RELATIONSHIP BETWEEN THE
ABSORPTION AND EMISSION SPECTRA AND
THE "RED DROP" IN THE ACTION
SPECTRA OF FLUORESCENCE IN VIVO

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ABSTRACT The Stepanov equation, relating the intensity of emission, $f_\nu(\nu)$, at a given frequency, and that of absorption, $k(\nu)$, at the same frequency, is applied, in its modified form (see equation 3 in text) to suspensions of Chlorella, Porphyridium, and Anacystis and to chlorophyll solutions. This application can reveal whether the yield of fluorescence, $\Phi(\nu)$, is constant, or changes with frequency. In Chlorella (green alga) a sharp drop of $\Phi(\nu)$ is indicated towards the lower frequencies (longer waves), beginning around $\nu = 1.48 \times 10^4 \text{cm}^{-1}$ (680 m$m$); the $\Phi(\nu)$ function calculated from the Stepanov equation is in fair agreement with the directly determined action spectrum for the excitation of chlorophyll fluorescence in this organism. In Porphyridium (red alga) and Anacystis (blue-green alga) application of the Stepanov equation supports the conclusions, derived from direct measurements, of a much earlier "red drop" of the fluorescence excitation spectra. Direct measurements suggest that the drop in Porphyridium may begin at about $1.53 \times 10^4 \text{cm}^{-1}$ (654 m$m$); in Anacystis, it may begin already above $1.57 \times 10^4 \text{cm}^{-1}$ (<637 m$m$). These results confirm the relation, postulated earlier by Duysens and others, between the action spectra of photosynthesis and of chlorophyll a fluorescence in algal cells. The relation of these findings to spectroscopic evidence, suggesting the existence of two main chlorophyll a components in vivo, in green as well as in red and blue-green algae, is discussed.

INTRODUCTION
Comparison of absorption spectra with the emission spectra of polyatomic molecules has given some important information about molecular structure. A first example had been the interpretation of the Stokes shift [by reference to the Franck-Condon principle (1)] and of the mirror-symmetry rule [in terms of similarity of the potential curves of the ground state and the excited state (2)]. More recently, another relationship, first formulated by Stepanov (3), was found to be useful. Stepanov pointed out that if, at the time of emission, the emitting molecules are
distributed among the vibrational levels of the excited electronic state in accordance with the temperature of the medium, \( T \), the ratio of the relative intensities of emission, \( f_e(\nu) \), and absorption, \( K(\nu) \), at a given wave number should be:

\[
\frac{f_e(\nu)}{K(\nu)} = D(T)\nu^3 \exp \left(-\frac{\hbar \nu c}{kT}\right)
\]  

(1)

where \( D(T) \) is a function of temperature, independent of the wave number \( \nu \); \( h \) and \( k \) are the Planck and Boltzmann constants, respectively. Equation (1) may be rewritten as:

\[
F(\nu) = 3 \log \nu - \log \left[\frac{f_e(\nu)}{K(\nu)}\right] = \frac{\hbar \nu c}{kT} \log e + \text{const.}
\]  

(2)

According to equation (2) the plot of \( F(\nu) \) vs. \( \nu \) should be a straight line, the slope of which is determined by the temperature, \( T \).

If thermal equilibrium distribution among the vibrational levels is not established during the lifetime of the excited electronic state, actual distribution may correspond, as suggested by Neporent (4), to a higher temperature, \( T^* \). In this case, Equation (2) would show a slope corresponding to this higher temperature. Borishevich and Gruzinskii (5) confirmed the validity of equation (2) with a temperature \( T^* > T \), for several low-pressure gaseous systems, where the approach to vibrational equilibrium is relatively slow.

In condensed systems, we can expect all excess vibrational energy to be dissipated long before the emission, because of repeated collisions with the molecules of the medium. Nevertheless, the temperature obtained from the slope of equation (2) had been sometimes found to be higher than the actual temperature of the medium (6). Some observers have considered this as proof that even in this case, no vibrational equilibrium had been reached by the time of the emission, despite the many collisions (6). There are, however, other factors which can influence the apparent temperature values obtained from equation (2). These are (a) reabsorption of primary fluorescence (7), (b) emission of secondary fluorescence (8), and (c) presence of two (or more) species of absorbing molecules (9). Borishevich et al. (10) and Kazachenko (11) suggested that if, after proper correction had been made for the reabsorption of primary fluorescence and for the emission of secondary fluorescence, the calculated temperature still is higher than the ambient temperature, light-absorbing impurities must be responsible for the difference. Kravcov and Rubinov (12) attempted to derive a relationship between \( \Delta T = T^* - T \) and the concentration and the absorption spectrum of an impurity.

