STABILITY OF THE ISOMERS OF DIHYDROTHIAMINE

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It was previously reported by the authors (1, 2) that dihydrothiamine (TH₂) was relatively stable in an alkaline solution, whereas it was unstable in an acidic solution, and that the compound was also unstable, being degraded easily in animal bodies or by incubation with the homogenate of animal and plant tissues.

The form and the pathway of the degradation of TH₂ in a dilute solution are still obscure in many respects and the difference among the isomers of the compound remains to be investigated. Hirano (3) assumed that the thiazole nitrogen of TH₂ occurs in an aqueous solution in a quaternary ammonium form similarly to thiamine hydrochloride (B₁), since normal (*n*-) and pseudo (*ψ*-) TH₂ were reduced to tetrahydrothiamine by NaBH₄ in an aqueous solvent but not in an anhydrous solvent. Asahi (4) suggested from the results of polarographic experiments that TH₂ would be in an equilibrium state among several forms including the quaternary ammonium form.

It is well known that *n*- and *iso*-forms of TH₂ have exactly the same planer structure and both of them are easily transformed into the *ψ*-form having the ring closure by acid or heat treatment (5). In the present experiment, the stabilities of *n*- and *ψ*-forms in a dilute aqueous solution were investigated and found a marked difference between the two isomers.

EXPERIMENTAL

1. *Stability in an Alkaline Solution*

After TH₂ solutions having pH 4—13² were incubated at 37° for 2 or 4 hours, the remaining amounts were determined by FeCl₃ methods (6).

As shown in Fig. 1, *n*-TH₂ was unstable in an alkaline solution. It was decreased to about 1/3 after two-hour incubation at pH 11—13, whereas *ψ*-TH₂ was stable, being hardly degraded. That TH₂ has been assumed to be stable in an alkaline solution, seems to be due to the conversion of the majority of TH₂ into the *ψ*-form, since it was dissolved in an acid. The comparison of the stability of both isomers in neutral (pH 7.0) and alkaline (pH 13) solutions at various temperatures are given in Fig. 2. *n*- and *ψ*-TH₂ showed no difference in stability

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² Crystal of TH₂ was dissolved in a small volume of ethanol and 0.5 ml of 1 *M* buffer solution of each pH level or 1 *N* NaOH was added and water was added to a final TH₂ concentration of 1 mg/ml.

FIG. 1 Stabilities of *n*- and ψ -TH₂ in Aqueous Solutions
—, *n*-TH₂; ----, ψ -TH₂; ○, 2 hr; △, 4 hr. Remaining TH₂ shows the ratio of the value after heating to that before heating.

FIG. 2 Stabilities of *n*- and ψ -TH₂ in Neutral and Alkaline Media
A, neutral solution (pH 7); B, alkaline solution (pH 13); —, *n*-TH₂; ----, ψ -TH₂; ○, 14°; ×, 35°; △, 50°; □, 65°. Reaction mixture, 5 ml TH₂ (1.11 mg/ml) + 0.6 ml water and 1 *N* NaOH.

at pH 7.0 and were stable at low temperatures, but it had the tendency to be degraded with the rise of temperature. On the other hand, *n*-TH₂ was unstable in an alkaline solution: 20 % was degraded at 14° and 50 % at 35° after 2 hours, whereas TH₂ was hardly degraded at 35° and only about 14 % at 65°. It is therefore possible to detect the two isomers in aqueous solution, considering the different behaviors in an alkaline solution between *n*- and ψ -forms. Kawasaki *et al.* (7) have already reported that oxidation of TH₂ to thiamine by dichlorophenol-indophenol was markedly different according to the difference of the procedure of dissolving TH₂ crystals. The authors have dissolved the *n*- and ψ -TH₂ crystals in three different ways as described in Table I and to 4.5 ml of each solution

TABLE I
Stabilities of Dilute TH₂ Preparations Against Alkali
 The figures indicate percentage recoveries of the amounts of TH₂
 after heating to those before heating the alkaline medium.

Solution	<i>n</i> -TH ₂	ψ-TH ₂
Dissolved in distilled water by heating	85	86
Dissolved in a little amount of 0.1 <i>N</i> HCl, diluted with water and neutralized with dilute bicarbonate	87	88
Dissolved in a little amount of ethanol and diluted with water	88	82

(corresponding to 4.5 mg TH₂) was added 1 *N* NaOH up to pH 13, the mixture was incubated at 50° for 1 hour and the remaining TH₂ was compared.

As shown in Table I, ψ-TH₂ as well as *n*-TH₂ which had been dissolved in hot distilled water or in an acidic solution gave higher values of remaining TH₂ (82–88 %), but *n*-TH₂ dissolved in a small amount of ethanol, and diluted with water was fairly labile, being much degraded upon heating in an alkaline solution. It can be explained that *n*-TH₂ is converted into the ψ-form by dissolution in acid or in hot water and it exists in the *n*-form in an aqueous solution only when dissolved in alcohol.

