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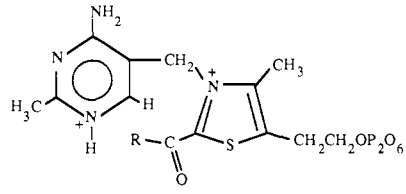
ABSTRACT: The synthesis of 2-acetylthiamin pyrophosphate (acetyl-TPP) is described. The synthesis of this compound is accomplished at 23 °C by the oxidation of 2-(1-hydroxyethyl)thiamin pyrophosphate using aqueous chromic acid as the oxidizing agent under conditions where Cr(III) coordination to the pyrophosphoryl moiety and hydrolysis of both the pyrophosphate and acetyl moieties were prevented. Although the chemical properties exhibited by acetyl-TPP are similar to those of the 2-acetyl-3,4-dimethylthiazolium ion examined by Lienhard [Lienhard, G. E. (1966) *J. Am. Chem. Soc. 88*, 5642–5649], significant differences exist because of the pyrimidine ring in acetyl-TPP. Characterization of acetyl-TPP by ultraviolet spectroscopy, ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR provided evidence that the compound in aqueous solution exists as an equilibrium mixture of keto, hydrate, and intramolecular carbinolamine forms. The equilibria for the hydration and carbinolamine formation reactions at pD 1.3 as determined by ^{1}H NMR are strongly dependent on the temperature, showing an increase in the hydrate and carbinolamine forms at the expense of the keto form with decreasing temperature. The concentration of keto form also decreases with increasing pH. Acetyl-TPP is stable in aqueous acid but rapidly deacetylates at higher pH to form acetate and thiamin pyrophosphate. Trapping of the acetyl moiety in aqueous solution occurs efficiently with 1.0 M hydroxylamine at pH 5.5–6.5 to form acetohydroxamic acid and to a much smaller extent with 1.0 M 2-mercaptoethanol at pH 4.0 and 5.0 to form thio ester. Transfer of the acetyl group to 0.5 M dihydrolipoic acid at pH 5.0 and 1.0 M phosphate dianion at pH 7.0 is not observed to any significant extent in water. The kinetic and thermodynamic reactivity of acetyl-TPP with water and other nucleophiles is compatible with a hypothetical role for acyl-TPPs as enzymatic acyl-transfer intermediates.

The work of Breslow (1958) on the chemistry of acylthiazolium salts implicated 2-acylthiamin pyrophosphates (acyl-TPP,[1] **1**) as possible intermediates in several TPP-dependent enzymatic reactions. Subsequent studies on the



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oxidation of 2-(1-hydroxyalkyl)thiamin pyrophosphates with artificial electron accepters catalyzed by phosphoketolase (Schroter & Holzer, 1963), pyruvate and α-ketoglutarate dehydrogenase complexes (Das et al., 1961; Fonseca-Wollheim et al., 1962), and pyruvate decarboxylase (Holzer & Beaucamp, 1961) led investigators to conclude that acyl-TPP compounds are intermediates in these TPP-dependent enzymatic reactions. The 2-oxoacid:ferridoxin oxidoreductase has been found to utilize a naturally occurring electron accepter to generate an acyl-TPP as an intermediate (Kersher & Oesterhelt, 1981), and phosphoketolase has long been known to catalyze the nonoxidative conversion of fructose-6-P to acetate and erythrose-4-P in the presence of arsenate via the enzyme-bound, catalytic intermediate acetyl-TPP (Goldberg & Racker, 1962). Recent findings in this laboratory have strongly suggested that acyl-TPPs are produced in the course of oxidation and decarboxylation of α-keto acids catalyzed by α-keto acid dehydrogenase complexes (Steginsky & Frey, 1984; CaJacob et al., 1985; Flournoy & Frey, 1986); however, it is not known whether these species are compulsory intermediates or exist in equilibrium with intermediates.

Several investigators synthesized acyl-TPP analogues for use in chemical model studies. Breslow and McNelis (1960) reported the preparation of crude 2-acetyl-3,4-dimethylthiazolium nitrate and investigated some of its chemical reactivities. They found that this compound is readily deacylated by water or methanol. White and Ingraham (1960, 1962) reported similar findings with 2-benzoyl-3,4-dimethylthiazolium iodide. Daigo and Reed (1962) purified 2-acetyl-3,4-dimethylthiazolium iodide and described the chemical lability of this compound in the presence of water, hydroxylamine, and mercaptide ions. Lienhard (1966), in a detailed kinetic analysis, investigated the hydrolytic and hydration mechanisms for the 2-acetyl-3,4-dimethylthiazolium ion. These studies established the kinetic and thermodynamic lability of 2-acylthiazolium salts toward nucleophiles, including water, and showed that the chemical properties of these compounds are compatible with a hypothetical role for acyl-TPPs

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[1] Abbreviations: TPP, thiamin pyrophosphate; HETPP, 2-(1-hydroxyethyl)thiamin pyrophosphate; HET, 2-(1-hydroxyethyl)thiamin; acetyl-TPP, 2-acetylthiamin pyrophosphate; acyl-TPP, 2-acylthiamin pyrophosphate; TCA, trichloroacetic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ATP, adenosine triphosphate; PEP, phosphoenolpyruvate; NADH, nicotinamide adenine dinucleotide, reduced form; EDTA, ethylenediaminetetraacetic acid.

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as enzymatic acyl-transfer intermediates.

It is reasonable to expect acetyl-TPP to exhibit chemical properties similar to those of the 2-acetyl-3,4-dimethylthiazolium ion; however, the pyrimidine and pyrophosphoryl substituents may perturb the chemical reactivities of the thiazolium center. An example of this effect is provided by the work of Kluger et al. (1981) compared with that of Lienhard and co-workers (Crosby et al., 1970; Crosby & Lienhard, 1970), where differences in the decarboxylation rates of 2-lactylthiamin and the model compound 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride were observed. Therefore, the identification of any species isolated from an enzymatic reaction mixture as acetyl-TPP will require quantitative knowledge of its chemical properties.

In this paper we describe the first synthesis and characterization of 2-acetylthiamin pyrophosphate. The compound exists in aqueous solution in equilibrium with its hydrate and a carbinolamine formed by internal addition of the pyrimidinyl amino group to the carbonyl function of the 2-acetylthiazolium moiety. We also describe certain chemical aspects of acetyl-TPP, such as the reactions of acetyl-TPP with thiols, hydroxylamine, and phosphate under a variety of conditions. We compare these results with those from model compounds and discuss their relevance to TPP-mediated enzymatic reactions that may involve acetyl-TPP as an intermediate.

Experimental Procedures

Materials. Chemicals, coenzymes, and enzymes were purchased from the following commercial sources: TPP, EDTA, NADH, imidazole, ATP, PEP, 2-mercaptoethanol, pyruvate kinase, lactate dehydrogenase, and column materials Sephadex SP-C25 and G-10, Sigma; acetate kinase, Boehringer Mannheim; 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt, acetaldehyde, acetaldehyde-d_4, D_2O, DCl, *n*-butyl mercaptan, and acetohydroxamic acid, Aldrich; acetaldehyde-*1,2*-^{13}C, MSD Isotopes; CrO_3, J. T. Baker; AG1-X8 anion-exchange resin, Bio-Rad; $Na_4P_2O_7 \cdot 10H_2O$, KH_2PO_4, and hydroxylamine HCl, Fisher Scientific. All other chemicals were of highest purity from readily available sources.

Instrumentation. Proton NMR spectra were obtained with Bruker WH-270 and Bruker AM-400 spectrometers field-frequency locked on the deuterium resonance of 99.8 atom % D_2O. All chemical shifts are reported downfield from 3-(trimethylsilyl)-1-propanesulfonic acid (internal). ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer field-frequency locked on the deuterium resonance of 20 atom % D_2O, chemical shifts being referenced to 60% dioxane in D_2O (external). ^{31}P NMR spectra were recorded on a Nicolet NT-200 WB spectrometer field-frequency locked on the deuterium resonance of 50–99.8 atom % D_2O, with chemical shifts referenced to 85% H_3PO_4 (external).

Solutions of TPP, HETPP, and acetyl-TPP in 30 mM HCl were scanned from 360 to 220 nm with a Hitachi 100-80A UV–vis spectrophotometer equipped with an electronic temperature control unit as described in the legend of Figure 1. All quantitative assays using UV–vis spectrophotometry were done with a Cary 118 UV–vis spectrophotometer.

Synthesis of 2-(1-Hydroxyethyl)thiamin Pyrophosphate (HETPP). HETPP was synthesized by a modification of the procedure of Holzer et al. (1961). Freshly distilled acetaldehyde (30 mmol) was added to a 5-mL aqueous solution of TPP (1.5 mmol) in a 50-mL pear-shaped flask. The pH of the solution was adjusted to 8.0 with 5.0 M NaOH and then to 8.7 with 1 M NaOH. The flask was sealed and incubated for 2 h at 45 °C. The solution was cooled to room temperature and its pH readjusted to 8.7 with 1 M NaOH. It was then resealed in the flask and incubated for an additional 1 h at 45 °C. The pH of the product mixture was adjusted to 2.5 with 5 N HCl and the solution concentrated by rotary evaporation in vacuo to approximately 1-mL total volume. The concentrated solution was desalted by passage through a 1.0 × 48 cm column of Sephadex G-10, preequilibrated and eluted with 1% formic acid. One-milliliter fractions were collected and the absorbance at 260 nm and conductivity measured. The fractions having absorption at 260 nm were pooled and concentrated by rotary evaporation in vacuo to about 2-mL total volume. The desalted product solution was then chromatographed through a 45 × 1.5 cm column of Sephadex SP-C25 cation-exchange resin, preequilibrated and eluted with 1% formic acid. Fractions 6 mL in volume were collected, and those containing HETPP (A_{260}) were pooled and freed of water and formic acid by rotary evaporation in vacuo. The colorless glassy product was dissolved in 2 mL of 0.10 M HCl and the solvent removed as before. The product was stored as a 0.2 M aqueous solution at 4 °C. The yield was typically 45%. The molar extinction coefficients of HETPP in 30 mM HCl at room temperature, referenced to total phosphate analysis, were found to be 14360 and 14050 at 246 and 260 nm, respectively. The ^1H NMR gave δ 1.69 (3 H, d), 2.42 (3 H, s), 2.58 (3 H, s), 3.33 (2 H, t), 4.22 (2 H, q), 5.43 (1 H, q), 5.55 (2 H, s), and 7.32 (1 H, s), in agreement with values reported by Kluger et al. (1985) and Mieyal et al. (1967).

