Ring Opening of Thiamine Analogs. The Role of Ring Opening in Physiological Function†

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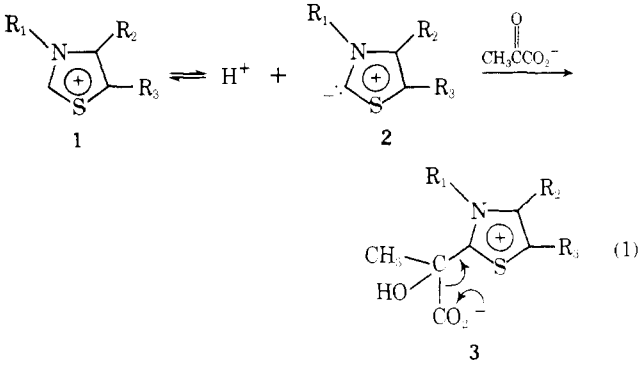
ABSTRACT: The stability of azolium species to ring-opening reactions has been examined. The second-order rate constants for the reaction between hydroxide and 3,4-dimethyloxazolium ion, 3,4-dimethylthiazolium ion, and 1,3-dimethylimidazolium ion were found to be in the relative order: $10^{8.6}$:$10^{4.9}$:1. The half-life for the ring-opening reaction of the oxazolium species at physiological pH is approximately 20 sec. At pH 7.4, the half-life for the ring-opening reaction of the thiazolium ion is approximately 33 hr, assuming the reaction irreversible. The imidazolium species, although more than $10^{8.5}$ times more stable than an oxazolium ion and nearly 10^5 times more stable to ring opening than a thiazolium ion, forms its ylide intermediate, known to be essential in thiamine catalysis, much more slowly than the thiazolium ion. Thus of the azolium species, the thiazolium ion is most suited for cocarboxylase function. It has the optimum thermodynamic and kinetic properties, both in regard to the lability of the C-2 proton and in regard to its integrity in the ring-closed form. The oxazolium ion would always be in its ring-opened form at pH 7.4, and thus inactive as a substitute for the thiazolium heterosystem of thiamine, and the imidazolium ion would be ineffective in generating the ylide required for thiamine function. Furthermore, at physiological pH, there is a dynamic conversion between ring-opened and ring-closed forms of thiamine. This interconversion may be important in thiamine transport across cell membranes and therefore may be involved in its evolution as a vitamin.

Thiamine (**1a**), the anti-beriberi vitamin (B_1), is composed of substituted pyrimidine and thiazolium heterocycles. The pyrophosphate derivative, cocarboxylase (**1b**), is the coenzyme for such important biochemical reactions as the decarboxylation of pyruvate, the synthesis of α-acetolactate, the transketolase, and the phosphoketolase reactions (Krampitz, 1969; Reed, 1974). Breslow (1957) demonstrated that the C-2 hydrogen of the thiazolium ring is kinetically labile, **1** ⇄ **2**, and that the ylide **2** is the reactive species which adds to the carbonyl group of substrates in cocarboxylase function (Breslow, 1958, 1962); this labilizes bonds to the carbonyl group as in decarboxylation (eq. 1).



(1)

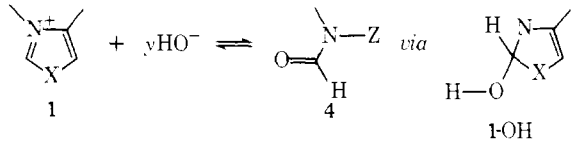
a. R_1 = 4-amino-2-methylpyrimidin-5-yl; $R_2 = CH_3$; $R_3 = CH_2CH_2OH$

b. R_1 = 4-amino-2-methylpyrimidin-5-yl; $R_2 = CH_3$; $R_3 = CH_2CH_2OP_2O_6^{3-}$

c. $R_1 = CH_3$; $R_2 = CH_3$; $R_3 = H$

In order to understand the natural selection of the thiazolium heterocycle as the active part of the vitamin, we have investigated the fundamental chemical nature of thiazolium ions, and have compared them to other azolium species (Haake *et al.*, 1969; 1971; Haake and Miller, 1963; Haake and Bausher, 1968; Haake and Duclos, 1970). Our results on the rate of DO^- catalyzed generation of **2** demonstrate that the oxazolium ion forms its ylide some 100 times faster than a thiazolium ion (Haake *et al.*, 1969). Why, then, are oxazolium ions unsuitable for use in biological systems?

