Effect of Excitation and Emission Wavelength on the Fluorescence Lifetimes of Chlorophyll *a*

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Abstract. The effect of excitation and emission wavelengths on fluorescence decay times of Chlorophyll *a* were measured in four solutions ($C \sim 10^{-5}$ mol . l^{-1}) and in polymethyl methacrylate films. The decay times observed were corrected with respect to self-absorption and re-emission effects. The fluorescence decay times of Chlorophyll *a* was found to depend on both the excitation and the emission wavelengths.

Key words: Chlorophyll *a* — Fluorescence — Lifetime

Introduction

In spite of an intensive study of photophysical properties of chlorophyll *a* (Chl *a*) and its derivatives over the last 20 years there several problems have still remained unresolved needing further investigation. No systematic study has been undertaken to explain e.g. the great discrepancies in published values of fluorescence lifetimes of Chl *a* in solutions, which range from 4.9 ns (Singhal and Rabinowitch 1962) to as high as 8.1 ns (Yuen et al. 1980). In a few cases only effects of experimental measurement conditions such as geometrical arrangement, pigment concentration or quenching by dissolved oxygen were taken into account. Some recent studies of fluorescence lifetimes of chlorophyll-like pigments (Avarmaa et al. 1977; Avarmaa and Tamkivi 1978; Yuen et al. 1980; Kaplanová and Parma 1981) have proved a clear depondence of experimental values of Chl *a* fluorescence, τ_f, on both the excitation and emission wavelengths.

Explanation and understanding of such observed the above spectral dependences of τ_f may be explained and understood only if corrections of experimental data with respect to secondary processes, i.e. reabsorption and reemission of fluorescence, are made.

A systematic study of the effect of spectral conditions on fluorescence decay times, τ_f, of Chl *a* in four solutions with different solvation properties has been

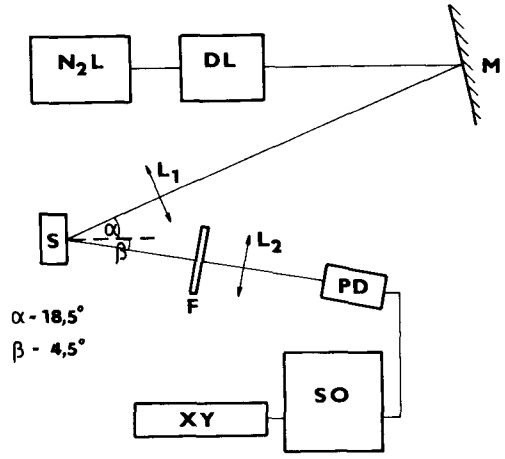

Fig. 1. Scheme of the optical and electrical set-up (N_2L — nitrogen laser, DL — dye-laser, M — mirror, S — sample, F — optical filter, L_1L_2 — lenses, PD — PIN diole, SO — Sampling Oscilloscope, XY — recorder).

undertaken. The concentrations of Chl *a* in solutions ranged from 2×10^{-5} mol . l^{-1} to 8×10^{-5} mol . l^{-1} and that in polymer matrix from 4×10^{-5} mol . l^{-1} to 8×10^{-4} mol . l^{-1}.

Materials and Methods

Solutions of chromatographically pure Chl *a* (Skorkovská and Vavřinec 1973) in acetone, dioxane, n-hexane, and methanol were prepared under dim light and nitrogen to minimize Chl *a* photooxidation during the preparation. Solvents (p.a.) were used without further purification and deoxygenation. The concentrations of Chl *a* in solutions were determined from absorption spectra using their molar extinction coefficients (Seely and Jensen 1965). The measurements were carried out in sealed 2 mm and 5 mm quartz cuvettes. Thin polymethyl methacrylate films (PMMA) containing Chl *a* ($d \sim$ 0.1—0.3 mm) were prepared from mixtures of Chl *a* solution and PMMA dissolved in trichloromethane. The concentration of Chl *a* in PMMA foils was evaluated using the molar extinction coefficient determined from accurately weighted samples.

