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Effect of Secondary Fluorescence on the Emission Spectrum and Quantum Yield of Fluorescence in Chlorophyll-a Solutions and Algal Suspensions

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The spectral effect of secondary fluorescence of chlorophyll-a in benzene is negligible up to $c \times d = 10^{-5}$ mole. cm/l (c and d are concentration and layer thickness of the solution). Under the same conditions the effect of secondary fluorescence on the absolute value of the intensity of fluorescence is, however, about 10 per cent. This effect is negligible only up to $c \times d = 10^{-6}$ mole. cm/l allowing for a difference of 2 per cent between the measured and true intensities. Practically the same results are obtained with chlorophyll-*a* in other solvents. In suspensions of Chlorella, Anacystis and Porphyridium there is no spectral effect of the secondary fluorescence and the effect on the quantum yield of fluorescence is negligible (less than 1 per cent even in suspensions of optical density of 0.5). From the saturation of the secondary fluorescence, an absolute maximum spectral effect of 5—6 per cent is estimated for the secondary fluorescence and 18 per cent for the quantum yield in chloroplast.

1. In complicated organic compounds of biological interest, the absorption and fluorescence spectra of their solutions strongly overlap. As a result, the fluorescence originating in the solution is reabsorbed and the measured intensity of fluorescence is smaller in this (overlap) spectrum range than the true intensity. Förster (1951) and Duysens (1952) have pointed out that the reabsorption can be taken into consideration by simple calculations.

The reabsorption of fluorescence photons give rise to new excited molecules. The secondary fluorescence of these photons has been studied by Budó and Ketskeméty (1956, 1957), Budó et al. (1957), Lavorel (1957), Agranovitch and Konobejev (1959), Melhuish (1961), Kravtsov (1963) and Rohatgi and Singhal (1966). In this paper we report investigations on the secondary fluorescence in chlorophyll *a* after the method of Budó and co-workers (Budó, Horvai, 1956; Dombi, Horvai, 1956; Budó, Ketskeméty, 1962). We have used this method bacause all the parameters and functions (needed for calculations by Budó's method) are available in a tabulated form in our laboratory (Szeged) and because some of the methods listed above are either tedious or inexact. Budó and Ketskeméty (1956) have shown that in case of longitudinal observation from the side of excitation (i.e. from the front face) the true fluorescence spectrum $f(\lambda')$ is correlated with the

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measured spectrum $dB(\lambda')$ in the following way:

$$dB(\lambda') = \frac{\rho}{4\pi n^2} E(\lambda)\Phi(\lambda) f(\lambda') \frac{\alpha}{\alpha+\beta} \frac{1-e^{-(\alpha+\beta)}}{1-\kappa} d\lambda'. \tag{1}$$

In equation (1), $E(\lambda)$, $\Phi(\lambda)$, n, λ and λ' denote the intensity of the exciting light, the true quantum yield of fluorescence, the refractive index of the solution, the wavelength of absorption (λ) and emission (λ'), respectively. $\rho < 1$ is a factor accounting for the reflection from the front surface of the cuvette. α and β are related to concentration (c), layer thickness (d) and molar decadic extinction coefficient $\varepsilon(\lambda)$ as shown below:

$$\alpha = 2.3 \times \varepsilon(\lambda)\, cd; \qquad \beta = 2.3 \times \varepsilon(\lambda')\, cd; \tag{2}$$

κ in equation (1) is the ratio of the intensity of secondary fluorescence (S) to the intensity of primary fluorescence (P). This equation becomes identical with the Förster-equation (Förster, 1951; equations 8 and 14 on page 41) for reabsorption when the secondary fluorescence is negligible compared to the primary fluorescence ($S \ll P$, $\kappa \to 0$).

When the directions of excitation and observation form an angle of ϑ with the normal of the front face of the cuvette, the path length of the exciting light and/or the thickness of the observed layer is increased. This can be considered by taking $d/\cos\left(\text{arc sin}\,\frac{\sin\vartheta}{n}\right)$ instead of d in Equ. (2). (For small ϑ, $d/\cos\vartheta/n$ should be introduced for d in Equ. (2) as a sufficient approximation; this holds if $\vartheta < 15°$ and an error of 1–2 per cent is allowed.)

According to the calculations in (9) and (11):

$$\kappa = \int_0^\infty \Phi(\lambda'') f(\lambda'') M(\alpha, \beta, m)\, d\lambda'', \tag{3}$$

where M is a function of the depth (d) and the radius (R) of the cylindrical cuvette and

$$\gamma = 2.3 \times \varepsilon(\lambda'')\, cd; \qquad m = \frac{R}{d}. \tag{4}$$

Actual measurements [i.e. in (12)] showed that κ increases with concentration and layer thickness from $\kappa = 0$ up to an upper limit from which it becomes constant.

