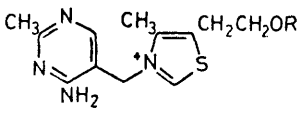
Luminescence Characteristics of Thiamine Derivatives

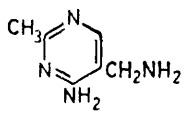
By **Ernest P. Gibson** and **James H. Turnbull,*** Chemistry Branch, Royal Military College of Science, Shrivenham, Swindon, Wiltshire SN6 8LA

A detailed study of the fluorescence and phosphorescence characteristics of thiamine (Vitamin B_1), thiamine pyrophosphate (cocarboxylase), and a number of related compounds has been undertaken. The attempt to enhance the phosphorescence of thiamine by several external heavy-atom species resulted in a new emission band, which has been assigned to a charge-transfer state.

In a previous paper [1] we described the detection of phosphorescence for the first time from thiamine (I) and thiamine pyrophosphate (III). We now present further spectroscopic data on a series of thiamine derivatives, in particular the monophosphate (II) and *O*-acetylthiamine (IV). The excited-state parameters of such systems are of interest in relation to photochemical studies [2] and also to investigate the effect of groups remote from a chromophore upon the photophysical characteristics of that chromophore.[3] In an attempt to enhance the phosphorescence of thiamine a variety of external heavy-atom perturbers were used. Thiochrome (IX) is of particular



(I) R = H
(II) R = PO_3H_2
(III) R = $P_2O_6H_3$
(IV) R = $COCH_3$



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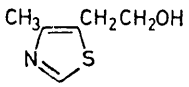

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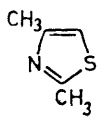

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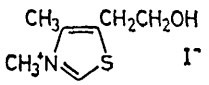

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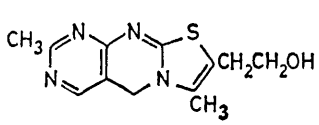

(IX)

interest as thiamine is routinely determined fluorometrically after oxidation to thiochrome and extraction into a suitable solvent, usually isobutyl alcohol.

Luminescence Characteristics.—Fluorescence spectra, quantum yields, and decay times were measured in 100% ethanol at 298 K, except where noted, and in clear ethanolic glasses at 77 K under anaerobic conditions. The spectral data are summarised in Tables 1 and 2. The luminescence of thiamine excited at 280 nm is shown in Figure 1.

No fluorescence was detected from any of the compounds studied except thiochrome and 4-amino-5-aminomethyl-2-methylpyrimidine (V) at 298 K. The fact that the pyrimidine emits at 298 K whereas thiamine does not, shows the importance of the thiazolium moiety in enhancing non-radiative decay processes.

The results shown in Table 1 indicate that the fluorescence of thiochrome is quenched by oxygen in eth-

TABLE 1

Fluorescence parameters of thiochrome and 4-amino-5-aminomethyl-2-methylpyrimidine at 298 K

	λ_{ex}/nm	λ_{em}/nm	ϕ_f	τ_f/ns
4-Amino-5-aminomethyl-2-methylpyrimidine	280	340	3.2×10^{-3}	0.6
Thiochrome	360	429	0.45	2.1
Thiochrome [a]	360	429	0.50	
Thiochrome [b]	360	429	0.72	2.9
Thiochrome [c]	360	422	0.61	4.8

[a] Degassed by bubbling white-spot nitrogen through the solution for 10 min. [b] Degassed by freeze–pump–thaw technique. [c] Non-degassed, solvent isobutyl alcohol.

anolic solution. In aqueous solution thiochrome fluorescence is quenched by chloride ions and the fluorescence intensity is a function of the pH of the solution. In routine assay work thiochrome is usually extracted into isobutyl alcohol in which chloride is insoluble. Therefore it is probable that while oxygen quenching of thiochrome fluorescence will occur in isobutyl alcohol,

