Synthesis and Chemical Properties of Ethenothiamin†

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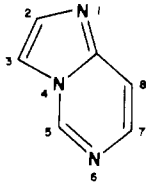
ABSTRACT: *N*-[(5-Methylimidazo[1,2-*c*]pyrimidin-8-yl)-methyl]-4-methyl-5-(2-hydroxyethyl)thiazolium bromide [ethenothiamin (V)] has been synthesized by two methods: (1) condensation of 5-methyl-8-(bromomethyl)imidazo[1,2-*c*]pyrimidine hydrobromide (IV) with 4-methyl-5-thiazoleethanol (VIII); (2) condensation of thiamin with chloroacetaldehyde, followed by extensive purification. Ethenothiamin was identified by elemental analysis and UV and proton NMR spectroscopy. The UV spectrum showed a maximum at 260 nm ($\epsilon = 6.5 \times 10^3$ M^{-1} cm^{-1}) with a shoulder at 295 nm ($\epsilon = 2.68 \times 10^3$ M^{-1} cm^{-1}). The ^1H NMR spectrum, in ^2H_2O (reference 2,2-dimethyl-2-silapentane-5-sulfonate), pH 7, had peaks at δ 7.95 (1 H, d), 7.93 (1 H, s), 7.69 (1 H, d), 5.88 (2 H, s), 3.84 (2 H, t), 3.14 (2 H, t), 2.89 (3 H, s), and 2.56 (3 H, s). Ethenothiamin is fluorescent; for solutions in 0.1 M phosphate, pH 7, excitation at 325 nm gives a broad emission maximum centered at ~410 nm. Ethenothiamin and 1-[(5-methylimidazo[1,2-*c*]pyrimidin-8-yl)-methyl]-2-methylpyridinium bromide [ethenoamprolium (VII)], prepared according to Kluge [Kluge, A. F. (1978) *J. Heterocycl. Chem. 15*, 119], and 8-(hydroxymethyl)-5-methylimidazo[1,2-*c*]pyrimidine (III) were compared with thiamin and the related 4-amino-5-hydroxymethyl-2-methylpyrimidine (II) with respect to acid–base properties. For solutions in ^2H_2O at 19 °C, compounds V and VII have pK_a' values of 2.3 and 2.4, compared to 5.36 for thiamin; the imidazo compound III has $pK_a' = 4.52$ compared with 6.76 for the corresponding pyrimidine compound II. The ethenopyrimidines are, therefore, 10^2–10^3 times less basic than the corresponding pyrimidines. Under strongly acidic conditions, the pyrimidinyl CH_3 of thiamin undergoes ^1H–^2H exchange [Hutchinson, D. W. (1971) *Biochemistry 10*, 542]; under equivalent conditions the CH_3 of the imidazopyrimidine compound III exchanges ~30 times as rapidly as that of thiamin. The protonated form of ethenothiamin is stable in the solid state for at least 10 months. In aqueous solution the protonated forms of ethenothiamin and ethenoamprolium are unstable. At ambient temperature, fully protonated ethenoamprolium decomposes with $t_{1/2} \simeq 100$ min. As a result of the decomposition, the ^1H NMR signals ascribed to the protons of the picolinium ring are displaced upfield by 0.10–0.15 ppm, while those ascribed to the ethenopyrimidine ring disappear from that region of the spectrum; free picoline is not found. When ethenothiamin decomposes under the same conditions, a substance appears whose proton NMR spectrum is identical with that of 4-methyl-5-thiazoleethanol.

The condensation of chloroacetaldehyde with aminopurines and aminopyrimidines, first described by Kochetkov et al. (1971), has been extensively employed in the modification of nucleic acid fragments and some coenzymes. The condensation products, known as etheno[1] derivatives,[2] are very useful as they



are strongly fluorescent and in some cases can replace the analogous purine or pyrimidine compounds that are the natural substrates or coenzymes. Ethenoadenine nucleosides and nucleotides, synthesized by Secrist et al. (1972), have been used in the study of aspartate transcarboxylase (Chien & Weber, 1973) and the myosin ATPase (McCubbin et al., 1973) among others. The ϵ-cytidine analogue of NAD in which ethenocytidine replaces adenosine has been shown to be active with NAD-requiring dehydrogenases (Greenfield et al., 1975). The corresponding ϵ-adenosine analogues of NAD and FAD have been prepared (Barrio et al., 1972; Harvey & Damle, 1972), and a number of etheno analogues are commercially available. Kluge (1978) has reported the synthesis of an etheno analogue of amprolium, an antagonist of thiamin, and found no anticoccidial activity against either *Eimeria tenella* or *Eimeria necatrix*. At present, no other etheno analogue of compounds related to thiamin, or of thiamin itself, has been described. Thiamin pyrophosphate is required as a coenzyme for many enzymes, including transketolase (de la Haba et al., 1955), α-keto acid dehydrogenases (Metzler, 1960), and pyruvate decarboxylase (Gounaris et al., 1971). In these systems, the function of thiamin involves interaction of the thiazolium moiety with the carbonyl groups of α-keto acids or of ketose substrates (Breslow, 1962; Gallo et al., 1978). Thiamin triphosphate has been implicated in nerve conduction (Cooper & Pincus, 1979), although the details of its function are not known; derangements of thiamin metabolism can be found in uremic neuropathy (Egan & Wells, 1979), Leigh's disease (Cooper & Pincus, 1972), maple syrup urine disease (Dancis & Levitz, 1972), and pyruvate dehydrogenase deficiency (Blass, 1979). Although the solution chemistry of thiamin has been studied intensively, there is little understanding of the interactions of various thiamin phosphate esters

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[1] Abbreviations used: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; ϵ-, etheno; ^1H NMR, proton magnetic resonance; PP, pyrophosphate; TLC, thin-layer chromatography.

[2] The numbering of the ϵ-pyrimidine ring is shown in the text. In ϵ-thiamin and ϵ-amprolium, the thiazolium or picolinium rings are senior, so the positions in the ϵ-pyrimidine moiety are numbered 1′, 2′, etc.

