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Quantification of Fluorescent Molecules in Heterogeneous Media by Use of the Fluorescence Decay Amplitude Analysis

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In heterogeneous media, including biological objects, fluorescent molecules of one kind often exist as a mixture of species with different fluorescence parameters. Fractional concentrations of these species can be measured by analyzing their fluorescence decay amplitudes. The amplitudes are linear functions of concentrations of actually fluorescent molecules, i.e., molecules whose fluorescence decay can be measured. Other (quenched) molecules do not influence these amplitudes. The other parameter that has to be measured to calculate these concentrations is the radiative rate constant. The parameter can be excluded by comparison of decay amplitudes of the sample studied and a standard. The comparison should be made taking into account the dependence of the radiation rates on emision wavelength. The method has been tested in experiments with the fluorescent probe 3-methoxybenzanthrone (MBA) bound with phosphatidylcholine bilayer membranes. The probe has a complex fluorescence decay in these membranes. The decay can be described as two exponentials, with decay times of 2 and 12 ns and a blue-shifted fluorescence spectrum of the short-life component as compared with long-life one. The shift was used to correct calculated radiative rate values. After this, about 100% of the MBA molecules were found to be fluorescent in these membranes. Thus, this approach can be used to measure absolute concentrations of subpopulations of fluorescent molecules in heterogeneous biological objects.

KEY WORDS: Fluorescent probes; fluorescence decay; concentration measurement.

INTRODUCTION

The fluorescence of organic molecules is very sensitive to environment properties. Therefore fluorescence in biological objects is often heterogeneous [1]: intrinsic [2,3] as well as extrinsic [4,5] fluorophore molecules in different parts of biological objects have different kinds of fluorescence. Nonexponential decay is often due to this heterogeneity.

Moreover, an appreciable fraction of fluorophores in biological media (perhaps, the majority of the total population in some cases) is strongly quenched and therefore takes no part in actually measured fluorescence. A contradiction between large decay times, on the one hand, and low mean quantum yields, on the other hand, can be considered evidence of the existence of the strongly quenched species of fluorescent molecules [2,3,6]. As a result, our description of the biological object studied actually relates to some indefinite part of the object, while its other parts have other properties. The fluorescence decay time is a commonly used parameter, while any quantification of its fractional weight in the whole fluorophore population is rather an exception.

The situation was recognized many years ago, and a method was proposed to calculate the concentration of the nonfluorescent fraction [2,6]. Nonexponential decay was described as the sum of several monoexponential components:

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$$F(t) = A_1 * \exp(-t/\tau_1) + A_2 * \exp(-t/\tau_2) + \ldots \quad (1)$$

where $A_1, A_2, \ldots$, are fractional amplitudes and $\tau_1, \tau_2, \ldots$, are fractional decay times. The fraction of nonfluorescent molecules was given as

$$F_{nf} = 1 - Q_r \tau_S / \sum \alpha_k \tau_k \quad (2)$$

where Q_r is the quantum yield of the sample studied relative to a certain standard, τ_S is the decay time of the standard, τ_k are fractional decay times of the sample, and α_k are their amplitudes normalized to 1 [3,7].

We have tried to estimate the nonfluorescent fraction of some fluorescent probes whose fluorescence decay in biological samples has a complex character. A method of amplitude analysis is proposed. It is based on the same ideas as those in the above works [2,3], but in our version only amplitudes $A_1, A_2, \ldots$, are used instead of the quantum yields and fractional decay times in Ref. 3 [Eq. (2)]: it is well-known that absolute quantum yield determination in biological objects is a very hard problem. Besides, a correction was made taking into account the spectral shifts of each fluorescent fraction: it has been found that in some cases the quantification can lead to significant errors if this correction is neglected.

The first case tested was the fluorescent probe 3-methoxybenzanthrone (MBA) in lipid membranes. The probe was used earlier in the study of protein conformational transitions [8], as a membrane probe for T- and B-lymphocyte detection [9,10] etc. (see review in Ref. 11 for details). In artificial lipid membranes its fluorescence is heterogeneous and can be fitted with two exponentials. After the quantification all its molecules were found to be actually fluorescent; i.e., their decay times were above the lower limit of the instrument time resolution.

