STABILITY OF THE ISOMERS OF DIHYDROTHIAMINE

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In order to investigate the chemical form of TH_2 ² in aqueous solution it was demonstrated in the previous paper (1) that the ψ -form was the stablest of three isomers of TH_2 by comparing the stability of the aqueous dilute solution of TH_2 . In this paper the stability of the ψ -form of TH_2 in aqueous solution is discussed from the standpoint of its change by some reagents.

EXPERIMENTAL

1. Time Course of the Change of TH_2 in Aqueous Solution at Various pH Levels

The aqueous solution of ψ - TH_2 was left standing at various pH levels and the stability was successively investigated. *n*-Form of TH_2 is dissolved in a small amount of *N*/10 HCl and diluted with a suitable amount of water. It was neutralized with a dilute NaOH solution and water was added to make a 2.0 mg/ml solution, in which TH_2 had been completely converted to ψ -form. 4 ml of this solution was mixed with 1.5 ml of the acid or *M*/10 buffer solution and the whole covered with a stopper was incubated at 35°. After various time intervals an aliquot was taken and the amounts of TH_2 and thiamine were determined by FeCl_3 and BrCN method respectively. (2) As shown in Fig. 1, TH_2 was relatively stable in a very weakly acidic to alkaline solution, and 70 to 80 % of it remained unchanged even after 4 hours, whereas it was unstable in an acidic solution and was quickly degraded, about 3 to 9 % being converted into thiamine. Besides, formation of triamine² was found in the reaction mixture by paper chromatography. Since TH_2 is so easily degraded in acid solution and a part of it is converted to thiamine by air oxidation, the change of TH_2 in the weakly acid solution after adding hydrogen peroxide or cyanide was investigated.

2. Effect of Hydrogen Peroxide on TH_2

Although TH_2 was easily oxidized to thiamine by such oxidants, as FeCl_3 both *n*- or ψ - TH_2 in acetic acid solution was relatively easily degraded to DAPm sulfate by hydrogen peroxide, and in neutral or alkaline solution no marked change was observed.

n- or ψ - TH_2 , 0.5 g, was dissolved in 5 ml of 50 % acetic acid, 0.6 ml of 30 %

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² Following abbreviations were used: TH_2 , dihydrothiamine; *n*, normal; ψ , pseudo; triamine, tris (2-methyl-4-amino-5-pyrimidylmethyl)-hexahydro-*S*-triazine; DAPm, 2-methyl-4-amino-5-amino-methyl-pyrimidine.

FIG. 1 *Stability of TH₂ Solution*
 A, 0.3 M HCl; B, 0.03 M HCl; C, 0.03 M CH₃COOH;
 D, 0.03 M acetate buffer, pH 3.2; E, 0.03 M phosphate
 buffer, pH 6.2; F, 0.03 M phosphate buffer, pH 8.4.
^a The amount of thiamine formed as estimated by FeCl₃
 was taken as 100%.
 —, remaining TH₂; ---, thiamine formed.

hydrogen peroxide was added, and after standing for 48 hours at room temperature 5 ml of ethyl alcohol was added, whereby colorless needles were precipitated, yielding 0.2 g. After recrystallization from diluted alcohol it decomposed at 265°, and the ultraviolet and infrared absorption spectra and elementary analyses were in good agreement with that of DAP_m sulfate. Elementary analyses: Calculated for C₆H₁₀N₄·H₂SO₄·2H₂O, C, 26.46, H, 5.92, N, 20.57, S, 11.84 and crystal water, 12.23. Found: C, 26.19, H, 5.84, N, 19.83, S, 11.83 and crystal water, 12.88.