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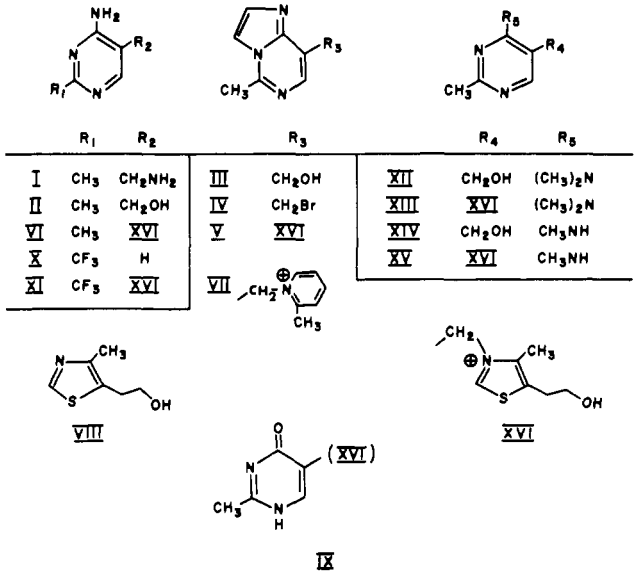
with proteins. The availability of ethenothiamin would be useful in further investigation of the biochemistry of thiamin.

The synthesis and characterization of ethenothiamin are reported in this work. Ethenothiamin was synthesized by two independent routes and the compound was characterized by spectroscopic methods. The solution chemistry of ethenopyrimidine compounds, principally ethenothiamin and ethenoamprolium, was investigated with special emphasis on acid–base properties and on the unexpected lability of the protonated species.

Experimental Section

Warning of Hazardous Substance. Chloroacetaldehyde is toxic, mutagenic, and carcinogenic [see Guengerich et al. (1979) and Malaveille et al. (1975)]. Experiments with this substance and its precursors should be carried out in a well-functioning hood, and suitable, impermeable gloves, aprons, and face shields should be worn.

Materials. Chloroacetaldehyde was purchased either from ICN as a 45% solution in water or from Aldrich as the dimethyl acetal. In the latter case, the free aldehyde was generated by refluxing the acetal with 0.10 N HCl for 3 h; the ~2 M solution contained some methanol. 2-Methyl-4-amino-5-(aminomethyl)pyrimidine dihydrochloride (I) was



obtained from Aldrich. Thiamin hydrochloride and 31% HBr in acetic acid were from Eastman. 4-Methyl-5-thiazoleethanol (VIII) was from Aldrich. Samples of 5-methyl-8-(hydroxymethyl)imidazo[1,2-*c*]pyrimidine (III), the hydrobromide of 5-methyl-8-(bromomethyl)imidazo[1,2-*c*]pyrimidine (IV), and 1-[(5-methylimidazo[1,2-*c*]pyrimidin-8-yl)methyl]-2-methylpyridinium bromide [ethenoamprolium (VII)] were prepared according to published procedures[3] (Kluge, 1978); in some of the experiments, compound III was purified by chromatography on silica gel columns eluted with $CHCl_3$–MeOH (9:1). In the conversion of the hydroxymethyl compound III to the bromo compound IV, the yields varied greatly, depending on the age of the brominating reagent.[3] All other compounds were reagent grade.

Table I: Thin-Layer Chromatographic Analyses[a]

compd	R_f on fluorescent cellulose, solvent A	R_f on fluorescent silica gel, solvent B
II	0.76	
III	0.82	
ϵ-thiamin (V)	0.77	0.23
partially degraded V	0.77, 0.82, 0.87	0.23, 0.70, 0.93
ϵ-amprolium (VII)	0.77	
partially degraded VII	0.77, 0.82, 0.92	
thiamin (VI)	0.75	
4-methyl-5-thiazole-ethanol (VIII)	0.9	0.93
α-picoline	*b*	

[a] Solvents: A, 1-propanol-H_2O-1 M NH_4OAc (65:20:15), pH 5.5; B, CH_3OH–$CHCl_3$ (1:1). [b] Evaporates.

Methods. NMR measurements were made variously on Bruker WH-270, Varian EM-390, and Varian A-60 spectrometers. The ambient temperature inside the probe of the A-60 spectrometer was 39 °C and that inside the WH-270 spectrometer was 19 °C. Chemical shifts are reported on the δ scale from internal 2,2-dimethyl-2-silapentane-5-sulfonate. This was achieved by adding 1 μL of acetone to the solution. In separate experiments the chemical shift of acetone with respect to DSS was found at δ 2.215 at 19 °C and δ 2.22 at 39 °C. Shifts in the downfield direction are positive. Fluorescence spectra were recorded on either Farrand Optical Co. Mk I or Spex Fluorolog fluorometers, and UV absorption spectra were recorded on a Cary 15 spectrophotometer. pH was measured with a Radiometer 26 pH meter. Two different methods were used for thin-layer chromatography. (a) ϵ-Thiamin (V) and ϵ-amprolium (VII) were applied to Avicel F cellulose and were developed in 1-propanol–water–1 M ammonium acetate (65:20:15), pH 5.5, and were visualized by excitation with a UV lamp. (b) Alternatively, Eastman silica gel coated sheets with a fluorescent indicator were used to monitor the preparation and purification steps, with MeOH–$CHCl_3$ (1:1) as the solvent. R_f values for the compounds of interest are shown in Table I.

2-Methyl-4-amino-5-(hydroxymethyl)pyrimidine (II) was prepared by the method of Andersag & Westphal (1937). To 2.11 g of I in 40 mL of water was added 0.75 g $NaNO_2$ in 10 mL of water with stirring. The solution was maintained at 60 °C for 1 h, during which there was rapid evolution of gas, and then the solvent was removed in vacuo. The solid was recrystallized from water, giving 1.2 g of a light yellow solid, which was characterized by its NMR spectrum and was used without further purification.