According to Ketskeméty and coworkers (13) equation (3), which includes the fluorescence yield, \( \Phi(\nu) \), and the index of refraction, \( n \), should be more generally
valid than equation (1):

$$\frac{f'(p)}{K(p)} = D(T)p^3\Phi(p)n^2(p) \exp (-hc/kT).$$  \hspace{1cm} (3)

This equation takes care of the presence of a nonfluorescent but light-absorbing impurity by introducing a term $\Phi(p)$, which makes $\Phi$ dependent on wave number.

The refractive index, $n$, also depends on $p$; however, in the region of the so-called "red drop" in the action spectrum of fluorescence, with which we are here concerned, this dependence can be neglected compared to that of $\Phi$. Equation (3) may be rewritten (assuming $n = \text{constant}$) as:

$$F'(p) = 3 \log p + \log \Phi(p) - \log [f'(p)/K(p)] = \frac{hc}{KT} \log e + \text{const.} \hspace{1cm} (4)$$

The plot of $F'(p)$ against $p$ should now yield a straight line, even in a spectral region where the function $F(p)$ deviates from linearity because of variations in $\Phi(p)$, e.g., because of the presence of a nonfluorescent impurity.

We have applied equations (2) and (4) to fluorescence and absorption of algal cells, in which the presence of several pigment components with different fluorescent properties has been often postulated (14–18).

**EXPERIMENTAL**

The green alga, *Chlorella pyrenoidosa* (Emerson's strain 3), the red alga, *Porphyridium cruentum*, and the blue-green alga, *Anacystis nidulans*, were grown in inorganic culture media (see reference 19 for details). *Chlorella* was grown over a combination of fluorescent and incandescent lamps at 22°C, *Porphyridium*, over fluorescent light at 18°C, and *Anacystis*, over fluorescent and incandescent lamps at 25°C. A mixture of 5% CO₂ and 95% air was bubbled through the cultures during growth. About three to six day-old cultures were used. Algae were harvested, when necessary, by centrifugation and diluted with their respective culture media to obtain optically thin suspensions (absorbance at 680 mμ ≈ 0.04 for 2 mm path length), appropriate for absorption and fluorescence measurements.

Absorption measurements were made with a Bausch and Lomb spectrophotometer (Spectronic 505; Bausch & Lomb Incorporated, Rochester, N.Y.) equipped with an integrating sphere, and fluorescence measurements with an automatic spectrofluorometer (20, 21). The fluorescence spectra (excited by blue light, $\lambda = 440$ mμ) were corrected for the spectral response of the photomultiplier (EMI 9558B) and for the transmission efficiency of the analyzing monochromator (Bausch and Lomb "large" spectrophotometer blazed at 750 mμ; band width 3.3 mμ). The intensity of fluorescence (measured at 740 mμ, i.e. in the second fluorescence band of chlorophyll a, to avoid reabsorption) were obtained as function of frequency of exciting light by means of a similar spectrophotometer. The action spectra for the excitation of fluorescence, obtained in this way, were corrected by reduction to a constant quantum flux. (The measurements were carried out in the low-intensity region, where the intensity of fluorescence of the cells is known to be proportional to that of incident light.) The resulting action spectra were divided by the per cent absorption spectra to obtain relative
quantum yields ($\Phi$) of fluorescence. These were plotted as function of the wave number of exciting light. The data were confirmed by means of another, also previously described, automatic spectrophotometer (22). All measurements were made at room temperature (25°C).

RESULTS AND DISCUSSION

A. Chlorella pyrenoidosa

Fig. 1 a shows the relative absorption spectrum [$K(\nu)/K(\nu)_{\text{max}}$] and the relative emission spectrum [$f_a(\nu)/f_a(\nu)_{\text{max}}$] of a Chlorella suspension. The absolute optical density of the suspension in the absorption maximum ($D_{\text{max}} = 1.482 \times 10^4\text{cm}^{-1}$) was, in four experiments, between 0.02 and 0.06; the results of the three experiments not shown in Fig. 1 were in good agreement with the one plotted in this figure. In Fig. 1 b the open circles, connected by a solid line, show the function $F(\nu)$ calculated from equation (2). This function is linear between $\nu = 1.470 \times 10^4$ and $\nu = 1.584 \times 10^4\text{cm}^{-1}$, but deviates from linearity below $1.470 \times 10^4\text{cm}^{-1}$ (680 mµ). We postulate that this deviation is caused by wavelength dependence of the quantum yield $\Phi$. If the linear portion of the $F(\nu)$ curve is extrapolated towards the smaller wave numbers the difference between the extrapolated values (full circles) and the plot of equation (2) (open circles) indicates the probable variation of the relative quantum yield of fluorescence, $\Phi(\nu)$. The $\Phi(\nu)$ values can be calculated from this deviation by using equation (4); they are represented in Fig. 1 b by black squares.

