ON THE OXIDATION OF DIHYDROTHIAMINE BY ORGANIC AND INORGANIC OXIDANTS¹

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The DHT² was synthesized first by Karrer and Krishna (1,2) in a crystalline form by reducing thiamine or thiamine-thiazolone with LiAlH_4 . Subsequently, Iwatsu (3) obtained DHT with a high yield by condensing 2-methyl-4-amino-5-aminomethyl-pyrimidine and γ -aceto- γ -mercaptopropyl alcohol with formaldehyde. He reported also the chemical characterization of three types of isomers, *i. e.*, *normal*, *iso*- and *pseudo*-forms.

Since the condition of the synthesis is not too different from a natural, physiological one and DHT in acidic solution is easily oxidized to thiamine by atmospheric oxygen as reported by Hirano *et al.* (4), Matsukawa and Iwatsu (5) suggested a possibility that the compound might be a precursor of thiamine in its biogenesis.

It has been shown that DHT is oxidized easily *in vivo*, resulting in an appearance of thiamine-like biological activity in several experiments using microorganisms, root of pisum (6), rice birds (3) and rats (1). In this paper, the fundamental experiments were carried out using inorganic and organic oxidants, in order to characterize the DHT-oxidizing reagent operative in nature.

EXPERIMENTAL

Methods

The samples of *normal*- and *pseudo*-DHT were synthesized by Iwatsu's method (3). The R_F -value of DHT was 0.83 and that of thiamine-HCl was 0.41, using the mixture of isopropanol and *M*/15 phosphate buffer, pH 7.0, (5:1) as a solvent system. The spots showed reddish orange color by Dragendorff reagent. In this experiment, 10.64 mg of DHT was usually dissolved in 50 ml of distilled water without heating and the solution was used as the initial solution.

¹ Studies on the Oxidation of Dihydrothiamine. I. The contents of this paper were presented before the 116th (January 23, 1960) and the 122th (October 15, 1960) Meetings of the Vitamin B Research Committee and the 12th Congress of the Vitamin Society of Japan (May 9, 1960).

² DHT, dihydrothiamine.

The buffer solutions used were 1 *M* HCl-sodium acetate over the range pH 1.1 to 5.0 and 0.1 *M* phosphate over the range pH 6.0 to 7.0 unless otherwise specified. Quantitative estimation of the thiamine produced was performed by the BrCN method which converts thiamine to thiochrome (7).

Results

1. The Oxidation by Dyes

Methylene blue, 2,6-dichlorophenol indophenol and toluylene blue were used as dyes, and the redox-potential of DHT was estimated from the degree of decolorization of the dyes and from the quantities of the thiamine produced. The results were shown in Tables I and II. DHT was oxidized by the

TABLE I
Decolourization of Dyes

Dye	Mb			2,6-D				Tb	
pH	2.2	3.5	5.0	3.5	5.0	6.0	7.0	2.2	5.0
E ₀ (V)	+0.33	+0.21	+0.10		+0.36	+0.29	+0.22	+0.39	+0.22
<i>min</i>	<i>normal-Dihydrothiamine</i>								
10	+	+	+	+	+	+	+	+	+
30	+	+	+	±	±	±	+	+	+
60	+	+	+	—	—	±	+	±	+
120	+	+	+	—	—	—	+	—	+
180	+	+	+	—	—	—	±	—	+
600	+	+	+	—	—	—	—	—	+
	<i>pseudo-Dihydrothiamine</i>								
10	+	+	+	+	+	+	+	+	+
30	+	+	+	—	±	+	+	±	+
60	+	+	+	—	—	±	+	—	+
120	+	+	+	—	—	—	+	—	+
180	+	+	+	—	—	—	±	—	+
600	+	+	+	—	—	—	—	—	+

Mb, methylene blue; 2,6-D, 2,6-dichlorophenol indophenol, Tb, toluylene blue. +, full color. —, colorless.

Each Thunberg tube contained 1 ml of 2×10^{-4} *M* dye, 1 ml of 4×10^{-4} *M* DHT and 3 ml of buffer solution. The oxidation was performed under nitrogen gas at 30°.

dyes having the potentials higher than +0.40, +0.36 and +0.23 V at pH 2.2, 5.0 and 7.0 respectively, nearly in agreement with the results obtained polarographically by Asahi (8). When the concentration of 2,6-dichlorophenol indophenol was increased to 2 to 10 folds excess, the quantities of the thiamine produced remained constant. But a strict stoichiometric correlation between the degree of the decolorization of the dye and the yield of thiamine could not be obtained.

2. The Aerobic Oxidation in Acidic Solution

By Hirano's method (4), using active carbon and dilute hydrochloric acid, the amounts of oxygen consumed were estimated by Warburg manometer.