The experimental set-up is shown in Fig. 1. Nitrogen-laser pumped dye laser (Lambda Physik M 100 A and FL 1000 T/IR) was used. The excitation pulse width was 1.9 ns, peak power reached approximately 10 kW. The sample luminescence was focused on a fast PIN photodiode (United Detector Technology 020 A). The electrical output signal from the PIN diode was fed directly to a sampling oscilloscope ORION 155. The sampled signal was displayed on an X—Y recorder. Following four wavelengths were chosen for excitation: 430 nm; 580 nm; 620 nm; and 652 nm. For the registration of fluorescence decay in the respective narrow spectral regions edge glass (IF 610 with transmittance over 90 % for $\lambda > 610$ nm) or interference filters IF 675 ($\lambda_{max} = 670$ nm, $\delta = 7.5$ nm), SIF 700 ($\lambda_{max} = 703$ nm, $\delta = 12$ nm), and IF 725 ($\lambda_{max} = 727$ nm, $\delta = 11$ nm) were used. The fluorescence decay time, τ_f, was determined from the fluorescence response function observed by means of the least-squares iterative method of reconvolution assuming a single exponential decay function (Čermák and Kaplanová 1980; Kaplanová and Čermák 1981), with the correlation coefficients being in the

Table 1. Experimental values of τ_f of Chl *a* in dependence on emission wavelength region

Solvent	Concentration (mol . l^{-1})	Cuvette thickness $d(10^{-2}$ m)	τ_f(ns) Filters F 610	IF 675	SIF 700	IF 725
ACETONE	3.0×10^{-5}	0.196	5.8	5.6	5.6	6.3
	8.0×10^{-5}	0.499	5.9	6.2	5.6	6.7
DIOXANE	2.3×10^{-5}	0.196	6.9	7.5	6.8	7.0
	7.9×10^{-5}	0.499	7.6	8.0	6.9	7.1
n-HEXANE	2.5×10^{-5}	0.196	6.1	5.9	5.0	6.2
	7.6×10^{-5}	0.499	6.2	6.6	5.7	7.5
METHANOL	2.3×10^{-5}	0.196	6 0	6.3	4.9	5.5
	7.9×10^{-5}	0.499	5.8	6.3	5.8	5.6
PMMA	8.0×10^{-4}	0.02	7.0	7.1	—	7.8
	4.9×10^{-5}	0.01	7.2	7.3	—	8.5

interval of 0.990—0.998. The probable τ_f error of the τ_f values presented in Table 1, 2 and 3 did not exceed ± 0.1 ns in any value.

The overal system was tested using Rhodamine B solution in concentration 10^{-5} mol . l^{-1} (in a mixture of 10 % methanol and 90 % ethanol). The value of the decay time obtained (3.2 ± 0.1 ns) is similar to that reported by Knof et al. (1978).

Results

At room temperature fluorescence spectra solutions of chlorophyll at low concentrations, at which molecule aggregation is insignificant, are characterized by two bands: one main with a maximum within 670—680 nm and a long-wavelength band within 720—730 nm. Experimental values of τ_f of Chl *a* fluorescence excited at 430 nm (the absorption Soret band), obtained in different spectral intervals of emission spectra are summarized in Table 1 and illustrated in Fig. 2. Table 2 presents experimental values of decay times of Chl *a* fluorescence excited in different bands of Chl *a* absorption spectra and detected in the spectral region of $\lambda > 610$ nm. As demonstrated in Table 1, chlorophyll *a* fluorescence exhibits different decay times in different spectral regions and consequently τ_f values presented in Table 2 and 3 are "average" fluorescence lifetimes.

Discussion

A more detailed observation of the data presented herein reveals a significant dependence of decay times, τ_f, on fluorescence wavelengths. The difference in the