The second example is the fluorescent probe K-35 for albumin study [12]. In contrast with the first example, three classes of fluorescent molecules were found in this case, and one of them—the majority of albumin-bound K-35 molecules—was strongly quenched.

Preliminary results of such quantification have been published recently in brief [13,14]. In this paper the first of these examples is described in detail.

EXPERIMENTAL

The fluorescent probe 3-methoxybenzanthrone (MBA) was synthesized by B. M. Krasovitsky and coworkers (Institute of Monocrystalls, Kharkov, Ukraine). Its optical properties in biological membranes were described earlier [15,16]. In *N,N*-dimethylformamide, its molar extinction in the long-wave absorption maximum is 9600 M^{-1} cm^{-1} [15] (similar values were obtained in other solvents [17,18]) and the fluorescence decay was described as one exponential with a decay time of 12.3 ns [16]. The MBA fluorescence quantum yield, Q, in the solvent was reported earlier to be 0.62 [14]. We measured the yield once more. A fluorescein solution in 0.1 *M* NaOH was used as a standard: in this medium its quantum yield is about 0.85–0.90 [19,20], and the mean of these published values (0.88) was used for calculation of Q. The new Q value for MBA in dimethylformamide is 0.61 ± 0.04, as earlier. The MBA quantum yield in other solvents and in membranes was measured by a comparison with the value in dimethylformamide. MBA fluorescence in membranes cannot be described with a single exponential, as shown earlier [21].

Artificial phospholipid membranes were prepared from egg phosphatidylcholine (Kharkov Plant of Bacpreparations, Ukraine) by fast injection of its ethanolic solution into 0.14 *M* NaCl–0.01 *M* Tris·HCl, pH 7.3 (Ref. 11, p. 193). The final phosphatidylcholine concentration in all solutions was 2 mg/ml.

MBA was dissolved in ethanol and added to the membrane solution with stirring. This fluorescent probe is rather hydrophobic and totally bound to membranes at these concentrations [13].

MBA absorption spectra in organic solvents and in membranes were corrected for solvent (membrane) absorption.

The corrected spectra of MBA steady-state fluorescence were monitored with a Hitachi F 4000 spectrofluorometer (Japan). The correction was made with a Hitachi standard incandescent lamp (Japan) of a known thread temperature.

Fluorescence decay was studied using a new station on a synchrotron beam of an S-60 synchrotron at the Lebedev Physical Institute of the Russia Academy of Sciences (Moscow) [22]. The time window was about 30 ps per one channel; the total accumulated count for any sample was about 10^6–10^7. Excitation was selected with a monochromator, and fluorescent light with interference filters (9- to 12-nm half-width). Excited light intensity was calibrated with an ethanolic MBA solution in a soldered quartz cell instead of the sample cell. Calculations of decay functions were made by the least-squares nonlinear method as described earlier [22,23].

RESULTS

Fluorescence Decay in Heterogeneous Media: Formalism

The case of nonexponential fluorescence decay after a very short excitation pulse is considered below. The

decay can be described as the sum of monoexponential components [Eq. (1)]. If each ith component of the sum corresponds to a definite species of fluorescent molecules, then each amplitude A_i has to be a function of the concentration C_i of these molecules [2,3,24]:

$$A_i = K_i * C_i \tag{3}$$

where K_i is an unknown coefficient. If K_i is the same for any ith species, then the value can be excluded and the fraction α_i of ith-class molecules may be calculated as

$$\alpha_i = C_i/\sum_k C_k = A_i/\sum_k A_k \tag{4}$$

Unfortunately, in the general case, the sum ΣA_k includes only molecules with measurable fluorescence. An indefinite but, perhaps, significant fraction can be strongly quenched and, as a result, excluded from the sum. Second, K_i is not the same for different fluorescent species because it is a function of at least the radiative rate constant [25], which depends on the molecule environment [26]. Thus, how to calculate the absolute concentrations of every kind of molecule, C_i, and their relative fractions in the whole population of fluorescent molecules is a difficult problem.