On neglecting κ in Equ. (1) we obtain the quantum yield $\Phi^\circ(\lambda)$ and fluorescence spectrum $f^\circ(\lambda')$ corrected for reabsorption. Consequently, the true fluorescence spectrum and the true quantum yield are given by the following equations:

$$f(\lambda') = N(1-\kappa) f^\circ(\lambda') \tag{5}$$

(where the constant N is given by the condition $\int_0^\infty f(\lambda')\, d\lambda' = 1$)

$$\Phi(\lambda) = \frac{\Phi^\circ(\lambda)}{1-\kappa}. \tag{6}$$

Instead of this a somewhat more complicated but more exact relationship is given by Budó, Dombi and Szöllősy (1956).

2. Our measurements and calculations were carried out for a solution of chlorophyll *a* in benzene and for suspensions of *Chlorella pyrenoidosa*, *Anacystis nidulans*, and *Porphyridium cruentum*. The corrections for secondary fluorescence were given at different concentrations or optical densities [$\alpha(\lambda)$]. The details of the experimental work are given by Szalay et al. (1967) and those of the spectrofluorometer by Govindjee and Yang (1966) and Govindjee (1966). The wavelengths of excitation are shown in Table 1. In the concentration range where the

Table 1

	λ_{exc} Å	λ_{max} Å	Φ_{max}	$\kappa(\alpha, 0)$	$\kappa(\alpha, \beta_{max})$
Chlorophyll-*a*	4 320	6 665	0.300	0.190	0.121*
Anacystis	4 400	6 750	0.002	0.000_6	0.005
Chlorella	4 400	6 750	0.030	0.017	0.014
Porphyridium	4 300	6 793	0.006	0.002	0.008

λ_{max} — location of the absorption maximum in the overlap region.
* This figure refers to a value of $\beta_{max} = \alpha$

effect of secondary fluorescence is not negligible (e.g. for higher concentration) κ does not depend on the radius of cuvette (more precisely on the ratio $m = R/d$) to a great extent. According to the Equs (2) and (4) κ depends on the excitation coefficients at the wavelength of excitation and observation and on the product $c \times d$, or (in algal suspensions where the extinction coefficient is unknown) on the optical density.

Chlorophyll-*a* in solution

3. κ as a function of log $c \times d$ for chlorophyll-*a* dissolved in benzene is shown in Fig. 1. The exciting wavelength $\lambda = 4320$ Å and therefore $\alpha(\lambda) = 2.3 \times \varepsilon(4320\text{ Å}) \times c \times d$. Since $\varepsilon(4320) = 1.02 \times 10^5$ (see Seely and Jensen, 1965), $\alpha(\lambda) \approx 2.3 \times 10^5$ *cd*. The α-values are given in terms of multiples of β-values, and we assumed that the quantum yield, $\Phi(\lambda) = 0.3$ [Weber and Teale, (1957) and Latimer et al. (1957)] was independent of the concentration. On account of the concentration quenching $\Phi(\lambda)$ begins to decrease with increased concentration from about $c = 2.10^{-3}$ mole/l (Rabinowitch, 1951). This means that the κ-functions are exact only for concentrations of $c < 2.10^{-3}$ mole/l. At higher concentrations the effect of secondary fluorescence with κ-functions calculated with $\Phi(\lambda) = 0.3$ is overestimated. This overestimation, however, should not be too much because self-quenching reduces the intensity of the fluorescence of chlorophyll-*a* in butyl ether only to about 70 per cent of the maximum (Rabinowitch, 1951), i.e. to about $\Phi = 0.20$ instead of $\Phi = 0.30$ even at a high concentration (10^{-2} mole/l).

In order to correct a given experimentally obtained result (e.g. a fluorescence spectrum or a quantum yield) for secondary fluorescence, first the correction for reabsorption should be made. Then the $\kappa(\alpha,\beta)$-values should be taken for the given $c \times d$ product from Fig. 1 and plotted versus $\beta(\lambda')$. From this new plot the

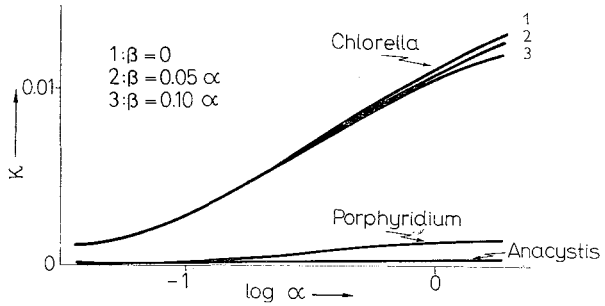

Fig. 1. The correction term κ (α, β) for calculating the true fluorescence characteristics as a function of log $c \times d$. (c — concentration of solution, d — layer thickness). α and β denote the optical densities of the solution at the excitation and observation wavelengths

