STUDIES ON THE SYNTHESIS AND THE PHYSICOCHEMICAL PROPERTIES OF THIOCHROME PHOSPHATES¹

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The concentrations of thiamine phosphates are usually determined fluorometrically as thiochrome (Thc) by means of the well-known thiochrome method. In this method, the thiamine phosphates are dephosphorylated and then oxydized to thiochrome which can be extracted from aqueous solution with butanol and determined fluorometrically. The butanol-extraction of Thc is carried out in order to avoid the interference arising from other concomitant fluorescent materials in aqueous solutions. However, when monochromatic light is used as an exciting light, it may be possible to determine the concentration of Thc in aqueous phase, because the background fluorescence of coexisting materials in aqueous phase may be decreased in a great measure.

The present investigation was undertaken in order to study the spectral properties of Thc, Thc-MP, and Thc-DP in aqueous solutions.

Although thiochrome triphosphate was previously prepared by Yamamoto (1), the ion-exchange chromatography was not applied for purifying the material. Shnol (2) reported the preparation of some radioactive thiochrome phosphates, but no detail of purification was given.

In this paper, therefore, the preparation of pure materials of Thc, Thc-MP, and Thc-DP and the fluorescence spectra of these compounds in aqueous solutions will be reported.

EXPERIMENTAL AND RESULTS

1. Preparation of Materials

Thiochrome—The preparation of Thc followed the process reported by Matsukawa (3). The purification procedure by Utsumi (4) yielded pure Thc, mp 228—229°. Elementary analysis. Found: C, 54.64; H, 5.49; N, 21.33. Calculated

¹ Following abbreviations are used: Thc, thiochrome; Thc-MP, thiochrome monophosphate; Thc-DP, thiochrome diphosphate.

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for $C_{12}H_{14}N_4OS$: C, 54.94; H, 5.38; N, 21.36.

Thiochrome Monophosphate — Ortho-phosphoric acid (85 %, 18.5 ml) was heated up to 310° to yield polyphosphoric acid. Thc (2.5 g) was added to the polyphosphoric acid cooled to 100° . After two hours' reaction at 100° , the reaction mixture was poured onto a small amount of ice. The resultant aqueous solution was then poured into 600 ml of acetone with stirring leading to a mushy precipitate of thiochrome polyphosphates. The treatment of dissolution in water and precipitation in acetone were repeated once more to remove some free phosphoric acid. The mushy precipitate was dissolved in 20 ml of 6 *N* hydrochloric acid and heated to hydrolyze thiochrome polyphosphates to Thc-MP at 60° for 6 hours. The solution was filtered through carbon. The pH value of the filtrate was adjusted to 4.0 with concentrated sodium hydroxide. Being kept standing, the solution gave yellow prisms of Thc-MP. The crystals collected by filtration were suspended in 27 ml of water and to this suspension a concentrated sodium hydroxide was added in portion to dissolve the crystals. The resultant solution was acidified to a pH of 4.0 with concentrated hydrochloric acid. This solution was allowed to stand to give pure Thc-MP, mp about 260° (decomposed), weighing 2.0 g. Elementary analysis. Found: C, 36.51; H, 5.44; N, 13.89; P, 7.88; H_2O , 13.54. Calculated for $C_{12}H_{15}N_4O_4PS \cdot 3H_2O$: C, 36.36; H, 5.34; N, 14.14; P, 7.81; H_2O , 13.63.

Thiochrome Diphosphate — Thc (7.8 g) was treated with polyphosphoric acid as described above and then with acetone to wash away some free phosphoric acid in a similar manner described above. The aqueous solution of thiochrome polyphosphates was adjusted to pH 5.5 and passed slowly through a column containing 200 ml (3×28 cm) Dowex 1-X8 (Cl^- type) resin. The adsorbed thiochrome phosphates was eluted with water, 0.01 *N* HCl, and 0.025 *N* NaCl in 0.01 *N* HCl successively, giving a chromatogram shown in Fig. 1. The fractions containing

FIG. 1 Separation of Thc-DP by Ion-exchange Resin, Dowex 1-X8 (Cl^- type), Column Chromatography

Thc-DP were collected and were concentrated under reduced pressure at room temperature. To this solution, whose pH value had been adjusted to 6–7, a concentrated calcium chloride solution was added to precipitate calcium salt of Thc-DP separated from other inorganic salts. One and half grams of this precipitate was suspended in water and dissolved in the solution with addition of sulfuric acid (pH 1.2). Precipitated calcium sulfate was collected by filtration and the filtrate was chromatographed over the ion-exchange resin once more. The fractions of Thc-DP were treated with calcium chloride to exclude other inorganic materials and with sulfuric acid to remove the calcium ion as described above. A large amount of acetone was added to the filtrate and the mixture was allowed to stand in a cold place to yield yellow plates of Thc-DP. The crude crystals were submitted to recrystallization from water by addition of acetone. The yield of pure Thc-DP, mp about 170° (decomposed), was 0.2 g. Elementary analysis. Found: C, 30.57; H, 4.43; N, 11.88; P, 12.90; H₂O, 9.11. Calculated for C₁₂H₁₆N₄O₇P₂S 2.5H₂O: C, 30.83; H, 4.53; N, 11.98; P, 13.25; H₂O, 9.64.