In the simplest case, there exists a single population of fluorescent molecules. At the initial moment ($t = 0$) all these molecules are illuminated with a fast light flash (wavelength λ_e). As a result, $N(0)$ molecules are converted to the excited state:

$$N(0) = b1 * C_f \varepsilon \tag{5}$$

where C_f is the molar concentration of these fluorescent molecules; ε, their molar extinction at λ_e; and $b1$, an instrument factor (it includes light intensity, etc). Subsequent deactivation has two paths: radiative (fluorescent, index "f") and nonradiative (nonfluorescent, index "n"; we combine all nonradiative processes). The number of excited molecules, $N(0)$, decreases to $N(t)$ in moment t. The decrease and the residual number of excited molecules $N(t)$ are described as

$$\begin{aligned} N(t) &= N(0)\exp[-(k_f + k_n)\,t] \\ &= b1 * C_f \varepsilon * \exp[-(k_f - k_n)\,t] \end{aligned} \tag{6}$$

where k_f and k_n are rate constants for radiative and nonradiative transitions, respectively. The total number of fluorescent quanta is expressed by the following equation:

$$\begin{aligned} N_f &= [k_f/(k_f + k_n)] * N(0) \\ &= [k_f/(k_f + k_n)] * b1 * C_f \varepsilon \end{aligned} \tag{7}$$

Fluorescence decay time τ and quantum yield Q are

$$\begin{aligned} \tau &= 1/(k_f + k_n); \qquad Q = N_f/N(0) \qquad k_f = Q/\tau \\ &= k_f/(k_f + k_n); \end{aligned} \tag{8}$$

The emitted quanta are distributed along the fluorescence spectrum $\Phi(\lambda)$. The major part of these quanta has wavelengths of λ_1 to λ_2. If the detector bandpath is $\Delta\lambda$, then the relative fraction of quanta in the interval $\Delta\lambda$ is

$$\Phi(\lambda) * \Delta\lambda/\int_{\lambda_1}^{\lambda_2} \Phi(\lambda) * d\lambda \equiv \Phi(\lambda) * \Delta\lambda/S\Phi \tag{9}$$

where $S\Phi$ is the integral. The measured fluorescence intensity at moment t at count accumulation time Δt is

$$\begin{aligned} F(t, \lambda) &= b2(\lambda) * N_f(t) * \Delta t[\Phi(\lambda) * \Delta\lambda/S\Phi] \\ &= b2(\lambda) * k_f * b1 * C_f \varepsilon * \exp \\ &\quad (-t/\tau) * \Delta t * [\Phi(\lambda) * \Delta\lambda/S\Phi] \end{aligned} \tag{10}$$

where $b2(\lambda)$ is a wavelength-dependent instrument factor including detector sensitivity. All instrument-dependent constants can be combined:

$$B(\lambda) = b2(\lambda) * b1 * \Delta t * \Delta\lambda \tag{11}$$

and then

$$F(t, \lambda) = B(\lambda) * k_f * C_f \varepsilon * [\Phi(\lambda)/S\Phi] * \exp(-t/\tau) \tag{12}$$

The function decays as a single exponential with the amplitude

$$F(0, \lambda) = B(\lambda) * k_f * C_f \varepsilon * [\Phi(\lambda)/S\Phi] \tag{13}$$

The amplitude is a direct function of the fluorescent molecule concentration C_f, which we must determine. This amplitude can be measured with a time-resolving instrument. The quantum-corrected fluorescence spectrum $\Phi(\lambda)$ and the ratio $\Phi(\lambda)/S\Phi$ can be measured in steady-state experiments. The molar extinction ε could be estimated for this kind of molecule. After that, however, it is necessary to determine two other unknown values—the instrument factor $B(\lambda)$ and the radiative rate constant k_f.

These molecules can be dissolved in a solvent (index "s") where their concentration C_s is known, absorption $C_s\varepsilon_s$ as well as the steady-state spectrum $\Phi_s(\lambda)$ can be measured. The instrument constant $B(\lambda)$ is the same for any sample; therefore, it can be excluded:

$$\frac{F(0, \lambda)}{F_s(0, \lambda)} = \frac{B(\lambda) * k_f\ C_f \varepsilon\ [\Phi(\lambda)/S\Phi]}{B(\lambda) * (k_f)_s * C_s\varepsilon_s * [\Phi_s(\lambda)/S\Phi_s]} \tag{14}$$

and thus

Table I. Decay Time (τ; ns) and Calculated Values of the Radiative Rate Constant $k_f = Q/\tau$ (s^{-1}) of MBA Fluorescence in Organic Solvents[a]

