LOW-TEMPERATURE FLUORESCENCE EXCITATION SPECTRA FOR LONG-WAVELENGTH EMISSION AS A FUNCTION OF GREENING IN *EUGLENA GRACILIS* AND CHLOROPHYLL *A* CONCENTRATION IN VITRO: A MATHEMATICAL MODEL TO DESCRIBE BOTH SYSTEMS

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ABSTRACT A systematic study was made of the spectrum for exciting long-wavelength fluorescence (at 77°K) during the first 100 hr of greening in *Euglena gracilis*. A band at 705–710 nm is observable after cells have been greening in light for 30 hr. The ratio of the 705-nm to the 675-nm peak increases during greening, reaching a maximum value at 85 hr, then declining. With concentrated solutions of chlorophyll *a*, fluorescence excitation spectra are similar to those observed in vivo. The ratio of aggregate to monomer bands increases with concentration of chlorophyll, reaching a maximum value in ethanol and in pyridine at about 3×10^{-2} M and 6×10^{-2} M respectively, then declining. Several model systems were analyzed. It is shown that the band observed in solution with maximum at 705–710 nm is not an artifact of the fluorescence apparatus; it does not arise from undissolved chlorophyll; it does not arise from a fluorescent or nonfluorescent impurity; it does not arise solely from light absorption by a dimer or larger aggregate of chlorophyll. Agreement is obtained between the experimental observations and the results of a mathematical model by including terms for the efficiency of energy transfer from monomeric to dimeric chlorophyll, as well as for the formation of dimers by an equilibrium reaction.

INTRODUCTION

Brody (1) reported that cooling photosynthetic organisms to 77°K results in the appearance of a fluorescence emission band at long wavelengths; since similar emission occurred from concentrated solutions of chlorophyll, he ascribed this band to aggregates. In the same work, it was suggested that the maximum of the associated absorption band was probably located at about 705 nm (and could, therefore, be correlated with Kok's (2) P700). Butler (3) not only showed such a shoulder at 705 nm in vivo by low-temperature absorption techniques, but also demonstrated it in the low-temperature spectrum for exciting long-wavelength fluorescence.

Such low-temperature (77°K) fluorescence excitation spectra for emission at long wavelengths (710–740 nm), henceforth referred to as F.E.S., have been measured for a variety of organisms (3–7). However, with the exception of Butler's (8) work, which extended only over a 5-hr period, there has been no systematic study of F.E.S. in greening organisms, in which chlorophyll content and concentration are increasing. Such a study is presented here, for *Euglena gracilis* strain Z. For purposes of comparison, F.E.S. have also been determined in vitro, as a function of increasing chlorophyll concentration. A mathematical model developed to fit the in vitro observations seems qualitatively to describe some of the in vivo observations as well.

METHODS AND MATERIALS

The apparatus used to determine F.E.S. has been described in detail previously (9). In summary, it consists of two monochromators, one used to monitor intensity of fluorescence at long wavelengths (usually at 736 nm), the other to excite in the range 400 to 730 nm. The sample is placed in a flat-bottomed, optically clear Dewar flask. For measurements at low temperature (77°K), the Dewar flask is filled with liquid nitrogen; the sample is frozen rapidly by immersing it in the flask. Fluorescence from the sample is detected by front-surface emission. In order to maintain a high signal-to-noise ratio in the various experiments, slits of both monochromators were adjusted so as to yield half-band widths of 4 to 9 nm.

In general, a 1600-w high-pressure xenon lamp was utilized as light source, but in some instances spectra were obtained with a 250-w tungsten iodide lamp; these have been noted in the body of the paper. For both lamps, the number of quanta incident on the sample, as a function of wavelength, is given in Fig. 1. The spectral distribution of light energy incident upon the sample with these lamps was detected with an Epply thermopile, and the signal was amplified with a DC amplifier (Beckman, model 14).

The intensity of fluorescence at 736 nm, resulting from excitation by light of wavelength λ, is represented by $E(\lambda)$. The corrected F.E.S. is obtained by calculating $E(\lambda)/I(\lambda)$, where $I(\lambda)$ is the number of quanta incident on the sample at wavelength λ. The spectra presented in this paper have not been corrected for $I(\lambda)$ unless so indicated.

The procedure followed for producing chlorophyll-less *Euglena gracilis* strain Z, and the subsequent conditions for culturing in the light, have been previously described (9) in detail. In summary, cells in the dark, at a temperature of 23–26°C, are continuously inoculated into fresh medium to keep them in the log phase of growth, for over fifty generations. They are then placed over cool-white fluorescent lamps which deliver an incident intensity of 92 ft-c. In the present work, the term "age" of cells is used to refer to the length of time they have been in the light (and is therefore equivalent to the greening or light-adaptation period).

In the course of preliminary measurements, it was found that the shape of the F.E.S. is dependent on the geometry of form of the sample vessel. If a beaker is used, for example, the sample tends to freeze unevenly, collecting along the vertical sides of the vessel, and this results in a shorter optical path through the center and a longer one at the sides. Since this problem could be largely circumvented by freezing samples in 1-mm capillary tubes, these were used in the present work. Exciting light impinged at right angles to the long axis of the tube, and the average optical path length, obtained by integrating over the circular cross-section of the tube, was 0.785 mm. In general, with cells "older" than 30 hr the density of the cell suspension in the capillary tube was chosen so that the light absorbed at the chlorophyll maximum was about 90%.

