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Time-Resolved Fluorescence Studies of the Wings of *Morpho sulkowskyi* and *Papilio xuthus* Butterflies

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ABSTRACT—Fluorescence from the wings of male *Morpho sulkowskyi* and *Papilio xuthus* butterflies has been investigated for the first time using time-resolved fluorescence spectroscopy. Intensity of emission spectra from the wings of *Papilio xuthus* decreased with increasing delay time after the application of exciting laser pulse and the intensity peak showed an interesting red shift from 480 to 520 nm. In contrast, such a peak shift was not observed from the wings of *Morpho sulkowskyi*. The decay times obtained from the wings of *Morpho sulkowskyi* and *Papilio xuthus* were given by two components, respectively; those of the former at 510 nm were about 0.545 ns and 2.74 ns and those of the latter at 480 nm were about 0.490 ns and 2.10 ns. Based on these results, the possibility of using time-resolved spectroscopy as a tool for the classification of butterflies is described along with a brief discussion of the origin of the 520 nm peak observed for *Papilio xuthus*.

INTRODUCTION

The observation that butterfly wings fluoresce when irradiated with ultraviolet (UV) light was first reported by Cockayne (1924). His extensive study of butterflies revealed that the wings of many Papilionid and Nymphalid species emit fluorescence under UV light, suggesting that an examination of the fluorescent wing pigments might be useful for the classification of butterflies. In the family Papilionidae, bright fluorescence is seen in the pale yellow parts of the wings of *Papilio xuthus*, *P. helenus*, *P. protenor* and others. Among the family Nymphalidae, bright fluorescence is seen in the wings of *Euripus*, *Parhestina*, *Myner* and other groups.

The wings of pierid butterflies, on the other hand, do not emit such bright fluorescence under UV light, even though their major wing pigments belong to fluorescent pteridine compounds. Based on results obtained with a micro-spectrophotometer (MSP), Shono (1983) reported that the wings of *Pieris rapae*, *Pieris melete* and *Papilio xuthus* fluoresce under UV light. However, it is noteworthy that fluorescence from pierid wings is less than that from *P. xuthus* by a factor of several hundreds.

Ford (1941, 1944) investigated the presence or absence of anthoxanthin in the wings of numerous butterfly species. Although the wings of many *Graphium* species generally contain anthoxanthin, some *Graphium* do not have such anthoxanthin pigments. Interestingly, these latter species have pale yellow parts and fluoresce under UV light. Cockayne included these *Graphium* species among *Papilio*. Ford (1947) also examined the presence or absence of pteridines in the wings using a modified murexide test, and reported that accumulation of pteridine pigments in the wings is a characteristic of the Pieridae.

Morpho sulkowskyi investigated in the present study belongs to the family Nymphalidae (Ackery, 1984). Several researchers have reported the presence of nonfluorescent pigments in the wings of *Morpho* species such as *Morpho cypris* and *Morpho menelaus* (Anderson and Richards, 1942; Hirata and Ohsako, 1966), but the occurrence of fluorescence from the wings of *Morpho* species had never been reported until our recent work (Kumazawa *et al.*, 1994, 1996; Tabata *et al.*, 1996). We reported that the blue region of the wings of male *Morpho sulkowskyi* was fluorescent when irradiated with UV or visible light, though the black region was nonfluorescent, and that there was a difference in the steady-state fluorescence spectra from the upper (dorsal) and lower (ventral) surfaces of the scales (Kumazawa *et al.*, 1994). In addition to an examination of the fluorescence spectra, compounds responsible for such spectra were identified on the basis of high performance liquid chromatographic (HPLC), high performance thin layer chromatographic (HPTLC) and ultra-violet (UV) spectrophotometric analyses. Pteridine compounds such as biopterin, pterin and isoxanthopterin were found for the first time in the wings (Tabata *et al.*, 1996).

The purpose of the present study was to obtain detailed information on the fluorescence from the wings of *Morpho sulkowskyi* showing typical structural colors and *Papilio xuthus* showing pigmentary colors. Using time-resolved spectroscopy, we first investigated the time-resolved emission spectra from the wings of both butterflies. The emission spectra from the wings of *Papilio xuthus* displayed a red shift from around 480 nm to 520 nm and the emission intensity decreased with

increasing delay time after exposure to an excitation laser pulse. Such a peak shift was not observed in the emission spectra of *Morpho sulkowskyi*.