The open squares in Fig. 1 b, connected by a dashed line, are the relative quantum yields of fluorescence obtained directly from the excitation spectra of chlorophyll a fluorescence and the per cent absorption spectra. Our calculated values (solid squares) agree satisfactorily with these experimental values. They show similarity with the data of Emerson et al. (23) on the red drop of the quantum yield of photosynthesis, but the red drop in the O₂ evolution begins only around 685 mµ.

The determination of the "red drop" of the fluorescence yield in Chlorella, used in Fig. 1 b, was more detailed than previous demonstrations of this drop. Duysens (24), using thick suspensions of Chlorella, noted a drop beginning at 675 mµ, but the use of such suspensions can lead to erroneous results, as pointed out by Weber (25). Teale (cited by Weber, 25) observed that $\Phi(\nu)$ in Chlorella remains constant, over a considerable spectral region up to 690 mµ; he found a sign of beginning drop at about 690 mµ, but made no measurements at longer wavelengths. However, in comparing the measurements of different observers one has to keep in mind that the shape of the fluorescence excitation curve may vary from culture to culture of the same algal species; the preillumination of the culture and the different intensity of exciting light may influence the shape of the excitation spectra.

It may be asked to what extent the shape of the action spectrum in the red drop region is affected by the so-called "sieve effect" (26), which deforms (widens and flattens) the absorption band of suspensions. We have made some observations on the red drop in the action spectrum of fluorescence of Chlorella sonicates (where
Figure 1 a. Absorption $[K(\bar{\nu})/K(\bar{\nu})_{\text{max}}]$ and emission $[f_e(\bar{\nu})/f_e(\bar{\nu})_{\text{max}}]$ spectra of a suspension of the green alga, Chlorella, in the overlap region of the spectra in relative units. b. Relative quantum yield of fluorescence, $\Phi(\bar{\nu})$, calculated from the excitation and absorption spectrum (open squares and dashed curve) and the lines obtained from equations (2) $[F(\bar{\nu}) + \text{constant}]$ and (4) $[F'(\bar{\nu}) + \text{constant}]$ for a Chlorella suspension. Solid squares show the calculated $\Phi(\bar{\nu})$ (see text). In this and the following figures, markings labeled 650, 675, and 700 (see top) are wavelength ($\lambda$) markings in $\mu$m.
the sieve effect is minimized by breaking the cells into fragments) (27). It was observed that the red drop disappears altogether in sonicates of Chlorella prepared under aerobic conditions, while sonicates prepared under argon at pH 7.8 retain the "drop" beginning at about 675–680 m\(\mu\). These observations suggest that aerobic sonication in acid media first affects chemically the minor, non-fluorescent chlorophyll a component with the absorption peak at 695–700 m\(\mu\) (Kok's "C700").

The temperature \(T\) (calculated from the slope of the straight line in Fig. 1 b), was 302, 302, 320, and 322\(^\circ\)K in the four experiments (the mean value = 311\(^\circ\)K) while the actual temperature of the medium was about 298\(^\circ\)K. This can be considered as satisfactory agreement. The accuracy of the temperature determination from the slope of the straight line in Fig. 1 b depends on the reliability of wavelength determinations. The accuracy of the wavelength setting was \(\pm 0.5\) m\(\mu\), in both the absorption and the emission measurements; the corresponding range of uncertainty in \(T\) is \(\pm 12^\circ\)K. The precision with which the location of the "red drop" can be determined by means of equations (2) and (4) is limited by the accuracy of the measurements at the long wavelength end (>710 m\(\mu\)) of the absorption bands, and of the emission at the short wavelength end of the emission bands.

B. Porphyridium cruentum

The relative absorption and emission spectra of the red alga Porphyridium are shown in Fig. 2 a. The optical density in the maximum of the absorption curve was, in three experiments, between 0.02 and 0.03.