The answer to this question and other relevant chemistry is given in this paper which presents a comparison of ring-opening reactions of azolium ions **1c, d,** and **e** (eq 2). We



(2)

c. X = S; R = CH_3; Z = $C(CH_3)$=CHS^-; $y = 2$

d. X = O; R = CH_3; Z = $C(CH_3)HCHO$; $y = 1$

e. X = NCH_3; R = H; Z = CH=$CHNHCH_3$; $y = 1$

show that the thiazolium ion is most suited for cocarboxylase function. Further, the results relate to the mechanism by which thiamine may be transported across cell membranes.

Detailed research on thiazolium ions and related heterocycles indicate that the ring-opening reaction proceeds through the pseudobase, **1**-OH (Duclos and Haake, unpublished results; Maier and Metzler, 1957).

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Experimental Section

General Procedures. Nuclear magnetic resonance spectra were recorded on a Varian Model A-60A spectrometer. For pH measurements, a Radiometer Model 26 pH meter was used and was standardized against phthalate, phosphate, and borate buffers. Analyses were performed by Spang Analytical Laboratories, Ann Arbor, Mich. All melting points are corrected.

Preparation of Compounds. The preparation of 3,4-dimethylthiazolium iodide, **1c,** has been described (Haake *et al.,* 1969).

3,4-Dimethyloxazolium p-Toluenesulfonate. According to the procedure of Cornforth and Cornforth (1953), 4.0 g (0.03 mol) of 4-methylcarboxyoxazole was heated with a small amount of cupric oxide. From this mixture, 4-methyloxazole distilled over as a colorless liquid, bp 86–89° (760 Torr). To 1.5 g of 4-methyloxazole dissolved in 10 ml of ether, 3.0 g (0.017 mol) of methyl *p*-toluenesulfonate was added. After standing overnight at ambient temperature a crystalline mass resulted. This was collected, washed four times with 25 ml aliquots of ether, and recrystallized three times from methanol–ethyl ether–ethyl acetate, yielding 1.0 g: mp 143.0–144.0°; nmr (D_2O) 2.32 (doublet, 3 H), 2.37 (singlet, 3 H), 3.87 (singlet, 3 H), 7.48 (A_2X_2 multiplet, 4 H), 8.08 (multiplet, 1 H). The C-2 hydrogen does not appear in D_2O solvent due to exchange with solvent deuterium.

Anal. Calcd for $C_{12}H_{15}NO_4S$: C, 53.52; H, 5.61. Found: C, 53.45; H, 5.51.

1,3-Dimethylimidazolium p-Toluenesulfonate. To an ethereal solution of 10.0 g (0.14 mol) of *N*-methylimidazole (Aldrich), 28.0 g (0.15 mol) of methyl *p*-toluenesulfonate was added. An exothermic reaction ensued (use caution). After the solution had cooled, the resulting white crystalline mass was collected, washed thoroughly with anhydrous ether, and dried. The product was recrystallized five times from ethanol–ethyl acetate–ether, yielding 15.0 g: mp 54.0–54.5°; nmr ($CDCl_3$) δ 2.37 (singlet, 3 H), 3.88 (singlet, 6 H), 7.51 (doublet, 2 H), 7.47 (A_2X_2 multiplet, 4 H), 8.65 singlet, 1 H).

Anal. Calcd for $C_{12}H_{16}N_2O_3S$: C, 53.71; H, 6.01; N, 10.44. Found: C, 53.51; H, 6.13; N, 10.31.