Synthesis of 2-Acetylthiamin Pyrophosphate (Acetyl-TPP). HETPP (80 μmol) was dried by rotary evaporation in vacuo and the residue dissolved in 100 μL of water. To this was added 300 μL of 1.5 M pyrophosphoric acid (prepared by combining 0.4 mL water with 7.5 mmol of $Na_4P_2O_7 \cdot 10H_2O$ followed by 3 mL of 10 N H_2SO_4 while mixing vigorously) followed by 50 μL of Jones reagent (Curtis et al., 1953). The latter was prepared by adding 2.30 mL of concentrated H_2SO_4 to 26.7 mmol of CrO_3, cooling the flask in an ice/water bath, adding 4.0 mL of water to dissolve most of the CrO_3, and then bringing the final volume to 10 mL with water. The oxidation proceeded for 6 h at 23 °C, after which 200 μL of water was added to the product mixture. The resulting solution was chromatographed at 4 °C on a 0.7 × 17 cm column of AG1-X8 anion-exchange resin, preequilibrated and eluted with 1% formic acid. One-milliliter fractions were collected and analyzed for A_{260}. Acetyl-TPP, TPP, and HETPP emerged in the void volume, while Cr^{III}(pyrophosphate)$_2$, pyrophosphoric acid, and unreacted Cr(VI) were retained on the column. The acetyl-TPP, TPP, and HETPP collected in two fractions and were combined and chromatographed at 4 °C on a 1.5 × 25 cm column of Sephadex SP-C25 cation-exchange resin, preequilibrated and eluted with 1% formic acid. Fractions 4 mL in volume were collected and analyzed for A_{260}. Those fractions containing acetyl-TPP, as determined by their UV absorption spectrum, were pooled and concentrated to approximately 300 μL by rotary evaporation in vacuo, keeping the water bath temperature below 30 °C. To this solution was added 500 μL of 0.1 N HCl. The solvent from this mixture was removed by rotary evaporation, again keeping the water bath temperature below 30 °C. The colorless glassy residue of acetyl-TPP was dissolved into 1.5 mL of 30 mM HCl and stored at −20 °C to prevent degradation. The yield was 40%.

The extinction coefficients of the product at 24 °C in 30 mM HCl were found to be $(1.21 \pm 0.07) \times 10^4$, $(8.27 \pm 0.49) \times 10^3$, and $(3.41 \pm 0.20) \times 10^3$ M^{-1} cm^{-1} at 246, 280, and 310 nm, respectively. The ^1H NMR gave δ 1.88 (s), 2.35 (s), and 2.85 (s) 3 H; δ 2.41 (s), 2.57 (s), and 2.58 (s) 3 H; δ 2.61 (s), 2.62 (s), and 2.63 (s) 3 H; δ 3.32 (t) and 3.50 (t) 2 H; δ 4.15

(m), 4.20 (m), and 4.27 (m) 2 H; δ 5.61 (d), 5.72 (s), 5.80 (s), and 6.39 (d) 2 H; δ 7.34 (s), 7.35 (s), and 8.59 (s) 1 H. The ^{1}H NMR assignments are given in Table I.

Synthesis of 2-(1-Hydroxyethyl-d_4)thiamin Pyrophosphate (HETPP-d_4) and 2-(Acetyl-d_3)thiamin Pyrophosphate (Acetyl-d_3-TPP). HETPP-d_4 was synthesized by the procedure described for HETPP on half the scale with respect to the starting materials TPP and acetaldehyde-d_4. The synthesis of acetyl-d_3-TPP from HETPP-d_4 was by the procedure described for acetyl-TPP synthesis with the exception that D_2O was used as solvent and to prepare reagents. The chromatography procedures for both HETPP-d_4 and acetyl-d_3-TPP purifications were the same as for their undeuteriated counterparts. The ^{1}H NMR spectrum of HETPP-d_4 was identical with that of HETPP except for the missing doublet at 1.69 ppm and the missing quartet at 5.43 ppm. The UV spectrum of HETPP-d_4 was identical with that of HETPP. The ^{1}H NMR spectrum of acetyl-d_3-TPP was identical with that of acetyl-TPP except for the missing singlets at 1.88, 2.35, and 2.85 ppm. The UV spectrum of acetyl-d_3-TPP was identical with that of acetyl-TPP.

Synthesis of 2-(1-Hydroxyethyl-1,2-^{13}C)thiamin Pyrophosphate (HETPP-$^{13}C_2$) and 2-(Acetyl-1,2-^{13}C)thiamin Pyrophosphate (Acetyl-$^{13}C_2$-TPP). HETPP-$^{13}C_2$ was synthesized by the procedure described for HETPP on half the scale with respect to TPP and acetaldehyde-1,2-^{13}C as starting materials. The synthesis of acetyl-$^{13}C_2$-TPP from HETPP-$^{13}C_2$ was by the same procedure described for acetyl-TPP synthesis. The chromatography procedures for both HETPP-$^{13}C_2$ and acetyl-$^{13}C_2$-TPP were the same as for HETPP and acetyl-TPP. The UV spectrum of HETPP-$^{13}C_2$ was identical with that of HETPP and proton-decoupled ^{13}C NMR of HETPP-$^{13}C_2$ gave δ 21.4 (d) for the C2 carbon and δ 64.1 (d) for the C1 carbon. The UV spectrum of acetyl-$^{13}C_2$-TPP was identical with that of acetyl-TPP. Proton-decoupled ^{13}C NMR of acetyl-$^{13}C_2$-TPP gave δ 26.9 (d), 27.3 (d), and 28.9 (d) for the C2 carbon and δ 78.2 (d), 93.4 (d), and 185.6 (d) for the C1 carbon.

Product Identification. The methods used for the identification of acetyl-TPP were ^{1}H NMR, ^{31}P NMR, ultraviolet spectroscopy, and analysis of hydrolysis products from acetyl-TPP by the above techniques and by enzymatic assay for acetate.

Acetyl-TPP was prepared for NMR analysis by first drying 20 μmol (from the pooled fractions off of the SP-C25 column), dissolving the residue in 700 μL of 25 mM DCl in D_2O, drying again, and then dissolving the residue in 0.75 mL of 50 mM DCl in D_2O. HETPP was prepared for NMR analysis in a similar manner. TPP was prepared for NMR analysis by dissolving 50 μmol into 0.75 mL of 50 mM DCl in D_2O. Fifty microliters of 20 mM EDTA (pH 2.0) was added to the sample prior to analysis by ^{31}P NMR.

The hydrolysis products of acetyl-TPP were prepared for NMR analysis by one of two procedures. For analysis by ^{31}P NMR, a 100-μL aliquot of 0.25 M sodium acetate, containing 0.010 M Na_2EDTA, was added to 400 μL of the acetyl-TPP NMR sample described above and allowed to stand overnight at room temperature. The final pH of the solution was approximately 6.0. Prior to recording the spectrum, 40 μL of 1 N HCl was added to bring the pH below 2.0. For ^{1}H NMR analysis, 50 μL of 1 M potassium phosphate (pH 8.0) and 50 μL of 1 M KOH were added to 10 μmol of acetyl-TPP in 0.75 mL of 50 mM DCl in D_2O, which brought the pH to approximately 7.0. The sample was held at room temperature for 30 min, dried by rotary evaporation in vacuo, and further dried by storing in a desiccator under vacuum overnight. The ^{1}H NMR spectrum of the sample was obtained after 0.75 mL of 0.1 M DCl in D_2O was added. The UV spectrum of the hydrolysis reaction products was recorded after 10 μL of the NMR sample was diluted into 0.99 mL of 30 mM HCl.

Acetate from the hydrolysis of acetyl-TPP was enzymatically assayed with a coupled system containing acetate kinase, pyruvate kinase, and lactate dehydrogenase (eq 1–4). Al-

$$\text{ATP} + \text{acetate} \rightleftarrows \text{acetyl phosphate} + \text{ADP} \quad (1)$$

$$\text{ADP} + \text{PEP} \rightleftarrows \text{ATP} + \text{pyruvate} \quad (2)$$

$$\text{pyruvate} + \text{NADH} \rightleftarrows \text{lactate} + \text{NAD}^+ \quad (3)$$

sum

$$\text{acetate} + \text{PEP} + \text{NADH} \rightleftarrows \text{acetyl phosphate} + \text{lactate} + \text{NAD}^+ \quad (4)$$

though the equilibrium for the acetate kinase catalyzed reaction lies in favor of ATP and acetate, the coupling of this reaction with those catalyzed by pyruvate kinase and lactate dehydrogenase under appropriate conditions results in quantitative production of acetyl phosphate, lactate, and NAD^+. The amount of acetate present was calculated from the change in A_{340} due to the consumption of NADH ($\epsilon_{340} = 6.22 \times 10^3$ cm^{-1} M^{-1}). Approximately 50 nmol of acetyl-TPP in 10 μL of 30 mM HCl was added to 990 μL of an assay solution at pH 7.2 containing 50 mM imidazole, 5.0 mM $MgCl_2$, 1.0 mM KCl, 5.0 mM ATP, 2.0 mM PEP, 0.20 mM NADH, 20 units/mL lactate dehydrogenase, 20 units/mL pyruvate kinase, and 10 units/mL acetate kinase. This solution was placed at room temperature for 30 minutes to complete the reaction. To get an accurate initial absorbance reading at 340 nm, a blank solution was prepared by adding 10 μL of 30 mM HCl to 990 μL of the assay solution and the A_{340} recorded.

