EFFECT OF REABSORPTION ON THE CONCENTRATION DEPENDENCE OF FLUORESCENCE LIFETIMES OF CHLOROPHYLL A

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Summary

The concentration dependence of mean fluorescence lifetimes of chlorophyll a in methanol was studied in the concentration interval 3×10^{-6} - 7.3×10^{-5} mol l^{-1} at room temperature. The values found experimentally were corrected for reabsorption of fluorescence using the average molar absorptivity $\bar{\epsilon}_F$ derived from spectral properties of overlapping fluorescence and absorption spectra of chlorophyll a.

1. Introduction

For a study of radiationless excitation energy transfer in chlorophyll a solutions using measurement of the mean fluorescence lifetimes it is necessary to correct the values of τ_{exp} determined experimentally for reabsorption of fluorescence. As there is a relatively small Stokes shift ($\Delta\nu$ = 216 cm^{-1}) between the main fluorescence band (λ_F = 675 nm) and the absorption band (λ_A = 665.7 nm) of chlorophyll a in methanol [1, 2], it is possible that the short-wavelength part of the fluorescent light will be effectively reabsorbed. Furthermore, the fluorescent radiation of the shortest wavelength, the intensity of which is minimum, has a high molar absorptivity in the overlapping spectral region.

Let us consider that the light incident on the cuvette containing the chlorophyll a solution has the same spectrum as the fluorescence of chlorophyll a. The total incident radiation energy within the wavenumber interval between ν_1 and ν_2 (the region of overlap of the absorption and fluorescence spectra) is given by

$$F_0 = \int_{\nu_1}^{\nu_2} I_0(\nu)\, d\nu \tag{1}$$

where $I_0(\nu)$ is the intensity of the incident light with wavenumber ν. The

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total energy F of the light transmitted through a layer of thickness d of the chlorophyll a solution with concentration C is lower because of absorption (eqn. (2)):

$$F = \int_{\nu_1}^{\nu_2} I_0(\nu) \exp\{-\epsilon(\nu)Cd\}\, d\nu \tag{2}$$

where $\epsilon(\nu)$ is the molar absorptivity of chlorophyll at wavenumber ν. The average molar absorptivity for reabsorption of fluorescence, which is constant for the whole region of overlap of the spectra, can be defined by eqn. (3) by analogy with the experimental method of Rohatgi and Singhal [3]:

$$\bar{\epsilon}_F = \frac{1}{Cd} \ln\left(\frac{F_0}{F}\right) \tag{3}$$

The average molar absorptivity $\bar{\epsilon}_F$ was derived [4] for the chlorophyll a fluorescence reabsorption assuming a gaussian shape for the overlapping fluorescence and absorption bands which have mirror symmetry. This average molar absorptivity for reabsorption of fluorescence is a decreasing function of the product of the solution concentration and the thickness of the absorbing layer through which the fluorescent radiation passes. To correct the mean fluorescence lifetimes we can use the relations derived for correction of the quantum yield for the effect of reabsorption [3, 5] and the known relations between the quantum yield and the lifetime of fluorescence [6]. In the absence of reabsorption and re-emission phenomena the intensity I of fluorescence reaching the detecting system is given by

$$I = K_{\text{reabs}} I_{\text{exp}} \tag{4}$$

where I_{exp} is the intensity observed in the presence of reabsorption phenomena. The correction factor K_{reabs} for the overlap of absorption and emission has been derived by Rohatgi and Singhal [5]:

$$K_{\text{reabs}} = \frac{\epsilon + \bar{\epsilon}_F}{\epsilon} \frac{1 - \exp(-\epsilon Cd)}{1 - \exp\{-(\epsilon + \bar{\epsilon}_F)Cd\}} \tag{5}$$

where ϵ, C and d are the chlorophyll a molar absorptivity at the excitation wavelength, the chlorophyll a concentration and the layer thickness for fluorescence absorption (d is the cell thickness in our experimental set-up) respectively.