In addition to these observation, the decay times from the wings of both butterflies were also measured. The measured decay times for *Morpho sulkowskyi* and *Papilio xuthus* were given by two components, respectively; those of the former at 510 nm were about 0.545 ns and 2.74 ns and those of the latter at 480 nm were about 0.490 ns and 2.10 ns. These results suggest that decay time measurement by time-resolved spectroscopy may be a useful method for the classification of butterflies, since decay times are thought to characterize the fluorescent pigments and/or compounds in the wings.

MATERIALS AND METHODS

Materials and preparations

Male *Morpho sulkowskyi* and *Papilio xuthus* butterflies were purchased from Okura Biological Lab., Tokyo, Japan. Fluorescence has been observed only from the blue color region of the wings of *Morpho sulkowskyi* and from the yellow color region of the wings of *Papilio xuthus* as described in our previous paper (Kumazawa *et al.*, 1994). Therefore, this investigation focused on fluorescence from these regions of the wings. This was done by removing the wings from the body with a cutter and mounting them on nonfluorescence quartz-glass (Matsunami Glass Co., Tokyo, Japan) for analysis.

Apparatus

Time-resolved emission spectra were obtained using an optical multichannel analyzer (OMA, Otsuka Electronics Co., Ltd., Tokyo, Japan) with the gate width of the image intensifier set at 10 ns. The monochromator used in the measurement covered a wavelength range from 310 to 590 nm. A 352-nm excimer laser was employed as an excitation source, and its pulsed beam was introduced into a quartz fiber. This fiber was used for both excitation and fluorescence detection.

Fluorescence decays were obtained with a picosecond fluorescence measurement system (Hamamatsu Photonics Co., Ltd., Shizuoka, Japan), consisting mainly of a monochromator (C5094), a streakscope (C4334-1), and a trigger unit (C4792). The excitation source was a N_2-gas laser (LN120), which produced a 300 ps, 337.1 nm excitation pulse, with a 20 Hz repetition rate. To avoid photodegradation of the butterfly wings, the beams from the excimer and N_2 lasers were attenuated using neutral density (ND) filters. Deconvolution of the measured fluorescence decay curves was accomplished with commercial software (U4790). The diameter of the irradiated area on the wings was adjusted to approximately 1 mm. All measurements were carried out at room temperature.

RESULTS AND DISCUSSION

Time-resolved emission spectra

Figure 1 shows the time-resolved emission spectra obtained from the upper surface (blue color region) of the wings of *M. sulkowskyi* when exposed to excitation at 352 nm. These time-resolved emission spectra were obtained by OMA measurements at different delay times with a 10 ns gate width.

After application of a 10-ns excitation pulse, the emission spectra broadened and the fluorescence intensity peak was located at around 510 nm (spectrum 1). The 510 nm peak showed a slight red shift at longer delay times than 20 ns. In addition to these features, the fluorescence intensity decreased with an increase in delay time and almost disappeared at 50 ns (spectrum 5). This behavior after irradiation at 352 nm is probably due to photobleaching. The shape and peak position of emission spectra after exposure to a 10-ns excitation were very similar to those of the static fluorescence spectra measured previously with a microspectrophotometer (MSP) (Kumazawa *et al.*, 1994). As reported previously, the 510 nm peak is thought to be mainly due to pteridine compounds such as biopterin and pterin (Kumazawa *et al.*, 1994; Tabata *et al.*, 1996).