Observation of the Ring-Opened Product of Oxazolium Hydrolysis. To 0.05 ml of distilled water, 53.2 mg (0.2 mmol) of 3,4-dimethyloxazolium *p*-toluenesulfonate, **1d,** was added. Sodium trimethylsilylpropanesulfonate was added as an internal reference. This solution was transferred to a thin-walled nmr tube, and the spectrum at 60 MHz was recorded. Then, 0.01 ml of 50% by weight NaOH was added with a micropipette. This amount is approximately 0.25 mmol. The spectrum was again recorded and is consistent with that predicted for 2-(*N*-methylformylamino)propanal (**4d**) (Figure 1).

Rate Measurements. The reaction between hydroxide and 3,4-dimethyloxazolium *p*-toluenesulfonate (**1d**) was followed using a Radiometer TTT11 apparatus equipped with a SBR2c Titrigraph, Model 26 pH meter, and a SBU 1c syringe. Constant temperature was maintained with a Haake Model FE thermostated circulator. The reaction vessel was purged with prepurified nitrogen (Matheson). Typically, 30 mg of substrate was dissolved in 5.0 ml of doubly distilled water and allowed to attain thermal equilibrium at 30.0°. After approximately 25 min, base addition was begun.

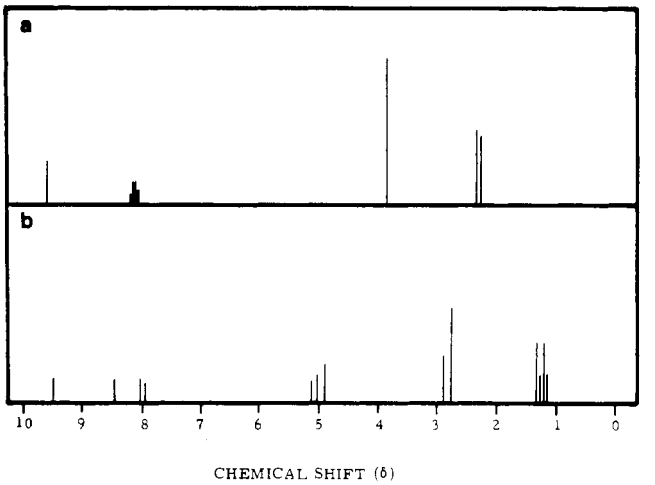


FIGURE 1: Pmr spectra for 3,4-dimethyloxazolium ion (**1d**) (a) and for the product of ring opening, 2-(*N*-methylformylamine)propanal, (**4d**) (b), in water at 37°.

The hydrolysis of 3,4-dimethylthiazolium iodide (**1c**) has been described (Haake and Duclos, 1970; Duclos and Haake, unpublished results; Duclos, 1972).

Reaction of 1,3-Dimethylimidazolium p-Toluenesulfonate (**1c**) *with Hydroxide in Strongly Basic Medium.* To a solution of 0.61 mol of dimethyl sulfoxide (53.3 mol %) and 0.53 mol of water (46.7 mol %), 48.9 mg (0.182 mmol) of 1,3-dimethylimidazolium *p*-toluenesulfonate (**1e**) was added. The total volume of the solution was 0.22 ml. This was transferred to a thin-walled nmr tube and the spectrum recorded at 60 MHz. Then 0.03 ml of 11.39 M KOH was added, bringing the total volume to 0.25 ml, and the signals of the C-4 and C-5 protons were integrated as a function of time. The hydrolysis was followed at approximately 40°, which is the temperature of the Varian A-60A probe, and was recorded without removing the nmr tube from the probe.

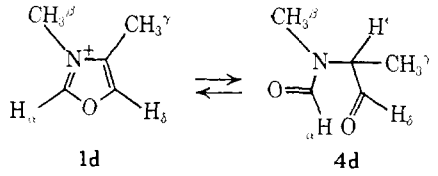
Calculations. For the hydrolyses of **1c** and **1d,** values of k_{obsd} were obtained from a least-squares program (Duclos, 1972). For the ring-opening reaction of **1e,** the assumption was made that 1 mol of **1e** reacts with 1 mol of hydroxide. The data were plotted as a second-order reaction using the integrated second-order equation, $k_2t = [1/(a - b)] \ln [b(a - x)/a(b - x)]$, where a is the concentration of substrate, b is the concentration of base, and x is the concentration of reacted substrate.

