EFFECT OF REABSORPTION ON THE FLUORESCENCE SPECTRA OF CHLOROPHYLL-*a*

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Received October 14th, 1982

Simple formula for the reabsorption correction was tested on the fluorescence spectra of thin polystyrene foils with built-in chlorophyll-*a*. The spectra were studied at various pigment concentrations, wavelengths and angles of incidence of the exciting radiation. It is shown that neglecting the reabsorption effect in dependence of the experimental arrangement can lead to a strong distorsion of the fluorescence spectrum. The derived formula yields a good qualitative picture of the fluorescence spectra.

Depending on the experimental arrangement, the fluorescence spectra are affected by secondary phenomena originating in the overlap of the fluorescence and absorption spectra. Among them is very important the reabsorption (for a review see ref.[1]). The attempts to correct the experiment provide either a constant useful for the correction of the experimental quantum yield or fluorescence lifetime only or general, complicated integral – differential expressions difficult to use in the experimental practice.

The aim of this work was to find the effect of reabsorption on the fluorescence spectra of chlorophyll-*a* built-in the solid foil in the experimental arrangement for transmission or reflection and to correct the experimental spectra by means of a simple formula derived for the particular experimental arrangement.

THEORETICAL

Starting point for the derivation of a correction formula for reabsorption is the experimental arrangement depicted on the Fig. 1*a* in which exciting and registrated beams are mutualy perpendicular. Sample has a refractivity index n and the shape of a thin foil of thickness d. It lies in the plane perpendicular to the plane made by exciting and emission beams. The sample plane makes an angle α with the exciting beam.

Exciting radiation having an intensity I_e (W/m^2) and wavelength λ_e is attenuated

after the passage through the sample to the distance x so that the volume $\mathrm{d}x\,\mathrm{d}y\,\mathrm{d}z$ absorbs $\mathrm{d}N_a$ quanta per second

$$\mathrm{d}N_a = (\lambda_e/\hbar c)\, I_e C k_e \exp(-k_e C x)\,\mathrm{d}x\,\mathrm{d}y\,\mathrm{d}z\,, \qquad (1)$$

where C is the pigment concentration and k_e is the molar absorption coefficient at the wavelength λ_e. The number of fluorescence quanta $\mathrm{d}N_F$ emitted by this element (for the unit wavelength interval $\mathrm{d}\lambda_F = 1$) is

$$\mathrm{d}N_F(\lambda_F) = \Phi_F f(\lambda_F)\,\mathrm{d}N_a\,, \qquad (2)$$

where Φ_F is the fluorescence quantum yield and $f(\lambda_F)$ is the molar fluorescence spectrum for which holds

$$\int_{\text{flurescence band}} f(\lambda_F)\,\mathrm{d}\lambda_F = 1\,.$$

Part of the emitted radiation is reabsorbed during the passage through the sample so that for the registered number of quanta in the perpendicular arrangement according to Fig. 1*a* we obtain

$$\mathrm{d}N_F^R(\lambda_F) = K\,\mathrm{d}N_F(\lambda_F(\exp[-k_F(\lambda_F)\,Cx\sqrt{(n^2 - \cos^2\alpha)}/\sqrt{(n^2 - \sin^2\alpha)}]\,, \qquad (3)$$

where $k_F(\lambda_F)$ is the molar absorption coefficient for the wavelength of emission λ_F. The refractivity index n is generally dependent on the wavelength but the dependence is neglected in this approximation. The geometric factors determining the measured part of fluorescence are contained in the coefficient K.