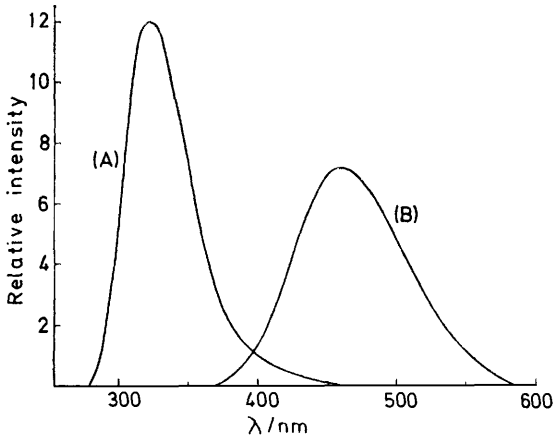

FIGURE 1 (A) Fluorescence and (B) phosphorescence (× 100) of thiamine in ethanol at 77 K, excited at 280 nm

impurity quenching will not. This point has not previously been made and may well contribute to the fluctuations in results between different laboratories.

The emission parameters of thiamine, thiamine mono-

phosphate, thiamine pyrophosphate, and *O*-acetylthiamine at 77 K are all similar. Significant differences are observed in the fluorescence and phosphorescence quantum yields and lifetimes. A notable feature of Table 2 is the observation of phosphorescence from thiamine derivatives for the first time. It had previously been reported that no phosphorescence was detectable,[4,5] and that the fluorescence emission was primarily due to the pyrimidine moiety energetically interacting with the thiazolium moiety. 2-(3,4-Dimethylthiazol-5-yl)ethanol iodide (VIII) and 4-amino-5-aminomethyl-2-methylpyrimidine (V) were used as models for the two halves of the thiamine molecule to determine which chromophore fluoresces and which phosphoresces. Comparing the fluorescence of thiamine with that of 4-amino-5-aminomethyl-2-methylpyrimidine between 350 and 550 nm with the maximum at 451 nm and a quantum yield of 5.5×10^{-3}; the phosphorescence was too weak to allow the lifetime to be measured using a manual shutter. Thiamine phosphorescence is a broad structureless band between 350 and 550 nm with a quantum yield of 9.4×10^{-3} and a lifetime of 2.4 s. Therefore it is concluded that the phosphorescence emission is from the thiazolium moiety after triplet–triplet energy transfer from the pyrimidine to the thiazolium, hence the correlation of the phosphorescence lifetimes of the pyrimidine moiety and the thiamine system. Further evidence concerning the nature of the emitting species might be obtained by increasing the separation of the two chromophores by increasing the length of the methylene bridge. Such compounds are not available commercially but their synthesis may be performed by a

TABLE 2

Luminescence parameters of thiamine and some derivative at 77 K in ethanol, excited at 270 nm

	Fluorescence			Phosphorescence		
Compound	$\lambda_{max.}$/nm	ϕ_f	τ_f/ns	λ_{max}/nm	ϕ_p	τ_p/s
Thiamine	325	3.0×10^{-1}	8.1	448	9.4×10^{-3}	2.4
Thiamine monophosphate	320	3.2×10^{-1}	8.1	390	2.4×10^{-2}	2.0
Thiamine pyrophosphate	320	2.7×10^{-1}	6.1	436	2.4×10^{-2}	2.2
O-Acetylthiamine	323	2.0×10^{-1}	4.5	455	5.4×10^{-3}	2.7
4-Amino-5-aminomethyl-2-methylpyrimidine	328	4.5×10^{-1}	9.2	391	2.6×10^{-1}	2.0
2,4-Dimethylthiazole	288	1.7×10^{-2}	1.1	448	8.7×10^{-3}	2.3
2-(4-Methylthiazol-5-yl)ethanol	285	3.3×10^{-2}	1.1	459	1.0×10^{-3}	0.23
2-(3,4-dimethylthiazol-5-yl)ethanol iodide	343	3.1×10^{-2}	5.1	451	5.5×10^{-3}	
Thiochrome *	416	7.2×10^{-1}	3.0	476, 496	3.0×10^{-2}	0.20