Results

Hydrolysis of 3,4-Dimethylthiazolium Iodide (**1c**). The sequence, **1c** $\rightleftarrows$ **4c,** consumes 2 equiv of hydroxide/equiv of substrate. The product of the ring opening of **1c** has been unambiguously established as **4c.** Further, the unusual pH–rate profile for the hydrolysis of **1c** provides evidence that the pseudobase **1c**-OH is an intermediate on the reaction pathway. In the pH region above pH 9.4, this reaction is first order in hydroxide. The effects of deuterated solvent, ionic strength, substituents at the C-2 position, and temperature point conclusively to rate-determining attack by hydroxide (eq 2) (Haake and Duclos, 1970; Duclos and Haake, unpublished results; Duclos, 1972).

The rates of the ring-opening reaction of **1c** were studied by the pH-Stat method in the pH range from 9.1 to 10.45 (Haake and Duclos, 1970; Duclos and Haake, unpublished results). In the pH range above 9.6, the reaction **1c** $\rightleftarrows$ **4c** is

TABLE I: Assignments of the Nmr Signals of **1d** and **4d**.[a]



(3)

1d ⇌ **4d**

Assignment		
α	9.63 (s)	7.95 (s)
		8.02 (s)[b]
β	3.87 (s)	2.78 (s)
		2.90 (s)[b]
γ	2.28 (d, J = 1.5 Hz)	1.19 (d, J = 7 Hz)
		1.26 (d, J = 7 Hz)[b]
δ	8.08 (q, J = 1.5 Hz)	9.45 (s)
		8.45 (s)[b,c]
ϵ		5.05 (q, J = 7 Hz)[d]

[a] The signals due to the counterion, *p*-toluenesulfonate, have been omitted. Values for the chemical shifts are in δ. [b] These two values are the chemical shifts of the conformers due to slow rotation about the C–N amide bond. [c] Upon further addition of base, the absorption at δ 9.45 disappears and that at δ 8.45 becomes a sharp singlet. The γ protons broaden. This effect is due to exchange of H_ϵ. [d] The signals due to the H_ϵ are partially masked by water absorption. It is possible to distinguish three of the four quartet signals.

first order in hydroxide with a second-order rate constant of 15.7 M^{-1} sec^{-1}. As an independent method of analysis the hydrolysis of 3,4-dimethylthiazolium methanesulfonate was followed spectrophotometrically in ethylenediamine buffer solutions, and confirmed the pH–rate behavior of **1c** (Duclos and Haake, unpublished results). The stoichiometry of the reaction was consumption of 2 equiv of HO^- above pH 9.6 (eq 2).

Hydrolysis of 3,4-Dimethyloxazolium p-Toluenesulfonate (**1d**). In the ring-opening reaction of **1d**, 1 equiv of hydroxide is used per equiv of **1d** from pH 5.0 to pH 6.0. Figure 1a is the nmr spectrum of **1d** in water at 40°. When 1 equiv of hydroxide is added, spectrum **1b** results. The assignments are presented in Table I. From these spectra (Figure 1) it is clear that the product of the reaction between **1d** and hydroxide is 2-(*N*-methylformylamino)propanal (**4d**). Figure 1b demonstrates that an amide moiety is present, because all signals appear in two sets due to the limited rotation about the C–N amide bond (Emsley *et al.*, 1966). Further evidence that **4d** is the product is given by the chemical shifts of both the formamide hydrogen at δ 7.95 and of the *N*-methyl hydrogens falling at δ 2.78 and 2.90. This is as predicted in analogy with the spectrum of *N,N*-dimethylformamide (Bhacca *et al.*, 1962).

The rates of hydrolysis of **1d** were studied at 30.0 ± 0.1° in the pH range from pH 5.0 to pH 6.0. A plot of the pseudo-first-order rate constants *vs.* the concentration of hydroxide is a straight line with slope 8.0 × 10^4 M^{-1} sec^{-1}. Within experimental error, the intercept is zero at zero concentration of hydroxide. The stoichiometry of the reaction was evaluated; 1.0 ± 0.1 equiv of HO^- was consumed (eq 2).

