Studies on Room Temperature Phosphorometric and Delayed Fluorometric Analysis. I. Delayed Fluorometric Analysis of Porphyrin Derivatives

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The spectral characteristics and its application to measurement of porphyrins were investigated by use of room temperature phosphorometry. Certain porphyrin compounds adsorbed on filter paper exhibit intense E-type delayed fluorescence. This could be utilized for the determination of porphyrin derivatives.

Papar and thin layer chromatography have been used for the separation and measurement of porphyrins present in biological and geological materials.[1-5] After chromatography, the porphyrin spots can be easily located on the layer by observing the orange—red fluorescence in ultraviolet radiation. However, quantitative detection of prompt fluorescence of the compounds adsorbed on the layer is tedious, because of the reflection of excitation light and the fluorescence from the support surfaces. This paper describes a new approach to the application of phosphorometric time-resolution of room temperature phosphorometry (RTP).[6,7]

Phosphorometry was usually carried out at 77 K in liquid nitrogen, a low temperature being necessary for maintaining a rigid matrix in order to minimize the effect of intermolecular collisional quenching. Walling and Schulman[8] observed the phosphorescence of various organic compounds adsorbed on filter paper, silica gel, alumina, and polycellulose at room temperature. This method (RTP) has advantages for analysis. No liquid nitrogen, cryogenic equipment, or high purity solvents are required, substances being identified in chromatographic separation without elution. In this article, application of RTP combined with paper chromatography to the analysis of porphyrins is described.

Experimental

Apparatus. Long-life emission was measured with a Hitachi-2A spectrofluorometer with a phosphorescence accessory. The sample holder was designed to fit into the standard MPF-2A phosphoroscope accessory instead of the normal cap and cylinder used for the dewar flask assembly (Fig. 1). The plate of holder was blackened to a dull, non-reflecting finish. A 150-W xenon arc lamp was used as the excitation light source, the signal being detected with a R-446 UR photomultiplier tube. The spectra were recorded on a Hitachi QPD 33 recorder.

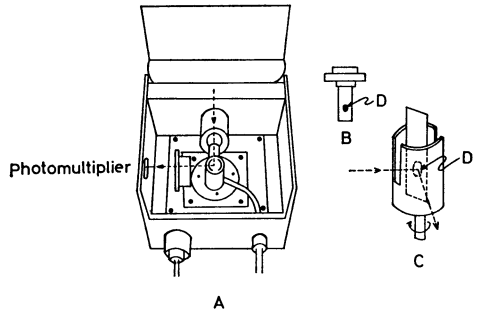

Fig. 1. Schematic diagram of filter paper cell system for room temperature phosphorescence studies.
A: Hitachi MPF-2A phosphoroscope accessory, B: sample holder, C: rotating can assembly, D: sample spot.

Reagents. Magnesium-protoporphyrin IX dimethyl ester (Mg-PPDE) was prepared by refluxing protoporphyrin IX dimethyl ester (Midori jūji Co. Ltd.) and anhydrous magnesium chloride in *N,N*-dimethylformamide.[9] Crude metal chelate was chromatographed on a cellulose column with a light-petroleum : acetone (20 : 3, v/v) eluant. Pure Mg chelate was obtained by recrystallization three times from benzene–chloroform (20 : 1, v/v) solvent. Chlorophyll a and b were extracted from spinach with a mixed solvent, methanol : acetone (1 : 1, v/v). After partial purification by the dioxane method,[10] crude pigments were chromatographed on a glucose column in order to separate chlorophyll a, b, and the other pigments. Chlorophyll c_1 and c_2 were isolated from *Undaria pinnatifida* and *Sargassum racemosum*, respectively, with methanol and acetone according to the Jeffrey method.[11] After being chromatographed, crude chlorophyll c_1 and c_2 were crystallized from pyridine-acetone. All other chemicals were of reagent grade. Toyo No. 51 filter paper was used as a support.

Procedure. The sample solution, four 0.5 μl portions, was allowed to drain slowly from the tip of a microliter syringe when it came into contact with the filter paper. After 1.0 μl of 1 mol dm^{-3} NaOH aqueous solution had been placed on a sample spot, the filter paper was dried for ca. 30 min with an infrared lamp. The samples were then directly mounted on a holder shown in Fig. 1. Since the emission is very sensitive to humidity and temperature, the sample compartment was continuously flushed with warm dried air.