The rate of degradation of ϵ-amprolium was monitored by both TLC and NMR methods. Samples (10 mg) of ϵ-amprolium were incubated in 350 μL of 50 mM phosphate–50 mM fumarate buffer at various pH values and left at ambient temperature (23 °C). At timed intervals samples were removed and spotted on TLC plates, and the chromatograms were developed immediately (Table I). At the same time, a 50-μL aliquot was diluted into 100 mM phosphate buffer, pH 7.0, to give a final pH of 6.5–7.0, for eventual analysis by NMR. The TLC analysis showed the appearance and gradual increase of two substances of high R_f, with concomitant decrease of the size of the ϵ-amprolium spot. For the analysis by NMR spectroscopy, the neutralized samples were lyophilized to dryness and redissolved in ^2H_2O. The amount of the degradation was calculated from the ratio of the signal of undegraded material (e.g., signals J or K of Figure 4 or the signal of the CH_2 bridge) to the sum of the signals of the

[3] HBr in glacial acetic acid is frequently used to convert primary alcohols into the corresponding bromo compounds. One of our laboratories has observed that when the reagent becomes old, even if the bottle had never been opened, the yields are very poor. In addition, an opened bottle, used again a week later for an identical reaction, gave significantly poorer yields. Consequently, the use of fresh reagent for each preparation in desirable.

Table II: Proton Chemical Shifts and Coupling Constants of Ethenothiamin and Ethenoamprolium[a]

	ethenothiamin			ethenoamprolium			
	protonated	unprotonated	J		protonated	unprotonated	J
C(4) CH_3	2.56, s	2.56		C(2) CH_3	2.89, s	2.89	
C(5') CH_3	3.08, s	2.89		C(5') CH_3	3.07, s	2.93	
C(5) CH_2-	3.20, t	3.15	5.8				
C(5) C-CH_2-	3.89, t	3.85	5.8				
bridge CH_2	6.09, s	5.88		bridge CH_2	6.23, s	6.04	
C(2')*H* or C(3')*H*	8.13, d	7.69	1.8	C(2')*H* or C(3')*H*	8.14, d	7.68	1.4
C(3')*H* or C(2')*H*	8.34, d	7.95	1.8	C(3')*H* or C(2')*H*	8.34, d	7.94	1.4
C(7')*H*	8.26, s	7.94		C(7')*H*	8.04, s	7.80	
C(2)*H*	9.76, s			*H*4 or *H*5	7.96, t	7.85	6.9
				*H*5 or *H*4	8.55, t	8.45	7.9
				*H*3 or *H*6	8.11, d	8.00	7.2
				*H*6 or *H*3	8.74, d	8.76	6.2

[a] Chemical shifts for protonated and unprotonated species are in parts per million. Coupling constants are first-order $^3J_{HH}$ in hertz.

degraded and undegraded material, e.g., J + J'.

Results

ϵ-Thiamin was synthesized by two distinct routes. In route A, the sequence I → II → III → IV described by Kluge (1978) was used, followed by the condensation of the bromo compound IV with the thiazole VIII to give ϵ-thiamin. In route B, thiamin (VI) was treated with chloroacetaldehyde to give ϵ-thiamin.

(A) N-[(5-Methylimidazo[1,2-c]pyrimidin-8-yl)methyl]-4-methyl-5-(2-hydroxyethyl)thiazolium Bromide [ϵ-Thiamin (V)]. A solution of 200 mg of IV in 4 mL of CH_3NO_2 was stirred at 55 °C, and 265 μL of 4-methyl-5-thiazoleethanol (VIII) was added; a precipitate began to form after 15 min. The solution was kept at 55 °C for 2 h and was then cooled and filtered, and the light brown precipitate was dissolved in 30 mL of ethanol plus 2 drops of water. Ether was added to produce faint turbidity, and the solution was stored at 0 °C for 24 h; 100 mg of dark yellow crystals was obtained. The yield, based on I, was 12%. A 40-mg sample was recrystallized from ethanol, yielding 15 mg of bright yellow crystals, presumed to be ϵ-thiamin hydrobromide. A small sample was dissolved in water, immediately spotted on a TLC plate, and, as soon as it had dried, developed as described above. A single spot was obtained. When such samples were allowed to remain in solution at room temperature for several hours before being spotted on the TLC plate, multiple spots were obtained. Due to the lability of ϵ-thiamin in acidic solution (see below), the NMR spectrum was measured on a solution prepared by dissolving the presumed hydrobromide in 100 mM phosphate buffer, pH 7.0 (Figure 1). The original sample of ϵ-thiamin hydrobromide was kept desiccated at 4 °C. After 2 months there was no indication of degradation by TLC analysis, and after 10 months an NMR spectrum showed no extraneous peaks (see below).

(B) Thiamin hydrochloride (8.55 g) was dissolved in 100 mL of H_2O, and 20 mL of 2 N chloroacetaldehyde was added with stirring. The solution was adjusted to pH 7.0 with Na_2HPO_4 and stirred for 24 h; the pH was maintained at 7.0 by the periodic addition of Na_2HPO_4. The reaction mixture was extracted 3 times with 100-mL portions of $CHCl_3$, and the aqueous phase was separated and concentrated almost to dryness on a rotary evaporator at a bath temperature of 40 °C. The residue was dissolved in 80 mL of 95% ethanol, and 2 g of activated charcoal was added. The mixture was stirred and filtered, and the filtrate was flash evaporated to dryness. The residue was suspended in 100 mL of absolute ethanol, stirred, and filtered, the insoluble material was discarded, and the filtrate was evaporated to dryness on a rotary evaporator.

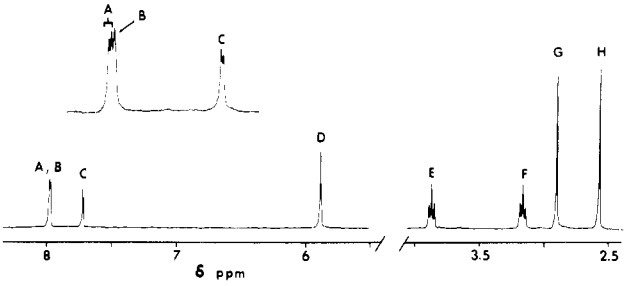


FIGURE 1: 270-MHz ^1H NMR spectrum of ϵ-thiamin at 19 °C. The chemical shifts of the lettered peaks are given in the text. The full spectrum was observed with a sweep width of 2500 Hz. The inset spectrum was recorded with a sweep width of 175 Hz to show details of signals A, B, and C. The coupling constant $^3J_{2',3'}$ = 1.6 Hz.