TABLE II
Formation of Thiamine by Dyes under Anaerobic Condition

Dye	Final concentration (M)		pH	Thiamine formed	
	DHT	Dye		Amount (μg)	Yield (%)
normal-Dihydrothiamine					
2,6-D	4×10^{-5} ^a	4×10^{-5}	5.0	22.66	42.6
	”	”	6.0	6.01	11.3
	”	”	7.0	4.84	9.1
	”	8×10^{-5}	5.0	24.10	45.3
	”	4×10^{-4}	5.0	23.62	44.4
Mb	”	4×10^{-5}	2.2	0.53	1.0
	”	”	5.0	0	0
Tb	”	”	2.2	14.36	27.0
	”	”	4.0	3.19	6.0
	”	4×10^{-4}	2.2	14.63	27.5
pseudo-Dihydrothiamine					
2,6-D	”	4×10^{-5}	5.0	26.01	48.9
	”	”	6.0	7.13	13.4
	”	”	7.0	5.69	10.7
	”	8×10^{-5}	5.0	25.54	48.0
	”	4×10^{-4}	5.0	26.3	49.5
Mb	”	4×10^{-5}	2.2	1.06	2.0
	”	”	5.0	0	0
Tb	”	”	2.2	18.35	34.5
	”	”	4.0	5.05	9.5
	”	4×10^{-4}	2.2	18.62	35.0

^a Corresponds to 53.2 μ g.

The condition was the same as that described in Table I, excluding the incubation for 600 min.

TABLE III
Aerobic Oxidation of Dihydrothiamine in Acidic Solution

DHT (mg)	Condition		O ₂ consumed (μ l)		Thiamine formed	
	Acid soln.	Active carbon (mg)	Theoretical	Found	Amount (mg)	Yield (%)
<i>normal-Dihydrothiamine</i>						
1.4	3 ml 2% HCl	1.0	58.9	55.0	0.675	48.2
0.7	"	"	29.5	30.0	0.357	51.0
5.0	"	10.0	211.0	216.0	2.670	53.4
<i>pseudo-Dihydrothiamine</i>						
1.4	"	"	58.9	57.0	0.714	51.0
0.7	"	"	29.5	31.0	0.353	50.5
5.0	"	5.0	211.0	218.0	2.530	50.6
"	3 ml 2% H ₂ SO ₄	"	"	211.5	2.400	48.0
"	3 ml 10% HCl	10.0	"	222.0	2.530	50.6
"	3 ml 10% H ₂ SO ₄	"	"	218.5	2.600	52.0
50.0	3 ml 2% HCl	18.0	2110.0	2190.0	23.950	47.9

When thiamine-HCl was treated under the same condition as DHT as indicated above, the recovery of thiamine-HCl was between 99 and 103%. No oxygen consumption was detected.

After completion of consumption, the thiamine produced was estimated. The yield of the vitamin was nearly 50 per cent as shown in Table III.³

3. The Oxidation by Organic and Inorganic Oxidants

The oxidizing reagents were selected considering the high redox-potential of DHT. The inorganic oxidants having $E_0 + 0.167$ to $+1.300$ V were allowed to react with DHT in 0.3 *N* HCl or in a buffer solution of pH 5.0 in Thunberg tube. Quinones were used as organic oxidants. But quinones of uniform chemical structure could not be obtained except for *p*-quinone. Therefore, quinone solutions were prepared in buffer solution (pH 7.0) by exposing to air, and the oxidation of DHT was coupled to quinone formation at the same time. The results were shown in Table IV. Among the oxidations of DHT by inorganic oxidants, production of thiamine by Cu^{2+} (having a low redox-potential) showed a rather high value, and it may not be a simple potentiometric oxidation.

TABLE IV
Formation of Thiamine by Inorganic Oxidants

Oxidant	E_0 (V)	Medium	Thiamine formed ^a		Thiamine formed ^b	
			Amount	Yield	Amount	Yield
			μg	%	μg	%
CuCl_2	$+0.167$	pH 5.0 0.3 <i>N</i> HCl	0.43	0.4	0.74	0.7
			4.89	4.6	12.56	11.8
$\text{K}_3\text{Fe}(\text{CN})_6$	$+0.36$	pH 5.0 0.3 <i>N</i> HCl	17.56	16.5	20.85	19.6
			42.99	40.4	48.31	45.4
FeCl_3	$+0.77$	pH 5.0 0.3 <i>N</i> HCl	2.98	2.8	3.51	3.3
			42.13	39.6	46.28	43.5
$\text{K}_2\text{Cr}_2\text{O}_7$	$+1.30$	pH 5.0 0.3 <i>N</i> HCl	5.32	5.0	7.13	6.7
			48.41	45.5	58.73	55.2
None	—	pH 5.0 0.3 <i>N</i> HCl	0.43	0.4	0.74	0.7
			0.64	0.6	1.06	1.0

^a from *normal*-DHT

^b from *pseudo*-DHT

Each reaction tube contained (in final concentration) 8×10^{-5} *M* DHT, 8×10^{-4} *M* oxidant and 0.6 *M* buffer or 0.3 *N* HCl. The oxidation was performed under nitrogen gas for 60 minutes at 30° .