Results and Discussion

As shown in Table 1, porphyrins exhibited a long-life emission (1—100 ms), but no long-life emission was observed for non-ionic porphyrin, Cu-porphyrin chelate and chlorins. The emission was studied for Mg-PPDE.

Spectral Studies of Mg-protoporphyrin IX Dimethyl Ester (Mg-PPDE). The emission spectra of Mg-PPDE adsorbed on the paper are shown in Fig. 2. The spectrum of Mg-PPDE obtained by RTP at 70 °C is almost the same as the usual prompt fluorescence spectra observed in chloroform solution and on filter paper. Phosphorescence of Mg-PPDE in solid solution at liquid nitrogen temperature (77 K) appears in the far red region as compared with the spectrum obtained by RTP. Very weak room tem-

Table 1. Properties of long-life emission from porphyrins adsorbed on filter paper[a)]

Compound	λ excitation nm	λ emission nm
Protoporphyrin IX	415	630
Protoporphyrin IX dimethyl ester	415	630
Mg-protoporphyrin IX dimethyl ester	428	600
Zn-protoporphyrin IX dimethyl ester	420	587
Cu-protoporphyrin IX dimethyl ester	—	—
meso-Tetraphenyl porphinetrisulfonate	465	654
meso-Tetrakis(4-methyl-1-pyridyl)porphyrin	427	650
meso-Tetraphenylporphyrin	—	—
Chlorophyll c_1	430	605
Chlorophyll c_2	430	605
Chlorophyll a	—	—
Chlorophyll b	—	—

a) Measured with phosphoroscope at *ca.* 1000 min^{-1} of rotating can assembly.

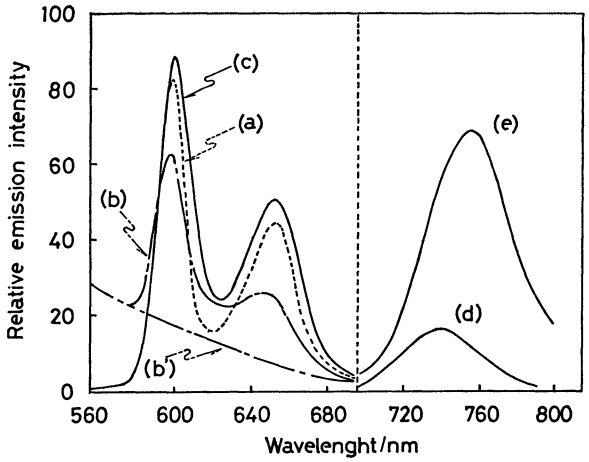

Fig. 2. Spectra of long-life emission from Mg-PPDE adsorbed on filter paper.
(a): Prompt fluorescence; 12 °C in chloroform, (b): prompt fluorescence; 12 °C adsorbed on filter paper, (b'): blank; prompt fluorescence from filter paper at 12 °C, (c): long-life emission; 70 °C adsorbed on filter paper, (d): long-life emission; 20 °C adsorbed on filter paper, (e): phosphorescence; −196 °C in methanol. Intensity scale for (d), (e) is *ca.* 100 times less than for (a)—(c).

perature phosphorescence of Mg-PPDE was also observed when a small amount of sodium iodide was present on the filter paper. The effect of temperature on the intensity of long-life emission is conspicuous. The emission intensity of Mg-PPDE increases with increase in temperature, in contrast to the case of normal prompt fluorescence and phosphorescence (Fig. 3).

Logarithm of the intensity of long-life emission, ln ϕ_e, obtained from the spectra is plotted against $1/T$

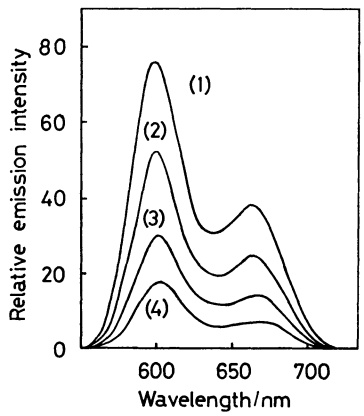

Fig. 3. Effect of temperature on long-life emission of Mg-PPDE (9.6 ng) adsorbed on filter paper.
(1): 72±1 °C, (2): 60±1 °C, (3): 55±2 °C, (4): 40±2 °C.