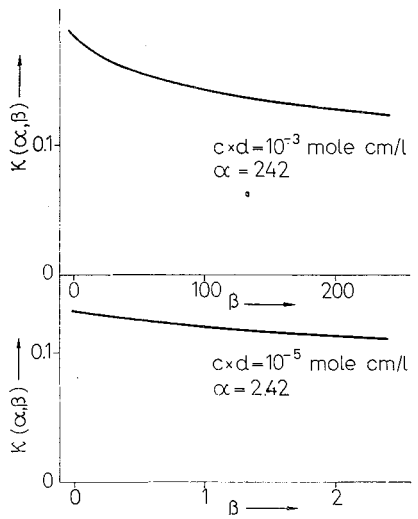

Fig. 2. The correction term κ (α, β) for calculating the true fluorescence characteristics as a function of $\beta = 2.3\ \varepsilon\ (\lambda') \times c \times d$ for two values of $c \times d$ (c — concentration of solution, d — layer thickness). and denote the optical densities of the solution at the excitation and observation wavelengths

values of $\kappa(\alpha,\beta)$ at the proper λ'-values can be read and the corrected fluorescence spectrum $f(\lambda')$ can be calculated with Equ. (5). (See Fig. 2 for $d = 0.1$ cm and $c = 10^{-4}$ or 10^{-2} mole/l). Similarly, from Equ. (6) the true absolute quantum yield can be calculated. These calculations were programmed and carried out by means of a Minsk-3 computer in the Laboratory for Cybernetics of the University of Szeged.

The spectral effect of secondary fluorescence in the case of chlorophyll *a* in solution is shown in Fig. 3. The corrected spectra are labeled f_1^0, f_2^0, f_3^0, whereas the uncorrected ones are labeled as f_1^*, f_2^*, f_3^*. All the spectra were "normalized" at 700 mμ since no spectral effect of the secondary fluorescence is expected in this wavelength range. The emission spectra corrected for reabsorption are given for $d = 0.1$ cm and for $c = 10^{-5}(f_1^0)$, $10^{-4}(f_2^0)$ and $10^{-2}(f_3^0)$ mole/l. [These were calculated from Equ. (5) assuming that the shape of the true spectrum was independent of the concentration.] In the concentration range considered this assump-

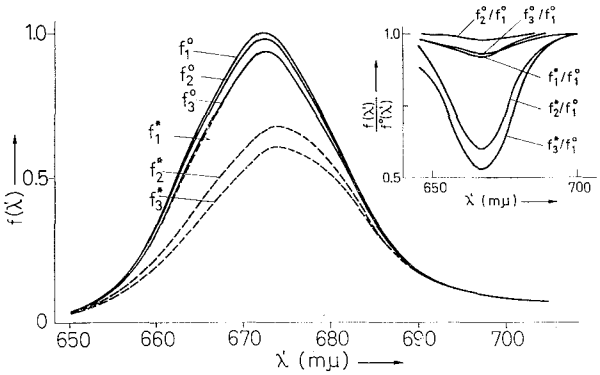

Fig. 3. The effect of reabsorption on the fluorescence spectrum of chlorophyll *a*. f_1^0, f_2^0, f_3^0: fluorescence spectra corrected for reabsorption at concentration of 10^{-5}, 10^{-4} 10^{-3} mole/l. f_1^*, f_2^*, f_3^*: uncorrected fluorescence spectra for concentrations of 10^{-5}, 10^{-4}, 10^{-3} mole/l, layer thickness 0.1 cm

tion may be valid. No significant difference between the absorption spectrum of chlorophyll *a* in dilute butyl ether solution and in a solution of 2.10^{-2} mole/l is observed (Rabinowitch, 1951; p. 774). However, Brody and Brody (1961) have reported increased absorption at 6820 Å in 3×10^{-2} M chlorophyll *a* in ethanol when compared with 10^{-5} M chlorophyll *a* in ethanol.

All the spectra denoted by f_1^0, f_2^0 and f_3^0 (Fig. 3) ought to coincide provided only the reabsorption exists. Since f_1^0, f_2^0 and f_3^0 do not coincide, there is the remaining spectral effect of secondary fluorescence. The shape of the true emission spectrum is not independent of the concentration as was assumed above; the measured spectrum does change with the concentration on account of reabsorption and at higher concentrations also because of secondary fluorescence. This spectral effect of the secondary fluorescence is, however, not great (see below). The insert in Fig. 3 shows the plot of f_2^0/f_1^0, etc. It shows that the spectral effect of secondary fluorescence does not exceed 2 per cent at 10^{-4} mole/l and 7 – 8 per cent at 10^{-2} mole/l. Fig. 1, however, shows that the secondary fluorescence in chlorophyll-*a* solution (in benzene) does not exert a spectral effect up to a log $c \times d$ value of about -5.00. This means that in a layer thickness of 0.1 cm up to a concentration of 10^{-4} mole/l there is no spectral effect of secondary fluorescence.