The degradation product formed by heating *n*-TH₂ in an alkaline solution was detected as a Dragendorff-reagent positive spot of *R_F* 0.30 in paper chromatography using acetic acid-*n*-butanol-water (4:1:5) as a solvent system. The compound was isolated as dipicrate (mp 202–203°).

2. Degradation of TH₂ by Sulfite

To 1 ml of *n*-TH₂ solution³ was added 1 ml of 1 *M* acetate buffer (pH 5.5). After addition of 1 ml 0.05–0.5 mM Na₂SO₃, the mixture was incubated at 35°

TABLE II
Sulfite Cleavage of Dilute n-TH₂
n-TH₂, 0.03 moles (equivalent to 16.9 μg B₁)

Time	Na ₂ SO ₃ used	TH ₂ detected	Percentage recovery of TH ₂	TH ₂ remaining ^a
<i>hr</i>	<i>μ</i> moles	<i>μ</i> g as thiamine	<i>per cent</i>	
1	0	4.7	27.8	100
	0.05	4.6	27.5	99
	0.15	4.4	26.0	94
	0.25	4.5	27.0	97
	0.35	4.5	27.0	97
	0.50	4.4	26.0	94
2	0	1.8	10.4	100
	0.05	1.8	10.4	100
	0.15	1.7	10.1	97
	0.25	1.8	10.4	100
	0.35	1.7	9.9	96
	0.50	1.5	8.8	85

^a The remaining TH₂ without addition of sulfite was taken as 100.

n-TH₂, 0.05 μmoles = 13.32 μg; Na₂SO₃, 0.05 μmoles = 6.30 μg.

³ *n*-TH₂ was dissolved in a small amount of ethanol and diluted with water to a final concentration of 0.05 mM.

and the remaining TH_2 was determined with 2,6-dichlorophenol-indophenol (8). As shown in Table II, $n\text{-TH}_2$ at such a dilute concentration (final concentration, $4.44 \mu\text{g/ml}$), decreased rapidly after incubation for 1–2 hours in the presence of 1–10 equivalent amount of sodium sulfite, but in the control experiment without sulfite it was also degraded remarkably; no difference between the two could be recognized. Therefore the degradation of TH_2 by various concentrations of sulfite was compared under the same condition. As listed in Table III, both $n\text{-}$ and $\psi\text{-}$

TABLE III
Sulfite Cleavage of $n\text{-}$ and $\psi\text{-TH}_2$
Reaction mixture: 2.5 ml TH_2 + 1.5 ml 1 *M* acetate
buffer (pH 5.5) + 1 ml Na_2SO_3 ; at 35° , 2 hr.

	Final conc. of TH_2 reacted	Final conc. of Na_2SO_3 reacted	TH_2 detected	Percentage recovery of TH_2	TH_2 remaining ^a
	μM	μM	$\mu\text{g as B}_1$	per cent	
$n\text{-TH}_2$	37.5	0	6.7	10.5	100
		1190	6.4	10.2	97
	375	0	169.9	26.8	100
		5950	97.2	15.4	57
	3750	0	4101	64.8	100
		27760	1125	17.8	27
$\psi\text{-TH}_2$	37.5	0	9.3	14.8	100
		1190	9.7	15.4	97
	375	0	254.8	40.2	100
		5950	103.7	16.2	41
	3750	0	4556	72.0	100
		27760	1068	16.9	23

^a The remaining TH_2 without addition of sulfite was taken as 100.

TH_2 were markedly degrade at a diluted concentration (final concentration, $37.5 \mu\text{M}$), no difference being observed between the main and control tests. With the rise in concentration the remaining TH_2 of the control became higher than that of the main test and the effect of sulfite markedly appeared. However, under this condition, no difference in degradation between $n\text{-}$ and $\psi\text{-TH}_2$ was recognizable. Thus the effect of sulfite on TH_2 was found only at higher concentrations, at which TH_2 was relatively stable, whereas in dilute solution, the effect of sulfite was scarcely recognized owing to a distinct degradation of TH_2 in a

TABLE IV
Effect of Sulfite on TH_2 Degradation
Reaction mixture: 2.5 ml $n\text{-TH}_2$ (2 mg/ml) + 1.5 ml
1 *M* acetate buffer + 1 ml Na_2SO_3 ; 35° , 2 hr.

Amount of Na_2SO_3	TH_2 detected	Percentage recovery of TH_2	TH_2 remaining
equivalents to TH_2	$\mu\text{g as B}_1$	per cent	
0	4514	71.3	100
1	1419	22.4	31
2	1071	16.9	24
4	868	13.7	19
6	620	9.8	14
8	680	10.7	15

weakly acidic medium. Comparison of the degradation of 18.8 moles n -TH₂ at varying concentrations of sulfite gave the results given in Table IV. The highest degradation of TH₂ took place in the presence of about 6 equivalents of sulfite. The degradation of n - and ψ -TH₂ at varying pH levels in the presence of 6 equivalents of sulfite was shown in Fig. 3. Both the isomers were most degraded at a weakly acid to neutral reaction, similarly to B₁, whereby no difference in degradation was observed between the two. On the other hand, ψ -TH₂ was hardly degraded in an alkaline medium and quite stable at pH levels higher than 10, whereas n -TH₂ was unstable even in an alkaline medium, being markedly degraded by sulfite.