2. Paper Chromatography

A small amount of these compounds (0.005 ml × 1 mg/ml) spotted on a filter paper (Toyo Roshi No. 51A) was developed with a mixture of 1 M formate buffer (pH 5.0), isopropanol, and water (15:65:20) at 0° with the descending method. Under irradiation of ultraviolet light, Thc, Thc-MP, and Thc-DP gave single fluorescent spots at the *R_f* values of 0.37, 0.16 and 0.05, respectively, showing the purity of these compounds.

3. Infrared Absorption Spectra

The infrared absorption spectra of Thc, Thc-MP, and Thc-DP were recorded in Nujol mull on a Perkin-Elmer infrared spectrophotometer Model 21 and are shown in Fig. 2. The spectrum of Thc is in good agreement with that reported by Utsumi. Thc-MP shows strong peaks at 1,170, 1,060, 1,030, 970, and 920 cm⁻¹ and Thc-DP at 1,210, 1,110, 1,085, 1,060, 1,010, 980, and 925 cm⁻¹. These peaks can be attributed to the phosphate groups.

4. Ultraviolet Absorption Spectra

The ultraviolet absorption spectra of these compounds were recorded at various pH values with a Beckman automatic recording spectrophotometer Model DK-2. The sample solutions were prepared in 0.01 N hydrochloric acid (pH 2.1), McIlvain buffer (pH 4.7 and 7.1), Clark-Lubs buffer (pH 10.0) and 5% sodium hydroxide (pH 13.6). Fig. 3 indicates the spectra of these compounds. These compounds show nearly the same dependence of the spectrum upon pH. In the neutral and alkaline solutions, the absorption maxima are located at 370 mμ but by acidification the maxima are shifted to 390 mμ accompanied with the appearance of shoulders at about 330 mμ. This fact means that the light absorption takes place at the thiochrome ring of Thc-MP and Thc-DP and that the phosphorylation of the hydroxyl group scarcely affects electronic structure of the thiochrome group. This seems to be very reasonable because the phosphate group is at a distant part from the conjugated system of the molecules.

5. Fluorescence Spectra

The fluorescence and excitation spectra of these compounds were measured with a Shimadzu spectrophotometer model QB-50 equipped with a Shimadzu

FIG. 2 *Infrared Absorption Spectra of Thc Phosphates*

TABLE 1
Molecular Extinction Coefficients of Thc Phosphates at Various pH Levels

pH	Thc		Thc-MP		Thc-DP	
	$\lambda_{\max}$	ϵ	$\lambda_{\max}$	ϵ	$\lambda_{\max}$	ϵ
	$m\mu$		$m\mu$		$m\mu$	
2.1	390	16,000	388	14,300	383	12,000
4.4	390	17,400	389	17,100	390	16,200
7.1	367	20,600	367	20,900	367	20,300
10.0	367	21,200	367	21,700	367	21,300
13.6	367	21,300	367	21,800	367	20,900

FIG. 3 *Ultraviolet Absorption Spectra of Thc Phosphates at Various pH Levels*

---, pH 2; ---, pH 4; ---, pH 7; —, pH 10.

fluorometric accessory Model GF-16. The concentration of these compounds was $4.0 \times 10^{-5} M$ in the acid solutions and $2.0 \times 10^{-5} M$ in the alkaline solutions. The fluorescence spectra of these three compounds are nearly the same as expected from the similarity of their absorption spectra. In the acid medium, the fluorescence spectral maxima of these compounds are located at about $500 m\mu$ and the fluorescence intensity is low. In the alkaline medium, on the other hand, strong fluorescence was observed at $450 m\mu$. As seen from Fig. 4, the appearance of the fluorescence is scarcely affected by phosphorylation, whereas the quantum yield of the emission is appreciably affected.

The excitation spectra of these compounds dissolved in the alkaline medium (pH = 10) show their peaks at $370 m\mu$ where the absorption maxima are located. Therefore, the best condition for the fluorometric determination of Thc derivatives is exciting the sample solutions with ultraviolet ray of $370 m\mu$ and observing the fluorescence at $450 m\mu$.

The dependence of the fluorescence of these compounds upon pH are shown in Fig. 5, indicating that (a) these three compounds fluoresce weakly in the acid

FIG. 4 *Excitation and Fluorescence Spectra of Thc Phosphates*
●, Thc; △, Thc-MP; ×, Thc-DP.

FIG. 5 *Relation between the Fluorescence intensities of Thc Phosphates and pH*
●, Thc; △, Thc-MP; ×, Thc-DP.

but strong in the alkaline medium and (b) the fluorescence intensity of these compounds are constant over the range of pH 9 to 12 with the order of intensity, $\text{Thc} > \text{Thc-DP} > \text{Thc-MP}$, but (c) the intensity of Thc is markedly weakened over pH 13.6.