3. Effect of Cyanide on TH₂

TH₂ is known to be converted to thiamine in some extent by air oxidation in the presence of hydrochloric acid or protein (3), and also in the presence of active carbon (4). Since TH₂ has a property to be easily oxidized by air, it was assumed that TH₂ might be stabilized in the presence of reductants such as potassium cyanide. However, cyanide was found to catalytically accelerate the air oxidation of TH₂. The reaction mixtures containing 2.5 ml of TH₂ solution (100 μg/ml), 2 ml of M/10 acetate buffer (pH 4.5) and 1 ml of KCN solution (10 mg/ml, pH 4.5), were left standing at room temperature, 37°, 60° and 80°, and the amount of thiamine formed was estimated. As shown in Fig. 2, about 9% thiamine was formed after the reaction at 37° for 5 hours. In order to investigate the effect of pH on the reaction, acetate buffer in the reaction mixture was replaced by N/100

FIG. 2 *Effect of Temperature and Time on Formation of Thiamine from TH₂ by KCN*

TH₂ equivalent to 113 μ g thiamine was added.

○, 37°; ×, room temperature; ●, 60°; ▲, 80°.

HCl (pH 2) and *M*/10 buffers (acetate for pH 3.5 to 4.5 and phosphate for pH 5.5 to 7.5), and the amounts of thiamine formed were compared. As given in Fig. 3,

FIG. 3 *Effect of pH on Formation of Thiamine from TH₂*

TH₂ equivalent to 234 μ g thiamine was added.

●, main experiment; ×, control (water was used in place of KCN solution)

thiamine formation increased in an acid solution below pH 4.5, but after correction for the control values it showed the maximum value at pH 3.5 to 4.5. As reported

in the previous paper (1) TH_2 is assumed to exist as a quaternary ammonium form in a weakly acid solution, which is easily partially oxidized by air to thiamine similarly to the case in the presence of protein or active carbon. To ascertain these facts, the reaction mixture was placed in the Thunberg tube. The reaction mixture contained 1 ml of 20 $\mu\text{g}/\text{ml}$ TH_2 , 2 ml of 100 mg/ml KCN solution (pH 4.5) and 2 ml of $M/10$ acetate buffer (pH 4.5). It was incubated after replacing the air by hydrogen, oxygen or nitrogen at 37° for 20 hours and the thiamine formation was compared. As a control, similar experiment was carried out using water in place of KCN solution. As summarized in Table I, thiamine formation

TABLE I
Effect of Gas Phase

Gas	Thiamine formed			
	Main experiment		Control	
	μg	(%)	μg	(%)
Hydrogen	3.0	(20.7)	0.3	(2.1)
Nitrogen	5.8	(40.0)	0.3	(2.1)
Oxygen	8.0	(55.2)	0.5	(3.5)
Air	6.8	(46.8)	0.4	(2.8)

TH_2 equivalent to 14.5 μg thiamine was added

was accelerated in the presence of oxygen, whereas it was inhibited by nitrogen or hydrogen, showing the reaction to be oxidation by air.

To investigate the relationship between the amount of KCN and its action on TH_2 , various amounts of KCN were added to a constant amount of TH_2 in Thunberg tubes and after oxygen-aeration for 2 minutes with ice-cooling, the tubes were incubated at 37° for 5 hours. As shown in Table II, from 0.25 mg TH_2 scarcely any thiamine formation was detected after addition of KCN in

TABLE II
Thiamine Formation from TH_2 by the Addition of Various Amounts of KCN

Experimental gas	KCN added (mg)								
	0	0.01	0.1	1	2	4	8	10	100
	Thiamine formed (μg)								
1 Oxygen aeration	4.8 (1.8) ^a	6.2 (2.3)	5.7 (2.1)	6.6 (2.4)				32.7 (12.2)	33.2 (12.6)
No aeration	1.3 (0.5) ^a	0.6 (0.2)	1.1 (0.4)	3.0 (1.1)				3.5 (1.3)	8.4 (3.1)
2 Oxygen aeration	3.9 (1.8) ^a				3.7 (1.7)	6.0 (2.8)	7.1 (3.3)	18.0 (8.4)	13.2 (6.2)