The extinction coefficients of acetyl-TPP at 246, 280, and 310 nm (the λ_{max}) were calculated by assaying for total phosphate using a malachite green phosphate assay (Toshima et al., 1975). Four samples of acetyl-TPP were prepared ranging in A_{310} readings from 0.101 to 0.397, and the A_{246}, A_{280}, and A_{310} values were recorded for each at 24 °C. Appropriate aliquots of each, in triplicate, were ashed and hydrolyzed by the method of Ames (1966) and then assayed for total phosphate. The number of nanomoles in each sample was found by reference to a standard curve. The spectrum of acetyl-TPP shown in Figure 1 was from the same sample used to calculate the extinction coefficients.

Equilibrium Measurements. The equilibrium constants governing the levels of hydrate, keto, and carbinolamine forms of acetyl-TPP were measured as a function of temperature in the range of 6.9–47 °C at pD 1.3. The equilibrium concentrations of the three forms were found from the acetyl-CH_3 peak areas in the ^{1}H NMR spectra. The equilibrium constants K_1 and K_2, where K_1 = [carbinolamine]/[keto] and K_2 = [hydrate]/[keto], were then calculated by using the equations

$$\text{carbinolamine} \overset{K_1}{\rightleftharpoons} \text{keto} \overset{K_2}{\rightleftharpoons} \text{hydrate} \quad (5)$$

$$K_1/K_2 = [\text{carbinolamine}]/[\text{hydrate}] \quad (6)$$

$$\alpha[\text{keto}] = [\text{keto}]/([\text{keto}] + [\text{hydrate}] + [\text{carbinolamine}]) = 1/(1 + K_1 + K_2) \quad (7)$$

Enthalpy and entropy terms for the equilibrium reactions were calculated by least-squares statistical analysis of log $K_{1,2}$ vs $1/T$ (K), where ΔH = −slope × 2.3R and ΔS = y intercept × 2.3R, where R is the universal gas constant (1.987 cal·deg^{-1}·mol^{-1}).

Hydrolysis of Acetyl-TPP. The hydrolysis rate of acetyl-TPP at pH 2.8 and 7.0 and 24 °C was measured by the de-

crease in absorbance at 310 nm due to the loss of the keto form of acetyl-TPP. A least-squares statistical analysis of ln ($A_{310} - A_{310\infty}$) vs time yielded the rate constants from the negative slope values. Five microliters of 20 mM acetyl-TPP was added to 995 μL of buffered solution and the reaction followed to completion in the spectrophotometer. The pH was maintained by the presence of 50 mM phosphate, and the ionic strength was adjusted to 0.5 M with NaCl. The average of triplicate determinations was accepted as the rate constant.

Reaction of Acetyl-TPP with Nucleophiles. Hydroxylamine, PO_4^{2-}, 2-mercaptoethanol, and dihydrolipoic acid were tested for their ability to trap the acetyl moiety of acetyl-TPP in aqueous solutions. *n*-Butyl mercaptan was tested as a nucleophile in ethanol. Acetyl transfer to these nucleophiles was measured by assaying for acetohydroxamic acid (Rose et al., 1954; Jencks, 1958) formed from reactions of hydroxylamine with acetyl-TPP, acetyl phosphate, or acetyl thio esters (formed by PO_4^{2-} or R-SH attack on acetyl-TPP, respectively). The absorbance at 540 nm of the reaction solutions was recorded and compared to a standard curve to quantitate the amount of acetohydroxamic acid formed. All reactions were carried out under conditions where 2% trapping could be detected. Neither TPP nor HETPP under similar assay conditions produced significant A_{540} above background. All measurements were made in duplicate.

Hydroxylamine (1.0 M) was tested as a nucleophile toward acetyl-TPP as a function of pH in the range of 5.5–6.5. A 20-μL aliquot of 6.5 mM acetyl-TPP in 30 mM HCl was diluted to 100 μL with H_2O; to this was added 100 μL of 2 M hydroxylamine at pH 5.5, 6.0, or 6.5. After 1 h at room temperature, 0.6 mL of H_2O, 100 μL of 20% TCA, and 100 μL of 10% $FeCl_3$ in 2 N HCl were added, and the absorbance at 540 nm was recorded.

Dihydrolipoic acid (0.5 M) was tested at pH 5.0 as a nucleophile toward acetyl-TPP by first diluting 10–40 μL of 20.0 mM acetyl-TPP in 30 mM HCl with H_2O to a volume of 50 μL. A 50-μL aliquot of 1.0 M dihydrolipoic acid at pH 5.0 was then added. After incubation for 2 h at room temperature, 100 μL of 2.0 M hydroxylamine, pH 6.0, was added and the resulting solution incubated for an additional hour at room temperature. The solution was acidified with 200 μL of 1 N HCl and the dihydrolipoic acid extracted with three 2-mL volumes of ether. After 500 μL of H_2O and 100 μL of 10% $FeCl_3$ in 2 N HCl were added to the aqueous phase, the A_{540} was recorded.

The nucleophilic reactivity of 2-mercaptoethanol toward acetyl-TPP was tested at pH 4.0 and 5.0. Aliquots (20–40 μL) of 20 mM acetyl-TPP in 30 mM HCl were diluted to 50 μL with H_2O. To this was added 50 μL of 2 M 2-mercaptoethanol in 1.5 M sodium formate (pH 4.0) or 2 M 2-mercaptoethanol in 1.5 M sodium acetate (pH 5.0). After an overnight or 2-h incubation, at pH 4.0 or 5.0, respectively, at room temperature, 100 μL of 2 M hydroxylamine, pH 6.5, was added, and the resulting solutions were incubated for an additional hour at room temperature. 2-Mercaptoethanol and water were removed by lyophilization, 200 μL of H_2O was added to dissolve the residue, and lyophilization was repeated. The residue was dissolved in 700 μL of H_2O and 200 μL of 1 N HCl added followed by 100 μL of 10% $FeCl_3$ in 2 N HCl. The A_{540} was then recorded.

Inorganic phosphate dianion was tested as a nucleophile toward acetyl-TPP in aqueous solution at pH 7.0. A 40-μL aliquot of 20 mM acetyl-TPP in 30 mM HCl was diluted with H_2O to a volume of 50 μL and combined with 50 μL of 2 M potassium phosphate at pH 7.0. After 30 min at room temperature, 100 μL of 2 M hydroxylamine at pH 6.0 was added and the resulting solution allowed to stand for an additional 30 min. Water (500 μL) was added followed by 200 μL of 1 N HCl and 100 μL of 10% $FeCl_3$ in 2 N HCl. The A_{540} was then recorded.

n-Butyl mercaptan was tested as a nucleophile toward acetyl-TPP in 95% ethanol in the presence of 10 mM sodium acetate. A 50-μL aliquot of 20 mM acetyl-TPP in 30 mM HCl was added to 950 μL of absolute ethanol containing 1 mmol of *n*-butyl mercaptan and 10 μmol of sodium acetate. After a 2-h incubation at room temperature, 1 mL of aqueous 2 M hydroxylamine at pH 6.5 was added. The solution was allowed to stand at room temperature for 30 min, and 1 mL of 3 N HCl was then added to acidify the solution. Unreacted *n*-butyl mercaptan was extracted with three 5-mL volumes of ether, and 500 μL of 10% $FeCl_3$ in 1 N HCl was then added to the aqueous phase and the A_{540} recorded.

The apparent extinction coefficient of acetyl-TPP at 310 nm was measured as a function of pH and in the presence of the nucleophiles used in the trapping experiments (refer to Table IV). A 10-μL aliquot of 20 mM acetyl-TPP was diluted with 990 μL of buffer and the absorbance reading at 310 nm immediately recorded prior to the onset of hydrolysis.

RESULTS

Synthesis of HETPP. The synthesis of HETPP by the method described under Experimental Procedures is an improvement over the procedure of Holzer et al. (1961), both in the yield and in the purification procedure. The modifications that account for these improvements include the readjustment of the pH of the reaction mixture after 2 h of reaction at 45 °C as well as the use of a different chromatographic scheme for the purification of the product. HETPP is cationic at low pH and is therefore retarded on a cation-exchange column of Sephadex SP-C25 eluted with 1% formic acid. HETPP emerges prior to TPP and is well separated under these conditions. Excellent resolution of HETPP is obtained first by passage through a Sephadex G-10 column and then by cation-exchange chromatography through Sephadex SP-C25. Purity is confirmed by the ^1H NMR spectrum of the product in D_2O, which only shows signals due to HETPP and a small residue of formic acid (see Experimental Procedures).

Synthesis of Acetyl-TPP. The synthesis of acetyl-TPP was achieved by oxidation of HETPP with excess chromic acid in 1 N aqueous sulfuric acid at room temperature. The additional presence of a large excess of pyrophosphoric acid prevented Cr(III) coordination to the pyrophosphate moiety of the product. Acetyl-TPP was purified by Dowex AG1-X8 anion-exchange chromatography followed by Sephadex SP-C25 cation-exchange chromatography, using 1% formic acid as the eluting buffer with both columns. For SP-C25 cation-exchange chromatography, UV spectral analysis of the major A_{260} peak showed that the very early fractions contained a small amount of HETPP, whereas the later fractions in the peak contained a small amount of TPP. Discarding the early and late fractions and combining the central fractions led to a product with little contamination by HETPP or TPP, as indicated by the ^1H NMR spectrum. As indicated by the UV spectrum, the integrity of acetyl-TPP is maintained when stored frozen at −20 °C for 3 months.