Values of the quantum yields and the fluorescence lifetimes in the presence (τ_{exp}, ϕ_{exp}) and in the absence (τ, ϕ) of reabsorption are related by

$$\frac{\tau_{\text{exp}}}{\tau} = \frac{\phi_{\text{exp}}}{\phi} \tag{6}$$

On the basis of all these relations it is possible to correct the values of τ_{exp} found experimentally using the following relation:

$$\tau = \tau_{exp}/K_{reabs} \tag{7}$$

2. Experimental

Chlorophyll a solutions of concentration 3×10^{-6} - 7.3×10^{-5} mol l^{-1} in methanol were prepared under nitrogen using conditions which minimized the possibility of chlorophyll photooxidation by atmospheric oxygen during the preparation of the samples. Measurements were carried out in closed quartz cuvettes. The concentrations of the solutions were determined from absorption spectra measured using a Specord Zeiss spectrophotometer [7]. For fluorescence excitation by a nitrogen laser in the UV spectral region (λ = 337.1 nm) it was necessary to purify the methanol (p.a., for UV spectroscopy) by drying it over calcium oxide with subsequent distillation.

For the measurement of the fluorescence decay we used an experimental arrangement with a nitrogen laser (with a pulse halfwidth of less than 2 ns and a peak power of about 150 kW) and a high speed SP 102 photodiode (with a rise time of less than 2 ns) for monitoring the time dependence of the fluorescence [8]. The exciting laser beam was focused onto the front wall of the cell containing the solution. An RG1 glass filter was placed between the back cell wall and the photodiode (transmission about 90% for $\lambda > 640$ nm) for removal of the exciting radiation. The system was calibrated using a solution of Rhodamine B of concentration 10^{-5} mol l^{-1} (in a mixture of 10% methanol and 90% ethanol). The value we obtained (τ = 3.24 ± 0.02 ns) agrees very well with that (3.2 ns) reported by Knof *et al.* [9] for a temperature of 300 K. The fluorescence decay curve observed for a molecule excited by a short pulse of laser light is a convolution of the excitation pulse shape (distorted by the detecting system) with the δ-pulse response of the luminescent system. The deconvolution of the experimental decay curves of chlorophyll a fluorescence was carried out in the time interval in which the intensity of the exciting pulse was negligible. Furthermore, we assumed a specific functional form for the decay law, *i.e.* a single-exponential form:

$$I_F(t) = I(0) \exp(-t/\tau_{exp}) \tag{8}$$

Statistical tests showed that such an assumption for the fluorescence decay of chlorophyll a (of concentration 10^{-6} - 10^{-5} mol l^{-1}) in methanol solutions was justified. The mean fluorescence lifetimes τ_{exp} were estimated from the linear regression function

$$\ln\left\{\frac{I(0)}{I_F(t)}\right\} = \frac{1}{\tau_{exp}}\, t \tag{9}$$

using a least-squares method. Correlation coefficients for the linear

regression function were in the range 0.9950 - 0.9990 for all measurements. A set of 12 subsequent fluorescence decay measurements of the same sample showed high reproducibility of the measurements and sample stability.

3. Results and discussion

Fluorescence spectra of chlorophyll solutions of low concentration (where aggregation of the molecules is insignificant) are characterized at room temperature by two bands: a main band with a maximum in the range 670 - 680 nm and a long-wavelength band at 730 nm (I_{670}/I_{730} = 4.5 [2]).

The wavelength (337.1 nm) of the nitrogen laser pulse used for the excitation of the fluorescence falls within the Soret absorption band of chlorophyll a (molar absorptivity $\epsilon = 3.18 \times 10^4$ mol^{-1} l cm). Figure 1 illustrates the dependences of the average molar absorptivity $\bar{\epsilon}_F$ for reabsorption of fluorescence and the correction factor K_{reabs} calculated from expression (5) on the product Cd of the concentration and the cell thickness. The values of K_{reabs} determined in this way were used for the correction of τ_{exp} according to eqn. (6). This method of correcting τ_{exp} for reabsorption effects was tested using fluorescence lifetime measurements of chlorophyll a solutions of two concentrations in cuvettes of various thicknesses. Table 1 shows that the deviations of the corrected values for such different cuvette thickness values are very small. The concentration dependence of both the experimental and the corrected mean fluorescence lifetimes of chlorophyll a