* Excitation wavelength 380 nm.

it is apparent that the wavelengths of the emission maxima are similar; however, the quantum yield and fluorescence lifetime of the pyrimidine moiety are greater than those of thiamine. A careful analysis of the fluorescence decay of thiamine failed to reveal a weaker, shorter-lived component corresponding to the fluorescence of the thiazolium moiety. Although the emission maximum of the thiazolium is at longer wavelength than that of the pyrimidine, it is not possible to determine accurately the onset of fluorescence, and thus it is not possible to exclude the possibility that the lowest energy singlet of the thiazolium might be at higher energy than that of the pyrimidine. Certainly singlet–singlet energy transfer from the thiazolium to the pyrimidine would account for the lack of the shorter-lived component in the fluorescence decay, and if energy transfer occurred from the pyrimidine to the thiazolium in thiamine then the emission from thiamine should correspond in wavelength more closely to that of the thiazolium. Therefore, it is proposed that in thiamine the fluorescence is due to the pyrimidine moiety, perturbed by the presence of the thiazolium ring which causes a quenching of the quantum yield and shortening of the fluorescence lifetime.

The phosphorescence emission from 4-amino-5-aminomethyl-2-methylpyrimidine (V) is a broad structureless band between 340 and 500 nm with the maximum at 391 nm, the phosphorescence lifetime is 2.0 s, and the quantum yield is 2.6×10^{-1}. 2-(3,4-Dimethylthiazol-5-yl)ethanol iodide (VIII) has a phosphorescence band

modification of the condensation method of synthesis of thiamine.[6]

A comparison of the luminescence characteristics of the thiamine derivatives for which data has been obtained (thiamine, thiamine monophosphate, thiamine pyrophosphate, and *O*-acetylthiamine) shows that whilst the fluorescence maxima are similar, the quantum yields and lifetimes are significantly different. The quenching effect of the thiazolium moiety upon the pyrimidine has already been discussed and it may be that the addition of one phosphate group causes the quenching process to be less efficient, resulting in an increase in the fluorescence quantum yield. However the addition of a pyrophosphate to thiamine quenches both the fluorescence quantum yield and lifetime, which indicates that the quenching process has been made more efficient. The increase in the phosphorescence quantum yields of thiamine monophosphate and thiamine pyrophosphate, and the decrease in the phosphorescence lifetimes, compared to thiamine, are indicative of increased spin–orbit coupling between T_1 and S_0, possibly caused by the phosphate groups, although no evidence for this sort of effect was forthcoming from previous work on adenosine phosphates.[3] *X*-Ray structural determination have been carried out which indicate that in thiamine pyrophosphate the pyrophosphate is folded back over the thiazolium ring.[7,8] This conclusion is supported by n.m.r. studies.[9,10]

The strong fluorescence emission (ϕ_f *ca.* 0.2) from

thiamine, thiamine monophosphate, thiamine pyrophosphate, *O*-acetylthiamine, and 4-amino-5-aminomethyl-2-methylpyrimidine is indicative that the emissive singlet state is (π,π^*), and the long phosphorescence lifetimes (>1 s) indicate that the radiative triplet state is also (π,π^*). The fluorescence emissions from 2,4-dimethylthiazole and 2-(4-methylthiazol-5-yl)ethanol at 77 K are weak (ϕ_f *ca.* 10^{-2}); in this case the emissive state might be $^1(n,\pi^*)$. Metzger *et al.*[11] calculated the energy of the lowest $^1(n,\pi^*)$ state of unsubstituted thiazole as *ca.* 4.5 eV corresponding to a transition at *ca.* 275 nm, compared with the emission maximum at 285 nm. The radiative lifetimes are 65 ns in the case of 2,4-dimethylthiazole and 33 ns in the case of 2-(4-methylthiazol-5-yl)-ethanol, which are somewhat short for $^1(n,\pi^*)$ state lifetimes. The triplet states for which calculations have been performed are (π,π^*) and the lowest is at *ca.* 3.2 eV and the second lowest at *ca.* 4.2 eV. This implies that the intersystem crossing from the singlet manifold to the triplet manifold will be facilitated, first by the fact that the $S_1 \rightarrow T_2$ energy difference is small and therefore the vibrational wave function overlap integral will be large, and secondly by the fact that the transition will be of $^1(n,\pi^*) \rightarrow ^3(\pi,\pi^*)$ nature, and thus the rate of intersystem crossing will be *ca.* 10^3 greater than the rate of intersystem crossing for a $^1(\pi,\pi^*) \rightarrow ^3(\pi,\pi^*)$ transition.[12]