The UV spectrum of acetyl-TPP in 30 mM HCl after purification on SP-C25 is shown in Figure 1, together with those of HETPP and TPP. It is evident that acetyl-TPP exhibits two absorption bands at wavelengths longer than the bands characteristic of HETPP and TPP. Acetyl-TPP does not

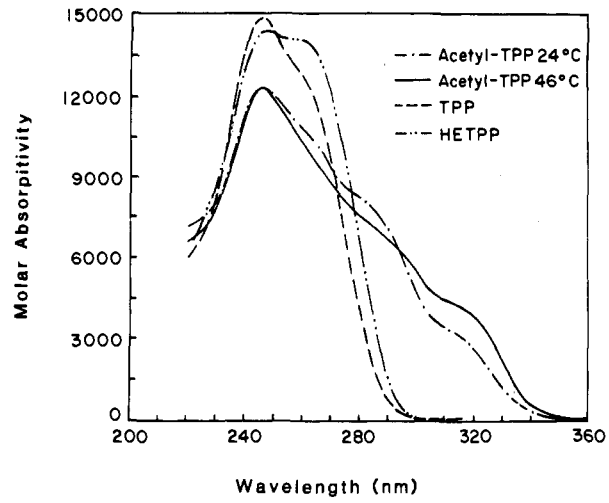


FIGURE 1: UV absorption spectra of acetyl-TPP, HETPP, and TPP in 30 mM HCl at room temperature unless indicated otherwise. Three microliters of 20 mM compound was diluted to 1.0 mL with 997 μL of 30 mM HCl and the spectrum recorded as described under Experimental Procedures.

absorb visible light. When the temperature is increased from 24 to 46 °C, the extinction coefficient at 310 nm increases, while that at 280 nm decreases. The apparent extinction coefficient at 246 nm is independent of temperature. The magnitude of change in extinction coefficient at 310 nm as a function of temperature is directly correlated with the change in the keto form of acetyl-TPP, as observed in the ^{1}H NMR temperature study described below. Therefore, the absorption band at 310 nm is assigned to the keto form of acetyl-TPP, in agreement with the spectral properties of the 2-acetyl-3,4-dimethylthiazolium ion under comparable conditions (Lienhard, 1966).

Acetyl-TPP under hydrolysis conditions should form acetate and TPP. The results of experiments aimed at identifying the hydrolysis products of the oxidized HETPP compound showed that the UV spectrum after hydrolysis at pH 7 was identical with the TPP spectrum shown in Figure 1. The same solution was assayed enzymatically for acetate, chemically for phosphate, and spectrophotometrically for TPP (ϵ = 14 700 M^{-1} cm^{-1} at 246 nm in 30 mM aqueous HCl). The results indicated the presence of equivalent amounts of TPP and acetate. In addition, the ^{1}H NMR spectrum of the hydrolysis products showed signals assignable to only TPP and acetate, which were present in equivalent amounts, and the ^{31}P NMR chemical shifts of the hydrolysis products were identical with those for the phosphorus resonances for TPP. From these data it was concluded that the product formed in the oxidation of HETPP is acetyl-TPP.

^{1}H NMR Spectrum of Acetyl-TPP. Figure 2 shows the expanded ^{1}H NMR spectrum of purified acetyl-TPP. This complex spectrum cannot be interpreted solely on the basis of the presence of the hydrate and keto forms of acetyl-TPP, which are expected to be present on the basis of the chemistry of the 2-acetyl-3,4-dimethylthiazolium ion (Lienhard, 1966). Identifiable impurities are formic acid (δ 8.23), remaining from the isolation procedure, and traces of acetic acid (δ 2.11) and TPP (C-4′ pyrimidine methyl group at δ 2.56, C-6′ pyrimidine proton at δ 7.94, and the bridging methylene protons at δ 5.57), the hydrolytic breakdown products. One additional form of acetyl-TPP must be present to account for all of the proton resonances, which are more than twice the number observed for HETPP.

The ^{1}H NMR spectrum of acetyl-d_3-TPP is identical with that shown in Figure 2 for acetyl-TPP except for the absence

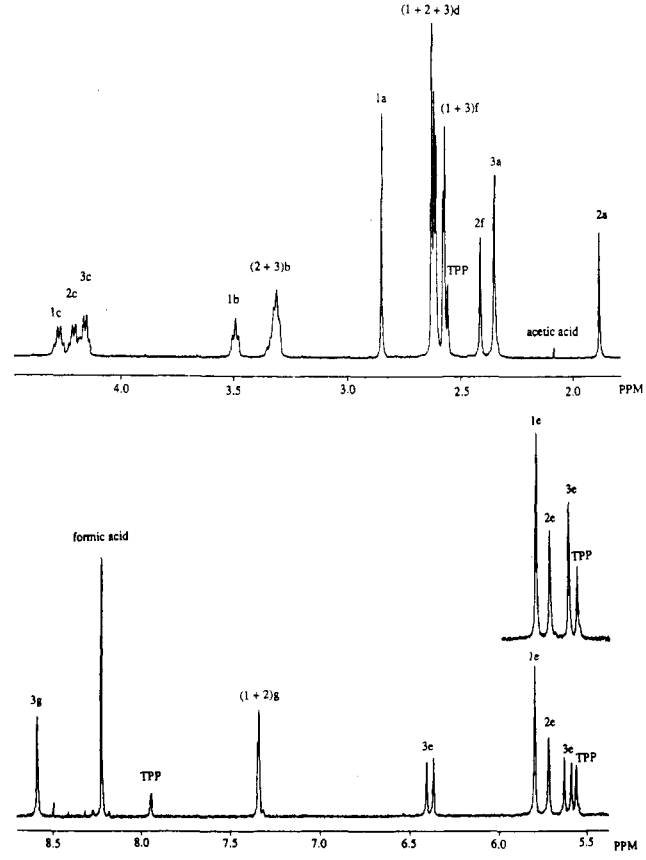
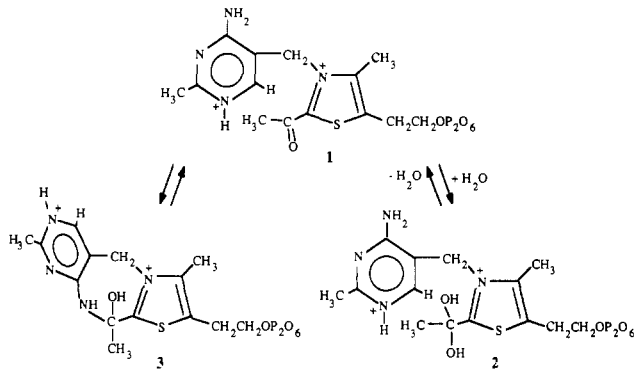


FIGURE 2: ^{1}H NMR spectrum of acetyl-TPP in 50 mM DCl in D_2O at 400 MHz. Proton peaks are assigned with reference to Table I (i.e., protons a–g) and the keto (**1**), hydrate (**2**), and carbinolamine (**3**) forms of the molecule. Peaks for the downfield portion of the spectrum (from 5.5 to 8.5 ppm) are twice the size relative to the peaks for the upfield portion of the spectrum. The raised spectrum from δ 5.3 to 6.0 is the result of a homonuclear decoupling experiment on the doublet at δ 6.39. The sample was prepared as described under Experimental Procedures.

of singlets at δ 1.88, 2.35, and 2.85 (spectrum not shown). Therefore, these three resonances are assigned to the acetyl methyl group of acetyl-TPP, and the third form of acetyl-TPP involves the acetyl group and exists in addition to keto and hydrate forms. The remaining signals appearing between δ 2.41 and 2.63 can be assigned to the methyl group protons of the pyrimidine and thiazolium rings. On the basis of signal integrations, the signals at δ 2.41, 2.57, and 2.58 belong to one methyl group of the three, and those at δ 2.61, 2.62, and 2.63 belong to the other methyl group in the three molecular forms. The three upfield signals are assigned to the pyrimidinyl methyl group and the downfield signals to the thiazolium methyl group for reasons set forth later. Based on integration and chemical shift position (compared to those of TPP and HETPP), the unresolved multiplets appearing at δ 3.32 and 3.50 are assigned to the methylene group α to the C-5 thiazolium ring carbon, and the unresolved multiplets at δ 4.15 to 4.27 are assigned to the β methylene group for the same reasons. By the same criteria, resonances at δ 5.59, 5.63, 5.72, 5.80, 6.37, and 6.41 are all assigned to the bridging pyrimidinyl thiazolium methylene protons, and the singlets at δ 7.34, 7.35 and 8.59 are assigned to the C-6′ pyrimidine proton.

The three acetyl methyl resonances of acetyl-TPP indicate the presence of one species in addition to the hydrate and keto forms of this molecule. This must be reconciled with the presence of six resonances assigned to the bridging methylene protons. By close inspection of the spectrum, it is seen that the two resonances at δ 5.59 and 5.63 have equal spacing and

Scheme I



peak intensity to the two resonances at δ 6.37 and 6.41. The spacing between each pair of signals is 16 Hz and is independent of instrument field strength. In addition, when the signals at δ 6.37 and 6.41 are irradiated and decoupled, the resonances at δ 5.59 and 5.63 converge to a singlet, as shown in the raised portion of the spectrum. These results show that the third form of acetyl-TPP is a closed structure that imposes different chemical shifts on the bridging methylene protons. The resonances at δ 5.59 and 5.63 and at 6.37 and 6.41 are two doublets generated by nonequivalent geminal protons coupled to each other by 16 Hz. This closed structure is shown in Scheme I and is proposed to be an internal adduct, a carbinolamine (**3**), in equilibrium with the keto (**1**), and hydrate (**2**) forms of acetyl-TPP. The pyrimidine ring for all three forms is shown protonated at the N-1 nitrogen in accord with the results of Cain et al. (1977).

