Differences in Thiochrome Fluorescence Produced by Thiamine and Its Mono-, Di-, and Triphosphate Esters

HANNU K. PENTTINEN

Department of Medical Chemistry, University of Helsinki,
Siltavuorenpenger 10 A, SF-00170 Helsinki 17, Finland

Equimolar amounts of thiamine and its mono-, di-, and triphosphate esters, when oxidized with potassium hexacyanoferrate(III) in an alkaline medium, were found to produce unequal intensities of thiochrome fluorescence. These differences were less pronounced if the oxidation medium contained ethanol. Assuming that the phosphate groups do not increase the fluorescence, then in 50 % ethanol (v/v); 74 % of thiamine and thiamine monophosphate, 85 % of thiamine diphosphate and 93 % of thiamine triphosphate are converted to thiochrome. The differences observed may be due to the formation of different amounts of nonfluorescent oxidation products, especially the disulfide derivative. This view is supported by the observation that thiamine and its phosphate esters differ in their stability to alkalinity.

In 1936 Jansen¹ found that oxidation of thiamine with potassium hexacyanoferrate(III) in an alkaline medium leads to the formation of the fluorescent thiochrome derivative. This reaction has frequently been used in the quantitative determination of thiamine. Methanol and ethanol, when present in the oxidation medium, favor the production of thiochrome from thiamine,^{2,3} but there is no study on their possible effect on the formation of thiochrome from thiamine phosphate esters. In addition to thiamine, its mono-, di-, and triphosphate esters are found in biological materials⁴⁻⁶ and can also be oxidized to fluorescent thiochrome derivatives. Lewin and Wei⁷ claimed that thiamine and its mono- and diphosphate esters in equimolar amounts produce fluorescence of the same intensity. This view seems not to be generally accepted, because most investigators still treat thiamine phosphate

esters with phosphatase⁸ to liberate the thiamine before oxidation. The aim of the present study was to characterize the formation of thiochrome from thiamine phosphate esters, and to examine some factors influencing this reaction.

EXPERIMENTAL

Abbreviations. TMP = thiamine monophosphate, TDP = thiamine diphosphate and TTP = thiamine triphosphate.

Reagents. Thiamine and its mono- and diphosphate esters were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Thiamine triphosphate was prepared as described elsewhere.⁹ All these preparations were crystallized three times and were free from inorganic phosphate. A standard solution of thiamine was made from the U.S.P. Reference Standard.¹⁰ Thiochrome was obtained from Pfalz & Bauer, Inc., New York, U.S.A., and crystallized twice before use.

The quinine standard was 0.01 % (w/v) quinine sulfate in 0.1 M H₂SO₄, diluted 1:10 and was taken to have a fluorescence of 100.

Formation of thiochrome. Thiamine and its phosphate esters were oxidized to thiochrome by the method of Lewin and Wei,⁷ except that the oxidation medium contained ethanol. A sample (5 μ l) of the compound was added to 3.0 ml of 50 % ethanol (v/v) and shaken, and after a few minutes 0.5 ml of alkaline hexacyanoferrate(III) reagent (15 ml of 15 % NaOH and 1 ml of 2 % potassium hexacyanoferrate(III)) was added. The mixture was agitated for 2 min, and 10 μ l of 30 % H₂O₂ was added to destroy the yellow color of hexacyanoferrate(III). The fluorescence was then measured with a Zeiss PMQ II spectrophotometer fitted with a ZFM 4 fluorometer attachment (Carl Zeiss, Germany). The excitation wavelength was 365 nm and the emission maximum 430 nm.

Determination of phosphate. Total and hydrolyzable phosphate were determined according to Fiske and SubbaRow.¹¹ The hydrolyzable phosphate was determined after the specimen had been incubated in 1 M HCl for 10 min in a boiling water bath.

Infrared absorption analysis. Pellets containing 1 mg of thiamine compound in 120 mg of KBr were analyzed with a Beckman IR 10 spectrophotometer.

RESULTS

Effect of different alcohols on thiochrome formation and fluorescence. The effects of methanol,

Fig. 1. The effect of different alcohols on formation of thiochrome from a mixture containing the same amounts of thiamine and its mono-, di-, and triphosphate esters.

ethanol and propanol on thiochrome formation from a mixture of thiamine and its phosphate esters were compared (Fig. 1). In the light of this experiment, 50 % ethanol was chosen for the oxidation medium. When thiamine compounds were separately oxidized in media containing different proportions of ethanol, it was noted that the increase in thiochrome formation due to the presence of ethanol was greatest with thiamine, and smaller with TMP, TDP, and TTP, in this order. This conclusion can be drawn from the values of the index F/F_w in Table 1 as well.