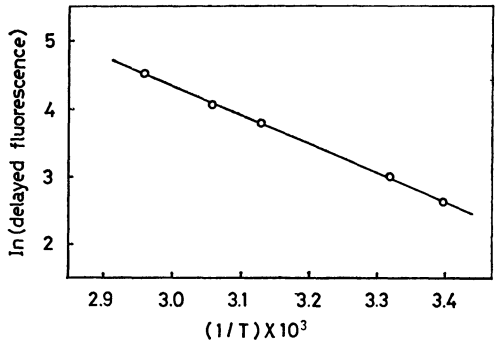

Fig. 4. E-type delayed fluorescence of Mg-PPDE (0.18 μg) adsorbed on filter paper as a function of temperature.

(Fig. 4). The plot is linear. The activation energy calculated from the slope is 0.37 eV, agreeing with the energy difference (0.38 eV) between the maxima of the two emission bands: 600 nm for specific emission, 736 nm for phosphorescence. In room temperature phosphorometry, in most cases, sodium iodide is efficient for increasing the phosphrescence quantum yield.[6)] In this study, however, the intensity of long-life emission decreased drastically in the presence of environmental sodium iodide. The long-life emission of Mg-PPDE could be attributed to E-type delayed fluorescence resulting from the thermal activation of molecules from the lowest triplet state to the first singlet state followed by radiative transition to the ground state.

The relationship between the intensity of E-type delayed fluorescence and the concentration of Mg-PPDE is linear over a wide range of ng—μg. The analytical curve indicates that E-type delayed fluorescence could be applied not only to the identification of porphyrins but also to their determination.

Application of E-type Delayed Fluorescence to Analysis of Chlorophyll c from Algae. Pigments of algae were extracted by freezing the algae fronds at −20 °C for 30 min, immersing the frozen tissue in methanol for 30 s and homogenizing in 100% acetone. Extraction was repeated several times until the cell res-

idue became colourless. The acetone and methanol extracts were separated from the tissue residues by centrifugation, and immediately mixed with diethyl ether. The mixture was shaken with 10% sodium chloride solution to wash out impurities and concentrate the pigments in the ether phase. The ether layer was made up to the designed volume. Two μl of the sample solution was spotted on Toyo No. 51 filter paper with a microsyringe. In applying the sample to the filter paper, care was taken to maintain the spot size (5 mm diameter) as constant as possible, the best reproducibility being attained when spot size was kept constant. The tip of the syringe just touched the filter paper, followed by spotting of four 0.5 μl portions. Each spotting was made at intervals to allow evaporation of the solvent. Chromatography was carried out with light petroleum ether:acetone:1-propanol (90:10:0.4, v/v). After the solvent front had ascended 10 cm, the spots were examined with a ultraviolet mineral light for orange—red fluorescence. Chlorophyll c_1 and c_2 and chlorophyllide having carboxyl side chain did not move from the origin and the spot size of chlorophyll c remained constant, the other pigments being developed. Existence of chlorophyllide, however, gave no influence on emission intensity of chlorophyll c, even when its amount was the same as that of chlorophyll c. After development, 1.0 μl of 1 mol dm^{-3} NaOH aqueous solution was added on the spot of chlorophyll c, and dried for *ca.* 10 min by infrared lamp heating, the emission intensity remaining constant for at least 20 min. The paper strip was mounted on the sample holder, and E-type delayed fluorescence of chlorophyll c was measured by use of rotating can phosphorescence assembly (Ex: 430 nm, Em:605 nm). Chlorophyll c in the concentration range 0.1 ng—μg order could be determined.

Conclusion

E-type delayed fluorescence of porphyrins observed by room temperature phosphorometry can be used for the determination of ng order of porphyrins, and it may be applied to the analysis of porphyrins in biological or geological samples, in combination with paper or thin-layer chromatographic technique.

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