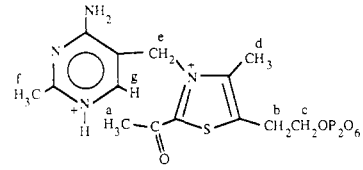
It is known that the extent of hydration of aldehydes and ketones decreases with increasing temperature (Bell & McDougall, 1960) and that the ^{1}H NMR chemical shift of the α-hydrogens from the keto form is 0.8–1.1 ppm downfield from the hydrate (Kokesh, 1976; Ahrens & Strehlow, 1965; Hine & Redding, 1970; Hine et al., 1965; Lombardi & Sogo, 1960). On the basis of these precedents, the acetyl methyl resonances at δ 1.88 and 2.85 can tentatively be assigned to the hydrate and keto forms of acetyl-TPP, respectively. Definitive assignments of these signals are made from the ^{1}H NMR temperature study discussed below.

Results from the ^{1}H NMR temperature study on acetyl-TPP showed that an increase in temperature effected an increase in the integrated intensity of the acetyl methyl signal at δ 2.85 and a corresponding decrease in the acetyl methyl resonances at δ 2.35 and 1.88. This is in agreement with the assignment of the peak at δ 2.85 to the keto form of acetyl-TPP. The acetyl methyl resonance at δ 2.35 can be assigned to the carbinolamine form and that at 1.88 to the hydrate on the basis of the chemical shift difference stated above and from a comparison of the ratios of peak areas of the acetyl methyl resonances to peak areas of the bridging methylene resonances as a function of temperature. From these results and peak area ratio analysis, the entire ^{1}H NMR spectrum can be interpreted and assignments made for the remaining resonances to their specific molecular form (i.e., hydrate, keto, or carbinolamine). The results are listed in Table I.

^{13}C NMR Spectrum of Acetyl-$^{13}C_2$-TPP. The proton-decoupled ^{13}C NMR spectrum of acetyl-$^{13}C_2$-TPP is shown in Figure 3. In agreement with the ^{1}H NMR spectrum described above and the ^{31}P NMR spectrum discussed below, the proton-decoupled ^{13}C NMR spectrum indicates that there are three molecular forms of acetyl-TPP in solution. The three doublets appearing at δ 26.9, 27.3, and 28.9 are assigned to the C2 carbon and the three doublets occurring at δ 78.2, 93.4, and 185.6 are assigned to the C1 carbon. Identification of which C1 resonance is coupled to which C2 resonance can be made by determining the C1–C2 coupling constant for each doublet. In addition, the chemical shift position and the peak integrations of the proton-coupled ^{13}C NMR spectrum, where NOE effects are eliminated (not shown), allow assignments to be made as to the molecular form of acetyl-$^{13}C_2$-TPP each resonance represents. The results are listed in Table II.

Table II also gives the average signal width for the two peaks in each doublet. Comparison of these values shows that the carbinolamine form of the molecule has the broadest lines in both the C1 and C2 carbons. The line widths for both the C1

Table I: Assignment of ^{1}H NMR Signals from Figure 2 for Hydrate, Keto, and Carbinolamine Forms of Acetyl-TPP



^{1}H	peak (δ)	assignment	^{1}H	peak (δ)	assignment
a	1.88 (s)	hydrate	e	5.61 (d)[a,b]	carbinolamine
a	2.35 (s)	carbinolamine	e	5.72 (s)	hydrate
a	2.85 (s)	keto	e	5.80 (s)	keto
b	3.32 (t[a])	hydrate + carbinolamine	e	6.39 (d)[a,b]	carbinolamine
b	3.50 (t)[a]	keto	f	2.41 (s)	hydrate
c	4.15 (m)[a]	carbinolamine	f	2.57 (s)	keto
c	4.20 (m)[a]	hydrate	f	2.58 (s)	carbinolamine
c	4.27 (m)[a]	keto	g	7.34 (s)	keto
d	2.61 (s)	hydrate	g	7.35 (s)	hydrate
d	2.62 (s)	carbinolamine	g	8.59 (s)	carbinolamine
d	2.63 (s)	keto			

[a] Chemical shift reported is to center of multiplet. [b] Coupling constant is 16 Hz.

Table II: Assignments, C1–C2 Coupling Constants, and Peak Width of ^{13}C NMR Signals for Hydrate, Keto, and Carbinolamine Forms of Acetyl-$^{13}C_2$-TPP Shown in Figure 3

peak[a] (δ)	coupling constant (Hz)	peak width[b] (Hz)	assignment
26.9 (d)	41.0	3.50	C2, carbinolamine
27.3 (d)	43.8	2.06	C2, hydrate
28.9 (d)	47.5	1.51	C2, keto
78.2 (d)	41.0	3.45	C1, carbinolamine
93.4 (d)	43.8	1.71	C1, hydrate
185.6 (d)	47.5	1.41	C1, keto

[a] Chemical shift reported is to center of doublet. [b] Average peak width at half-height of the two peaks in the doublet.

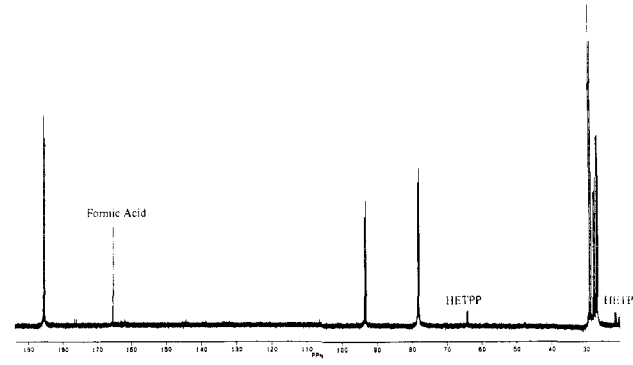


FIGURE 3: ^{13}C NMR spectrum of 15 mM acetyl-$^{13}C_2$-TPP in 50 mM HCl and 20% D_2O. The sample was prepared as described under Experimental Procedures. Assignments, peak widths, and coupling constants for acetyl-$^{13}C_2$-TPP are given in Table II.

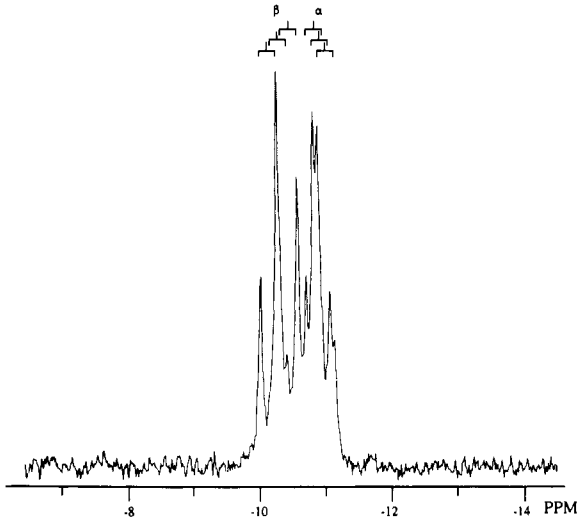


FIGURE 4: ^{31}P NMR spectrum of acetyl-TPP in 50 mM DCl and 1.25 mM EDTA in D_2O. Chemical shifts of the coupled α and β phosphates are indicated at the top of the figure. From left to right the order of the different forms of acetyl-TPP are, for the β phosphate, carbinolamine, hydrate, and keto, and, for the α phosphate, hydrate, carbinolamine, and keto. The sample was prepared as described under Experimental Procedures.

and C2 carbons are about twice as broad in the carbinolamine as the carbon resonances in the keto and hydrate forms. This is consistent with the carbinolamine adduct structure for reasons addressed under Discussion.

^{31}P NMR Spectrum of Acetyl-TPP. Figure 4 shows the proton-decoupled ^{31}P NMR spectrum of acetyl-TPP. Similar to the proton spectrum and ^{13}C NMR spectrum, the phosphorus spectrum is also complicated by the presence of three different forms of acetyl-TPP. The α and β phosphorus coupling constant is 20 Hz, and so by close inspection one can identify doublets that are part of an AB pattern. A proton-coupled ^{31}P NMR spectrum of acetyl-TPP (not shown) showed that the upfield doublets are due to the α phosphate resonances and the downfield doublets to the β phosphate as indicated in Figure 4. If the chemical shifts of the α and β phosphorus for all three forms of acetyl-TPP were well resolved, then one would expect 6 signals, each a doublet, or 12 in all. At the pH at which the spectrum was recorded (a low pH where acetyl-TPP remains stable), the signals are not completely resolved. Nine distinct signals are seen with the remaining apparently in overlapping positions.

Equilibrium Constants between Hydrate, Keto, and Carbinolamine Forms of Acetyl-TPP as a Function of Temperature. The effect of temperature on the equilibrium among hydrate, keto, and carbinolamine forms of acetyl-TPP in D_2O at pD 1.3 is given in Table III. The equilibrium constants show that the carbinolamine and hydrate species decrease in concentration as the temperature is increased. It is interesting that the ratio K_1/K_2, which is equal to [carbinolamine]/[hydrate], remains nearly constant throughout the temperature range at an average value of 3.0 ± 0.3. The enthalpy and entropy values for these two processes are given in the legend of Table III.

Hydrolysis of Acetyl-TPP at 24 °C. At pH 2.8 and 7.0, the observed first-order rate constants for the hydrolysis of acetyl-TPP are (2.63 ± 0.03) × 10^{-5} s^{-1} and (1.18 ± 0.01) × 10^{-2} s^{-1}, respectively. The rate of hydrolysis continues to decrease with decreasing pH such that at pH 1.5 and room temperature the amount of acetyl-TPP hydrolyzed over a 24-h period is less than 10%. It is apparent from these results that acetyl-TPP in water is rapidly hydrolyzed at neutral pH but is relatively stable under acidic conditions.

