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THE FLUORESCENCE LIFETIME AND QUANTUM YIELD OF CHLOROPHYLL *a* IN TRITON X-100 SOLUTIONS

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The fluorescence of chlorophyll (Chl) *a* in 0.007–0.1% Triton X-100 was investigated by a phase-shift technique. The Chl *a* concentrations varied from 0.7 to 25 μM. Parallel measurements of fluorescence lifetime (τ) and quantum yield (ψ) were made. It was concluded that homogeneous energy transfer takes place at detergent concentrations above 0.025%: (i) the transfer between uniform molecules of the pigment solubilized in Triton X-100 micelles, when τ and ψ are constant; (ii) the transfer towards the quenching centers, resulting in a proportional decrease in τ and ψ. At a Triton X-100 concentration of about 0.025% the Chl *a* emission becomes heterogeneous. It is evident from the disproportional decrease in τ and ψ (greater in ψ than in τ) and also from the rise of the fluorescence at 730–750 nm. As the Triton X-100 concentration becomes lower than the critical one (0.021%), the number of micelles drops abruptly and Chl *a* forms colloid particles in the aqueous medium. This manifests itself as a decrease in τ and as a certain stabilization of ψ. Having analyzed the complex pattern of the τ/ψ ratio, we concluded that under these conditions more than 90% of Chl *a* is in a weakly fluorescent form ($\tau < 30$ ps) and about 1% is in an aggregated state fluorescing at 732 nm with τ about 0.7 ns.

Introduction

Studies on the fluorescence of pigments incorporated in detergent micelles are important from two points of view. On the one hand, with these micelles one can simulate the process of greening in etiolated plant leaves, i.e., formation of the light-harvesting antenna. A number of researchers have confirmed this hypothesis by studying the concentration depolarization of fluorescence in Triton X-100 micelles stained with Chl *a* [1–3]. Detergent micelles appeared to concentrate Chl *a* molecules to such an extent that energy transfer occurred between them.

On the other hand, detergents are usually employed for disintegration of photosynthetic membranes and isolation of pigment-protein complexes. For this reason, detergent micelles may be present in an incompletely dialyzed preparation, which results in partial solubilization of pigments and, hence, in distortion of the optical characteristics of the complexes obtained. This is what we observed on studying the fluorescence of the Chl *a/b* antenna complex from pea chloroplasts [4]. It was demonstrated that the presence of micellar Chl *a* gives rise to a fluorescent component with τ = 5–6 ns, which shows as a marked increase in the total τ value measured with a phase-type fluorometer. At a certain concentration of Triton X-100, the fluorescence of the complex proved to be as high as that of micellar Chl *a*, i.e., most of the complex pigment passed into detergent micelles. A partial quenching of the fluorescence was observed upon dilution of the above-mentioned sample with buffer. It may be due to distribution of the

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Abbreviations: Chl, chlorophyll; τ and ψ, fluorescence lifetime and quantum yield, respectively.

solubilized pigments among three phases, namely (i) accumulation of pigments in the remaining micelles; (ii) release of pigments into the aqueous phase; (iii) reverse adsorption of pigments on the protein.

In this respect, the purpose of the present work was to study the mechanism of the fluorescence quenching of Chl *a* in aqueous Triton X-100 solutions. Parallel measurements of τ and ψ enabled us to reveal the cases of homogeneous and heterogeneous emissions.

Materials and Methods

Chl *a* solutions in Triton X-100 were obtained by gradual addition of certain amounts of stock Chl *a* dissolved in acetone to an aqueous detergent solution of a required concentration which ranged from 0.007 to 0.1%, including the critical micellar concentration (approx. 0.021% [5]). The Chl *a* content of solutions was determined from the absorbance in the Soret band peak at 436—437 nm using the molar extinction coefficient of about $1.12 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ [6]. The Chl *a* concentrations varied from 0.7 to 25 μM. The probes with a Chl *a* concentration above 10 μM contained about 10—15% acetone.

Absorption spectra were measured with an SF-10 spectrophotometer (LOMO, U.S.S.R.), the fluorescence intensity and τ with a phase-type fluorometer [7]. The fluorescence was excited by 404 and 436 nm mercury lines and detected through a glass filter transmitting either $\lambda > 620$ nm or $\lambda > 700$ nm wavelength ranges. The measurements were made in a 0.55 cm path-length cell with 90° excitation geometry. The exciting light of an intensity of about 10^5 erg $\cdot$ $cm^{-2} \cdot s^{-1}$ was focused on a cell as close as possible to its wall facing a photomultiplier. The relative ψ value was measured as the ratio of the fluorescence photocurrent (a measure of the fluorescence intensity) in microamps to the absorbance of the object at exciting light wavelengths. The accuracy was about ±0.4 ns for τ, ±0.005 μA for fluorescence intensity and ±0.01% for absorbance.