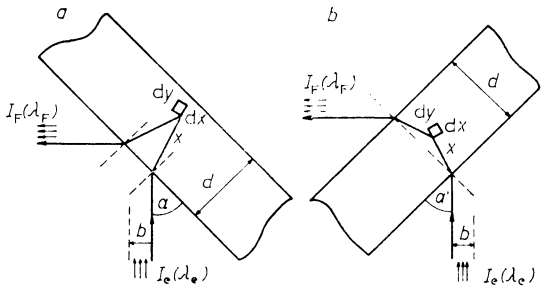

FIG. 1

Paths of the exciting and the emitted beams through the sample *a*) in the reflection arrangement *b*) in the transmission arrangement

Assuming the homogeneous excitation in the y and z directions, we obtain upon integration for the measured fluorescence intensity the expression (*4*) where b and v are beam width and the beam height:

$$I_R(\lambda_F) = (\lambda_e/\lambda_F)\, K\Phi_F f(\lambda_F)\, I_e k_e R(\lambda_e, \lambda_F, C, \alpha)\, bv/n \sin\alpha\,, \tag{4}$$

where

$$R(\lambda_e, \lambda_F, C, \alpha) = [1 - \exp\{-Cnd[k_e/\sqrt{(n^2 - \cos^2\alpha)} + k_F(\lambda_F)/\sqrt{(n^2 - \sin^2\alpha)}]\}]\,.$$
$$.\, k_e/\sqrt{(n^2 - \cos^2\alpha)} + k_F(\lambda_F)/\sqrt{(n^2 - \sin^2\alpha)}]^{-1}\,. \tag{5}$$

The spectral distribution of the molecular fluorescence intensity $F_R(\lambda_F)$ in the reflection arrangement is

$$F_R(\lambda_F) = f(\lambda_F)\, hc/\lambda_F\,. \tag{6}$$

Substituting from (*4*) and (*5*) we obtain

$$F_R(\lambda_F) = L(\alpha, \lambda_e)\, I_R(\lambda_F)/R(\lambda_e, \lambda_F, C, \alpha)\,, \tag{7}$$

where

$$L(\alpha, \lambda_e) = (n \sin\alpha\, hc)/(K\Phi_F I_e \lambda_e k_e bv) \tag{8}$$

is the coefficient dependent on the excitation wavelength λ_e and on the angle α. However, this coefficient does not affect the shape of the corrected spectrum.

For the transmission arrangement (Fig. 1*b*) where α ($= 180° - \alpha'$) is larger than 90°,we obtain in a similar way the expression (*9*) for the measured intensity of fluorescence:

$$I_R(\lambda_F) = (\lambda_e/\lambda_F)\, K\Phi f(\lambda_F)\, I_e k_e\, R'(\lambda_e, \lambda_F, C, \alpha')\, bv/n \sin\alpha'\,, \tag{9}$$

where

$$R'(\lambda_e, \lambda_F, C, \alpha') = [1 - \exp\{MCnd[k_e/\sqrt{(n^2 - \cos^2\alpha')} -$$
$$- k_F(\lambda_F)/\sqrt{(n^2 - \sin^2\alpha')}]\} \exp[Mk_F(\lambda_F)\, Cnd/\sqrt{(n^2 - \sin^2\alpha')}]\,.$$
$$.\, [k_e/\sqrt{(n^2 - \cos^2\alpha')} - k_F/\sqrt{(n^2 - \sin^2\alpha')}]^{-1} \tag{10}$$

in which $\alpha' = 180° - \alpha$ is the deflection angle (Fig. 1*b*). The molecular spectrum of fluorescence $F_T(\lambda_F)$ is then

$$F_T(\lambda_e) = L(\alpha', \lambda_e)\, I_T(\lambda_F)/R'(\lambda_e, \lambda_F, C, \alpha')\,. \tag{11}$$

To describe the magnitude of the reabsorption effect on the spectral shape, it is useful to introduce the quantity $w(\lambda_F)$ as the ratio of the spectral distribution of the fluores-

cence intensity $I_R(\lambda_F)$ or $I_T(\lambda_F)$, respectively, and the same function $I_R^0(\lambda_F)$ or $I_T^0(\lambda_F)$, respectively, obtained by solving the relation (*4*) or (*9*), respectively, for the case of an absorption coefficient $k_F(\lambda_F)$ equal to zero. Substituting from (*4*) or (*9*), respectively, we obtain

$$w(\lambda_F) = R_0(\lambda_e, \lambda_F, C, \alpha)/R(\lambda_e, \lambda_F, C, \alpha) \qquad (12)$$

and

$$w'(\alpha_F) = R'_0(\lambda_e, \lambda_F, C, \alpha')/R'(\lambda_e, \lambda_F, C, \alpha') \qquad (13)$$

respectively, where R_0 and R'_0 are the values of the expressions (*5*) and (*10*), respectively, for $k_F(\lambda_F) = 0$. The function $w(\lambda_F)$ has the values greater than one with increasing reabsorption.