	Wavelength (nm)					
	479	503	528	553	582	k_f (s^{-1})
Acetone			12.0 ± 0.3	11.7		(5.4 ± 0.5) * 10^7
Butanol		12.7	12.6 ± 0.3	12.6	12.7	(4.1 ± 0.3) * 10^7
Chloroform			12.0 ± 0.2			(6.0 ± 0.2) * 10^7
Cyclopentanone	10.9	10.6	11.5 ± 0.2	11.0	11.9	(3.7 ± 0.2) * 10^7
Dimethylformamide	12.3	12.2	12.5 ± 0.1	12.5	12.7	(4.8 ± 0.3) * 10^7

[a]Typical τ errors are $\pm(0.1 \div 0.3)$ ns (not shown except for the error at 528 nm.) MBA concentration was 10–25 μM. Excitation at 425 nm.

$$C_f = \frac{F(0, \lambda)}{F_S(0, \lambda)} * \frac{[\Phi_S(\lambda)/S\Phi_S]}{[\Phi(\lambda)/S\Phi]} * \frac{(k_f)_S}{k_f} * \frac{\varepsilon_S}{\varepsilon} * C_S \quad (15)$$

In this case, it is not necessary to know the instrument parameter $B(\lambda)$, the radiative constants k_f and $(k_f)_S$, extinctions ε and ε_S because the ratios $[(k_f)_S/k_f]$ and $[\varepsilon_S/\varepsilon]$ are sufficient. As a first approximation, these ratios may be assumed to be 1. Then the measurement of the decay amplitudes $F(0, \lambda)$ and $F_S(0, \lambda)$ at a *single* wavelength allows us to calculate the unknown concentration C_f of actually fluorescent (not fully quenched) molecules in biological samples.

The approach can be also used for complicated situations: one kind of fluorescent molecule is distributed among sites with different molecule environments, which leads to a complex character of summary fluorescence. At any wavelength λ each population (index i) has its own amplitude $F_i(0, \lambda)$ and decay time τ_i:

$$F(t, \lambda) = F_1(0, \lambda) * \exp(-t/\tau_1) + \ldots + F_i(0, \lambda) * \exp(-t/\tau_i) + \ldots \quad (16)$$

If $[(k_f)_S/k_f]$ and $[\varepsilon_S/\varepsilon]$ are the same in any population, then

$$C_i = \frac{F_i(0, \lambda)}{F_S(0, \lambda)} * \frac{[\Phi_S(\lambda)/S\Phi_S]}{[\Phi_i(\lambda)/S\Phi_i]} * \frac{(k_f)_S}{(k_f)_i} * \frac{\varepsilon_S}{\varepsilon_i} * C_S \quad (17)$$

Further, if the ratio $[\Phi_i(\lambda)/S\Phi_i]$ is the same for any population, then each fractional concentration C_i can be calculated very simply:

$$C_i \approx \frac{F_i(0, \lambda)}{F_S(0, \lambda)} * \frac{[\Phi_S(\lambda)/S\Phi_S]}{[\Phi(\lambda)/S\phi]} * C_S \quad \text{at} \quad [(k_f)_S/k_f] = 1 \quad \text{and} \quad [\varepsilon_S/\varepsilon] = 1 \quad (18)$$

where the ratio $[\Phi(\lambda)/S\Phi]$ can be taken from the whole steady-state fluorescence spectrum of the mixture. Moreover, if $\Phi(\lambda)$ is close to the standard spectrum $\Phi_S(\lambda)$, then

$$C_i \approx \frac{F_i(0, \lambda)}{F_S(0, \lambda)} * C_S \quad (19)$$

But, perhaps, each population has its own spectrum $\Phi_i(\lambda)$, which is rather far from the spectra of other populations and from $\Phi_S(\lambda)$. In this case individual spectra $\Phi_i(\lambda)$ can be calculated after time-resolving experiments as the spectra of individual decay amplitudes $F_i(0, \lambda)$ (the so-called DAS; decay-associated spectrum [5]), corrected with a wavelength-dependent parameter $b2(\lambda)$ of the instrument used:

$$\Phi_i(\lambda)/S\Phi_i = [1/b2(\lambda)] * [F_i(0, \lambda)/SF_i(0)] \quad (20)$$

where $SF_i(0)$ is the corrected integral

$$SF_i(0) = \int_{\lambda_1}^{\lambda_2} [1/b2(\lambda)] * F_i(0, \lambda) * d\lambda \quad (21)$$

The total concentration of all fluorescence-emitting molecules is $C_f = C_1 + C_2 + \ldots$.