The absolute value of the fluorescence intensity can be very much influenced by the secondary fluorescence. For $\kappa > 0.1$ for $d = 0.1$ cm and $c = 10^{-4}$ mole/l,

the true intensity of fluorescence is about 10 per cent less than the intensity of fluorescence obtained by correcting only for the reabsorption. Fig. 1 shows that $\kappa > 0.02$ in solution of $\log c \times d = -6.00$! This means that in a layer thickness of 0.1 cm, even at a concentration of 10^{-5} mole/l, a secondary fluorescence of about 2 per cent (in the absolute value of the intensity of emission) is found.

Chlorophyll-*a* in Algae

4. Calculations – similar to those with solutions – were made in the case of suspensions of *Chlorella pyrenoidosa, Anacystis nidulans* and *Prophyridium cruentum.* Since the true concentration of the pigment and the optical density value for cells are unknown, it is *not* possible to apply corrections for reabsorption and secondary fluorescence within single cells. However, it is possible to apply "outer" corrections for reabsorption and secondary fluorescence. The correction for secondary fluorescence given in the above manner yields the "true" emission spectrum of a suspension which is considered to be a true solution in the sense of the two consecutive applications of Beer's law by Rabinowitch (1951).

On assuming a concentration of 10^{-2} mole/l in the chloroplast (or the whole cell of Anacystis, since blue-green algae do not contain chloroplast) a further correction would be necessary. For 10^{-2} mole/l chlorophyll *a* and 10 μ maximum layer thickness in the chloroplast, $\log cd = -4.00$. According to Fig. 1, this is about the saturation of κ. This means that the maximum value of $\kappa = 0.18$ to 0.19 is attained for chlorophyll *a* for $\beta = 0$ (at a wavelength in emission where there is no absorption) and $\kappa = 0.12$ for $\beta = \alpha$ (at a wavelength in emission where the optical density is equal to that at the wavelength of excitation). Even if we assume a concentration of 10^{-1} mole/l, the situation does not change because the saturation of κ has already been attained. Consequently in any case (different concentration of chlorophyll and different size of chloroplast), the effect of secondary fluorescence on the spectral distribution of fluorescence should not exceed 5–6 per cent and that on the quantum yield should not exceed 18 per cent. This is a fairly conservative estimate.

On account of the small quantum yield of fluorescence by algae (Latimer, Bannister and Rabinowitch (1957)) the effect of secondary fluorescence was found to be comparatively small though the overlap of the two spectra is considerable. Fig. 4 shows the κ-factor as a function of α at different β-values. In Chlorella for $\log \alpha = -1.0$ ($\alpha = 0.10$), $\kappa = 0.003$, in Porphyridium, $\kappa = 0.000_4$ and in Anacystis much less. In all cases the effect of secondary fluorescence is negligible. If the optical density of suspension is as high as 0.50 ($\log \alpha = 0.70 - 1$), $\kappa = 0.010$ in Chlorella, $\kappa = 0.002$ in Porphyridium, and $\kappa = 0.000_3$ in Anacystis. There is no spectral effect of secondary fluorescence. This means that even in these dense suspensions the effect of secondary fluorescence on the quantum yield is less than 1 per cent. Of course, we have considered only the "outer" correction. The comments made above are valid for the "inner" correction.

Table 1 summarizes some of the data. $\kappa(\alpha, 0)$ denotes the κ-function at a wavelength of observation where there is no reabsorption, $\kappa(\alpha, \beta_{max})$ means a

κ-function for a wavelength of observation where the reabsorption is maximum; α represents the optical density for the exciting wavelength.

The effect of secondary fluorescence is practically independent of the observation wavelength. Thus the shape of the emission spectrum is not influenced by

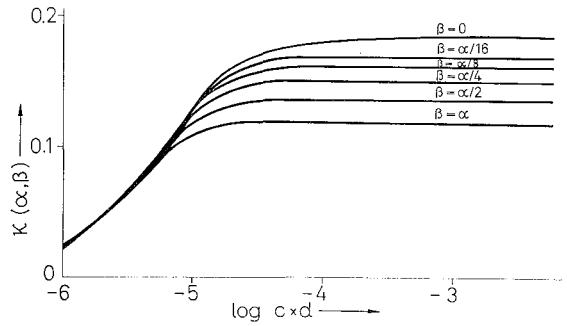

Fig. 4. The correction term κ (α, β) for calculating the true fluorescence characteristics as a function of α (optical density of the suspension) at different β-values (β-optical density at the observation wavelength) 1 : $\beta = 0$; 2 : $\beta = 0.05$; 3 : $\beta = 0.10$ α

secondary fluorescence even at longer waves but the absolute quantum yield is influenced, especially at higher optical densities.

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