These steps were then repeated. To the solid remaining after the second evaporation of the ethanol, 125 mL of $CHCl_3$ was added and mixed well to dissolve. The mixture was filtered, the insoluble material was discarded, and the filtrate was evaporated at 0 °C on a rotary evaporator. The solid residue was dissolved in 30 mL of absolute ethanol containing 1 drop of 4 N HCl, and the solution was stored for 1 week at −20 °C. A total of 2.1 g of pink crystals was obtained. The net yield was 26%.

The purified materials prepared by the two methods have identical NMR, UV, and fluorescent spectra. A sample of the material prepared by method B was used for elemental analysis. Anal. Calcd for $C_{14}H_{17}N_4OSCl$ (324.8): C, 51.8; H, 5.28; N, 17.25. Found: C, 51.4; H, 5.48; N, 16.43. The 270-MHz ^1H NMR spectrum of ϵ-thiamin is shown in Figure 1. The sample used for this spectrum was prepared by method A. Material prepared by method B gave an identical spectrum. The resonances were assigned as shown in Table II. The assignments of the two methyl signals are made by analogy with thiamin. The following criteria are used. The resonances assigned to the two CH_3 groups of thiamin were identified unequivocally previously (Hutchinson, 1971; Biaglow et al., 1969; Mieyal et al., 1971). The signal assigned to the C-2' CH_3 group of thiamin moves downfield (Sable & Biaglow, 1965) when the pyrimidine ring is protonated.[4] In the case of ϵ-thiamin, one CH_3 resonance moves from δ 2.89 in the unprotonated species to δ 3.09 in the protonated species. The other CH_3 resonance is essentially immobile at δ 2.55–2.57 over the same range of pH. Since only the ϵ-pyrimidine ring can be protonated, the mobile signal is assigned to the CH_3

[4] The CH_3 resonances were misassigned in the original study of thiamin by Sable & Biaglow (1965).

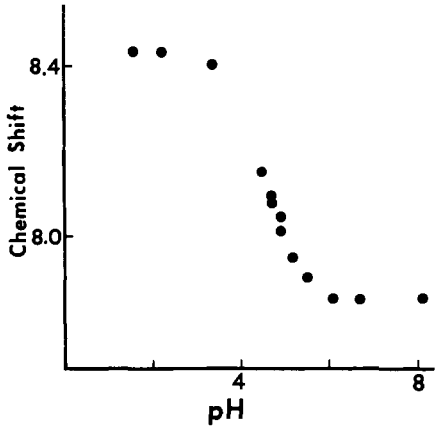


FIGURE 2: pH dependence of the chemical shift of the C(7) proton in III. Spectra were recorded at 270 MHz, and the chemical shift is reported relative to internal DSS, with 1 μL of acetone as an internal reference.

group attached to that moiety. Verification of the assignment is given by the relative breadths of the CH_3 signals. The signal of the C(4) CH_3 group of thiamin is broadened by long-range coupling of the protons with those of the C(5) CH_2 group (Mieyal et al., 1971). A spectrum of the high-field region of ethenothiamin was obtained at a resolution of 11 data points/Hz, instead of the usual 3 points/Hz. This spectrum shows that the CH_3 resonance at δ 2.56 is lower and broader than the mobile signal, in agreement with these assignments. The respective assignments of signals to C(2′)H and C(3′)H are purely arbitrary, as there is no easy means for distinguishing between the two signals.

The UV absorption spectrum of ϵ-thiamin shows a maximum at 260 nm ($\epsilon = 6.5 \times 10^3$ M^{-1} cm^{-1}), with a shoulder at 295 nm ($\epsilon = 2.68 \times 10^3$ M^{-1} cm^{-1}). ϵ-Thiamin is fluorescent in neutral solution at room temperature. For solutions in 0.1 M phosphate, pH 7, excitation at 325 nm gave a broad emission maximum at 410 nm. The intensity of this fluorescence was compared to that of an equal concentration of thiochrome, and the latter was found to give 600-fold stronger fluorescence (Wittorf & Gubler, 1970).

Acid–Base Properties of the Etheno Compounds. The relationship of the amino group and the pK of thiamin to its catalytic function, binding properties, etc. remains unsettled (Schellenberger, 1967; Gallo et al., 1978). It was of interest, therefore, to determine the pK_a of the protonated forms of the etheno derivatives and to compare them with those of the unmodified compounds. In view of the small amounts of the pure compounds that were available, some method other than potentiometric titration was desirable. Earlier studies (Suchy et al., 1972) established the linear dependence of the chemical shifts of the NMR signals of thiamin upon the state of protonation of the molecule. Buffers composed of various nonvolatile components were prepared for a series of pH values and lyophilized to dryness and stored desiccated until needed. Small amounts, generally 1–2 mg of the compound under investigation, were added, and the solutions were reconstituted with ^2H_2O just before being placed in the NMR probe. In this way, spectra were obtained before appreciable degradation of the labile compounds had occurred. After the spectra were recorded, the pH of the samples was measured. For each compound, one particular, well-resolved resonance was chosen, such that there would be little chance of confusion with other resonances. The chemical shifts of the resonances in the fully protonated[5] and fully deprotonated species were determined

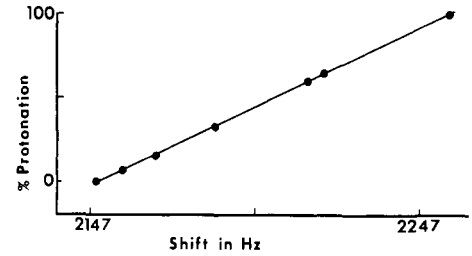


FIGURE 3: Relationship between the chemical shift of the C(3′) proton of ϵ-amprolium and the percent protonation of the ring. Samples consisted of 2 mg of ϵ-amprolium in 0.5 mL of 50 mM phosphate–50 mM fumarate buffer. All solutions were prepared immediately prior to use. The abscissa is the chemical shift in hertz downfield from DSS.

in preliminary experiments, and the data could then be displayed graphically in the usual way (Figure 2). The pK was calculated from the data for each experimental point by

$$pH = pK + \log ([B]/[BH^+])$$

The ratio $[B]/[BH^+]$ is obtained directly from the values of the chemical shift for intermediate degrees of protonation. As a further check on the accuracy of the measurements and the validity of the assumption, graphs were constructed for percent protonation vs. chemical shift (Figure 3); such graphs must be linear. It must be emphasized that all of these determinations were carried out in solutions of 99% ^2H_2O, and the pK values reported are uncorrected for possible differences between solutions in ^2H_2O and those in ^1H_2O for the reasons noted below.