Spectra of fluorescence emission were recorded with an Aminco Bowman spectrofluorimeter with excitation at 436 nm. The object was placed into a 1 mm path-length cell at an angle of about 40° to the exciting beam. The spectra measured were corrected for self-absorption of fluorescence.

All experiments were carried out at room temperature.

Chl *a* was purchased from Sigma Chemical Co. (St. Louis, MO), Triton X-100 from Ferak (Berlin).

Results and Discussion

The decrease in τ as a function of the concentration of Chl *a* solubilized in Triton X-100 is displayed in Fig. 1. A number of Triton X-100 concentrations, lower and higher than the critical one, were chosen. (In a recent work [4], we observed that τ drops from 5.3 to 3.4 ns upon 20-fold dilution of the Chl *a/b* antenna complex in 0.07% Triton X-100. This decrease can be simulated by curves analogous to those in Fig. 1.) As seen from these curves, the smaller the

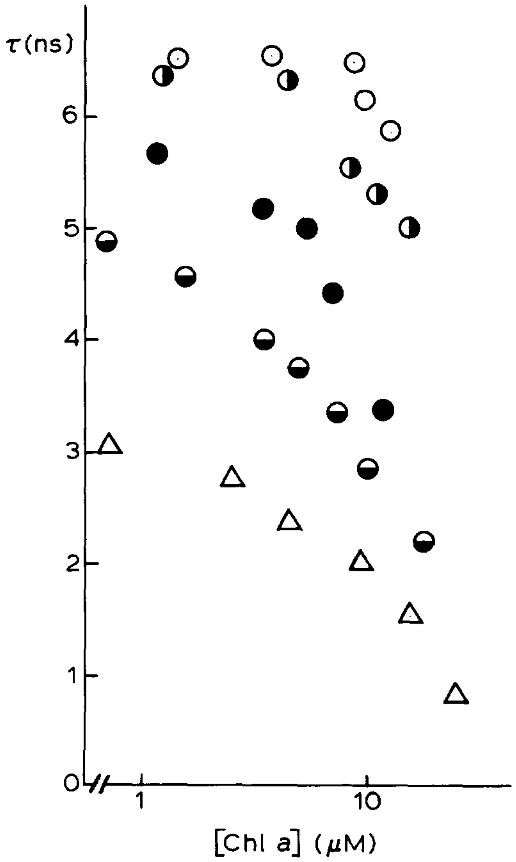

Fig. 1. Fluorescence lifetime (τ) as a function of the concentration of Chl *a* solubilized in Triton X-100 at five detergent concentrations: ◑, 0.1%; ○, 0.05%; ●, 0.025%; ◒, 0.015%; △, 0.007%.

detergent content, the lower the τ value and the higher the degree of its decrease upon increasing the Chl *a* concentration from 1 to 25 μM. To reveal the mechanisms of the decrease in τ, the changes in three fluorescence parameters, τ, ψ and the τ/ψ ratio, were analyzed for each Triton X-100 concentration.

Simultaneous measurements of τ and ψ allow information to be obtained about the character of the emission studied by the phase-shift method. In the case of homogeneous emission, the τ/ψ ratio is constant and equal to τ_0, the radiative lifetime, i.e., the value which is the reciprocal of the constant of intramolecular radiative deactivation. By analyzing the absorption spectra of Chl *a* solution and chloroplasts, τ_0 is inferred to be the same value with an accuracy of 30% for Chl *a* in vitro and in vivo [8]. In the case of heterogeneous emission, the τ/ψ ratio has no physical meaning. According to the theory of phase fluorometry [9], the rise in the τ/ψ ratio compared to that for homogeneous emission indicates that components with highly quenched fluorescence appeared.

Fig. 2a and b shows the dependences of τ, ψ and the τ/ψ ratio on the concentration of Chl *a* in 0.1 and 0.05% Triton X-100 solutions, respectively, at detergent concentrations higher than the critical one. Under these conditions, Triton X-100 forms oblate ellipsoidal micelles with semiaxes of 27 and 52 Å at room temperature [10]. Each micelle contains about 140 detergent molecules and about 6000 water molecules [11]. In our experiments, the content of Chl *a* did not exceed 3% of Triton X-100. Therefore, the structure and the size of detergent micelles seem to remain unchanged after dissolution of the pigment. The Chl *a* molecules are distributed randomly within a micellar volume and are, apparently, disoriented with respect to each other [1]. The concentration depolarization of fluorescence observed in these systems is due to excitation energy transfer [1–3]. Using previous data [3], we determined Chl *a* concentrations at which energy transfer begins. At first, this process does not affect the fluorescence of the sample, as described in Refs. 1 and 2. Actually, τ and ψ remain constant until the Chl *a* concentration exceeds 10 μM