This approach was used to estimate the fractional concentrations of the fluorescent species of the membrane fluorescent probe MBA in lipid bilayers.

Fluorescent Probe MBA in Organic Solvents

MBA was dissolved in a set of organic solvents where its fluorescence had monoexponential decay. The decay time in those solvents was independent of the fluorescence wavelength within measurement errors (Table I).

As can be seen, the actual k_f values are rather variable, but their deviation from the mean value $\langle k_f \rangle = 4.8*10^7$ s^{-1} does not exceed ±25%. Thus, the ratio $[(k_f)_S/k_f]$ in Eq. (15) is close to 1 (with an error of <25%) for MBA fluorescence in any pair of the solvents. The ratio $[\varepsilon_S/\varepsilon]$ at 425 nm is about 1.0 too, and the maximal deviation is ±9% (not shown).

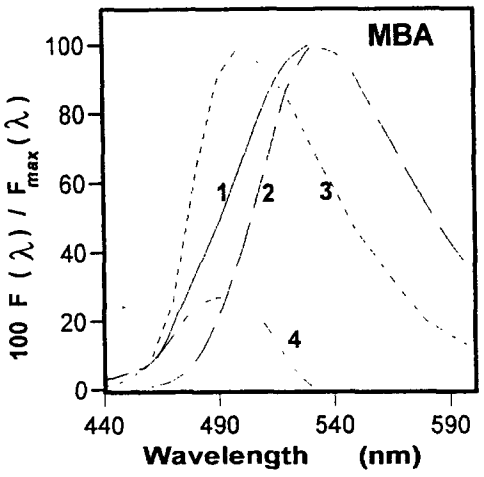

Fig. 1. MBA fluorescence spectra in lipid membranes (1), butanol (2), and chloroform (3) and the difference between curve 1 and curve 2 (4). Fluorescence intensity is normalized to the maximal one (curves 1–3). Concentrations: MBA—10 μ*M*; phosphatidylcholine liposomes—2.0 g/L, in 0.14 *M* NaCl–0.01 *M* Tris·HCl, pH 7.3. Excitation at 425 nm.

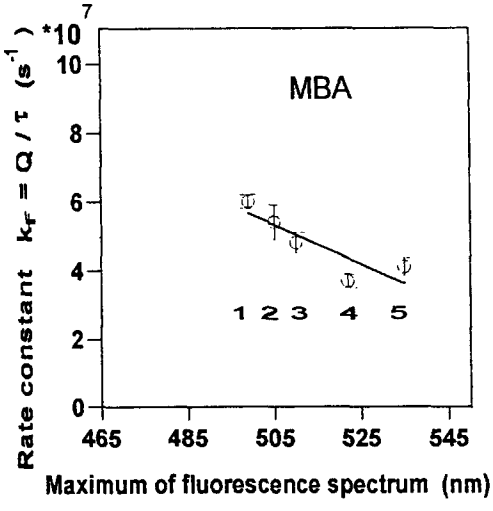

Fig. 2. Spectral maximum and experimental radiative rate constant k_f = (Q/τ) of MBA fluorescence in organic solvents: (1) chloroform, (2) acetone, (3) dimethylformamide, (4) cyclopentanone, and (5) butanol. Conditions are the same as in the legend to Fig. 1.

Thus, there are reasons to suppose that MBA behavior in various media can be described on the base of Eq. (15) with a probable error of the calculated concentration, C_f, less than several tenths percent.

Einstein's theory of radiative transition rates shows their strong dependence on transition wavelength. The Einstein–Strickler–Berg equation for k_f [25,26] shows that

$$(k_f)_S = \text{const}1/(\lambda_S)^a, \quad \text{where} \quad a = 3 \quad (22)$$

Our results are in accordance with this idea (Fig. 2): the lower λ_S, the higher k_f. It seems that in the case of MBA this dependence is more strong ($a > 3$), but this discrepancy can be insignificant because of some variations of Q/τ values in different solvents.