EXPERIMENTAL

Samples of chlorophyll-*a* (m.w. = 893·6) were prepared as amorphous solid solutions in polystyrene having the form of thin foils. Chlorophyll-*a* was obtained chromatographically[2] and was incorporated into polystyrene as already described[3]. The absorption spectra were measured at 185 K on a Specord UV-VIS (Zeiss, Jena) spectrophotometer equipped by a cryostat. The fluorescence spectra at 185 K were measured using the equipment already described[4]. Maximal pulse power of the exciting dye laser was 80—100 kW with the pulse duration of 2 ns. The spectral width of the exciting radiation was 0·1 nm. Sample luminescence was measured at 90° angle with respect to the exciting beam. The angle between the exciting beam and the surface of the sample was set up with an accuracy of 1°. The spectra were digitized for further work up in 15 cm^{-1} steps.

RESULTS AND DISCUSSION

Effect of reabsorption of the fluorescence spectra was studied on samples of chlorophyll-*a* (concentration 0·16 mM, 1·5 mM, and 9 mM) in polystyrene matrix (foil thickness 110 μm, 60 μm, and 80 μm) using the excitation wavelength 650, 621, and 434 nm. Fluorescence spectrum was recorded in the reflection arrangement (Fig. 1*a*, angles 60°, 30°) and in the transmission arrangement (Fig. 1*b*, angles 120° and 150°) for each sample and each wavelength.

Reabsorption causes the largest decrease in intensity of fluorescence in the region of maximal overlap of the absorption and fluorescence spectra. That leads in the case of chlorophyll-*a* to a decrease in the fluorescence intensity at the short-wavelength maximum, eventualy to its apparent shift to the longer wavelengths.The effects of reabsorption is one order of magnitude smaller for the long-wavelength maximum. Therefore, the intensity ratio of these bands can be used as an approximate measure of the reabsorption.

The variation of the reabsorption effect was realised in this work by changing the angle between the direction of the exciting beam and the plane of the sample or by the variation of wavelength of the exciting radiation (the different absorption coefficients). Correction formulas (*7*) and (*11*) can be considered as suitable if their application corrects different experimental shapes of the fluorescence spectra for different incidence angles to the same shape. With an additional assumption that the fluorescence spectrum is independent on the wavelength of excitation, it is desirable for a correction to provide the same shape in the whole series of measurements with varying angles and excitation wavelengths. Moreover, if the Beer's law holds, the corrections (*7*) and (*11*) are functions of concentration. Otherwise the concentration is a parameter only.

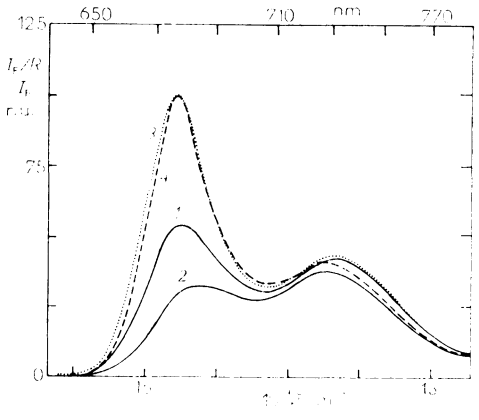

FIG. 2

Experimental spectra of polystyrene foils with built-in chlorophyll-*a*, concentration 0·16 mM and 1·5 mM (full line). The corrected spectra (dashed line) are within 2% identical in all cases. Corrected spectra were normalized to the short-wavelength maximum. The ratio in the experimental spectra is governed by the degree of reabsorption according to (*12*) and (*13*). *1* C = 1·5 mM, λ_e = 434 nm, α = 150°; *2* C = 1·5 mM, λ_e = 621 nm, α = 60°; *3* C = 1·5 mM, λ_e = = 434 nm, α = 60°; *4* C = 0·16 mM, λ_e = = 621 nm, α = 30°. *C* concentration, λ_e excitation wavelength, α angle between the sample plane and the exciting beam