Heavy-atom Perturbation.—In an attempt to enhance

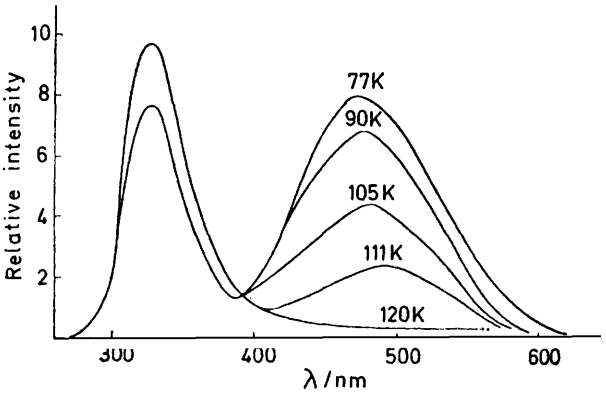

FIGURE 2 Luminescence spectra of thiamine and sodium iodide in ethanol excited at 280 nm at the indicated temperatures

the phosphorescence from thiamine, the external heavy-atom perturbation technique, using xenon and sodium iodide as perturbers, was utilised. Using xenon some difficulty was experienced in obtaining a stable glass, but some enhancement of the phosphorescence was observed. Using sodium iodide caused fluorescence quenching, the expected enhancement of phosphorescence, and the appearance of a new emission band with a maximum at *ca.* 470 nm. The same effects were also observed using sodium bromide and potassium thiocyanate, although to a lesser degree; sodium chloride and caesium fluoride did not cause similar effects. The luminescence spectra of thiamine and sodium iodide in exthanol excited at 280 nm at various temperatures are shown in Figure 2. It should be noted that as the temperature increases, the maximum of the long wavelength emission shifts to the red. Similar effects were observed with thiamine mono- and pyro-phosphate, *O*-acetylthiamine, and 2-(3,4-dimethylthiazol-5-yl)ethanol iodide with sodium iodide, but not with 4-amino-5-aminomethyl-2-methylpyrimidine, 2,4-dimethylthiazole, or 2-(4-methylthiazol-5-yl)-ethanol. The intensity of the long wavelength emission was insufficient in the cases of thiamine pyrophosphate and *O*-acetylthiamine with sodium iodide to allow measurement of the emission spectra as a function of temperature.

The results may be analysed in two ways; the first assumes that a ground state complex is formed between the quenching species, Q, and the molecule, M, under study [reaction (1)]. The second analysis assumes that

$$M + Q \longrightarrow MQ \overset{h\nu}{\rightleftharpoons} MQ^* \longrightarrow M + Q + h\nu' \quad (1)$$

the quencher forms an exciplex with an excited molecule, M* [reaction (2)]. In the first case it is necessary

$$M \xrightarrow{h\nu} M^* + Q \longrightarrow MQ^* \longrightarrow M + Q + h\nu' \quad (2)$$

to assume that the absorption spectrum of the ground state complex overlaps the absorption spectrum of the molecule, M. In the second case it is necessary to assume that diffusion can occur to allow the exciplex to form. In both cases Arrenhius-type relationships (3) and (4) [13] are obtained where I_E and I_M are the intensities

$$I_E \propto \exp(-E_F/RT) \quad (3)$$

$$I_E/I_M \propto \exp(-E_F/RT) \quad (4)\ [13]$$

of the emission from the complex and the molecule respectively, E_F is the energy of formation of the excited state complex, R is the gas constant, and T is the absolute temperature. Estimates of the energy of formation of the excited state complex have been made and are in Table 3. The appearance of the new band is suggestive