The Fluorescent Probe MBA in Artificial Lipid Membranes

MBA fluorescence spectra in lipid membranes are presented in Fig. 1 (curve 1). In contrast to organic solvents, MBA fluorescence decay in these membranes can be described as two components—with long and short decay times (Table II):

$$F(t, \lambda) = F_1(0, \lambda) * \exp(-t/\tau_1) + F_2(0, \lambda) * \exp(-t/\tau_2) \quad (23)$$

The same decay times at any wavelength, in spite of very large differences in the amplitude ratio, allow us to assume that there are two fluorescent species of MBA molecules in these membranes. Evaluation of the partial yields of the first and second components on the basis of the data in Table II (by summation of amplitudes multiplied by decay times at all wavelengths) shows that the first one is responsible for about 80% and the second one for 20% of the total fluorescence:

$$F1 = \text{const} * \sum_{\lambda} \tau_1 * F_1(0, \lambda); \quad F_2 = \text{const} * \sum_{\lambda} \tau_2 * F_2(0, \lambda) \quad (24)$$

$$FT = F1 + F2; \quad F1/FT = 0.8; \quad F2/FT = 0.2 \quad (25)$$

Now fractional concentrations of these species can be estimated. MBA in butanol was used as a reference solution (standard). The fractional fluorescence spectrum $\Phi_1(\lambda)$ was calculated with Eqs. (20) and (21) using decay-associated spectra $F_1(0, \lambda)$ and $F_S(0, \lambda)$ from Table II. A relative fraction of the species is $C_1/CT = 0.51 \pm 0.16$, where CT is the total concentration of all membraneous (all added) MBA. The result is independent of wavelength λ in Eq. (20) in the wide range of 503 to 582 nm.

The same procedure could be used for the second species of MBA molecules. If $\lambda = 528$ nm, then $C_2/CT \approx 0.87$. If $\lambda = 503$ nm, then $C_2/CT \approx 1$. In both cases the sum $C_1 + C_2$ is significantly larger than the total concentration CT. It is clear that some sources of this discrepancy must exist.

Perhaps the main cause of the discrepancy is the blue shift of the short-life species spectrum relative to

Table II. Decay Time (τ; ns) and Corrected Amplitudes ([1/*b*2 (λ)] * F_i (0, λ); Arbitrary Units) of Two Decay Components of MBA Fluorescence in Lipid Membranes and an Organic Solvent (Butanol)[a]

	Wavelength (λ) (nm)				
	479	503	528	553	582
MBA in butanol					
τ_S	—	12.7	12.6	12.6	12.7
$F_S(0, \lambda)$	0	53 ± 5	100	102 ± 3	65 ± 4
MBA in membranes					
τ_1	9.8	12.0	12.3	12.1	12.4
$F_1(0, \lambda)$	20 ± 1	61 ± 8	100	111 ± 13	65 ± 11
τ_2	2.0	1.8	2.1	—	—
$F_2(0, \lambda)$	143 ± 19	200 ± 40	170	0	0

[a]Amplitudes at current wavelengths are normalized to the amplitude of the first decay component measured at 528 nm both in butanol and membranes. These relative values are expressed as percentages. Typical τ errors are ±(0.1 ÷ 0.3); ns (not shown). MBA concentration was 10–25 μM. Excitation at 425 nm.

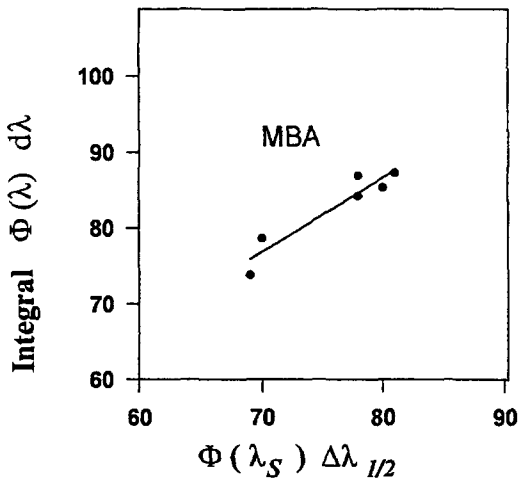

Fig. 3. Relationship between the integral $S\phi_S$ and the value ϕ (λ_S) ($\Delta\lambda_{1/2})_S$, where λ_S is the spectral maximum and ($\Delta\lambda_{1/2})_S$ is the half-width of MBA steady-state fluorescence spectra in organic solvents. Conditions are the same as in the legend to Fig. 1.

the long-life one (Table II). This is a very frequent event [1,2,5]. In our case, their spectral maxima are λ_1 = 540 nm and $\lambda_2 \approx$ 500 nm. The steady-state spectra are in accordance with these data: there exists a difference between the MBA spectra in membranes and butanol (Fig. 1, curve 4), which has a maximum close to 490 nm and presents about 17% of the total spectrum.