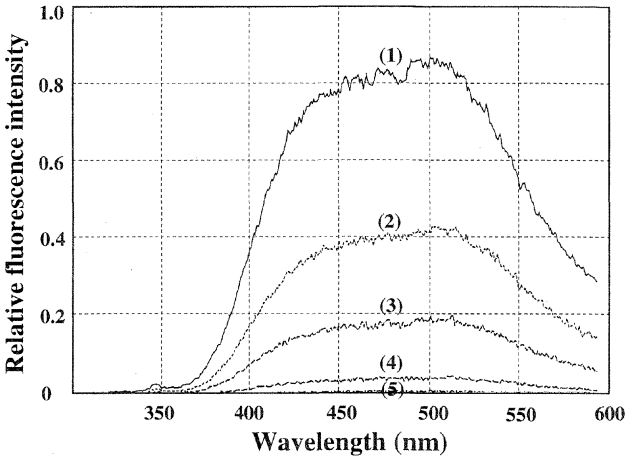

Fig. 1. Time-resolved emission spectra obtained by OMA measurements from the wings of *M. sulkowskyi* at a gate width of 10 ns. Excitation wavelength was 352 nm. Delay times after application of an excitation laser pulse: (1) 10 ns; (2) 20 ns; (3) 30 ns; (4) 40 ns; (5) 50 ns.

The time-resolved emission spectra from the upper surface (pale yellow color region) of the wings of *P. xuthus* are shown in Fig. 2. After application of a 10-ns excitation pulse the emission spectra rose more sharply than those of *M. sulkowskyi* and the fluorescence intensity peaked at around 480 nm (spectrum 1). This behavior was also similar to that observed in our previous study (Kumazawa *et al.*, 1994) and in Shono's report (1983).

Umebachi (1975, 1985, 1988) investigated the chemical and physical properties of the pigments in the wings of *P. xuthus* in detail and reported that one of them consisted of kynurenine and N-β-alanyldopamine. He named this pigment Papiliochrome II; it emits greenish-yellow fluorescence. From these results, the fluorescence peak at around 480 nm was thought to be caused by the Papiliochrome II pigment.

The 480 nm peak displayed a red shift to 520 nm at longer delay times than 20 ns and the intensity decreased noticeably with an increase in delay time. Similar to the results seen for *M. sulkowskyi*, the fluorescence intensity had nearly disappeared at 50 ns (spectrum 5). These results suggest that, in addition to a photobleaching process, a new fluorescent band (photoproduct) was also formed at around 520 nm during irradiation at 352 nm.

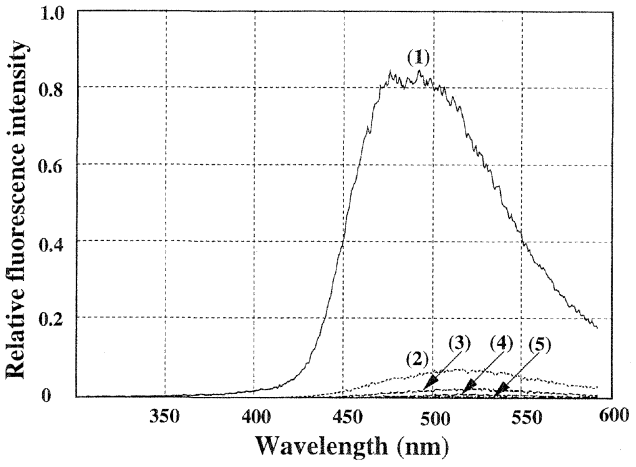

Fig. 2. Time-resolved emission spectra obtained by OMA measurements from the wings of *P. xuthus* at a gate width of 10 ns. Excitation wavelength was 352 nm. Delay times after application of an excitation laser pulse: (1) 10 ns; (2) 20 ns; (3) 30 ns; (4) 40 ns; (5) 50 ns.

Fluorescence decay curves

Time-resolved fluorescence decay measurements can provide detailed information concerning the origin of complex fluorescence decay in compounds (Beehem and Brand, 1985; Holzwarth *et al.*, 1985; Schneckenburger *et al.*, 1988). To obtain information on the process of photoproduct formation, therefore, we investigated the decay curves of the fluorescence wavelength, focusing in particular on the peak wavelength. The fluorescence decay function was assumed to be the sum of exponentials written as

$$\Sigma A_i \exp\left(-\frac{t}{\tau_i}\right)$$

where A_i is the preexponential factor (amplitude) of the *ith* component and τ_i is the decay time (lifetime) of *ith* component (Holzwarth *et al.*, 1985; König *et al.*, 1990; Menna *et al.*, 1993).