In Fig. 2 b, the straight line \(F(\nu) + \text{const.}\) (open circles connected by a solid line) was calculated from equation (2). The temperature \(T\) calculated from its slope is 402\(^\circ\)K \(\pm 12^\circ\)K, markedly different from the true ambient temperature (298\(^\circ\)K). The experimental points suggest that a "nonfluorescent" component is present in this case practically in the whole absorption region covered by Fig. 2 b, from 640 to 700 m\(\mu\).

The open squares of Fig. 2 b, which represent new direct measurements of the relative quantum yield of fluorescence, confirm that in Porphyridium the yield of chlorophyll fluorescence (measured at 740 m\(\mu\)) begins declining already at about \(\nu = 1.53 \times 10^4\text{cm}^{-1}\), i.e. \(\lambda = 654\) m\(\mu\), in analogy to the well-known early drop of the yield of photosynthesis in red algae, noted by Haxo and Blinks (28) and Brody and Emerson (29). In order to convert the \(F(\nu)\) line in Fig. 2 b into a straight line corresponding to \(T \approx 300^\circ\)C (lower solid line in Fig. 2 b), the \(\Phi(\nu)\) values marked by full squares must be introduced into equation (4). There is a rough agreement, over most of the spectral region covered, between the directly measured \(\Phi\) values (open squares), and the \(\Phi\) values (represented by full squares) needed to fit the \(F(\nu)\) curve to a straight line with proper slope. The fact that in red algae, light absorbed by chlorophyll a itself is much less efficient in producing chlorophyll a fluorescence than light absorbed by phycoerythrin, was already noted by Duysens (24) and French and Young (30).
FIGURE 2  a Absorption and emission spectra of a suspension of the red alga, *Porphyridium*, in the overlap region of the spectra, in relative units. b, Relative quantum yield of fluorescence, $\Phi(\nu)$, calculated from the excitation and absorption spectrum and the lines obtained from equations (2) and (4) for a *Porphyridium* suspension (see text and legend of Fig. 1).

C. *Anacystis* *nidulans*

The results obtained with the blue-green alga *Anacystis* are shown in Fig. 3. In this case, four samples were investigated, with optical densities (in the absorption maxima) between 0.02 and 0.07. There were variations, both in the emission and in the
Figure 3. Absorption and emission spectra of a suspension of the blue-green alga, *Anacystis*, in the overlap region of the spectra, in relative units. b. Relative quantum yield of fluorescence, $\Phi(\nu)$, calculated from the excitation and absorption spectrum and the straight lines obtained from equations (2) and (4) for an *Anacystis* suspension (see text and legend of Fig. 1).

The absorption spectra of the different samples, which led to considerable variations in the temperatures calculated from the slope of the line obtained by using equation (2). The samples used differed in their physiological age and in the culture conditions, in particular the light intensities used in their growth; Ghosh and Govindjee (31) have shown that the intensity and color of light, during the growth of *Anacystis*,
has a marked effect on the shape of its emission spectrum. Fig. 3b represents the results for one sample. (The variations were so wide that averaging was inappropriate.) In one culture, $T$ was found to be $474 \pm 12^\circ K$, while in another culture, it was $372^\circ K$.

To obtain the lower straight line (with solid points) in Fig. 3b corresponding to a temperature of $T = 316 \pm 12^\circ K$, the values of $\Phi$ indicated by solid squares had to be postulated (equation 4). These agreed fairly well with the directly measured $\Phi$ values (open squares connected by a broken line). They exhibit a considerable drop in fluorescence yield already at or above $1.57 \times 10^4 cm^{-1}$, i.e., below 637 m$\mu$. Comparison with the action spectrum of photosynthesis was difficult; the “red drop” curves of photosynthesis (32, 33) may be distorted, in blue-green algae, by the inhibitory effect of far-red light on $O_2$ uptake (34).