The relationship between p^1H and p^2H is reported (Glasoe & Long, 1960) to be

$$p^2H = pH_{obsd} + 0.4$$

It is difficult to determine whether, and if so how, this relationship can be used to convert the pK values measured on the acid–base couple dissolved in ^2H_2O into corresponding values for samples dissolved in ^1H_2O, for at least two reasons. First, p^2H is a quantity that refers to measurements with a pH meter in which the reference solutions would be 1 M ^2HCl in ^2H_2O in the glass electrode and saturated KCl in ^2H_2O as the flowing solution. Second, the dissociation constants for the acids $B_i{}^2H$ in ^2H_2O may not have any simple relationship to the dissociation constants for the acids $B_i{}^1H$ in ^1H_2O. To resolve this difficulty, we have measured the pK of thiamin by the NMR method both in ^1H_2O and in ^2H_2O. Solutions of fully protonated and fully unprotonated thiamin in H_2O and 11 solutions of intermediate degrees of protonation were prepared. Each solution was divided into two equal aliquots and lyophilized. One set was reconstituted with ^1H_2O, and the other was reconstituted with ^2H_2O. The chemical shift of the C(2′) CH_3 protons was measured relative to internal acetone, and the pH of each solution was measured immediately after the spectrum was recorded. The respective chemical shifts of the signals for the fully associated and dissociated forms were unaffected by the solvent. For solutions in ^1H_2O at 39 °C, $pK_a = 5.17 \pm 0.02$, and for solutions in ^2H_2O at 18 °C, pK_a'

[5] Full protonation and deprotonation were assumed to be achieved when further change of pH in the appropriate direction caused no further change in chemical shift of the signal being monitored. In the case of V and VII, full protonation was achieved by use of 0.1 N ^2HCl as the solvent. The high correlation coefficient for the straight lines obtained (e.g., Figure 3) is a self-consistent criterion for the validity of the titration. In practice, pK + 2 to pK − 2 is a satisfactory range over which to measure the spectra.

Table III: Dissociation Constants of Some Pyrimidine and Imidazopyrimidine Compounds

compd	temp (°C)	pK_a'	solvent	method	ref
II	19	6.76	^2H_2O	NMR	*a*
III	19	4.52	^2H_2O	NMR	*a*
V (ϵ-thiamin)	19	2.3	^2H_2O	NMR	*a*
VI (thiamin)	19	5.36	^2H_2O	NMR	*a*
VI (thiamin)	39	5.17	^1H_2O	NMR	*a*
VII (ϵ-amprolium)	19	2.41	^2H_2O	NMR	*a*
IX (oxythiamin)		2.3	^1H_2O	potent.[f]	*b*
X		1.39	^1H_2O	UV	*c*
XI		<1.6	^1H_2O	potent.	*d*
XII	19	7.66	^2H_2O	NMR	*a*
XIII (*N*,*N*-dimethylthiamin)	19	5.94	^2H_2O	NMR	*a*
XIV	19	7.56	^2H_2O	NMR	*a*
XV (*N*-methylthiamin)	19	6.02	^2H_2O	NMR	*a*
ϵ-adenosine		3.9	^1H_2O	potent.	*e*
ϵ-cytidine		3.7	^1H_2O	potent.	*e*

[a] Present study. [b] Suchy et al. (1972). [c] Barone (1963). [d] Unpublished observation of W. Rosenberg, J. E. Stuehr, and H. Z. Sable. The potentiometric study showed that the pK, if it exists, is below 1.6. [e] Secrist et al. (1972). [f] Potent., potentiometric.

= 5.36 ± 0.03. In a separate experiment, a 0.1 M solution of thiamin was titrated potentiometrically with 0.100 N NaOH, while constant temperature was maintained in a constant temperature cell. Under these conditions, pK_a' at 40 °C was 0.2 pH unit lower than that at 22 °C. Consequently, one may conclude that pK_a' for thiamin $^1H^+$ in ^1H_2O is the same as that for thiamin $^2H^+$ in ^2H_2O. Roberts et al. (1969) observed that pK_a' values determined from chemical shifts of histidine resonances of RNase dissolved in ^2H_2O were identical with those for solutions in ^1H_2O. They considered that this equivalence was due to a coincidental balancing of the isotope effect on the glass electrode by the isotope effect on the ionization equilibrium of the acids in question. We believe that further studies are needed to resolve this matter, but since the present study deals with a series of closely related compounds, we assume, without proof, that similar relationships exist between the values measured in ^2H_2O and the true values in ^1H_2O.

For several compounds prepared in this study (Table III), pK_a' was determined from the chemical shift of the proton attached to C(6′) of the pyrimidine ring of thiamin or the corresponding proton in the other compounds. A number of related compounds for which pK values have been reported are also listed in the table. Several points are demonstrated by the values listed. (1) The well-known delocalization of the electrons of the NH_2 group (Breslow, 1962; Mason, 1958; Jardetzky et al., 1963) contributes to the basicity of N(1′) of thiamin relative to that of oxythiamin. In the case of trifluorothiamin, the electron-withdrawing capacity of the CF_3 group is so great that the compound probably should be regarded as a strong acid. (2) The replacement of the 5-CH_2OH group of the pyrimidine compound II by the 5′-methylthiazolium of thiamin (VI) causes an increase in the acidity of N(1′) of ~1.5 pH units. The quaternary nitrogen, separated from the pyrimidine ring by a single methylene group, must therefore exert a strong electron-withdrawing effect on the ring. This is not unexpected and is merely the obverse of the ability of the pyrimidine ring to stabilize charges on the thiazolium ring (Breslow & McNelis, 1959). (3) The conversion of the N(3)–C(4)–NH_2 segment into an imidazo ring has an even greater effect: ~2.2 pK units greater acidity in the imidazopyrimidine compound III relative to that in the corresponding pyrimidine compound II, and ~3 units in ϵ-thiamin and ϵ-amprolium relative to that in thiamin.