Hydrolysis of 1,3-Dimethylimidazolium p-Toluenesulfonate (**1e**). Because the imidazolium cation, **1e**, is not susceptible to hydrolysis even at high [HO^-] in water, a strongly basic solution of dimethyl sulfoxide and water (Me_2SO–H_2O) was used. Such solutions increase the activity of hydroxide (Bowden, 1966). The ring-opening reaction of **1e** was followed by integrating the nmr signals of the C-4 and C-5 hydrogens as a function of time. The data were analyzed using the integrated form for the second-order reaction. We expected (eq 2) that 1 equiv of hydroxide would react with 1 equiv of **1e**. Figure 2 is based on this stoichiometry; the assumption of stoichiometry appears correct. The slope of the line is 4.4 × 10^{-4} M^{-1} sec^{-1} at 40°. In order to compare the second-order rate constants for the ring-opening reactions of **1c**, **1d**, and **1e**, the value for k(HO^-) of **1e** at 30° is estimated to be about 2 × 10^{-4} M^{-1} sec^{-1}.

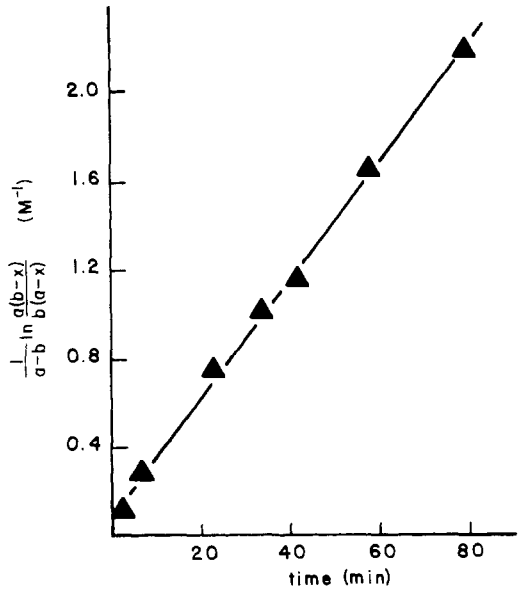


FIGURE 2 A plot of $1/(a-b)\ln[a(b-x)/b(a-x)]$ *vs.* time for the hydrolysis of 1,3-dimethylimidazolium *p*-toluenesulfonate (**1e**) in 53.3 mol % Me_2SO–46.7 mol % H_2O at approximately 40°.

Discussion

Structure and Function of Thiamine

2-Ylide Stability and Catalysis. Breslow's (1957) discovery of facile formation of thiazolium ylides and their importance in thiamine catalysis leads to the question: Is the rate of ylide formation related to the catalytic effectiveness of thiamine and thiamine analogs? Haake *et al.* (1969) have shown that the relative rates for the formation of the ylides of azolium ions with homologous sturcture (**1** ⇄ **2**) are in the order: **1d: 1c: 1e:** = $10^{5.5}$: $10^{3.5}$: 1 = oxazolium: thiazolium: imidazolium. That is, the oxazolium ion, **1d**, forms its ylide 100 times more easily than the thiazolium ion, **1c**, and 30,000 times faster than the imidazolium species, **1e**. Therefore, if rate of ylide formation correlates with catalytic effectiveness, oxazolium ions should be more effective than thiazolium ions.

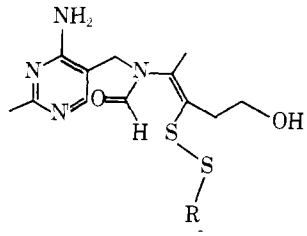
Although there are no data for *in vitro* catalysis by simple oxazolium ions, Breslow (1958) studied the benzoin condensation catalyzed by a number of azolium species, including benzoxazolium ions. In all cases, the benzoxazolium species showed no catalysis. Codington and Wuerst (1957) synthesized an oxazolium analog of thiamine. They found that this compound gave no cocarboxylase activity. Thus the oxazolium species appears to be ineffective in both

TABLE II: Second-Order Rate Constants for the Hydrolyses of Azolium Ions at 30°.