Table III: Equilibrium Constants between Hydrate, Keto, and Carbinolamine Forms of Acetyl-TPP in D_2O at pD 1.3 as a Function of Temperature[a]

carbinolamine $\overset{K_1}{\rightleftarrows}$ keto $\overset{K_2}{\rightleftarrows}$ hydrate

K_1 = [carbinolamine]/[keto] K_2 = [hydrate]/[keto]

temp (°C)	K_1	K_2	temp (°C)	K_1	K_2
6.85	1.6	0.64	31.9	1.3	0.42
11.9	1.6	0.50	36.9	0.96	0.36
16.9	1.4	0.55	41.9	0.95	0.28
21.9	1.4	0.43	46.9	0.83	0.25
26.9	1.3	0.43			

[a] Equilibrium constants were calculated as described under Experimental Procedures by using the equilibrium concentrations of each form found in the ^1H NMR spectrum. The enthalpy and entropy values calculated from these data are for K_1, ΔH = −3.0 ± 0.4 kcal mol^{-1} and ΔS = −9.4 ± 1.3 cal·deg^{-1}·mol^{-1}, and for K_2, ΔH = −3.8 ± 0.5 kcal mol^{-1} and ΔS = −14.3 ± 1.5 cal·deg^{-1}·mol^{-1}.

Table IV: Apparent Extinction Coefficient of Acetyl-TPP at 310 nm[a]

solvent	pH	$\epsilon_{310\ nm}$ (M^{-1} cm^{-1})
0.050 M sodium phosphate[b]	2.81	3589 ± 22
0.050 M sodium formate[b]	3.52	3573 ± 10
0.050 M sodium formate[b]	4.01	3445 ± 31
0.050 M sodium acetate[b]	4.54	3148 ± 15
0.050 M sodium acetate[b]	5.02	2800 ± 25
0.050 M sodium succinate[b]	5.57	2552 ± 10
0.050 M sodium succinate[b]	6.13	2400 ± 11
0.050 M sodium phosphate[b]	6.68	2329 ± 22
0.75 M sodium formate	4.0	3190
0.75 M sodium formate + 1.0 M 2-mercaptoethanol	4.0	2870
0.75 M sodium acetate	5.0	2740
0.75 M sodium acetate + 1.0 M 2-mercaptoethanol	5.0	2530
1.0 M hydroxylamine	6.0	567
0.50 M hydroxylamine	6.0	763
0.25 M hydroxylamine	6.0	954
0.125 M hydroxylamine	6.0	1280
1.0 M dipotassium phosphate	7.0	1920
50 mM HCl in 95% ethanol		2230

[a] Apparent extinction coefficients were found as described under Experimental Procedures. Each value is the average of three measurements except where standard deviation is not given, in which case the value is the average of two measurements. [b] The ionic strength of these solutions was adjusted to 0.50 M by the addition of NaCl.

Reaction of Acetyl-TPP with Nucleophiles. One molar hydroxylamine in H_2O reacts as a nucleophile with acetyl-TPP, trapping the acetyl moiety to the extent of 56, 67, and 74% at pH 5.5, 6.0, and 6.5, respectively. At pH 6.0 the observed first-order rate constant for the disappearance of acetyl-TPP in the presence of 1.0 M hydroxylamine (measured by the decrease in absorption at 310 nm as a function of time) is (9.7 ± 0.7) × 10^{-3} s^{-1} as calculated by the observed half-life, while the measured observed first-order rate constant for the formation of acetohydroxamic acid is (3.37 ± 0.15) × 10^{-3} s^{-1}. In contrast to hydroxylamine, the other nucleophiles tested did not trap the acetyl moiety of acetyl-TPP at all or were not nearly as effective. Phosphate and dihydrolipoic acid under the conditions outlined under Experimental Procedures did not react as nucleophiles with acetyl-TPP, nor did *n*-butyl mercaptan in 95% ethanol. One molar 2-mercaptoethanol reacts poorly as a nucleophile toward acetyl-TPP, trapping the acetyl moiety only 5.3 and 24% at pH 5.0 and 4.0, respectively.

The apparent extinction coefficients of acetyl-TPP at 310 nm under a variety of conditions are given in Table IV. The table shows that the fraction of keto form is strongly dependent upon the pH. In fact, a plot of ϵ vs pH for the first eight values

describes a titration curve (not shown) with an apparent pK_a of approximately 4.8, the value associated with the N-1 pK_a of the pyrimidine ring (Cain et al., 1977). The results also show that 1.0 M 2-mercaptoethanol has a rather small effect on the extinction coefficient at both pH 4.0 and 5.0, indicating binding of this nucleophile, presumably as a mercaptol adduct. Hydroxylamine in the range of 0.125–1.0 M, however, has a profound effect on the extinction coefficient. The adduct formed by reaction of 1.0 M hydroxylamine with acetyl-TPP prior to cleavage probably accounts for a large portion of the total acetyl-TPP in solution.

Discussion

Synthesis of Acetyl-TPP. The use of chromic acid in the oxidation of HETPP might appear to be a harsh approach to the synthesis of acetyl-TPP, considering the known chemical lability of the molecule. The thiazolium ring opening reaction, tricyclic species formation, and air oxidation of the sulfur anion in the open ring form, to name a few, are some examples of the coenzyme's chemical lability under alkaline conditions (Maier & Metzler, 1957). In the presence of strong oxidizing agents such as chromic acid, one might expect oxidative susceptibility in both the pyrimidine and open thiazolium rings. These reactions would not be favorable under acidic conditions, however, since the pyrimidine ring is protonated and the hydroxide concentration is too low to open the thiazolium ring. It is clear then that under acidic conditions the most susceptible group for oxidation in HETPP is the secondary alcohol of the hydroxyethyl moiety.

Synthesis of acetyl-TPP in aqueous solution must be carried out under acidic conditions to prevent hydrolysis to acetate and TPP. The use of a different solvent is not feasible because of solubility limitations. The solvating capacity of water offers an advantage in addition to dissolution of HETPP, which is that Cr(VI) is a slower and thus more selective oxidizing agent in water than in a solvent such as acetic acid, presumably because of the greater degree of solvation of the Cr(VI) ion in water.

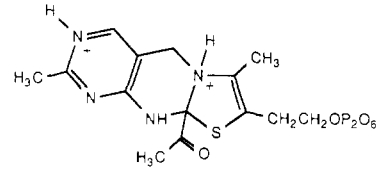
Two deleterious side reactions that had to be overcome were Cr(III) coordination to the pyrophosphoryl moiety and hydrolysis of the pyrophosphate. The former was prevented by the addition of a large excess of pyrophosphoric acid that trapped the Cr(III) as $Cr^{III}(pyrophosphate)_2$. The latter side reaction was controlled by working at concentrations of reactants such that the time required for complete oxidation was short relative to the rate of pyrophosphate hydrolysis. The pyrophosphoryl moiety in HETPP actually acts as a protecting group of the primary alcohol—which is hydroxyethyl in HET—preventing oxidation of this group.

Solution Structure of Acetyl-TPP. The characterization of acetyl-TPP by means of chemical properties is straightforward, but the NMR and UV spectra of Figures 1–4 reveal the existence of a complex mixture of species, including the keto and hydrate forms and an internal adduct. Given the precedent provided by the keto and hydrate species of the 2-acetyl-3,4-dimethylthiazolium ion, the existence of keto and hydrate forms of acetyl-TPP is easily understood. The nature of the unexpected third species is less clear. As stated under Results, a clue to the structure of this species is the nonequivalence of the geminal protons associated with the bridging methylene group. These protons must be involved in a rigid structure. In our view, the most reasonable structure is that resulting from addition of the 4′-amino group of the pyrimidine ring to the acetyl carbonyl group for reasons stated below.

In support of the above proposal is the observation that this amino group can act as an internal nucleophile as evidenced by the yellow sulfur anion form of TPP where nucleophilic addition has occurred at the C(2) thiazolium ring carbon (Maier & Metzler, 1957). Additional support based on structural considerations is provided by the crystal structure of thiamin thiazolone and by the confirmed structure of fawcettimine (a compound isolated from *Lycopodium fawcetti*). The 4′-amino group in thiamin thiazolone provides the hydrogen in the N–H···O intramolecular hydrogen bond that stabilizes the V conformation (Shin & Kim, 1986). This V conformation, where the 4′-amino group is close to the C(2) active center, is the same conformation of the proposed carbinolamine **3**. The more characteristic conformation of other C(2)-substituted thiamins is the S form, where the 4′-amino group is quite distant from the catalytic action occurring at the C(2) carbon (Shin et al., 1977). In the case of fawcettimine, a carbinolamine adduct as part of a seven-membered ring has been confirmed as the preferred structure for this molecule (Heathcock et al., 1986, and references cited within), thus providing literature support for such a structure being possible for acetyl-TPP.

Possible alternative intramolecular nucleophiles are the N-1 and N-3 nitrogens of the pyrimidine ring. These are poor candidates, however, even though the N-1 pyrimidine nitrogen is known to be more basic than the primary amine group (Cain et al., 1977). This is because the steric constraints imposed by planarity of the conjugated pyrimidine ring will not allow bonding of either of these nitrogens to the acetyl carbonyl carbon.

There is an alternative structure for the third species. Since the pyrimidine amino group can react as a nucleophile at the C(2) carbon of TPP and C(2)-substituted thiamins, a six-membered center ring structure may form rather than a carbinolamine. This structure is shown in **4**.