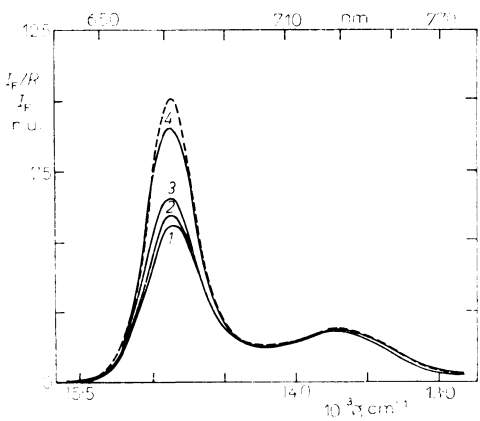

FIG. 3

Experimental spectra of 9 mM sample (full line) and their reabsorption corrected form. *1* λ_e = 434 nm, α = 60°; *2* λ_e = 621 nm, α = 30°; *3* spectrum *1* corrected for reabsorption (dotted line), *4* spectrum *2* corrected for reabsorption (dashed line). Symbols and normalization are same as in Fig. 2

Fig. 2 shows the measured and corrected spectra for pigment concentrations 0·16 mM and 1·5 mM. With samples if the lowest concentration, the corrections according to equations (*12*) and (*13*) are 10 – 15% and bellow 2% for the short-wavelength and the long-wavelength maximum, respectively. In the series of 1·5 mM samples these corrections represent 93% of the original intensity for the short-wavelength maximum but do not exceed 3% for the long-wavelength one. Applying the corrections according to relationships (*7*) and (*11*), a good agreement in the spectral shape is obtained for the whole ansamble of samples of both concentrations. The relative deviation in the intensity ratio of both bands is 2%, their frequencies are determined with accuracy of 1 nm.

With the 9 mM sample measured in the reflection arrangement, the reabsorption diminishes the intensity of the main short-wavelength maximum below the intensity of the long-wavelength band (Fig. 3). Despite the marked difference in the experimental shape, spectra with dominant short-wavelength band and with a good intensity ratio are again obtained when applying the correction for reabsorption according to relation (*7*). The relative deviation of the intensity ratio is 10%, the difference in the main maximum wavelength amounts 4 nm. In the transmission arrangement, the fluorescence intensity in the region of absorption and fluorescence spectra overlap is so diminished that the measured fluorescence signal is at the noise level.

The application of reabsorption correction to all three samples allows to obtain more accurate data from the fluorescence spectra. A quantitative agreement of the corrected spectra shape was obtained for 0·16 mM and 1·5 mM samples in which the product of optical density for the excitation wavelength and quantum yield is smaller than 0·3 (Fig. 2). With 9 mM sample, where the product of optical density and quantum yield is greater than 0·3, it appears that neglection of reabsorption leads to considerable distortion of the spectrum. In that case even the simple correction formula produces the same course of the corrected spectra for different angles of deflection (Fig. 3). The reason why the correction in this case did not lead to the same decrease of the intensity ratio of both bands similarly to that at lower concentrations might be physico-chemical concentration events, *e.g.* the aggregation with bathochromically shifted spectra and the transfer of he excitation energy to those long-wavelength spectral species[5,6]. Large intensity ratio of the long-wavelength and the short-wavelength bands can be also due in part to the neglection of the secondary fluorescence that is another correction affecting the experimental spectrum shape. However, the good agreement of the intensity ratio for various angles of reflection measured with the most concentrated samples indicates the concentration changes in the sample as the more probable explanation.

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Translated by P. Sedmera.