This blue shift gives rise to two problems in the practical use of Eq. (17):

(1) The radiative rate k_f is a function of emission wavelength. This problem is not appreciable for the first MBA species: the amplitude spectrum $F_1(0, \lambda)$ and its maximum λ_1 in membranes are very close to the amplitude spectrum $F_S(0, \lambda)$ and its maximum (λ_S) in butanol. Therefore, butanol was used as a reference (standard) medium to calculate the fractional concentration C_1. But for the second species, a significant difference between $(k_f)_2$ and $(k_f)_S$ can exist, in contradiction with condition (18).

(2) The second problem is the value $\Phi_2(\lambda)/S\Phi_2$. It cannot be measured correctly because the decay-associated spectrum $F_2(0, \lambda)$ of this minor fluorescence component is lost on the right edge of the spectrum (>540 nm), where its part in the total fluorescence is very small. But in organic solutions, a tight relationship among the spectral maximum (λ_S), the half-width $(\Delta\lambda_{1/2})_S$, and the integral $S\Phi_S$ is expected to exist (Figs. 3 and 4) (a very frequent case). As a result, the value $F_2(0, \lambda)$ can be measured at λ close to the maximum λ_2, and other parameters can be calculated:

$$(\Delta\lambda_{1/2})_S \approx \text{const2} * \lambda_S; \quad \text{const2} \approx 0.15 \quad \text{(see Fig. 4)} \quad (26)$$

$$S\Phi_S \approx \text{const3} * \Phi_S(\lambda_2) * (\Delta\lambda_{1/2})_S; \quad \text{const3} \approx 1.09 \quad \text{(see Fig. 3)} \quad (27)$$

Then

$$[\Phi_S(\lambda_2)/S\Phi_S] \approx (1/1.09)\,(1/\Delta\lambda_{1/2})_S \approx \text{const4} * (1/\lambda_S); \quad \text{const4}=6.1 \quad (28)$$

[These constants are not significant for our goals, because only relative values are used in the final equation (29).] The same can be related to each species of MBA molecules in membranes. Then Eq. (17) can be rewritten

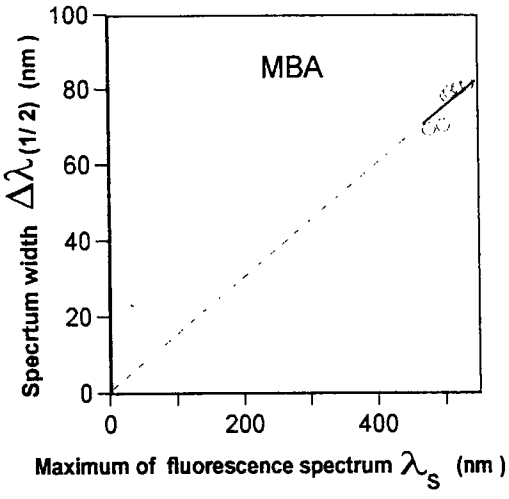

Fig. 4. The width (on the level of half-height) and the position of the center of MBA steady-state fluorescence spectra in organic solvents. Conditions are the same as in the legend to Fig. 1.

so that only the amplitude $F_i(0, \lambda_i)$ in the spectral maximum (λ_i) of any decay species is used:

$$C_i \approx \frac{F_i(0, \lambda_i)}{F_S(0, \lambda_S)} * \frac{B(\lambda_S)}{B(\lambda_i)} * \frac{1/\lambda_S}{1/\lambda_i} * \frac{1/(\lambda_S)^3}{1/(\lambda_i)^3} * \frac{\varepsilon_S}{\varepsilon} *$$

$$C_S \approx \frac{F_i(0, \lambda_i)}{F_S(0, \lambda_S)} * \frac{B(\lambda_S)}{B(\lambda_i)} * \frac{(\lambda_i)^4}{(\lambda_S)^4} * \frac{\varepsilon_S}{\varepsilon} * C_S \qquad (29)$$

where the ratio of the correction coefficients $B(\lambda)$ at wavelengths of two maxima (of the species and the standard) have to be taken into account.