Figure 3 shows the fluorescence decay curves from the wings of *M. sulkowskyi* monitored at two fluorescence wavelengths (510 and 560 nm) together with the excitation laser pulse profiles. The residual (Ri) plots are also shown at the top of the figure. These residual plots indicate whether a fit with the sum of some exponentials is sufficient or not (Holzwarth *et al.*, 1985). It can be seen that the respective residual plots are well distributed around zero, indicating that the fit with the two exponentials was adequate. The decay times and the amplitudes of the two components at both wavelengths are shown in Table 1. Deconvolution of the decay curves yielded two decay times of about 0.545 ns (first component: τ_1) and 2.74 ns (second component: τ_2) at 510 nm (curve a), and about 0.541 ns (τ_1) and 3.06 ns (τ_2) at 560 nm (curve b). In addition, the contribution of the amplitudes of the first and second components at 510 nm was approximately 84.7% (A_1) and 15.3% (A_2), respectively. On the other hand, that of the amplitudes of the first and second components at 560 nm was approximately 84.8% (A_1) and 15.2% (A_2), respectively.

Wang *et al.* (1990) reported that the fluorescence decay time (lifetime) provides another diagnostic tool since fluorescence materials which have similar spectral features often have different decay times, while the fluorescence spectrum does not always allow unambiguous identification. In line with their observation the decay times and amplitudes of the components, in addition to the fluorescence peak, may characterize some fluorescent pigments and/or compounds

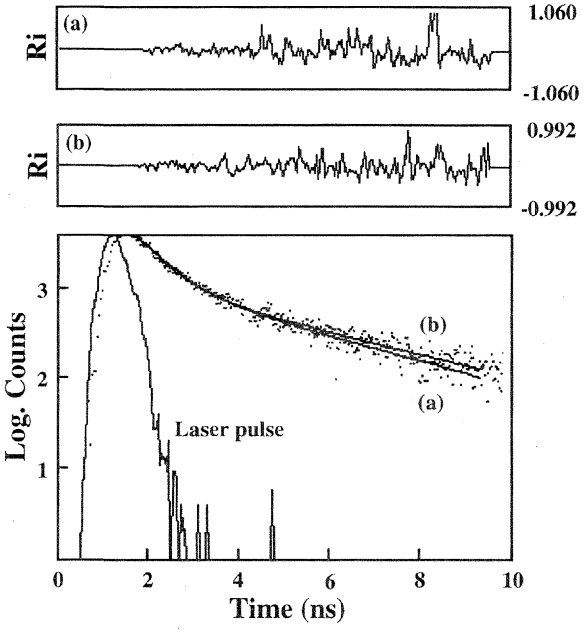

Fig. 3. Fluorescence decay curves from the wings of *M. sulkowskyi* monitored at 510 nm (curve a) and at 560 nm (curve b). Excitation wavelength was 337.1 nm. The upper frames show the residual (Ri) plots.

Table 1. Decay times and relative preexponential factors (amplitudes) in the fluorescence decay components at two wavelengths

Sample	Wavelength (nm)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)
M. sulkowskyi	510	0.545	84.7	2.74	15.3
	560	0.541	84.8	3.06	15.2
P. xuthus	480	0.490	93.7	2.10	6.3
	520	0.565	87.2	2.50	12.8

τ_i and A_i indicate the fluorescence decay times and the associated preexponential factors, respectively.

included in the blue region of the wings of *M. sulkowskyi*. Moreover, the results indicate that the decay times and amplitudes of the components at both wavelengths were essentially the same, because they remained virtually unchanged at 510 and 560 nm. In the case of the wings of *M. sulkowskyi*, therefore, it can be concluded that photoproducts did not form as a result of radiation-induced fluorescence under the conditions used in this study.

Fluorescence decay curves from the wings of *P. xuthus* at two fluorescence wavelengths are shown in Fig. 4. The faster relaxation at about 480 nm (curve a) in comparison with the fluorescence maximum at about 520 nm should be noted. Similar to the results seen for *M. sulkowskyi*, deconvolution of the decay curves yielded two decay times. The results are also summarized in Table 1. The decay times of the first and second components at 480 nm were about 0.490 ns (τ_1) and 2.10 (τ_2) ns, respectively, and about 0.565 ns (τ_1) and 2.50 ns (τ_2) at 520 nm, respectively. The contribution of the amplitudes of the first and second components at 480 nm was approximately 93.7% (A_1) and 6.3% (A_2), respectively. That of the amplitudes of the first and second components at 520 nm was approximately 87.2% (A_1) and 12.8% (A_2), respectively. In the case of the wings of *P. xuthus*, therefore, the decay times of the first and second components at 520 nm were greater than those at 480 nm. Moreover, the contribution of the amplitude of the second component was twice as large as at 480 nm. Judging from these results, the Papiliochrome II pigment, which is thought to be present in the pale yellow region of the wings of *P. xuthus*, may be characterized by such data as the decay times and amplitudes of the components.