D. Chlorophyll a In Vitro

In solutions of chlorophyll a, in ether or pentene, with concentrations of about $10^{-6}$ moles/liter, no deviations from the straight line, predicted by equations (2), were noted over the whole region studied, down to $\bar{\tau} = 1.44 \times 10^4 cm^{-1}$ (695 m$\mu$). The temperature, $T$, calculated from the slope of this plot (see Fig. 4), was $318^\circ K$ in ether, $316^\circ K$ in pentene, and $315^\circ K$ in ether-pentene (2:1) mixture. In these solutions, the overlapping of the absorption spectrum with the emission spectrum is somewhat greater than in Chlorella suspension; therefore, the errors in the calculation of $T$, caused by an uncertainty of 0.5 m$\mu$ in the wavelength determination, are greater; the precision of the results is estimated to be $\pm 25^\circ K$. Thus, the calculated T values do not differ significantly from the actual temperature of the medium (298 K). According to these experiments, there is no “red drop” in these dilute solutions of chlorophyll a (at least up to 695 m$\mu$, where the optical densities become too small for reliable calculations). A red drop in chlorophyll a fluorescence in $1.4 \times 10^{-4}$ M solution was noted by Forster and Livingston (35) at 698 m$\mu$; Livingston (36) later suggested that this may be due to the formation of dimers at the concentration used.

E. The Red Drop and the Spectral Components of Chlorophyll a In Vivo

French (17) has used “derivative spectroscopy” to confirm the (previously suspected) fact that the red absorption band of chlorophyll a in vivo is a doublet (and, perhaps, a triplet) of overlapping bands. Later, Cederstrand et al. (14) concluded, from computer analysis of absorption curves measured with extreme precision, of green, red, and blue-green algae, that in all these types of cells the red band is essentially a doublet, consisting of two “Gaussian” components of approximately equal intensity and width, with peaks located at 668 and 683 m$\mu$, respectively. In blue-green algae, a third component, due to allophycocyanin, may be present at 643 m$\mu$. One uncertainty of this analysis is the flattening influence of the accumulation of the pigments.
within chloroplasts ("sieve effect"), first discussed by Duysens (24, 37) and by Rabinowitch (38). If the red band were a single "Gaussian" band, the sieve effect would flatten its top, and thus suggest interpretation in terms of two overlapping Gaussian bands. However, the analysis is not substantially altered by removing the "sieve effect" by sonication, although the two components move somewhat closer together (26).

We now ask whether the red drop curves chlorophyll a fluorescence can be ex-
plained by assuming that "chlorophyll a 668" is fluorescent, while "chlorophyll a 683" is nonfluorescent. Constructing a theoretical "red drop curve" on the basis of this assumption, one obtains a curve declining to one-half of its peak about half-way between 668 and 683 μ, i.e. near 676 μ, while in green algae, the experimental curve (Fig. 1 b) declines to one-half only at 688 μ. Thus, nonfluorescence of the 683 component cannot be made responsible for the red drop of fluorescence in green algae (also see reference 39). One can try postulating that chlorophyll a 683 is not totally nonfluorescent and calculate its fluorescence yield (which turns out to be about 30-40% of that of the 668 component), needed to make the calculated "half-point" coincide with the observed one. However, the so-calculated red drop curve falls off much less steeply than the experimental one (in Chlorella). The need for an explanation of the "red drop" not related to the doublet structure of the red absorption band of chlorophyll a is demonstrated even more clearly by the early red drop in red and blue-green algae, in which Cederstrand's analysis suggested the presence of the same two main chlorophyll a components. [An alternative interpretation of these two components of the red chlorophyll a band in vivo is to ascribe them not to two different forms of Chl a, but to doublet splitting of a single band through the type of "dimerization" discussed by Hochstrasser and Kasha (40).]

For green algae, other hypotheses can be tried, for example, postulating a third long-wave component, which absorbs significantly only above 680-685 μ, with an absorption peak somewhere between 690 and 700 μ. Because of low total absorption at 690-700 μ, this component (perhaps identical with that postulated by Kok (41) and called by him "C700") would have to be present in a considerably smaller concentration than Chl a 668 and Chl a 683; however, its concentration could not be as low as ~0.3% of that of total chlorophyll a (as that of Kok's "P700") because if this were the case, the red drop could not begin already at 680-685 μ close to the peak of the over-all red band. For red and blue-green algae, additional hypotheses are needed to explain the much earlier "red drop."

To sum up, we must acknowledge that, so far, no simple explanation of the position (and shape) of the "red drop" in the action spectra of chlorophyll a fluorescence in different algae appears on hand. The main contribution of the present paper is a more precise redetermination of the quantum yield of fluorescence as a function of wavelength in the "red drop" region by two independent methods, the application of the Stepanov-Ketskeméy equation, which uses data on the absorption and the emission intensities in their "overlap" region, and direct measurements of the fluorescence yield at different wavelengths of excitation. The results of the two methods are in good agreement, particularly in the case of Chlorella.

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