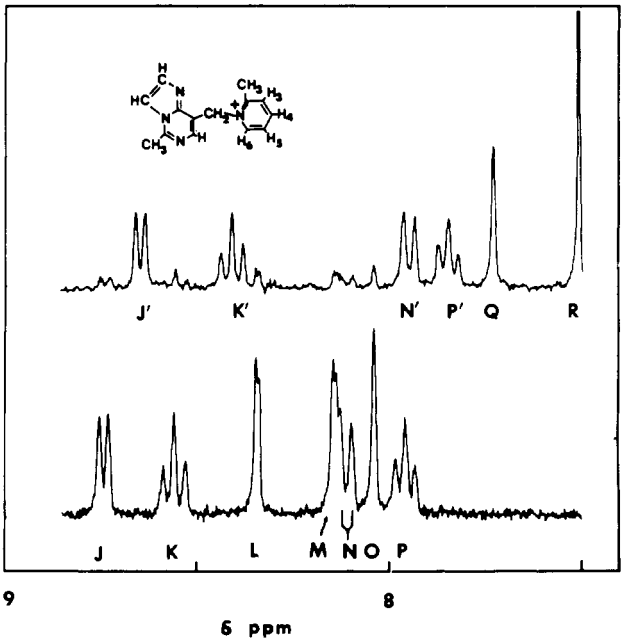


FIGURE 4: Low-field region of the 270-MHz ^1H NMR spectrum of ϵ-amprolium at 19 °C. In each case 2 mg of VII was dissolved in 0.5 mL of 0.1 N ^2HCl in ^2H_2O. The lower spectrum is of a sample that was prepared 5 min before the spectrum was recorded. The upper spectrum is that of a sample that was left at room temperature for 18 h. The chemical shifts and identification of the resonances are given in Table II. Under the conditions used, only the ortho couplings of the protons of the picolinium ring are resolved.

Lability of the Protonated Forms of the Etheno Analogues. Dilute (10–15 mM) aqueous solutions of ϵ-thiamin and ϵ-amprolium, pH 6.5–7.0, left at room temperature for 48 h, show no decomposition when analyzed by NMR or TLC; some concentrated solutions (100 mM) of V, pH 7.0, did show a small amount of degradation. At pH 2–3 the dilute solutions showed degradation; after 3 h of incubation three, approximately equal spots were observed on TLC plates (Table I). In each case the R_f of the fastest moving component suggested that the thiazole moiety VIII of thiamin or α-picoline, respectively, was one of the products. A large-scale degradation of ϵ-thiamin was carried out and the fastest moving component was isolated. NMR spectra of degraded ϵ-thiamin, authentic thiazole VIII, and the fast-moving component showed that the chemical shifts of the CH_3 group and the two CH_2 groups in all three spectra were identical, confirming the assumption that thiazole VIII was formed. Similar experiments with ϵ-amprolium gave a different result. Figure 4 shows the low-field region of the NMR spectrum of intact and degraded ϵ-amprolium at low pH. When degradation occurs, the resonances assigned to the picolinium protons are displaced upfield but remain in the same region of the spectrum. The fastest moving spot on TLC plates of degraded ϵ-amprolium was not identified; it cannot be α-picoline since the latter apparently evaporates from the plates. Further, the new resonances in the spectrum of degraded ϵ-amprolium do not correspond to those of protonated α-picoline.

Concomitantly with the degradation of ϵ-thiamin and ϵ-amprolium the low-field signals assigned to the protons of the ϵ-pyrimidine ring disappear from that region of the spectrum, and the signals of the bridge CH_2 and the CH_3 protons also undergo large displacements. There is no rational basis at present for assigning the new signals. It is conceivable that the etheno bridge disintegrates under acidic conditions or that

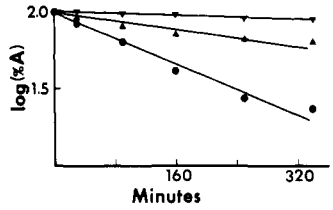


FIGURE 5: Degradation of ϵ-amprolium. The fraction of ϵ-amprolium remaining, calculated as described in the text, is shown as a function of time; (●) pH 2.1; (▲) pH 3.1; (▼) pH 4.2.

even more deep-seated changes, such as disintegration of the whole ϵ-pyrimidine ring system, occur.

Figure 5 shows the relative rates of degradation of ϵ-amprolium at three pH values. The data gave a good fit to a first-order rate equation and a very poor fit when second-order kinetics were assumed, and the rate constants[6] were calculated from the slopes of the lines in Figure 5. A sample incubated at pH 5.4 showed no degradation during 6 h. The percent of unprotonated ϵ-amprolium at each pH was calculated from the previously determined pK and compared with the rate constant determined from the degradation study. These points are consistent with a straight line which also shows that the rate of breakdown approaches zero as the molecule becomes deprotonated. Extrapolation of the data gives $t_{1/2}$ for the fully protonated form of $\simeq$ 100 min. Preliminary studies show that under similar conditions ϵ-thiamin decomposes with $t_{1/2}$ = 6–8 h.

When samples of III in acidified ^2H_2O were allowed to stand for several hours at room temperature prior to observation, the NMR spectra showed a diminution of the methyl resonance. To examine the possibility of acid-catalyzed hydrogen exchange, we dissolved a 5-mg sample of III in 500 μL of 2 N ^{2}HCl in ^2H_2O. The NMR spectrum was recorded repeatedly, at timed intervals, and the integrated area of the CH_3 resonance was compared to that of the three low-field signals. A linear relationship is observed, from which a half-time of 176 min was determined by a linear least-squares method.

Discussion

Several previous attempts had been made in two of the collaborating laboratories to convert thiamin and thiamin phosphate esters into the corresponding etheno analogues. Analysis by NMR and TLC suggested that although the desired condensation had occurred, extraneous compounds were also present in the reaction mixture, and attempts at purification were uniformly unsuccessful. The primary difficulty in purification was the unexpected lability of the products in dilute aqueous acid, and when this lability was realized to exist, it became possible to obtain pure ϵ-thiamin by either route with little difficulty. For routine preparations, route B is preferable, since it is the simpler procedure and gives a larger yield.