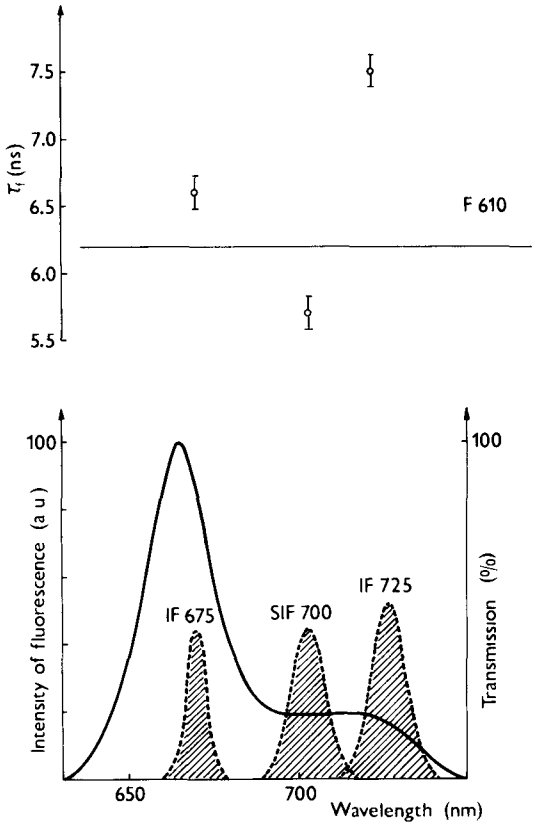

Fig. 2. Fluorescence spectrum of Chl *a* in n-hexane ($C = 7.6 \times 10^{-5}$ mol . l^{-1}), transmission curves of interference filters, and the corresponding τ_f values.

lifetimes measured in the short-and long-wavelength band of Chl emission, respectively, exceeds considerably the experimental error. Decay time measurements for different concentrations and/or different cuvette thickness (see Table1) demonstrated the effect of secondary processes on decay time, τ_f. Due to the relatively small Stokes shift between the main fluorescence band and the red absorption band of Chl *a*, the short wavelength part of the fluorescent light may effectively be reabsorbed. Such self-absorption is followed by secondary fluorescence. This effect results in a prolongation of the decay time, τ_f.

We tried to make correction of τ_f for secondary processes using three different methods, assuming

$$\tau_f = K_{reabs} \cdot \tau_c, \quad (1)$$

Table 2. Experimental values of τ_f of Chl a in dependence on excitation wavelength (emission filter F 610)

Solvent	Concentration (mol . l^{-1})	Cuvette thickness (10^{-4})	τ_f(ns) Excitation wavelengths			
			430 nm	580 nm	620 nm	652 nm
ACETONE	8.5×10^{-5}	0.499	5.9 (4.5)*	6.9 (4.3)	7.2 (4.7)	7.6 (5.2)
DIOXANE	7.9×10^{-5}	0.499	7.6 (6.0)	8.1 (4.7)	8.0 (5.1)	8.3 (6.1)
n-HEXANE	7.6×10^{-5}	0.499	6.2 (4.8)	6.8 (5.5)	6.7 (5.5)	7.5 (6.3)
METHANOL	7.9×10^{-5}	0.499	5.8 (4.6)	6.3 (4.4)	5.8 (4.2)	5.6 (4.1)
PMMA	8.0×10^{-4} 4.9×10^{-5}	0.02 0.01	7.0 7.2	7.2 7.3	7.8 7.2	7.7 7.7

*τ_c corrected by Eqs. (1) and (5)

where K_{reabs} is the correction coefficient, and τ_c is the corected fluorescence lifetime.

According to the theory of Budó and Ketskeméty (1957), the correction coefficient can be written as follows

$$K_{\text{reabs}} = (1-\chi)^{-1} \tag{2}$$

where χ represents the ratio of the intensity of secondary to primary fluorescence. This χ correction is a very complicated function of optical density of the solution at

Table 3. Experimental (τ_f) and corrected (τ_c) values of Chl *a* in PMMA fluorescence lifetimes ($C = 4.9\times10^{-3}$ mol . l^{-1}, $d = 1\times10^{-4}$ m, emission filter F 610)

Excitation wavelength	τ_f(ns)	K^{reabs}*	τ_c(ns)	K^{reabs}**	τ_c(ns)
430 nm	7.2	1.0104	7.12	1.060	6.79
580 nm	7.3	1.0104	7.22	1.046	6.98
620 nm	7.2	1.0104	7.12	1.0608	6.79
652 nm	7.7	1.0104	7.62	1.056	7.29

*K_{reabs} calculated from Eq. (3)

** K_{reabs} calculated from Eq. (5)

both the exciting and emission wavelengths, of the optical density between the short-wavelength part of the fluorescence and the long-wavelength part of the absorbance and, finally, of the parameter $m = R/l$, where R denotes the radius of the exciting light, and l denotes the thickness of the cuvette. We did not succeed in evaluating the correction coefficient according to Budó's and Ketskeméty's (1957) general formula under our experimental conditions ($R/l \ll 1$).