TABLE 3

Energy of formation of excited-state complexes of certain thiamine derivatives with sodium iodide

	Reaction (1)	Reaction (2)	
Thiamine	−33.5	−37.0	kJ mol^{-1}
Thiamine monophosphate	−21.3	−24.7	
2-(3,4-Dimethylthiazol-5-yl) ethanol iodide	−3.0	−3.9	

of exciplex formation, especially as thiocyanate ions are not regarded as heavy-atom perturbers. It has, however, been shown by many workers [14-18] that thiocyanate is an efficient quencher of fluorescence, through a postulated charge-transfer species. Selinger [19,20] has pointed out that the analysis of exciplex emission [reaction (2)] is the result of simplification by using limiting conditions and that many workers have assumed that these conditions were met when analysing their results, without ensuring that the limiting conditions had been met. Selinger [19] has shown that in order to determine the heat of formation of an exciplex (E_F) the complex must have a low value of E_F and the solvent must be of low viscosity; both these factors ensure that the

rate of exciplex dissociation, $MQ^* \longrightarrow M^* + Q$, is large. It is apparent that the values obtained from this model [reaction (2)] will be incorrect because the data were obtained in the temperature range 77—120 K where the viscosity of ethanol is of the order of 10^{14} centipoise.[21] Therefore it was decided to analyse the data on the basis that the long wavelength emission was from an excited-state complex [reaction (1)],* not an exciplex. Evidence for the excited state complex being responsible for the long wavelength emission is found in the shift of the emission maximum to red with increasing temperature, according to the prediction of Nagakura, whereas exciplex emission does not exhibit a temperature-dependent red shift.[22] The shift in the maximum of the long wavelength band is from 470 at 77 to 495 nm at 111 K, whereas the fluorescence emission maximum attributed to thiamine does not undergo a red shift as the temperature increases from 77 to 120 K. Additional evidence in favour of an excited state complex, rather than an exciplex, being responsible for the long wavelength emission, is found in the excitation spectra of the uncomplexed thiamine fluorescence ($\lambda_{max.}$ 283 nm) and the long wavelength emission ($\lambda_{max.}$ 289 nm). No differences were observed in the absorption spectrum of thiamine and sodium iodide at room temperature compared to the absorption spectrum of thiamine alone. Unfortunately experimental limitations prevented the study of the excited state complex emission from the thiamine–bromide ion and thiamine–thiocyanate ion systems.

The observation of excited state complex emission from the 2-(3,4-dimethylthiazol-5-yl)ethanol iodide–iodide ion system and not from the 4-amino-5-aminomethyl-2-methylpyrimidine–iodide ion system leads to the conclusion that the interaction in the thiamine–iodide ion system is between the thiazole ring with its formal positive charge and the anion. Therefore it is concluded that the long wavelength emission is due to a charge-transfer state. The tabulated heats of formation of the excited-state complexes are only estimates, as it was not possible to extend the temperature range beyond 120 K. Thus the values for thiamine and thiamine monophosphate are in reasonable agreement, both with themselves and with values from other systems, ranging from −5.7 to −42.6 kJ mol^{-1}.[13] The third system studied produced a value outside this range; this may be due to the inability to study a wide enough temperature range. (Initial estimates of the heat of formation of the excited-state complex in the thiamine–iodide ion system were −0.9 kJ mol^{-1} because the temperature range chosen was too small.)