After these corrections, the calculated relative concentration of the second MBA species in membranes is $C_2/CT = 0.65 \pm 0.19$. The sum of the first and second species is $(C_1 + C_2)/CT = 0.51 + 0.65 = 1.16 \pm 0.27$. Thus, about 100% of the membrane-bound MBA molecules are found to be fluorescent.

The Strickler–Berg equation (22) does not coincide fully with the MBA data (Fig. 2). Therefore, another method can be used to increase the accuracy of the C_2 estimation. We can calculate C_2 in a way similar to C_1 (see above), but taking another solvent (chloroform; Fig. 1, curve 3) as a standard for C_2. In this case the simplest Eq. (18) is used, because both rate constants can be considered as the same. This leads to $C_2/CT = 0.56 \pm 0.21$, and the sum $(C_1 + C_2)/CT = 0.51 + 0.56 = 1.07 \pm 0.27$. The fraction of actually fluorescent MBA molecules is again about 100%.

Now the fractional quantum yields can be calculated, and the mean yield $\langle Q \rangle$ can be compared with the steady-state one:

$\alpha_1 = C_1/(C_1 + C_2) = 0.46$; $\alpha_2 = C_2/(C_1 + C_2) = 0.54$;
$Q_1 = (k_f)_1 \tau_1 \approx (k_f)_S \tau_1 = 4.1 * 10^7 * 12.6 * 10^{-9} = 0.51$;
$Q_2 = (k_f)_2 \tau_2 \approx 0.11$; $\langle Q \rangle \approx \alpha_1 Q_1 + \alpha_2 Q_2 = 0.46 * 0.51 + 0.54 * 0.11 = 0.30$

(We do not discuss here the errors of these Q values, regarding them as approximate values.) In the calculation of the mean value $\langle Q \rangle$ it was assumed that all species of membrane-bound MBA molecules had equal molar absorptivities (extinction ε). This is not so obvious in the general case [2]. But the experimentally measured mean quantum yield in steady-state experiments (where the mean actual absorption was determined) was $Q = 0.33 \pm 0.04$, which is very close to the calculated $\langle Q \rangle$. Thus, this fact can be considered as evidence of small variations of the probe absorptivity in different areas of these membranes, in contrast to the fluorescent properties.

DISCUSSION

Thus, in biological objects, where the fluorescence of a probe has a complex decay, the amplitudes of the decay components can be used to estimate the amount of fluorescent molecules. The decay can be measured at a single wavelength but more correct results were obtained on the basis of the whole decay-associated spectrum.

In the case studied, the fluorescent probe MBA had a complex decay in phospholipid membranes. The decay can be described as two exponential. The independence of fractional decay times from the emission wavelength allowed us to consider the components as two species of probe molecules. The analysis of their amplitudes gives about 100% recovery of fluorescent molecules.

These results were in accordance with the steady-state data. Thus the mean calculated quantum yield of the decay-measured components after the amplitude analysis was very close to the steady-state one.

There was a great difference in the behavior of MBA fluorescence in organic solvents (simple decay) and in membranes (complex decay). However, it is well-known that fluorescent probes can be localized in different parts of biological objects, and the complex character of their fluorescence is due to the heterogeneous environment. The long-life component of MBA in membranes has a decay time as well as spectral maximum close to those in a polar organic solvent (butanol). Both these parameters of the other, short-life, component can be attributed to nonpolar hydrocarbon media (for

example, MBA has a decay time of 1.8 ns and an emission maximum at 480 nm in toluene). It can be assumed that the first kind of MBA fluorescence may be attributed to molecules located near the polar lipid–water interface, where, perhaps, MBA molecules can interact with water and/or lipid polar groups. The second kind of MBA fluorescence, perhaps, is related to the probe molecules immersed in the hydrocarbon interior of the lipid bilayer, where MBA is inaccessible to polar molecules.

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