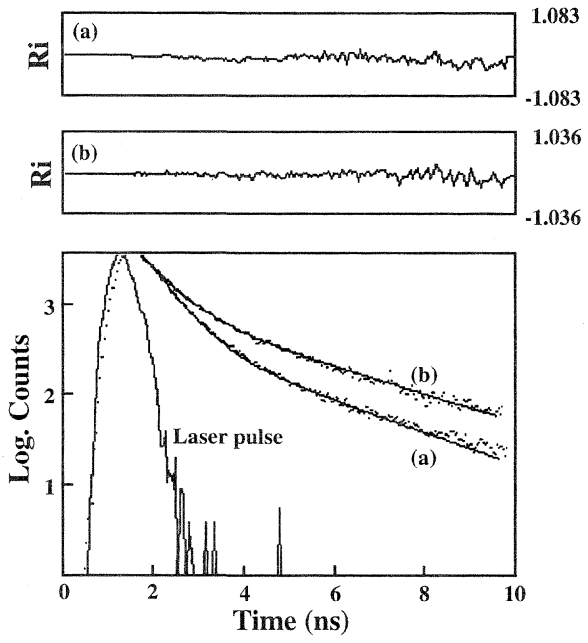

Fig. 4. Fluorescence decay curves from the wings of *P. xuthus* monitored at 480 nm (curve a) and at 520 nm (curve b). Excitation wavelength was 337.1 nm. The upper frames show the residual (Ri) plots.

According to Umebachi (1985, 1988), the Papiliochrome II pigment is also present in the pale yellow region of the wings of male *P. demoleus*, *P. protenor* and *P. dardanus* in the family Papilionidae. Therefore, similar decay times and behavior for the Papiliochrome II pigment might also be observed for species other than *P. xuthus*, though this has yet to be examined. The results discussed here are thus interesting from the biochemical and taxonomical points of view.

Various methods such as morphological, chemical, and genetic analyses have been employed to conduct taxonomic studies of the butterflies. These methods, however, in many cases involve complicated experimental procedures. The measurement of fluorescence decay times using time-resolved spectroscopy, on the other hand, can be performed rather easily and nondestructively, and yields information about fluorescent pigments and/or compounds of various parts of butterflies. A comparison of the decay times of the two butterfly species used in this study indicates that decay time measurement by this method may be a useful tool for the classification of butterflies, although much more data on the other butterfles must be collected.

Moreover, interestingly, these results also suggest that the 520 nm peak seen in the time-resolved spectra of *P. xuthus* may have been due to the formation of new photoproducts as a consequence of radiation-induced fluorescence. What is the new fluorescent band (photoproducts) observed at 520 nm in the time-resolved spectra? Although it is difficult to answer this question at the present time, our tentative explanation is as follows. It is generally known that oxygen plays a major role in the photochemical reactions involved in the bleaching of fluorescence and the formation of photoproducts during exposure to light (Kinoshita *et al.*, 1988; König *et al.*, 1990, 1992). Although there is no direct proof at this time, the broad peak (photoproducts) observed at 520 nm in the time-resolved spectra may have been caused by the presence of singlet oxygen. Similar to the case for hematoporphyrin derivative (HpD) in living cells (Kinoshita *et al.*, 1988; König *et al.*, 1990), such photoproducts may be due to the production of singlet oxygen by energy transfer from the triplet excited state of the Papiliochrome II molecule. Since the kinetics of photoproduct formation associated with the 520 nm peak are complex, including the protein-bound Papiliochrome II molecule, their origin is not yet known. More work is needed to clarify the origin of such photoproducts fully.

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