6. Calibration Curves

Fig. 6 shows the relationship between the fluorescence and the concentration of these compounds. The straight calibration curves (solid lines in Fig. 6) were obtained by carrying out the analysis of variance of experimental values with 3 levels and 5 replications of each sample. From this results it was concluded that the ratio of fluorescence intensity is 10:8:9 for Thc: Thc-MP: Thc-DP. It is of interest to note that monophosphate group quenches the fluorescence emission of the molecules but diphosphate group recovers it partly.

FIG. 6 Calibration Curves of Thc Phosphates and Thiamine Phosphates

7. Fluorescence Intensity of Thiochrome Phosphates Derived from Thiamine Phosphates

The dependence of fluorescence intensity upon the concentration was also studied for Thc, Thc-MP, and Thc-DP derived from thiamine, thiamine monophosphate (TMP), and thiamine diphosphate (TDP) respectively. Dilute solutions (2 to $8 \times 10^{-6} M$) of thiamine hydrochloride, TMP phosphate and TDP chloride were treated with cyanogen bromide and potassium ferricyanide according the procedure shown in Table 2. Measurements of fluorescence intensity were carried out directly on the reaction mixture (Fig. 6). When cyanogen bromide was used as an oxidizing agent, a reasonable result was obtained considering the reaction yields.

On the other hand, when potassium ferricyanide was used, the fluorescence intensities are about a half of pure Thc, Thc-MP and Thc-DP. In addition, the order of fluorescence intensity is different from that obtained in section 6. In this case, therefore, cyanogen bromide is preferable to potassium ferricyanide as an oxidizing agent.

TABLE 2
The Analytical Procedure of Thiamine Phosphates without Dephosphorylation

<i>Cyanogen bromide method</i>		<i>Potassium ferricyanide method</i>	
1. Sample solution (2 to $8 \times 10^{-6}M$, pH 4.5)	3.0 ml	1. Sampl solution (2 to $8 \times 10^{-6}M$, pH 4.5)	3.0 ml
2. Add BrCN solution Shake for 30 sec.	6.0 ml	2. Add $\left\{ \begin{array}{l} 1\% \text{ K}_3\text{Fe}(\text{CN})_6 \text{ 1 ml} \\ + \\ 15\% \text{ NaOH 29 ml} \end{array} \right\}$	4.0 ml
3. Add 15% NaOH Shake for 30 sec. and keep standing for 10 min.	4.0 ml	Shake for 30 sec. and keep standing for 10 min	
4. Add 10% H_3PO_4 (pH 10 to 11)	4.0 ml	3. Add 10% H_3PO_4 (pH 10 to 11)	4.5 ml
5. Measure the fluorescence intensity		4. Measure the fluorescence intensity	

The effect of potassium ferricyanide on the fluorescence intensity of thiochrome phosphates studied in order to understand the fact described above. Measurements was made of the pH 10 buffer solution containing $1 \times 10^{-6}M$ of thiochrome phosphates and potassium ferricyanide of the same concentrations with analytical procedure described in Table 2. As indicated in Table 3, the presence of potassium ferricyanide quenches the emission of thiochrome phosphates to a great extent. The shift of fluorescence maxima was also observed ($450 \rightarrow 460 m\mu$). The depression of fluorescence intensity and the wavelength shift of fluorescence maxima are due to a quenching effect of potassium ferricyanide showing a absorption maximum at near $420 m\mu$. The cause of the quenching effect is likely due to the absorption by potassium ferricyanide of a part of fluorescence of Thc derivatives.

TABLE 3
Quenching Effect of Potassium Ferricyanide on the Fluorescence Intensity of Thc Phosphates (concentration = $1 \times 10^{-6}M$)

Compound	Fluorescence intensity		Quenching rate
	pH 10 buffer solution	pH 10 buffer soln. containing $\text{K}_3\text{Fe}(\text{CN})_6$	
	<i>per cent</i>		<i>per cent</i>
Thc	36.2	16.7	53.6
Thc-Mp	28.0	14.0	50.0
Thc-DP	32.0	16.7	47.8

The fluorescence intensity of $1 \mu\text{g/ml}$ quinine sulfate solution was taken as 50 %.

SUMMARY

Thiochrome, its mono- and diphosphate were synthesized and their physicochemical properties such as paper chromatography, ultraviolet absorption, infrared absorption and fluorescence spectra were investigated. It was found that the ultraviolet

absorption and fluorescence maxima of these compounds were shifted to longer wavelengths with decreasing pH values. In alkaline solutions, the ultraviolet absorption and fluorescence maxima are located at 370 and 450 m μ , respectively. It is of interest to note that the phosphorylation induces no changes in the absorption and the appearance of emission spectra but induces an appreciable decrease in the fluorescence intensity. The ratio of the fluorescence intensity, thiochrome: thiochrome monophosphate: thiochrome diphosphate, is 10:8:9 in the alkaline solution (pH 9 to 12).

The concentration of thiamine, thiamine monophosphate, and thiamine diphosphate were determined fluorometrically without dephosphorylation. A reasonable results was obtained when cyanogen bromide was used as an oxidizing agent, whereas an unreasonable result was obtained when potassium ferricyanide was used because of its quenching effect.

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