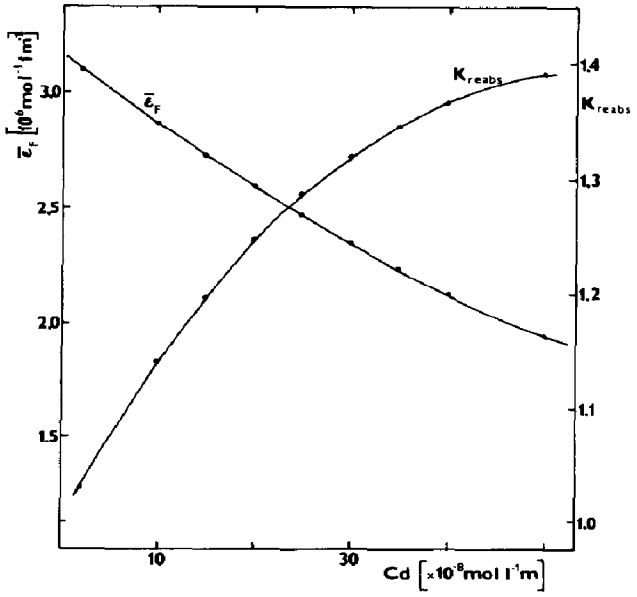

Fig. 1. The dependences of the average molar absorptivity $\bar{\epsilon}_F$ for reabsorption of fluorescence and the correction factor K_{reabs} on the product of the chlorophyll a concentration C and the layer thickness d.

TABLE 1

Test of the method of correcting fluorescence lifetimes of chlorophyll a for the reabsorption effect

Concentration (mol l^{-1})	*d = 0.198 cm*		*d = 0.491 cm*		*d = 0.998 cm*		τ (ns)
	τ_{exp} (ns)	τ (ns)	τ_{exp} (ns)	τ (ns)	τ_{exp} (ns)	τ (ns)	
1.22×10^{-5}	6.12	5.89	6.41	5.88	6.90	5.92	5.897
2.16×10^{-5}	6.19	5.81	6.67	5.81	7.30	5.79	5.803

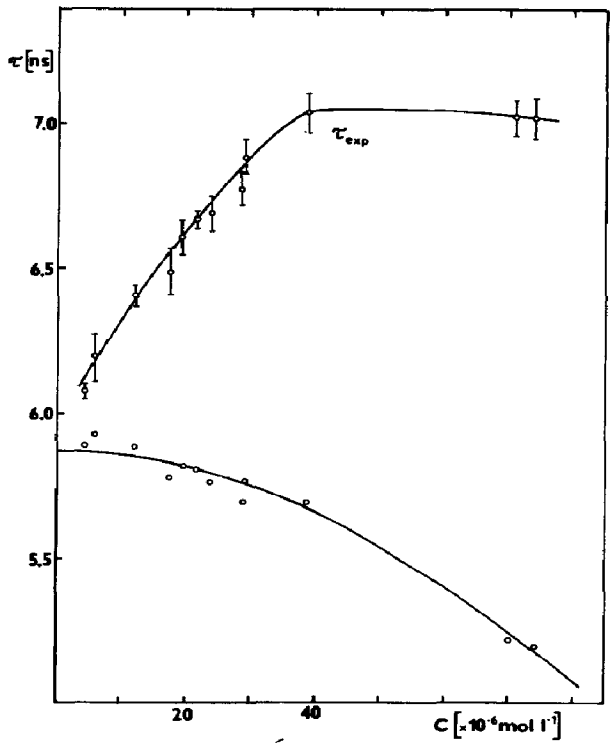

Fig. 2. The concentration dependence of experimental (τ_{exp}) and corrected (τ) mean fluorescence lifetimes of chlorophyll a in methanol solution.