Another reagents such as arsenic acid, mercuric nitrate, potassium iodate and potassium permanganate were used, but the production of thiamine failed to be recognized, probably due to complex formation of DHT or of the thiamine produced with the oxidants, or due to further oxidative degradation.

In the cases of organic oxidants, appreciable amount of thiamine was produced under physiological conditions (at pH 6.0), as shown in Table V. When

³ In Hirano's report (4) the absolute yield of thiamine was not given. According to his latest reexamination carried out under the same condition, the absolute yield of thiamine was 48.8 per cent (private communication).

TABLE V
Formation of Thiamine by Organic Oxidants

Oxidant	Final concn.		pH	Thiamine formed ^a		Thiamine formed ^b	
	Oxidant	DHT		Amount	Yield	Amount	Yield
	<i>M</i>	<i>M</i>		μg	%	μg	%
<i>p</i> -Quinone ^d	4×10^{-4}	4×10^{-5c}	1.1	16.22	30.5	26.39	49.6
	"	"	6.0	2.18	4.1	5.80	10.9
	"	"	0.3 <i>N</i> HCl	23.35	43.9	26.55	49.8
Hydroquinone ^e	7×10^{-3}	6.66×10^{-5}	7.0	0.85	1.6	0.85	1.6
Catechol ^e	"	"	"	2.13	4.0	4.15	7.8
Adrenaline ^e	"	"	"	2.50	4.7	2.87	5.4
Ascorbic acid ^e	"	"	"	0.37	0.7	0.47	0.9

^a from *normal*-DHT ^b from *pseudo*-DHT

^c Corresponds to 53.2 μg .

^d Anaerobic condition (Thunberg tube)

^e Aerobic condition (Warburg manometer)

The oxidation was performed for 120 minutes at 30°.

the experiments were carried out under a similar condition at pH 1.0, the yield of thiamine was nearly 50 per cent. As the redox-potential of ascorbic acid is obviously low, the production of thiamine was negligible as compared to the control.

DISCUSSION

DHT was oxidized by participation of $[\text{H}^+]$ in the presence of potentially oxidizable oxidants resulting in the production of thiamine. Therefore, it has been known that DHT in acidic solution was unstable when exposed to air. It has some resemblance to the stability of DPNH and DPN^+ in acidic and alkaline solutions. The maximum yield of thiamine was nearly 50 per cent when DHT was oxidized to thiamine as shown in Tables II to IV. On the other hand, half of DHT is degraded, probably hydrolytically, to unknown substances⁴. This inference is based on a hydrogen sulfide-like smell when the oxidation was effected with active carbon.

Since the yield of thiamine was about 50 per cent, the DHT solution dissolved in distilled water was always used in this experiment. If the DHT solution dissolved in 0.01 *N* NaOH was oxidized by ferric chloride under the same condition, the yield of thiamine was not constant.

The difference between the oxidations of the two isomers, *normal*- and *pseudo*-DHT, was hardly recognized, as shown in Table III. But the rate of

⁴ Recently, they were identified by Matsukawa and Yurugi (9) to be tris-[2-methyl-4-aminopyrimidyl-(5)] methyl-trimethylene-triamine, 2-methyl-4-amino-5-aminomethylprimidine, and γ -aceto- γ -mercaptopropyl alcohol.

thiamine production from *pseudo*-DHT was somewhat faster than the other as given in Table II and IV. These findings resembled those obtained by Kawasaki and Okada (10), possibly related to the isomeric structures of these substances. If DHT is oxidized in plant tissues, it does not seem to be oxidized by metallic ions, because the inorganic oxidants can act only in higher hydrogen ion concentrations. It is probable that the oxidation of DHT is coupled with the enzymes of polyphenol oxidizing system. Appreciable amounts of thiamine was produced from DHT by adding the oxidized form of adrenaline, suggesting a possible role of DHT in animal tissues.

SUMMARY

1. *normal*- and *pseudo*-Dihydrothiamine were oxidized by various organic and inorganic oxidants having high redox-potential.

2. *normal*- and *pseudo*-Dihydrothiamine were oxidized by the dyes having the redox potentials higher than +0.40, +0.36, and +0.23 V at pH 2.2, 5.0, and 7.0 respectively.

3. The absolute yield of thiamine produced by the oxidation of dihydrothiamine was about 50 per cent under the experimental conditions described in this paper.

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