When thiochrome was dissolved in 50 % ethanol its fluorescence was 2.5 times as intense as in water. Inclusion of potassium hexacyanoferrate(III) and NaOH in the assay medium was found to reduce the fluorescence of thiochrome to 70 % of the maximal. The presence of ethanol did not alter the pH of the assay medium, which was found to be 13.4 under all conditions used.

Differences in absorbance and in thiochrome fluorescence between thiamine and its phosphate esters. In Table 1 the absorbance in 0.1 M HCl is collated with the fluorescence of thiochrome derived from thiamine and its phosphate esters in water and in 50 % ethanol. When oxidized in 50 % ethanol, the thiamine compounds gave equal intensities of fluorescence, but after oxidation in an aqueous medium the intensities were unequal. The absorbances were also unequal. Because of these differences the concentrations were estimated in another way: by determining the total and hydrolyzable phosphate of the thiamine phosphate esters. As the data in Table 2 show, equimolar amounts of thiamine and its phosphate esters have the same absorbance, but do not produce the same

Table 1. Differences in absorbance and in thiochrome fluorescence between thiamine and its phosphate esters oxidized in 50 % ethanol and in water. The preparations were diluted to get equal thiochrome fluorescence in 50 % ethanol. The index F/F_w is the amount of fluorescence when the oxidation was performed in 50 % ethanol compared with that in water. $N=6$.

	Absorbance $\times 10^{-3} \pm$ S.D. In 0.1 N HCl at 248 nm	Fluorescence $\pm$ S.D. In 50 % ethanol	F/F_w	In water
Thiamine	434 ± 6.34	27.60 ± 0.90	3.60	7.66 ± 0.25
TMP	422 ± 7.23	28.10 ± 0.57	3.34	8.41 ± 0.23
TDP	379 ± 6.47	28.00 ± 1.11	2.88	9.72 ± 0.15
TTP	370 ± 10.23	28.14 ± 0.16	2.84	9.91 ± 0.11

Table 2. The relationship between molarity, absorbance and fluorescence of thiochrome, thiamine and its phosphate esters. The preparations were diluted to get equal absorbance in 0.1 M HCl at 248 nm.

	Molarity $\pm$ S.D. ^a according to Weight	Total phosphate		Relative absorbance $\pm$ S.D. ^b	Relative fluorescence $\pm$ S.D. ^b in 50 % ethanol		Correction index
			Hydrolyzable phosphate		Before hydrolysis	After hydrolysis	
Thiochrome	1.0	—	—	—	100 $\pm$ 0.5	—	—
Thiamine	1.0	—	—	100 $\pm$ 0.9	74 $\pm$ 0.3	74 $\pm$ 2.4	1.35
TMP	1.0	1.0 $\pm$ 0.01	—	100 $\pm$ 0.9	74 $\pm$ 0.4	74 $\pm$ 3.7	1.35
TDP	1.1	1.9 $\pm$ 0.02	1.0 $\pm$ 0.02	100 $\pm$ 0.6	85 $\pm$ 1.6	75 $\pm$ 2.5	1.18
TTP	1.0	3.2 $\pm$ 0.16	2.0 $\pm$ 0.03	100 $\pm$ 0.12	93 $\pm$ 1.3	79 $\pm$ 1.1	1.08

^aN = 3. ^bN = 4.

intensity of thiochrome fluorescence. The molar absorption coefficient of thiamine and its mono-, di-, and triphosphate esters at 248 nm in 0.1 M HCl was 13 400.

When the oxidation medium contained 50 % ethanol, the fluorescence intensities produced by thiamine and thiamine monophosphate were equal, but the intensities from thiamine di- and triphosphate were higher (Table 2). To correct for the deficient thiochrome formation, the intensities produced by thiamine, TMP, TDP, and TTP must be multiplied by 1.35, 1.35, 1.18, and 1.08, respectively. On the other hand, if thiamine is taken as the standard, the fluorescence values of TMP need no correction but that of TDP and TTP must be multiplied by 0.87 and 0.80, respectively. The correction indices were found to be constant and independent of the concentration of thiamine compounds.