^a The amount of thiamine formed by oxidation with FeCl_3 from TH_2 (Exp. 1, 270 μg , Exp. 2, 214 μg) was expressed as 100%.
Reaction mixture, 2.5 ml of $n\text{-TH}_2$, 1.0 ml of KCN and 2.0 ml of acetate buffer (pH 4.5).

amount less than 2 mg. With the increase of the amount of KCN thiamine formation rose gradually, reached to the constant value after addition of 8 to

10 mg KCN, and no more increase was observed by further addition of KCN. These results show that KCN at higher than a certain level is needed for thiamine formation. Under the above condition at least 8 to 10 mg KCN was needed for 4 to 5 ml of the reaction mixture. The change in thiamine formation with the increase of the amount of TH_2 in the presence of 10 mg KCN showed, that with the increase of the amount of TH_2 , the percentage formation of thiamine was reduced, as shown in Table III, and the amount of thiamine production was

TABLE III
*Effect of Various Amounts of TH_2 on the
Formation of Thiamine and the Change in Cyanide*

Time		TH_2 added (mg)			
		5	10	15	20
hr		Thiamine formed (μg)			
0	B_1 Main Control	10.3 (0.2) ^a 5.6 (0.1)	10.4 (0.1) 11.3 (0.1)	15.2 (0.1) 14.9 (0.1)	18.4 (0.1) 20.5 (0.1)
	CN^- Main Blank	89	88 100	100	105
5	B_1 Main Control	144.8 (2.6) 16.3 (0.3)	40.2 (0.4) 28.3 (0.3)	31.2 (0.2) 36.7 (0.2)	35.0 (0.2) 42.2 (0.2)
	CN^- Main Blank	89	86 98	93	91
20	B_1 Main Control	158.0 (2.8) 39.5 (0.7)	93.9 (0.9) 56.2 (0.6)	44.0 (0.3) 82.2 (0.5)	64.7 (0.3) 44.5 (0.2)
	CN^- Main Blank	87	— 94		

^a Thiamine formation in percent

Reaction mixture consisted of 0.5–2.0 ml TH_2 (10 mg/ml), 0.8 ml KCN (10 mg/ml, pH 4.5) and 1.6 ml of *M*/10 acetate buffer (pH 4.5) in a final volume of 4.4 ml, incubated at 37° (main).

In the control, water was used in place of KCN in the reaction mixture.

In the blank, water was used in place of TH_2 in the reaction mixture.

CN^- was estimated by colorimetric method (5) using mercuric thiocyanate and the value of the control at zero time was used for reference.

far greater after addition of a small amount of TH_2 . As can be seen from Table II, at least 30 to 40-fold amount of KCN is necessary for significant production of thiamine, from 0.25 mg TH_2 and in the presence of 10 mg KCN, with the rise of TH_2 from 5 to 20 mg, thiamine formation decreases at higher levels of TH_2 , suggesting the need of much KCN in comparison with TH_2 . As shown in Table III, the oxidation of TH_2 to thiamine is not directly dependent of the amount of cyanide.

These findings show that formation of thiamine by cyanide is marked in acetate buffer, pH 3.5–4.5, and KCN is partially changed to HCN. In the presence of HCN together with acetic acid and acetate, TH_2 is possibly oxidized by air. As given in Table IV, NaCN and KCNO also showed a catalytic effect on the

TABLE IV
The Effect of Cyanide, Cyanate and Rhodanate on the Oxidation of TH₂
 (), thiamine formation, %.

Amount added	Thiamine formed						CH ₂ (CN)-COOC ₂ H ₅
	Without addition	NH ₄ SCN	KSCN	KCNO	NaCN	KCN	
mg				μg			
1		8.8 (4.1) ^a	6.7 (3.1)	6.7 (3.1)	6.6 (3.1)	6.4 (3.0)	14.3 (6.7)
10	6.9 (3.2)	5.8 (2.7)	4.7 (2.2)	19.4 (9.0)	30.5 (14.4)	29.5 (13.8)	8.9 (4.1)
100		0.5 (0.2)	6.7 (3.1)	25.9 (12.1)	25.1 (11.7)	35.0 (16.3)	0.2 (0.1)

^a Thiamine formation in per cent.