The correction coefficient suggested by Birks (1970) is

$$K_{\text{reabs}} = (1 - aq)^{-1} \tag{3}$$

where a is the probability of reabsorption of an emitted photon, and q is the fluorescence quantum yield. The aq value is obtained from the equation (Birks 1970)

$$aq = \int F(\lambda)\,(1 - 10^{-\varepsilon(\lambda)lC})\mathrm{d}\lambda \tag{4}$$

where $\varepsilon(\lambda)$ is the molar extinction coefficient in the fluorescence wavelength region, C is the concentration of Chl a, $q = \int F(\lambda)\mathrm{d}\lambda$, and l is the distance that fluorescence passes within the cell from the plane (parallel to the front face of the cell) at which the exciting radiation has been reduced by absorption to 1/e its intensity incident on the front face. Decay times corrected for self-absorption by the method described by Birks (1970) are illustrated in Table 3 for Chl a in PMMA. Under our experimental conditions values of K_{reabs} were almost independent of excitation or registration wavelengths.

The third method employed for the correction was that of "average molar absorptivity" for reabsorption of polychromatic fluorescence light, originally derived by Rohatgi and Singhal (1968) for fluorescence quantum yield correction. The "average molar absorptivity", $\bar{\varepsilon}_F$, was calculated (Kaplanová and Čermák 1981) for Chl a fluorescence reabsorption assuming a gaussian shape for the overlapping fluorescence and absorption bands which have mirror symmetry. The correction factor K_{reabs} derived by Rohatgi and Singhal (1968) is as follows:

$$K_{\text{reabs}} = \frac{\varepsilon + \bar{\varepsilon}_f}{\varepsilon} \frac{1 - \exp(-\varepsilon Cd)}{1 - \exp[-(\varepsilon + \bar{\varepsilon}_f)Cd]} \tag{5}$$

where ε, C, and d are the Chl a molar absorptivity at the excitation wavelength, Chl a concentration and the layer thickness for fluorescence absorption, respectively. The correction factor K_{reabs} derived by Eq. (5) depends on excitation wavelength only. It follows from Tables 2 and 3 that the effect of excitation wavelength on the fluorescence decay time is somewhat lowered by the correction for self-absorption the trend of the lifetime prolongation with raising excitation wavelength remaining unchanged. None of the correction methods mentioned above was able to correct the effect of secondary processes on fluorescence decay time in dependence on emission wavelength.

Let us suppose both dependences of fluorescence decay time on spectral conditions of measurement to occur. Apparently longer lifetimes of long-wavelength emission (725 nm) as compared with the 675 nm emission were observed by Yuen et al. (1980) for chlorophyll-like pigments pheophytin *a* and pyropheophytin *a* in pyridine at room temperature. Spectral dependence of fluorescence decay time of Chl *a* in diethyl ether has been reported previously by Avarmaa et al. (1977) at different temperatures. Several hypotheses may be proposed to explain the photophysical basis of these phenomena, similar wavelength lifetime effects may be associated with emission from different levels in the excited state manifold (Yuen et al. 1980), or they may be due to different degrees of fluorescence polarization in two emission bands of chlorophyll-like pigments (Gurinovich and Sevchenko 1968; Vacek et al. 1977) also, similar effects may reflect the presence of more than one emitting species in the solution, since the solutions used in the experiments were not water free, and chlorophyll is able to form different solvates or aggregates. Further possible explanations of the observed dependences may be associated with the relatively high energy density of Chl *a* fluorescence excitation with a laser beam. Especially, the amount of photodestructed Chl molecules in the illuminated volume of solution during the time of the fluorescence decay measurement cannot be negligible due to the presence of oxygen in the solution. Finally, the observed phenomena of Chl *a* fluorescence lifetime may be associated with the non-linear absorption and emission processes of Chl *a* reported recently by Hoffmann et al. (1978) and Jahnke and Leupold (1981) for excitation energies higher than 10^{13} photons per cm^2.

Undoubtedly, a more detailed analysis of the proposed hypothesis is required and further experimental work on this point is indicated.

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