The pK values of the etheno compounds we have studied (Table III) are considerably lower than those for the corresponding amino compounds. The pK of ϵ-thiamin is closer to that of oxythiamin IX (Suchy et al., 1972) than it is to the pK of thiamin. It is conceivable that the decreased basicity of the ring nitrogen is caused by decreased ability of the lone pair of electrons on the N atom corresponding to the exocyclic amino group to assist in delocalizing the charge on the protonated ring.

The finding that the protonated forms of both ϵ-thiamin and ϵ-amprolium in aqueous solution were unstable at ambient temperature was unexpected. Such behavior has not been described for any other etheno analogue; on the contrary, Barrio et al. (1976) report that ϵ-cytidine is stable in 2 N HCl at reflux temperature, so that the extreme instability is hard to assign to the imidazopyrimidine ring itself. The decomposition appears to follow first-order kinetics, and although only a small number of points were available, the data are consistent with a linear relationship between the degree of protonation and the rate of reaction. This implies that it is only the protonated form of the molecule that is unstable. The lability of protonated ϵ-thiamin is of the same order of magnitude as is that of ϵ-amprolium, whereas under the same conditions only a few percent of degradation is observed in the fully protonated species of the imidazopyrimidine III after 24 h. The lability of protonated ϵ-thiamin and ϵ-amprolium must, therefore, depend to a large extent on the presence of the strongly electronegative quaternary nitrogen attached to the CH_2 bridge between the two rings. It will be interesting to investigate the effect of other electronegative groups at that position on the lability of the protonated ϵ-pyrimidine ring and also to identify all the products of degradation of ϵ-thiamin and ϵ-amprolium.

Hutchinson (1971) first reported that thiamin and oxythiamin undergo ^{1}H–^{2}H exchange of the pyrimidine methyl protons under strongly acidic conditions. In thiamin, for example, this exchange occurs with a half-time of ~20 h in 6 N ^{2}HCl and 6–7 days in 1 N ^{2}HCl. The methyl protons of III are far more labile, with a half-time of 3 h in 2 N ^{2}HCl. If the observed rate may be used to predict a half-time of 6 h in 1 N ^{2}HCl, this exchange occurs ~30 times as rapidly as that for thiamin. The fact that the pyrimidine methyl protons of thiamin exchange with solvent has been used previously to prepare tritiated thiamin (Zoltewicz & Kauffman, 1977). The enhanced lability of the CH_3 protons in the ϵ-pyrimidine analogues should allow the facile production of radioactively labeled etheno compounds. The lability of the C–H bonds of these methyl groups suggests that there may be a substantial degree of hyperconjugation in the resonance hybrid that describes the protonated forms of these compounds. Such hyperconjugation would also result in a partial negative charge on the ring nitrogen atoms. As these N atoms would tend to have a partial positive charge when the molecule is protonated, hyperconjugation would tend to stabilize the cation. In a homologue in which the methyl group is replaced by an ethyl group, hyperconjugation would be much less important an effect. If the ^{1}H–^{2}H exchange we have observed depends upon hyperconjugation, the homologue would exchange much more slowly than the imidazopyrimidine III, if at all. The possible synthesis of such a compound is now being considered.

Although all ϵ-purine analogues are fluorescent at neutral pH, ϵ-cytidine fluoresces only in acid solution. Barrio et al. (1976) hypothesized that a π^*–n transition associated with the carbonyl bond of ϵ-cytidine is responsible for its fluorescent behavior and showed that compounds lacking the carbonyl group, such as imidazo[1,2-a]-pyridine, were fluorescent in neutral solution. The observed behavior of ϵ-thiamin conforms to this analysis.

The question of the biological activity of ϵ-thiamin has been investigated.[7] Injected ϵ-thiamin can substitute for thiamin for growth in rats, but greater concentrations are needed in order to achieve normal growth. Since the protonated forms

[6] The integrated form of the rate equation for first-order reactions is $kt = \ln(c_0/c)$. Consequently, the slopes of the lines in Figure 5 are $-k/2.303$.

[7] Gibby & Gubler (1980).

Table IV: Fluorescence Properties of Etheno Compounds[a]

compound	pH	λ_{Cex}[b]	λ_{Cem}^{max}[b]	Φ[c]	ξ[c]
ϵ-adenosine	7.0	300	595	0.55	11 ± 1
ϵ-adenosine	0.1 N HCl	300	594	0.63	9 ± 1
ϵ-ADP	7.0	272	538	0.30	6 ± 2
ϵ-ADP	0.1 N HCl	273	541	0.30	2 ± 1
ϵ-cytidine	7.0	270	539	0.38	12 ± 2
ϵ-cytidine	0.1 N HCl	270	536	0.17	8 ± 2
IV	7.0	260	519	0.20	19 ± 2
IV	0.1 N HCl	261	518	0.12	8 ± 3
ϵ-thiamin	7.0	274	544	0.37	8 ± 1
ϵ-thiamin	0.1 N HCl	276	544	0.34	8 ± 1

[a] See Added in Proof for details. [b] λ_{Cex} and λ_{Cem}^{max} are "corrected" excitation and maximum emission wavelengths, respectively, in nanometers. [c] Φ is the quantum yield; ξ is the fluorescence lifetime in nanoseconds.

have p$K \simeq 2$, it is likely that if ϵ-thiamin were fed in the diet, some of it would be degraded at the acidic pH prevailing in the stomach. Nevertheless, a considerable amount would reach the more alkaline environment of the upper intestine and would then be available for absorption into the blood stream. The finding that ϵ-thiamin can substitute for thiamin in maintaining normal growth suggests that ϵ-thiamin is being converted into ϵ-thiamin pyrophosphate, which then serves as a coenzyme for thiamin PP requiring enzymes. Clearly, one would have to be certain that even the injected ϵ-thiamin was not, somehow, being converted into thiamin, although there is no present indication that such degradation does or does not occur. If ϵ-thiamin PP does indeed serve as a coenzyme, that fact alone would prove that the amino group of thiamin PP does not participate directly in the catalytic process. Current studies are directed toward the preparation of ϵ-thiamin PP for testing with thiamin PP requiring enzymes and for testing ϵ-thiamin as a substrate for thiamin pyrophosphokinase.