4

There are good reasons for discarding **4** as a possible alternative to the carbinolamine **3** despite its favorable six-membered ring compared with the unfavorable seven-membered ring in the carbinolamine **3**. Nucleophilic addition at the C(2) carbon results in the loss of aromaticity in the thiazolium ring and because of this occurs only at pH values above 10—where the thiazolium ring nitrogen loses a proton—with TPP and C(2)-substituted thiamin compounds (Maier & Metzler, 1957). Thus, it seems unlikely that under the acidic conditions at which acetyl-TPP was examined by NMR analysis (pD 1.3) structure **4** would form. Above all, structure **4** is not consistent with the NMR data. One would expect **4** to be in equilibrium with its hydrate, which, if true, should result in four structures of acetyl-TPP being evident in the NMR spectra. All of the data show only three different forms for the molecule. In addition, the ^{13}C NMR spectrum of acetyl-$^{13}C_2$-TPP shows that with the C1 acetyl carbon only one resonance occurs in the region associated with sp^2 hybridization, and this is assigned to the keto form of the molecule **1**. The chemical shift of the other two C1 resonances clearly occur in the range expected of sp^3 adducts containing electron-withdrawing heteroatoms (i.e., a hydrate and carbinolamine). Structure **4** could possibly exist if it is 100% hydrated, but there would then need to be an alternative explanation for the line broadening in the ^{13}C NMR spectrum

at δ 26.9 and 78.2 (discussed below). The hydrate of **4** occurring at 100% also seems unlikely since, if anything, one would expect the equilibrium to favor the keto form.

The assignments in the ^{13}C NMR spectrum of acetyl-$^{13}C_2$-TPP at δ 26.9 and 78.2 to the carbinolamine C2 and C1 carbons, respectively, are strengthened by the line-width calculations reported in Table II. Since ^{14}N is quadrupolar ($I = 1$) and relaxes quickly, nuclei that are spin–spin coupled to it can show a broadened singlet or a triplet depending on the ratio of the T_1 of nitrogen to the coupling constant between the two nuclei. While the larger line widths could possibly result from other factors, they are at least consistent with a C1–N bond and our assignments to a carbinolamine structure.

The stability of the proposed carbinolamine is at least in part attributable to the electrophilicity of the acetyl moiety and the fact that the compound is a result of an intramolecular nucleophilic addition. This apparently compensates for the strained seven-membered ring. The effectiveness of the intramolecular nucleophile over an intermolecular nucleophile at 55.5 M concentration is demonstrated by the data in Table III. The carbinolamine form of acetyl-TPP is preferred over the hydrate by an average factor of 3 within the temperature range of 6.85–46.9 °C. It is also interesting to note that the thermodynamic properties of the hydrate and carbinolamine formation reactions are somewhat similar, according to the calculated ΔS and ΔH values, but that a larger negative entropy term for hydrate formation compared to carbinolamine formation is the reason for greater carbinolamine concentration in the temperature range tested.

The proposed carbinolamine is a structural type normally not sufficiently stable to be observed in solution, since in most cases the loss of water to form a ketimine is highly favorable (Forlani et al., 1984; Diebler & Thorneley, 1973). Although one might expect the formation of this carbinolamine to lead to a ketimine, the absence of absorption in the visible spectrum and the sp^3 character of the C1 carbon in the adduct seen in the ^{13}C NMR spectrum do not support such a structure. Absorption in the visible spectrum would be an expected spectroscopic feature of a ketimine form of the molecule because of extended conjugation that would exist in such a structure. The lack of detectable ketimine formation can be rationalized by the structural limitations of the molecule; the addition of a double bond cannot be tolerated in the already strained seven-membered ring. It should be noted that in the case of fawcettimine its seven-membered ring carbinolamine also does not dehydrate for presumably the same reasons given here for the carbinolamine form of acetyl-TPP (Heathcock et al., 1986).

The UV absorption band at 310 nm was assigned to the keto form of acetyl-TPP on the basis of the parallel relationship between the increase in A_{310} with increasing temperature and the corresponding increase in keto form with increasing temperature seen by ^1H NMR. The absorption band at 280 nm could be due to either the carbinolamine or hydrate forms of the molecule since the A_{280} decrease with increasing temperature is a result expected from both the hydrate and carbinolamine forms. On the basis of structural similarities, however, one would expect the UV spectrum of the hydrate to resemble that of HETPP, which exhibits no band at 280 nm. Moreover, the hydrate of the 2-acetyl-3,4-dimethylthiazolium ion does not exhibit a 280-nm UV band. Therefore, it seems reasonable to assign this band to the carbinolamine form of the molecule.

The only questionable assignments in the ^1H NMR spectrum of acetyl-TPP are those for the pyrimidine and thiazolium ring methyl groups. One rationale for the assignments is based on the nearly identical chemical shift at δ 2.42 for the pyrimidine methyl resonance of HETPP and the hydrate methyl group resonance for acetyl-TPP at δ 2.41. The structural similarity between HETPP and the hydrate form of acetyl-TPP supports this assignment. Another rationale for the assignments is based on the observation that with both TPP and HETPP the pyrimidine methyl resonance appears upfield from the thiazolium methyl resonance.

Hydrolysis of Acetyl-TPP. The results of the hydrolysis of acetyl-TPP showing lability at neutral pH and relative stability under acidic conditions is a general feature expected of the compound and similar to results seen with model compounds (Breslow & McNelis, 1960; White & Ingraham, 1960, 1962; Daigo & Reed, 1962; Lienhard, 1966). However, preliminary results of a more detailed kinetic analysis of the hydrolysis reaction have shown that significant differences exist between the model compound, 2-acetyl-3,4-dimethylthiazolium ion (Lienhard, 1966), and acetyl-TPP.[2] These differences appear to be a direct result of the presence of the pyrimidine ring and will be presented in a future paper along with mechanistic details of the hydration and adduct formation reactions.

Reaction of Acetyl-TPP with Nucleophiles. The ability of a nucleophile to trap the acetyl moiety of acetyl-TPP in water is most likely dependent upon three factors relative to those for the water reaction: the ability of the nucleophile to form a tetrahedral adduct, the acidity of the hydroxyl group in the adduct, and the rate of decomposition of the alcoholate resulting from deprotonation of the adduct. The thiazolium group is common to all adducts, water included, and probably has a major effect on the pK_a. This fact along with the similar inductive effects of the HO and RS groups (Taft et al., 1958) (with that for hydroxylamine probably being similar) suggests that the acidities of the hydrate and nucleophile adducts are similar. This leaves the reactivity of the nucleophile toward the keto form and the rate of decomposition of the alcoholate anion as the controlling factors in governing the amount of trapping with a particular nucleophile.

The data in Table IV provide information regarding the contribution of each of the above-mentioned factors to the cleavage capability of a particular nucleophile. In the case of hydroxylamine, the ability to form an adduct is quite strong. Because of this, there is probably little free keto form available for hydrate formation. The increase in trapping with increasing pH around the pK_a of hydroxylamine also reflects greater hydroxylamine adduct formation as the concentration of the free base increases. Much of its effectiveness as a nucleophile is, therefore, based on its ability to form an adduct with acetyl-TPP. The decomposition of the hydroxylamine adduct is also a significant contributing factor, since the rate of disappearance of acetyl-TPP at pH 6.0 in the presence of 1.0 M hydroxylamine is close to the hydrolysis rate at pH 7.0.

The calculated rate constant for acetohydroxamic acid formation in the reaction of hydroxylamine with acetyl-TPP was measurably smaller than the rate constant for acetyl-TPP disappearance. A partial explanation for this difference is that hydrolysis proceeds in addition to hydroxylamine-mediated cleavage. However, a stated above, in the presence of 1.0 M hydroxylamine little hydrate is present for breakdown to hydrolysis products. The difference in rates is not surprising, however, when one considers the known chemistry of hydroxylamine attack on activated acyl groups. Jencks (1958)

[2] K. J. Gruys and P. A. Frey, unpublished results.

has pointed out that caution must be used when rates of hydroxylamine reactions with activated acyl groups are measured since the rate of acylhydroxamic acid formation is slower than the rate of breakdown of the nucleophile adduct. This is because the initially formed product is *O*-acylhydroxylamine, which gives no color with ferric chloride. In the presence of a large excess of hydroxylamine the *O*-acylhydroxylamine is then cleaved to form the acylhydroxamic acid.

One molar 2-mercaptoethanol partially traps the acetyl moiety of acetyl-TPP, though not nearly as effectively as hydroxylamine. The data in Table IV indicate that 2-mercaptoethanol depresses the extinction coefficient at 310 nm at pH 4.0 and 5.0 much less than 1 M hydroxylamine. This reflects the smaller tendency of 2-mercaptoethanol to compete with water in forming a tetrahedral adduct and probably accounts for its poor trapping ability. It is interesting, however, that the increase in the percent of trapping at pH 4.0 compared with pH 5.0 is greater than a factor of 4. Since there is no known difference in the properties of 2-mercaptoethanol and water at pH 4.0 and 5.0, the large difference in the percent of trapping must reflect a significant increase in the rate constant ratio, k (for decomposition of the hemithioketal)/k (for decomposition of the hydrate) from pH 5.0 to 4.0.

The absence of any trapping by dihydrolipoate under the conditions outlined under Experimental Procedures is not surprising when the results seen with 2-mercaptoethanol are considered. The concentration of dihydrolipoate in the reaction mixture was only half that of 2-mercaptoethanol, and at pH 5.0 trapping to even half the extent of 2-mercaptoethanol at that pH would have been at the minimum detection limit. Unfortunately, the low solubility of dihydrolipoic acid at pH below 5.0 did not permit us to explore trapping at low pH. Phosphate dianion does appear to add to the keto form of acetyl-TPP by comparison of the molar extinction coefficients of 1.0 vs 0.050 M nucleophile, but the effect on the extinction coefficient is small. The lack of any trapping with this nucleophile is at least partially due to little adduct formation. *n*-Butyl mercaptan in 95% ethanol had little chance of trapping the acetyl moiety of acetyl-TPP since ethanol is even more effective in forming a hemiketal than water is in forming a hydrate. The molar extinction coefficient of acetyl-TPP at 310 nm in 95% ethanol and 50 mM HCl is only 65% that in aqueous 30 mM HCl (refer to Table IV).