Compd	$k(OH^-)$ (M^{-1} sec^{-1})
1c	15.7
1d	8.0×10^4
1e	2.2×10^{-4}

model and *in vivo* reactions where thiamine is effective. Why?

RING-OPENING OF AZOLIUM RINGS. At pH 7.4, the ring-opening reaction of 3,4-dimethyloxazolium ions (**1d**) has a half-life of approximately 20 sec, and the equilibrium favors the ring-opened form (**4d**, eq 3). That is, oxazolium



5a, R = $CH_2CH_2CH_3$
b, R = $CH_2CH{=}CH_2$
c, R = $CH_2C_4H_8O$

ions are kinetically and thermodynamically unstable at physiological pH. In the pH region, 5.0–6.0, the reaction between hydroxide and **1d** consumes 1 equiv of base/equiv of substrate. This can be contrasted with the hydrolysis of thiazolium ions, in which 2 equiv of base are required in the region where the reaction is first order in hydroxide (Haake and Duclos, 1970). These results explain why oxazolium ions are inactive *in vitro* and *in vivo:* oxazolium ions are unstable at pH near neutrality and hydrolyze to their ring-opened forms, *e.g.*, **4d**, which are ineffective in catalysis. Casey *et al.* (1973) have studied benzothiazolium species and found that they are more susceptible to ring opening than their monocyclic analogs. This can explain why Breslow observed no catalysis with benzothiazolium species (Breslow, 1958). In regard to the oxazolium analog of thiamine (Codington and Wuerst, 1957), at pH 7.4 it would certainly exist in the ring-opened form, rendering it an ineffective thiamine substitute.

The second-order rate constants for the hydrolysis of **1c**, **1d**, and **1e** (Table II) give the following relative rates of ring opening: **1d: 1c: 1e** = $10^{8.6}$: $10^{4.9}$: 1 = oxazolium: thiazolium: imidazolium. The thiazolium ion is thermodynamically stable at physiological pH, but as the pH increases, a significant amount of ring-opened form is present and above pH 9.5 the ring-opened form predominates.

Imidazolium ions require very strongly basic solutions for ring opening. In fact, the data in Table II minimize the difference between imidazolium ions and the other ions because the activity of HO^- is increased in Me_2SO–H_2O solutions. Imidazolium ions, although stable to ring opening, form ylides more than 10^3 times more slowly than corresponding thiazolium ions (Haake, *et al.*, 1969).

Therefore, of the azolium species, only the thiazolium ion is suited for function in cocarboxylase. It is thermodynamically stable at pH 7.4, and the rate of formation of the thiazolium ylide enables it to be an effective catalyst. The oxazolium and imidazolium heterosystems fail in one of the two

TABLE III: Effect of Heteroatoms on Logs of Rate Constants for Ylide Formation and Ring Opening.

		Log of Relative Rate Constant	
	Heteroatom	Ylide Formation	Ring Opening
Oxazolium	O	5.5	8.9
Thiazolium	S	3.5	4.6
Imidazolium	N	0	0

criteria established above: the oxazolium ion is not stable to ring opening at physiological pH; the imidazolium ion is stable to ring opening, 10^5 times more stable than at thiazolium ion, but its relatively slow rate of ylide formation renders it a poor thiazolium substitute.

RELATIVE RATES. Equations 1 and 2 compare azolium ions in ylide formation and ring-opening reactions. Since E_a is proportional to log k, it is apparent that there is a proportional effect of the heteroatoms on activation energies for these reactions (Table III). There is a need to understand the reasons for these differences. The oxazolium ion is probably more labile in both reactions because of the greater electronegativity of oxygen compared to nitrogen and sulfur. The same reason cannot be the source of the greater reactivity of thiazolium compared to imidazolium ions since sulfur is less electronegative than nitrogen. The reason appears to lie with d-orbital participation in thiazolium ions (Haake and Nelson, unpublished results).