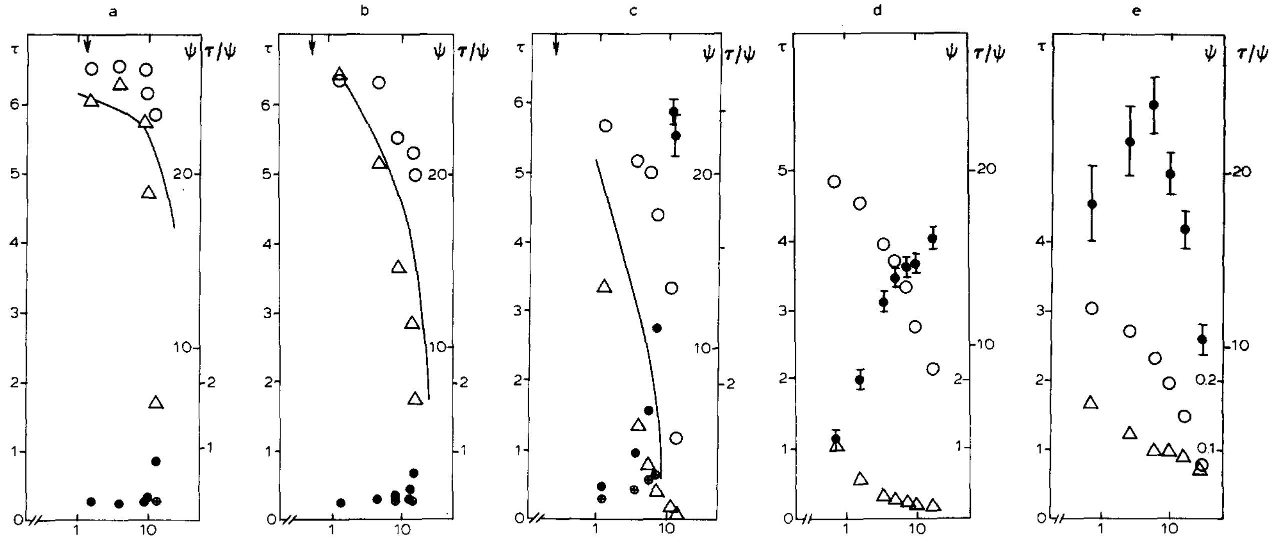

Fig. 2. Effect of the concentration of Chl *a* in Triton X-100 solutions on fluorescence lifetime (τ), fluorescence quantum yield (ψ) and the τ/ψ ratio. [Triton X-100]: a, 0.1%; b, 0.05%; c, 0.025%; d, 0.015%; e, 0.007%. The errors in τ/ψ determinations in a, b and partially in c are smaller than the size of the closed circles. The curves of ψ vs. Chl *a* concentration in a–c are constructed on the basis of the data from Ref. 2. Crossed circles indicate τ/ψ values calculated with the help of the ψ values taken from Ref. 2. The arrows in a–c show the beginning of the concentration depolarization of fluorescence according to Ref. 3. Axes: ○, τ (ns); △, ψ (relative units); ●, ⊕, τ/ψ (relative units).

(Fig. 2a) or 5 μM (Fig. 2b). Thus, homogeneous energy transfer occurs between uniform molecules of the pigment (e.g., monomers).

Then, at Chl *a* concentrations above 10 μM, a decrease in τ and ψ is observed. The rise in the τ/ψ ratio, which is seen in Fig. 2a and b, does not prove to be true, since this ratio becomes constant over the entire Chl *a* concentration range studied if the ψ values from Ref. 2 corrected for fluorescence self-absorption are used. Therefore, the real decrease in ψ is proportional to that in τ. Apparently, the proportional change in τ and ψ is due to formation of quenching centers and energy transfer to them, rather than to an increase in regular orientation of pigment molecules within micelles and to acceleration of energy transfer, since Chl *a* molecules are disoriented in Triton X-100 micelles [1]. The quenching centers are, most likely, Chl *a* associates characterized by an effective non-radiative channel of energy deactivation. The emergence of the quenching centers does not alter the homogeneous character of the fluorescence measured. This may be explained in two ways: either the emission of these centers is negligible, or their τ and ψ values coincide with those of the total pigment, but the latter is hardly probable.