In Lienhard's study of the kinetics and mechanism of the hydrolysis and hydration reactions of the 2-acetyl-3,4-dimethylthiazolium ion (Lienhard, 1966), the model compound displayed general properties similar to those of acetyl-TPP that have been very helpful in characterizing the latter compound. The properties of the 2-acetyl-3,4-dimethylthiazolium ion nonetheless differed significantly in detailed respects from those of acetyl-TPP. Contrary to the results presented in Table IV, mercaptans and phosphate dianion added to the carbonyl group of the 2-acetyl-3,4-dimethylthiazolium ion to a significant extent. There also was no pH effect on the molar extinction coefficient of the 2-acetyl-3,4-dimethylthiazolium ion at the wavelength associated with the keto form of the model compound, a result opposite to that of acetyl-TPP. Because of the observed pK_a, the pH effect on acetyl-TPP is concluded to reflect the protonation state of the pyrimidine ring. Therefore, the differences in chemical properties between the model compound and acetyl-TPP can be largely attributed to the presence of the pyrimidine ring.

Conclusions. One criterion needed to support the hypothesis that acetyl-TPP is viable as an enzymatic reaction intermediate is that the molecule be of high enough energy to acetylate compounds such as phosphate in the reaction catalyzed by phosphoketolase. The kinetic and thermodynamic reactivity of acetyl-TPP with water and other nucleophiles such as mercaptans and hydroxylamine are evidence that the properties of this molecule are indeed compatible with a hypothetical role for acyl-TPPs as enzymatic acyl-transfer intermediates. The susceptibility of acetyl-TPP toward hydration and hydrolysis also indicates that as an enzyme-bound reaction intermediate acetyl-TPP must be shielded from bulk water.

The presence of an intramolecular carbinolamine form of acetyl-TPP in aqueous solution is intriguing. It seems unlikely that this structure has relevance to enzyme-bound acetyl-TPP, but the resulting V conformation of the carbinolamine has been suggested to be the conformation of the "active" enzyme-bound thiamin (Shin & Kim, 1986; Schellenberger, 1967, 1982). Therefore, it cannot necessarily be ruled out as a possible enzyme-bound form of the molecule. This of course would result in a direct involvement of the 4′-amino group in catalysis.

A major goal of this investigation was to better understand the spectroscopic properties and chemical characteristics of acetyl-TPP to aid our identification of it as an intermediate in reactions catalyzed by pyruvate dehydrogenase complex. This has been accomplished, and the results will be useful for interpreting further mechanistic work with this and other enzymes. In addition, this chemical information and the synthesis of the molecule in relatively large quantities will allow it to be investigated as a substrate with the pyruvate dehydrogenase component of the complex. Studies of other enzymes postulated to produce acetyl-TPP as a catalytic intermediate might be advanced by the same type of analysis.

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REFERENCES

Ahrens, M. L., & Strehlow, H. (1965) *Discuss. Faraday Soc. 39*, 112–120.
Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.
Bell, R. P., & McDougall, A. O. (1960) *Trans. Faraday Soc. 56*, 1281–1285.
Breslow, R. (1958) *J. Am. Chem. Soc. 80*, 3719–3726.
Breslow, R., & McNelis, E. (1960) *J. Am. Chem. Soc. 82*, 2394–2395.
Cain, A. H., Sullivan, G. R., & Roberts, J. D. (1977) *J. Am. Chem. Soc. 99*, 6423–6425.
CaJacob, C. A., Gavino, G. R., & Frey, P. A. (1985) *J. Biol. Chem. 260*, 14610–14615.
Crosby, J., & Lienhard, G. E. (1970) *J. Am. Chem. Soc. 92*, 5707–5716.
Crosby, J., Stone, R., & Lienhard, G. E. (1970) *J. Am. Chem. Soc. 92*, 2891–2900.
Curtis, R. G., Heilbron, I., Jones, E. R. H., & Woods, G. F. (1953) *J. Chem. Soc.*, 457–464.
Daigo, K., & Reed, L. J. (1962) *J. Am. Chem. Soc. 84*, 659–662.
Das, M. L., Koike, M., & Reed, L. J. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 753–759.
Diebler, H., & Thorneley, R. N. F. (1973) *J. Am. Chem. Soc. 95*, 896–904.
Flournoy, D. S., & Frey, P. A. (1986) *Biochemistry 25*, 6036–6043.
Foneseca-Wollheim, F. da, Bock, K. W., & Hoizer, H. (1962) *Biochem. Biophys. Res. Commun. 9*, 466–471.

Forlani, L., Marianucci, E., & Todesco, P. E. (1984) *J. Chem. Res., Synop.* 126–127.
Goldberg, M. L., & Racker, E. (1962) *J. Biol. Chem. 237*, 3841–3842.
Heathcock, C. H., Smith, K. M., & Blumenkopf, T. A. (1986) *J. Am. Chem. Soc. 108*, 5022–5024.
Hine, J., & Redding, R. W. (1970) *J. Org. Chem. 35*, 2769–2772.
Hine, J., Houston, J. G., & Jensen, J. H. (1965) *J. Org. Chem. 30*, 1184–1188.
Holzer, H., & Beaucamp, K. (1961) *Biochim. Biophys. Acta 46*, 225–243.
Holzer, H., Goedde, H. W., & Ulrich, B. (1961) *Biochem. Biophys. Res. Commun. 5*, 447–451.
Jencks, W. P. (1958) *J. Am. Chem. Soc. 80*, 4581–4584.
Kerscher, L., & Oesterhelt, D. (1981) *Eur. J. Biochem. 116*, 595–600.
Kluger, R., Chin, J., & Smyth, T. (1981) *J. Am. Chem. Soc. 103*, 884–888.
Kluger, R., Stergiopoulos, V., Gish, G., & Karimian, K. (1985) *Bioorg. Chem. 13*, 227–234.
Kokesh, F. C. (1976) *J. Org. Chem. 41*, 3593–3599.
Krampitz, L. O. (1969) *Annu. Rev. Biochem. 38*, 213–240.
Lienhard, G. E. (1966) *J. Am. Chem. Soc. 88*, 5642–5649.
Lombardi, E., & Sogo, P. B. (1960) *J. Chem. Phys. 32*, 635–636.
Maier, G. D., & Metzler, D. E. (1957) *J. Am. Chem. Soc. 79*, 4386–4391.
Mieyal, J. J., Votaw, R. G., Krampitz, L. O., & Sable, H. Z. (1967) *Biochim. Biophys. Acta 141*, 205–208.
Rose, I. A., Grunberg-Manago, M., Korey, S. R., & Ochoa, S. (1954) *J. Biol. Chem. 211*, 737–756.
Schellenberger, A. (1967) *Angew. Chem., Int. Ed. Engl. 6*, 1024–1035.
Schellenberger, A. (1982) *Ann. N.Y. Acad. Sci. 378*, 51–62.
Schroter, W., & Holzer, H. (1963) *Biochim. Biophys. Acta 77*, 474–481.
Shin, W., & Kim, Y. C. (1986) *J. Am. Chem. Soc. 108*, 7078–7082.
Shin, W., Pletcher, J., Blank, G., & Sax, M. (1977) *J. Am. Chem. Soc. 99*, 3491–3499.
Steginsky, C. A., & Frey, P. A. (1984) *J. Biol. Chem. 259*, 4023–4026.
Taft, R. W., Jr., & Lewis, I. C. (1958) *J. Am. Chem. Soc. 80*, 2436–2443.
Toshima, Y., & Yoshimura, N. (1975) *J. Biochem. (Tokyo) 78*, 1161–1169.
White, F. G., & Ingraham, L. L. (1960) *J. Am. Chem. Soc. 82*, 4114–4115.
White, F. G., & Ingraham, L. L. (1962) *J. Am. Chem. Soc. 84*, 3109–3111.

Thyroid Hormones Selectively Modulate Human Alcohol Dehydrogenase Isozyme Catalyzed Ethanol Oxidation†

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ABSTRACT: Thyroid hormones are potent, instantaneous, and reversible inhibitors of ethanol oxidation catalyzed by isozymes of class I and II human alcohol dehydrogenase (ADH). None of the thyroid hormones inhibits class III ADH. At pH 7.40 the apparent K_i values vary between 55 and 110 μM for triiodothyronine, 35 and >200 μM for thyroxine, and 10 and 23 μM for triiodothyroacetic acid. The inhibition is of a mixed type toward both NAD^+ and ethanol. The binding of the thyroid hormone triiodothyronine to $\beta_1\gamma_1$ ADH is mutually exclusive with 1,10-phenanthroline, 4-methylpyrazole, and testosterone, identifying a binding site(s) for the thyroid hormones, which overlap(s) both the 1,10-phenanthroline site near the active site zinc atom and the testosterone binding site, the latter being a regulatory site on the γ-subunit-containing isozymes and distinct from their catalytic site. The inhibition by thyroid hormones may have implications for regulation of ADH catalysis of ethanol and alcohols in the intermediary metabolism of dopamine, norepinephrine, and serotonin and in steroid metabolism. In concert with other hormonal regulators, e.g., testosterone, the rate of ADH catalysis is capable of being fine tuned in accord with both substrate and modulator concentrations.

Metabolic pathways are regulated by a variety of mechanisms. The most immediate form of regulation is through specific interactions of substrates, products, and effectors with allosteric enzymes. Other levels of metabolic control are exerted by hormonal regulation and through the biosynthesis and degradation of constituent enzymes.

These considerations underlie the study of human alcohol dehydrogenase (ADH).[1] Three classes of structurally similar physicochemically distinct, dimeric isozymes are expressed by at least five genetic loci (Vallee, 1985). The catalytic properties of these isozymes differ markedly with respect to a number of alcohols and aldehydes in the intermediary metabolism of dopamine (Mårdh & Vallee, 1985), norepinephrine (Mårdh et al., 1985, 1986a), serotonin (Consalvi et al., 1986), and steroids (Frey & Vallee, 1980). Their number and distribution also vary both with the complexity of the organism

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[1] Abbreviations: ADH, alcohol dehydrogenase; K_i', apparent inhibition constant at fixed ethanol and NAD^+ concentrations; OP, 1,10-phenanthroline; 4-MePz, 4-methylpyrazole.

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