FIG. 3 Effect of pH on Sulfite Cleavage of TH₂
 1, 1', n -TH₂; 2, 2', ψ -TH₂; 3, 3', thiamine; —, main; ----, control.
 Reaction mixture: 2.5 ml TH₂ or thiamine (2 mg/ml) and 1 ml 1 M
 acetate buffer + 1 ml Na₂SO₃ (17.5 mg/ml); 35°, 2 hrs.
 The amount of remaining TH₂ at zero time was taken as 100.
 In control, water was used instead of Na₂SO₃.

DISCUSSION

TH₂ (Fig. 4, I, II) having three isomers may be easily transformed in an acidic solution, giving an ammonium form (III) which is readily degraded. In an alkaline solution it is assumed to take a thiol form (Fig. 4, IV). Considering, however, the different stabilities and behaviors of n - (Fig. 4, I) and ψ -TH₂ (Fig. 4, II) against sulfite degradation in an alkaline solution, the forms of both isomers in an aqueous solution are supposed to be different. On the other hand, in a weakly acidic solution, the stabilities of both isomers on heating and the behaviors against sulfite degradation are quite the same, the finding suggesting that both the isomers take the same structure (possibly the ammonium form as in Fig. 4, III). In an alkaline solution, the behaviors of both isomers are fairly different, suggesting the occurrence of different forms. Hirano (3) presumed from the findings of NaBH₄ reduction that n - or iso -TH₂ (Fig. 4, I) usually takes an ammonium form in an aqueous

FIG. 4 Degradation of B_1H_2

solution (III), whereas ψ -TH₂ takes a proper cyclic structure, different from ψ -form (II). Our findings given above show that both the isomers take in an acidic aqueous solution the same structure (III) which is unstable contrary to B_1 , being decomposed gradually in a lapse of time, but it is stabilized in an alkaline solution after converting to a cyclic structure (II) or its hydrolyzed form (II').

The finding that heat or sulfite degradation of *n*- or *iso*-TH₂ dissolved in an alkaline solution is definitely greater than ψ -form and that a considerable amount of a thiol form appears at the beginning of heating in an alkaline medium (7), suggest that the form of *n*-isomer in a neutral to alkaline solution is different from ψ -form (II), possibly the structure I' (Fig. 4), which is degraded further by heating through such a thiol form as IV (Fig. 4).

It has been reported by Hennessy *et al.* (9) that TH₂ is converted by sulfite ion in an acidic medium, similarly to B_1 , to pyrimidine-methanesulfonic acid (Fig. 4, V). In general, the sulfite cleavage of thiamine derivatives is assumed to be a reaction specific to the quaternary nitrogen in thiazole nucleus. The decompsing effect of sulfite on different concentrations of TH₂ was definitely observed at higher concentrations, suggesting that TH₂ *per se* is stable at higher concentrations but easily affected by sulfite. Generally, it is most easily degraded by sulfite in a neutral to weakly acidic solution, having a tendency to become more resistant with the rise in pH values, a finding quite similar to B_1 . It is therefore assumed that TH₂ takes a quaternary form (III) in a weakly acidic solution, the thiazole moiety of which is labile and readily degraded, but it is not the case in an alkaline solution, since TH₂ does not take a quaternary form in such a solution. Further, B_1 was never detected in the solution of dilute TH₂ and sulfite, and conversion to B_1 as in the case of thiothiamine in the reaction with sulfite has never taken place (10).

SUMMARY

Stabilities of dilute solutions of *n*- and ψ -TH₂ in an alkaline solution and degradation by sulfite were investigated.

1. The stabilities of two isomers upon heating or standing at room temperature showed no difference; they were both unstable in a weakly acidic medium but

relatively stable in a neutral medium. However, the *n*-form becomes labile in an alkaline medium, especially, it was readily degraded by heating, whereas the ψ -form remained fairly stable upon heating.

2. Dilute solutions (final concentration, 17—33 μM) were quite unstable and the effect of sulfite was scarcely detectable, but at higher concentrations (final concentration, 3.7 mM) both *n*- and ψ -TH₂ were readily degraded (about 88 % decomposed) by sulfite at pH 5.5—7.0 in the presence of 6 equivalents of sulfite. In more acidic or alkaline solution, the degradation was decreased. ψ -TH₂ was stable in an alkaline medium, being scarcely affected by sulfite, whereas *n*-TH₂ had a tendency to be degraded readily in the presence of sulfite.

3. These findings suggest that both *n*- and ψ -TH₂ take the same, probably a quarternary form in a dilute aqueous solution, whereas in an alkaline solution, the ψ -form takes a stable cyclic structure, and *n*-form is degraded gradually through a dihydro-form of thiamine and its labile thiol form.

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