Comparison of the ψ values obtained in Ref. 2 and in this work (Fig. 2a and b) shows that the effect of fluorescence self-absorption on the ψ measured is observed when the overall Chl *a* concentration exceeds 10 μM. However, in acetone solution, ψ remains constant (accuracy 13%) when the Chl *a* concentration reaches 100 μM (data not shown). This may be due to (i) elongation of optical paths in Triton X-100 solutions because of the scattering of the light by micelles; (ii) increase in the local Chl *a* concentration in micelles [2,3] causing a greater overlap between absorption and fluorescence spectra.

The changes in τ, ψ and the τ/ψ ratio at the Triton X-100 concentration of 0.025%, which is close to the critical one, are represented in Fig. 2c. At a Chl *a* concentration above 10 μM, the red absorption band shifts from 668 to 670 nm and is broadened by 25%, whereas the fluorescence maximum shifts from 678 to 688 nm and the fluorescence at 730–750 nm increases (cf. spectra a and b in Fig. 3). The τ/ψ ratio tends to increase even if we take into account the fluorescence self-absorption. These data suggest that the Chl *a* emission becomes heterogeneous.

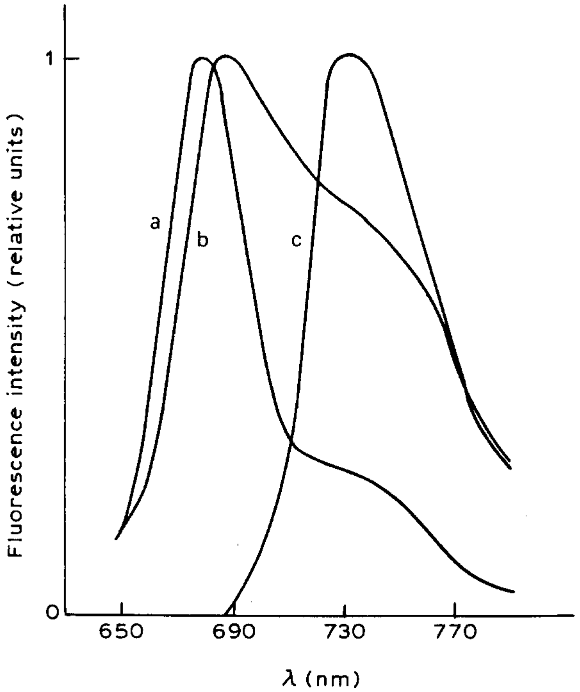

Fig. 3. Fluorescence emission spectra of Chl *a* in Triton X-100 at three detergent concentrations: 0.1% (a), 0.025% (b), 0.007% (c). [Chl *a*]: a, 5 μM; b, 30 μM; c, 25 μM.

Fig. 2d and e shows analogous dependences at Triton X-100 concentrations (0.015 and 0.007%) below the critical one. There are few micelles in these solutions and detergent monomers predominate. A further decrease in τ with a certain stabilization of ψ is observed (the scale of ψ in Fig. 2e increases 40-fold compared to that in Fig. 2a). The minimum value of the τ measured in a 0.007% Triton X-100 solution is 0.8 ns for the fluorescence detected at $\lambda > 620$ nm and 0.7 ns for that at $\lambda > 700$ nm. The τ/ψ ratio rises slowly, reaches its maximum and then drops.

In order to explain the complex pattern above of the τ/ψ changes, the two-component emission studied with a phase-shift fluorometer was analyzed theoretically. Fig. 4 demonstrates that τ, ψ and the τ/ψ ratio are functions of the relative concentration of either of the two components for three values of the τ_2/τ_1 ratio, where τ_1 and τ_2 are their fluorescence lifetimes. We suggest that each component had the same value of τ_0 (i.e., τ/ψ ratio). According to the well known equation, the τ_0 value depends on the area of the