Ring-Opened Disulfide Derivatives and the Biological Role of Ring Opening. Although alteration of the thiamine molecule usually results in loss of cocarboxylase activity (Metzler, 1960; Rogers 1962, 1970; Kawasaki, 1963) certain ring-opened disulfide derivatives (RODD's) of thiamine (**5**) are more easily absorbed from the intestines than thiamine itself. Further, these RODD's are easily converted to cocarboxylase upon or subsequent to passage across the intestinal membrane, resulting in a greater therapeutic value than thiamine (DeReno *et al.*, 1954, Kasper, 1966; Fujimoto, 1968; Kawasaki, 1963; L'vova Kruglikova, 1970; Thomson *et al.*, 1971).

Our observations indicate that the chemical dynamics of ring opening of the thiazolium ring of thiamine are consistent with transport of RODD's across cell membranes: at physiological pH, the ring-closed, catalytic form of thiamine is thermodynamically favored, but thiazolium ions are kinetically labile to ring opening. Consequently, **4a** can react with a sulfhydryl compound in an oxidation step, forming an analog of **5**. This has been demonstrated in the *in vitro* formation of the disulfide of thiamine (Zima and Williams, 1940) and the disulfide of 3-ethyl-4-methylthiazolium iodide (Clarke and Gurin, 1935). From our kinetic analysis of the ring-opening reaction of **1c** (Haake and Duclos, 1970; Duclos, 1972), we are able to calculate the half-life for the sequence **1c** $\rightleftarrows$ **4c**. At pH 7.4, assuming the reaction irreversible, $t_{1/2}$ = 33 hr. With enzymic catalysis, transport *via* the ring-opened form would be feasible.

Therefore, the evolutionary selection of thiamine and its evolution as a vitamin may be due to the thermodynamic perference for the ring-closed form of thiazolium ions at physiological pH and the kinetic lability toward ring opening which may permit transport as a ring-opened disulfide.

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The Formation and Characterization of the Nicotine Analog of Triphosphopyridine Nucleotide†

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ABSTRACT: A nicotine analog of TPN (nicotine-TPN) was isolated and purified from an incubation mixture containing nicotine, TPN, and a rabbit liver microsomal fraction. Physical, chemical, and immunochemical evidence indicates that the nicotinamide moiety of TPN has been replaced by nicotine in this analog. Nicotine-TPN is considerably more stable than TPN in the presence of the liver microsomal fraction. The analog could also be formed in incubation mixtures containing pig brain or beef spleen DPNase instead of the liver microsomal preparation. Nicotine-TPN cannot substitute for TPN in the reactions carried out by TPN-specific isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, or glutamate dehydrogenase. Competitive inhibition with respect to TPN is observed with the last two enzymes.

Nicotine undergoes extensive metabolic changes in mammalian tissues to give at least eight known products (McKennis, 1965; Dagne and Castagnoli, 1972a,b). The liver is particularly active in the oxidation and demethylation reactions which produce metabolites such as cotinine (Hucker *et al.,* 1960), nicotine *N'*-oxide (Papadopoulos, 1964; Booth and Boyland, 1971), or nornicotine (Papadopoulos and Kintzios, 1963). However, little is known about intermolecular reactions of nicotine with other constituents of living cells. In particular, products resulting from the exchange of nicotine or its metabolites for the nicotinamide moiety in DPN or TPN have not been reported. Yet the enzyme, DPNase, exists in most animal tissues and is capable of exchanging the nicotinamide moiety of DPN and TPN for other pyridine containing compounds (Kaplan, 1960). For example, 3-acetylpyridine (Kaplan and Ciotti, 1956; Kaplan *et al.,* 1954) and 6-aminonicotinamide (Dietrich *et al.,* 1958) can replace the nicotinamide moiety of DPN and TPN to produce analogs of these coenzymes whose structures have been elucidated and whose physiological effects have been described. Nicotine has been shown to be an inhibitor for sheep brain DPNase (McIlwain, 1950) but not for beef spleen enzyme (Zatman *et al.,* 1954).

This paper describes the formation and characterization

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