EXPERIMENTAL

Thiamine hydrochloride, thiamine monophosphoric acid, thiamine pyrophosphate, and 2,4-dimethylthiazole were supplied by Sigma, *O*-acetylthiamine by ICN, 4-amino-5-aminomethyl-2-methylpyrimidine and 2-(4-methylthiazol-5-yl)ethanol by Aldrich, and thiochrome by Hoffman-La Roche. 2-(3,4-Dimethylthiazol-5-yl)ethanol iodide was prepared by reacting 2-(4-methylthiazol-5-yl)ethanol with methyl iodide. After repeated washing of the product with acetone the m.p. was 83.5—84.5 °C (Found: C, 29.35; H, 4.3; N, 4.85; I, 44.75; S, 11.25. Calc. for $C_7H_{12}INOS$: C, 29.45; H, 4.2; N, 4.9; I, 44.55; S, 11.25%). Ethanol used as a solvent was purified by distilling through a Widmer fractionating column at a reflux ratio of 20 : 1 (the first and last 20% of the charge were discarded).

The spectrofluorimeter used has been described previously.[23] The system utilises a 2-kW xenon-arc lamp (Mazda XE/D), two 500-mm grating monochromators (Bausch and Lomb), and an EMI 9558 QB photomultiplier tube in a housing cooled to 203 K. For ambient-temperature measurements samples were mounted in a thermostatically controlled cell block at 298 K. Low-temperature measurements were made at 77 K by use of a cylindrical quartz sample cell mounted in a quartz Dewar flask containing liquid nitrogen. At intermediate temperatures the cylindrical quartz sample cell was inserted into an Oxford Instruments Ltd. DN 704 liquid nitrogen cryostat with an Oxford Instruments Ltd. DTC 2 temperature programmer. A rotating-can phosphoroscope attachment inserted around the Dewar flask was used to eliminate fluorescence when phosphorescence emission was being studied. Phosphorescence lifetimes were determined by photographing the cathode ray oscilloscope trace of the phosphorescence decay at the wavelength of maximum intensity.

Fluorescence lifetime measurements were made using a time-correlated single photon counting system employing a thyratron-controlled gated lamp. The excitation pulse had a typical width at half-height of 4 ns and a repetition rate of 20—50 kHz. The method of calculating the decay time from the experimental data has been described previously.[24]

Quantum Yield Determinations.—Luminescence quantum yields were determined by the comparative method using tryptophan in water (ϕ_f 0.13 [25]) and quinine sulphate in 1N-H_2SO_4 (ϕ_f 0.56 [26]) as reference standards at 298 K and benzophenone (ϕ_p 0.74 [27]) and 9,10-diphenylanthracence (ϕ_f 1.0 [28]) as reference standards at 77 K. Additionally it was assumed that the change in optical density with temperature was not significant between different samples. The quantum yield (ϕ) is calculated from relationship (6), where I is the area under the corrected emission curve, A the absorbance at the exciting wavelength, θ the relative photon output of the excitation system at the exciting wavelength, and n the refractive index, the subscripts refer to the standard (st) and the unknown (x).

$$\phi_x = \phi_{st} \cdot \frac{I_x}{I_{st}} \cdot \frac{A_{st}}{A_x} \cdot \frac{\theta_{st}}{\theta_x} \cdot \frac{n^2_x}{n^2_{st}} \qquad (6)$$

Procedure.—For fluorescence and phosphorescence measurements 10^{-4} M solutions were used in order to eliminate distortion caused by inner filter effects. For low-temperature-measurements solutions in clear ethanolic glasses were used, which were degassed by the cyclic freeze–pump–thaw technique to minimise oxygen quenching and to reduce the frequency of cracking of the glass. The spectra were corrected for the spectral response of the emission monochromotor and photomultiplier by the method of Melhuish.[29]

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* Various terms have been used to describe complexes in excited states, in this instance the term 'excited-state complex' is used to describe the product of the process (5).

$$M + Q \longrightarrow MQ \xrightarrow{h\nu} MQ^* \qquad (5)$$

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