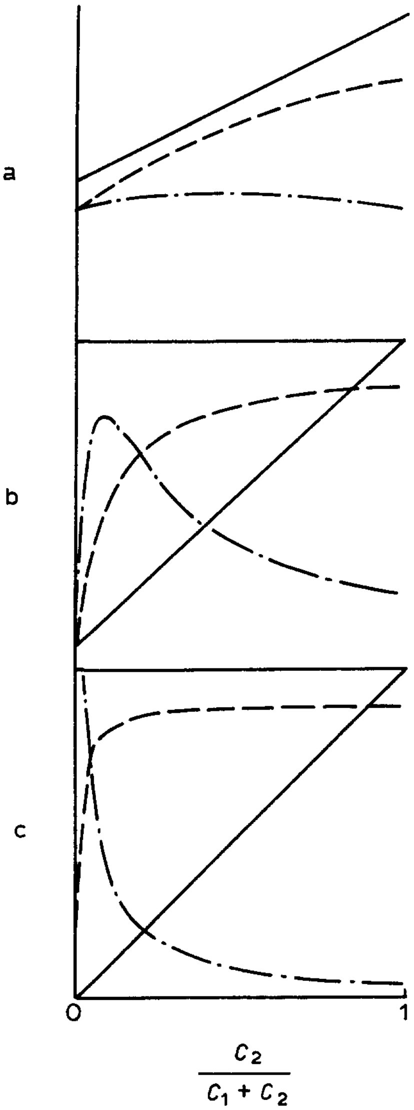

Fig. 4. Theoretical analysis of two-component emission studied by the phase-shift technique: the dependence of the fluorescence lifetime measured (τ_m) (– – –), the fluorescence quantum yield (ψ_m) (———) and the τ_m/ψ_m ratio (· – · – ·) on the relative concentration of either of two components. The calculations were made according to the equations of phase-shift fluorometry [9]: $\mathrm{tg}\,\phi_1 = \omega\tau_1$; $\mathrm{tg}\,\phi_2 = \omega\tau_2$;

$$\psi_m = \frac{C_1\psi_1 + C_2\psi_2}{C_1 + C_2};$$

$$\mathrm{tg}\,\phi_m = \frac{C_1\psi_1 \cdot \sin\phi_1 \cdot \cos\phi_1 + C_2\psi_2 \cdot \sin\phi_2 \cdot \cos\phi_2}{C_1\psi_1 \cdot \cos^2\phi_1 + C_2\psi_2 \cdot \cos^2\phi_2} = \omega\tau_m;$$

where C_1 and C_2 are the concentrations of two types of fluorescent molecules; ϕ_1 and ϕ_2, phase shifts between the exciting light and the fluorescence of each type; τ_1, τ_2 and ψ_1, ψ_2, fluorescence lifetimes and quantum yields of two emissions, respectively; ω, cyclic modulation frequency, 76.5 MHz. The τ_2 value was about 4–4.5 ns. (a) $\tau_2/\tau_1 = 2$, (b) $\tau_2/\tau_1 = 13$, (c) $\tau_2/\tau_1 = 100$.

absorption band, its peak position and the refractive index of the medium. There are no reasons to expect any significant changes in τ_0 of Chl *a* in various states. Earlier we confirmed this experimentally [8].

If τ_1 and τ_2 are comparable, the τ_m/ψ_m ratio hardly changes (Fig. 4a). If $\tau_2 \gg \tau_1$, the τ_m/ψ_m ratio rises infinitely as a portion of the short-lived component increases (Fig. 4c). In the intermediate case, the τ_m/ψ_m ratio goes through the maximum (Fig. 4b). The latter dependence does take place, although qualitatively, at a Triton X-100 concentration of 0.007% (Fig. 2e). We believe that there exist two emissions: of Chl *a* in the remaining detergent micelles with τ about 5 ns and of Chl *a* aggregates in a water/acetone solution fluorescing at 732 nm (Fig. 3c) with τ about 0.7 ns. When the overall Chl *a* concentration reaches 10 μM, the emission of the aggregates predominates. However, the maximum increase in the τ/ψ ratio in the experiment (Fig. 2e) is more than 20-times higher than that in the corresponding theoretical dependence. To bring the theoretical dependence into line with the experiment, one should admit the existence of more than 90% of weakly fluorescent pigment ($\tau < 30$ ps) and about 1% of fluorescent aggregates ($\tau \approx 0.7$ ns). This is consistent with the fact that in water Chl *a* forms polymer colloid particles characterized by a very low fluorescence intensity [5,12,13]. The chemical structure of these aggregates remains obscure and can be the subject of further investigations.

Conclusion

Porphyrin molecules are known to have a tendency for association which often results in electron excitation quenching. Our experiments with Chl *a* in Triton X-100 solutions were another proof of this phenomenon. While studying the fluorescence of the Chl *a*/*b* antenna complex in aqueous solution, we observed τ/ψ changes indicative of emission heterogeneity [4]. A portion of quenched pigment forms and, consequently, the τ/ψ ratio decreased upon dissociation of the complex by a detergent. Reduction of the useless association to a minimum is necessary for Chl *a* and *b* to form effective light-harvesting antenna without quenching centers. This is attained, e.g., by adsorption of chlorophylls on proteins and by incorporation of pigment-proteins into a lipid membrane. The latter weakens hydrophobic interactions between proteins and thereby diminishes interaction between pigments from protein to protein.

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