Reaction mixture containing 2.5 ml of TH₂, 100 μg/ml, 1.0 ml of the cyanide compounds of various concentrations and 2.0 ml of M/10 acetate buffer (pH 4.5) was aerated with oxygen under ice cooling for 3 minutes, incubated at 37° for 5 hr.

The amount of thiamine formed from 250 μg of TH₂ by FeCl₃ was taken as 100%.

oxidation of TH₂, but thiocyanate was ineffective. CH₂(CN) COOC₂H₅ accelerated the formation of thiamine with a small amount, whereas it inhibited the reaction with larger amounts similarly to that of NH₄SCN.

DISCUSSION

The findings that TH₂ was converted to DAP_m sulfate by oxidative degradation with hydrogen peroxide in a weakly acid solution and in the presence of cyanide it is partially converted to thiamine by air, and that it was most easily degraded by sulfite in a weakly acid solution as reported in the previous paper (1) suggest that TH₂ may be present as a quaternary ammonium form in a weakly acid solution similarly to thiamine.

However, contrary to thiamine, quaternary ammonium salt of TH₂ is labile and easily degraded, especially it is rapidly degraded by contact with protein or in animal body. Thiothiamine is known to form thiamine sulfate by oxidation with H₂O₂ in an acid solution, whereby the sulfur in position 2 of the thiazole moiety is removed and oxidized. In TH₂, the oxidation of the thiazole moiety is preceded by degradation and the mercapto compound simultaneously produced is oxidized to sulfuric acid and DAP sulfate is thus produced. The conversion of TH₂ to thiamine in the presence of much cyanide may be compared with the finding that thiothiamine is oxidized to thiamine by the dissolved oxygen with simultaneous oxidation of SO₃⁻ (7). The acceleration of thiamine formation by cyanide is due to the oxidation by air dissolved in water. Supposing the saturation of O₂ in water to be 30 ppm, and TH₂ + O → thiamine + H₂O, 5 ml of the solution will produce about 3 mg thiamine in the case of 100% yield. Considering that the percentage of thiamine formation is high at lower concentration of TH₂ and the highest amount of thiamine formation was 0.15 mg per 5 ml, oxidation of TH₂ by dissolved oxygen is significantly low in yield as compared with that in the oxidation of thiothiamine by dissolved oxygen. Although it has been reported that oxidation of TH₂ by active carbon in air gave a good yield (4), it was corrected by Mitsuda *et al.* (8)

to be less than 50%. The cause of a low yield of thiamine formation from TH_2 may be the unstability of TH_2 . The assumption that TH_2 may be a precursor of thiamine biosynthesis in plants is unpalusible, since TH_2 is more easily degraded than is converted to thiamine as reported by the authors (10). Thiothiamine seems to be a more palusible precursor of thiamine biosynthesis.

SUMMARY

1. Degradation of ψ -dihydrothiamine at 35° in aqueous solution was successive-ly investigated. It was shown to be stable in alkaline solution, but it rapidly de-graded in acid solution, being partially converted to thiamine.

2. Addition of hydrogen peroxide to either n - or ψ -dihydrothiamine in acid solu-tion resulted in 2-methyl-4-amino-5-aminomethylpyrimidine sulfate formation.

3. Dihydrothiamine was partially converted to thiamine by addition of cyanide in a weakly acid solution. Thiocyanate showed no effect. For the reaction a large amount of cyanide as compared with dihydrothiamine was required and the dissolved oxygen accelerated the reaction, whereas hydrogen or nitrogen inhibited the reaction. These findings suggest that the reaction is a kind of air oxidation catalyzed by cyanide.

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