UV and IR absorption spectra of thiamine and its phosphate esters. The UV absorption spectra of the different thiamine phosphate esters were recorded in 0.1 M HCl, 0.1 M NaOH, and 0.05 M potassium phosphate buffer, pH 7.0. Under the same conditions, all thiamine compounds showed identical spectra. At 234 and 265 nm (the absorption maxima) molar absorption coefficients of thiamine and its phosphate esters in 0.05 M potassium phosphate buffer were found to be 10 900 and 8100 at pH 7.4 and 11 300 and 8250 at pH 7.0, respectively. These results are comparable with the corresponding values reported earlier for thiamine.^{10,12-15}

The stabilities of the thiamine compounds were tested under conditions in which the UV spectra were measured. In 0.1 M HCl, absorbance at 248 nm was unchanged, ability to form fluorescent thiochrome was not reduced and no inorganic phosphate was liberated. In 0.1 M NaOH, in contrast, the ability to form fluorescent thiochrome disappeared within 1 min, but returned if the sample was acidified with HCl before oxidation. Neither a decrease in the absorbance at 230 nm nor liberation of inorganic phosphate occurred during the time needed for measurement of the UV spectra.

Characteristic differences, however, were found in the IR spectra of the different thiamine phosphate esters. Thiamine had a unique peak at 1050 cm⁻¹, whereas all three phosphate esters showed peaks at 1160 cm⁻¹. In the spectra of TMP, TDP, and TTP, additional peaks at 940, 1000, and 2560–2700 cm⁻¹ became amplified as the number of phosphate groups increased. These results are in keeping with the structures of these esters presented in Fig. 2.

Inhibitory effect of alkalinity on formation of thiochrome from thiamine and its phosphate esters. Table 3 shows the effect of changing the pH values of the preincubation medium on formation of thiochrome from thiamine compounds. When the pH of the medium or preincubation time or both were increased, less thiochrome was formed, the effect being greater with thiamine than with its esters. The ester least susceptible to alkali was TTP, followed by TDP and TMP. At pH 9.0 the decrease in ability to form fluorescent thio-

Fig. 2. Structures of thiamine, thiochrome and thiamine disulfide. R^1 =thiamine monophosphate (TMP), R^2 =thiamine diphosphate (TDP) and R^3 =thiamine triphosphate (TTP).

chrome after incubation for 48 h was irreversible, whereas at pH 8.0 it was reversible, provided the sample was acidified with HCl before oxidation.

DISCUSSION

It is not clear why thiamine esters give rise to different intensities of thiochrome fluorescence. They may produce different amounts of thiochrome, or the phosphate groups may

intensify the fluorescence, or both mechanisms may be involved. Several observations support the first alternative. The other major oxidation product is the nonfluorescent disulfide (Fig. 2).³ This is formed when thiamine is exposed to air in an alkaline medium, but can be reduced to thiamine with hydrochloric acid.¹⁶ When incubated in an alkaline buffer, thiamine and its phosphate esters may well show the same tendency to form disulfide (Table 3). The experimental results suggest that on mild oxida-

Table 3. The stability of 1 mM thiamine and its phosphate esters in 0.5 M potassium phosphate buffer of different pH values at 24 °C.

Relative fluorescence										
pH of buffer	9.0				8.0			7.0		
Incubation time	5 min	1 h	24 h	48 h	2 h	24 h	48 h	2 h	24 h	48 h
Thiamine	73	37	36	8 ^a	90	93	78 ^b	100	97	72
TMP	94	89	79	66 ^a	95	91	87 ^b	94	91	94
TDP	100	91	60	45 ^a	99	96	102	97	93	100
TTP	101	96	79	70 ^a	99	97	101	99	99	101

^a Irreversible change. ^b Reversible change.

tion in an aqueous medium, thiamine is most easily converted to the disulfide, whereas TMP, TDP, and TTP, in this order, show weaker tendencies to form corresponding disulfide derivatives. Thus possibly, the proportions of thiochrome and disulfide may differ. This notion is indirectly supported by observations on the effect of ethanol on thiochrome formation. In the oxidation of thiamine, Risinger and Pell² have suggested that solvents of low dielectricity will increase the formation of thiochrome, whereas those with a high dielectric constant will favor the production of disulfide. Comparison of the thiochrome fluorescence values derived from the oxidation of thiamine compounds in media with and without ethanol (Table 1, index F/F_w) showed that in the presence of ethanol, fluorescences of thiamine, TMP, TDP, and TTP were increased 3.6, 3.3, 2.9, and 2.8-fold, respectively, whereas that of thiochrome increased only 2.5-fold. The differences are small, but the tendency is clear: the more phosphorylated the thiamine compound, the smaller the effect of ethanol. Thus ethanol has the greatest effect on thiamine because thiamine has the greatest tendency to form disulfide. Furthermore, the weaker tendency of phosphorylated thiamines to form the disulfides may be due to the electrostatic repulsion between the phosphoric acid chains in the disulfide structure (Fig. 2).

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