The fluorescent nature of ϵ-thiamin makes it a potentially valuable probe for thiamin-requiring biological systems. In addition, the potential availability of tritiated ϵ-thiamin will allow its use in systems requiring very high dilution. Further investigations in this area are in progress.

Added in Proof (by Jesse C. Haggerty, III)

Fluorescence measurements have been carried out on ϵ-adenosine, ϵ-ADP, and ϵ-cytidine (Sigma), the bromomethyl-ϵ-pyrimidine IV, and ϵ-thiamin (V) (Table IV). "Corrected" fluorescence excitation and "corrected" fluorescence emission spectra were obtained from a Farrand Optical Inc., MK 1 spectrofluorometer with the Farrand excitation and emission correction modules. The fluorescence measurements were made at 21 °C, in neutral and in acidic solutions; the quantum efficiences were compared with that of ϵ-adenosine in 0.025 M phosphate buffer, pH 7.0 [$\Phi = 0.56$ (Secrist et al., 1972)], and against standard quinine taken as $\Phi = 0.70$ (Scott et al., 1970). Under the latter conditions, ϵ-adenosine gave $\Phi = 0.55$, as noted in Table IV. The fluorescence lifetimes were determined by phase measurements.

Acknowledgments

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Cloning and Determination of a Putative Promoter Region of a Mouse Ribosomal Deoxyribonucleic Acid Fragment†

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ABSTRACT: An endonuclease *Eco*RI digest of mouse DNA was subjected to molecular cloning, after partial purification with respect to the ribosomal RNA sequence, using λgtWES·λB with an in vitro packaging technique. Twelve positive clones were obtained from approximately 2×10^4 plaques. One of the clones transferred to the plasmid pBR322 (pMrEL-1) was about 14.9 kb long, hybridizing only with 18S rRNA but not with 28S rRNA. Hybridization of restriction fragments and electron microscopic studies of the R-loop confirmed that this fragment carried about half of the 18S rRNA sequences at one end, suggesting that it contained the initiation site for the 45S preribosomal RNA (pre-rRNA). S_1-nuclease protection mapping with hybrids between restriction fragments of the cloned DNA and the 45S pre-rRNA indicated that at least major transcription of the 45S RNA started at a site approximately 4.0 kb upstream from the 5′ end of the 18S rRNA. This was confirmed by electron microscopic observations of these hybrids.

One approach to the study of molecular mechanisms of gene regulation in eukaryotes may be the establishment of an in vitro transcriptional system that mimics the in vivo mechanisms with high fidelity. For this purpose, isolation and characterization of a specific gene are the first prerequisites. The ribosomal RNA gene (rDNA) has several interesting features in that it is arranged in tandem in the order of several hundreds and transcribed in vivo by RNA polymerase I, which is one of the three types of eukaryotic RNA polymerases. Its transcription is affected strongly by protein synthesis and, in some cases, cell growth.

Genes coding for rDNA have been cloned from yeast (Kramer et al., 1976), *Drosophila* (Thomas et al., 1974), *Bombyx mori* (Manning et al., 1978), *Xenopus* (Morrow et al., 1974), chicken (McClements & Shalka, 1977), mouse (Tiemeier et al., 1977), and human cells (Wilson et al., 1978). The mouse rDNA fragment isolated by Tiemeier et al. (1977) was an internal fragment containing both 18S and 28S rRNA gene regions; it did not contain initiation or termination regions.

To study the regulatory mechanisms of rDNA transcription, isolation of a fragment containing the 5′-terminal region of the rDNA was essential. In this paper, we report the isolation of such a fragment from mouse DNA. We also describe attempts to locate the initiation site for 45S pre-rRNA on this fragment with biochemical and electron microscopic methods.

Materials and Methods

Preparation of High Molecular Weight DNA and RPC-5 Column Chromatography. The preparation of DNA from the newborn Balb/c mice was described elsewhere (Kataoka et al., 1979). In brief, the method involved treatment with $NaDodSO_4$[1] and $NaClO_4$, phenol–chloroform extraction followed by RNase treatment, and extensive dialysis against 0.1 × SSC. DNA was digested completely with *Eco*RI, extracted with phenol–chloroform, and precipitated with 2 volumes of ethanol. Forty milligrams of DNA digested with *Eco*RI was chromatographed on an RPC-5 column as described previously (Tiemeier et al., 1977).

Preparation and Screening of the Recombinant Phage and Plasmid. Bacteriophage λgtWES·λB, an EK-2 vector, was propagated in *Escherichia coli* strain ED8656. Introduction of DNA into ED8656 by an in vitro packaging technique was carried out according to Blattner et al. (1978) on the basis of other reports (Becker & Gold, 1975; Hohn & Murray, 1977; Sternberg et al., 1977). Cloning experiments were performed in a P2 facility according to the proposed Japanese Guidelines (1978) and revised NIH Guidelines (1976).

The λgtWES arms were purified by 5–25% sucrose density gradient centrifugation after digestion with *Eco*RI and *Sac*I. The ligation of λgtWES arms and mouse DNA fractionated by the PRC-5 column was carried out according to the method of Tiemeier et al. (1977). Five micrograms of λgtWES arms was ligated with 1.5 μg of DNA in a volume of 0.5 mL at 15 °C for 16 h.

About 2000 plaques on each L-broth supplemented agar plate (Bacto-trypton (10 g), yeast extract (5 g), NaCl (5 g), agar (15 g), and distilled water (1 L)) were transferred to the Millipore filter essentially according to Benton & Davis (1977). Filters were hybridized with ^{32}P-labeled 18S cDNA (see below) and autoradiographed. Positive plaques were propagated as described by Tiemeier et al. (1977) and DNA was extracted.

To re-clone the fragment obtained above, *Eco*RI-digested recombinant DNA was ligated with plasmid pBR322 DNA, which had been digested with the same enzyme and treated

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; SSC, 0.15 M NaCl and 0.015 M sodium citrate; AMV, avian myeloblastosis virus; sarcosyl, sodium dodecyl sarcosinate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); kb, kilobase.

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