is illustrated in Fig. 2. The values of τ_{exp} are influenced by two factors which have opposing effects. The process of reabsorption of fluorescence results in an increase in the lifetime; this effect is predominant at concentrations lower than 4×10^{-5} mol l^{-1}, as shown by the marked increase in τ_{exp} in this region. With increasing concentration, however, the probability of radiationless energy transfer from the primary sites of excitation will increase, resulting in a decrease in the mean fluorescence lifetime as shown by the decreasing concentration dependence for the corrected values τ. For chlorophyll a molecules, the excited singlet states of which are weakly coupled [10], the excitation is transferred via the Förster resonance mecha-

nism [11]. The Förster transfer rate is directly proportional to R^{-6}, where R represents the mean intermolecular distance, and concentration quenching of this type depends on the second power of the concentration. The mechanism proposed for this type of concentration quenching is energy migration by a Förster-type resonance transfer between like molecules, followed by trapping at a pair of molecules, *i.e.* at a statistical pair trap [12]. The statistical pair is any two chlorophyll molecules which do not interact appreciably in their ground states but interact strongly when one of the pair becomes excited. At low concentrations, where the mean distance between molecules is large, the energy migration is slow, the trap concentration is low and we observe little quenching. Figure 2 shows that the corrected values τ may be fitted to an empirical equation of the form [12, 13]

$$\tau = \frac{\tau_0}{1 + kC^2} \tag{10}$$

with $\tau_0 = 5.872$ ns and $k = 4.15 \times 10^6$ mol^{-2} l^2.

The values reported for the fluorescence lifetimes of chlorophyll solutions (10^{-6} - 10^{-5} mol l^{-1}) in diethyl ether or ethanol vary between 4.9 and 7.8 ns [14, 15]. The decisive majority of these experimental values of τ were not corrected for the reabsorption effect, although in the experimental arrangement described the effect of reabsorption must have affected the results of the measurement. The value (τ = 6.8 ns) for the fluorescence lifetime of chlorophyll a solutions in diethyl ether ($C = 4 \times 10^{-5}$ mol l^{-1}; T = 300 K) given by Avarmaa *et al.* [15] is in good agreement with our experimental values (see Fig. 2). Compared with other correction methods involving complicated mathematical correction factors [16], our procedure for correcting the mean fluorescence lifetimes (or quantum yields) using the average molar absorptivity $\bar{\epsilon}_F$ enables rapid evaluation of experimental results. Spectral properties of chlorophyll a in a number of polar solvents (10^{-6} - 10^{-5} mol l^{-1}), *e.g.* almost perfect mirror symmetry of the spectra and not very large differences in the halfwidths and Stokes shifts of the fluorescence and absorption bands [1, 2], allow the values of $\bar{\epsilon}_F$ and K_{reabs} derived for methanol solutions to be used for other solvents.

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References

1 M. Kaplanová, *Sb. Věd. Pr., Vys. Sk. Chemickotechnol., Pardubice, 40* (1979) 39.
2 K. Vacek, E. Vavřinec and M. Kaplanová, *Acta Univ. Carol., Math. Phys., 17* (1976) 45.

3 K. K. Rohatgi and G. S. Singhal, *Anal. Chem., 34* (1962) 1702.
4 M. Kaplanová, *Sb. Věd. Pr., Vys. Sk. Chemickotechnol., Pardubice, 43* (1980) 33.
5 K. K. Rohatgi and G. S. Singhal, *Photochem. Photobiol., 7* (1968) 361.
6 R. S. Becker, *Theory and Interpretation of Fluorescence and Phosphorescence*, Wiley, New York, 1969.
7 G. R. Seely and R. G. Jensen, *Spectrochim. Acta, 21* (1965) 1835.
8 K. Čermák and M. Kaplanová, *Czech. J. Phys. B, 30* (1980) 713.
9 J. Knof, F. J. Theiss and J. Weber, *Z. Naturforsch., Teil A, 33* (1978) 98.
10 L. L. Shipman and D. L. Housman, *Photochem. Photobiol., 29* (1979) 1163.
11 T. Förster, *Discuss. Faraday Soc., 27* (1959) 7.
12 G. S. Beddard, S. E. Carlin and G. Porter, *Chem. Phys. Lett., 43* (1976) 27.
13 J. Dalton, *J. Chem. Soc., Chem. Commun., 3* (1980) 78.
14 G. P. Gurinovich, A. N. Sevchenko and K. N. Solov'ev, *Spectroscopy of Chlorophyll and Related Compounds*, Nauka i Tekhn., Minsk, 1968.
15 R. Avarmaa, T. Soovik, R. Tamkivi and V. Tonisoo, *Stud. Biophys., 66* (1979) 213.
16 A. Budo and I. Ketskemety, *J. Chem. Phys., 25* (1961) 595.