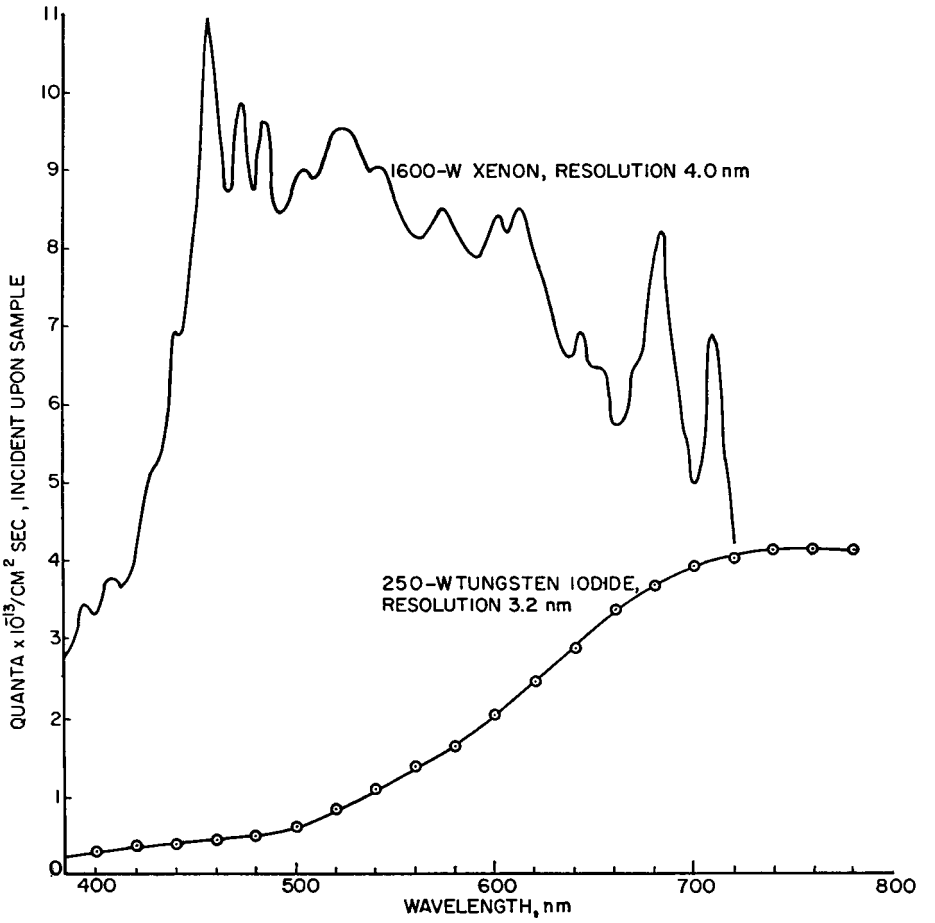


FIGURE 1 Spectral distributions and intensity of light incident on the sample in quanta/cm² sec for tungsten iodide and high-pressure xenon lamps; monochromator slit widths set to give the indicated resolution.

Crystalline chlorophyll *a* was prepared chromatographically by a procedure (see reference 12) based on a combination of the methods described by Jacobs et al. (10) and by Anderson and Calvin (11). Solvents, used without further purification, were reagent grade pyridine (Fisher Scientific Co., Pittsburgh, Pa.) and absolute reagent grade ethanol (U. S. Industrial Chemicals, Flushing, N. Y.). Chlorophyll concentration was determined by dissolving a weighed crystalline sample in a measured amount of solvent.

Since fluorescence intensities were measured at 736 nm, it was necessary to determine the relative contributions of monomer and aggregate to emission at this wavelength. Their respective fluorescence yields at 77°K, Φ' and Φ'', were used for this purpose. In the case of the aggregate, the contribution was equated to the published (13) value of $\Phi'' = 0.9$, because fluorescence measurements were made at (or close to) its emission maximum. However, in the

case of the monomer, measurements were made in the "tail" of its fluorescence emission band, and an adjustment was therefore necessary. Keeping in mind that, in vitro, intensity of monomer emission at 736 nm compared with its maximum at 685 nm is about 0.40 and that Φ' is equal (13) to 0.38, a value of 0.15 was calculated for the relative contribution of the monomer to emission at 736 nm. In this paper, Φ' and Φ'' are used to represent these relative fluorescence contributions, where single and double prime represent monomer and aggregate, respectively.

RESULTS

In Vivo

As a function of greening in *Euglena gracilis*, F.E.S. were monitored. See Fig. 2, in which F.E.S., for emission at 736 nm, are given for 69- and 96-hr cells. The three

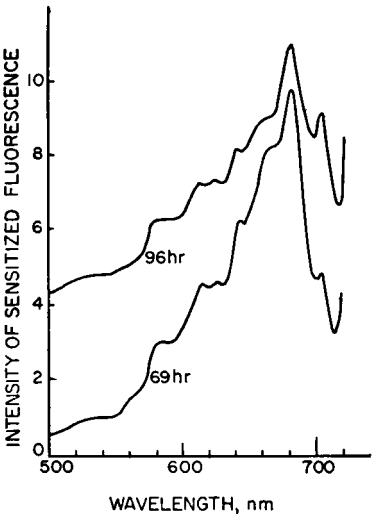


FIGURE 2 Low-temperature (77 K) fluorescence excitation spectra of *Euglena gracilis* strain Z, monitored at 736 nm. Curves were obtained with dark-grown cells which had been allowed to green for 69 and 96 hr, as indicated. Spectrum for 96-hr cells has been displaced upward 3 units to facilitate comparison of data. Fluorescence-intensity is given in arbitrary units.

bands observed in the red end of the spectrum will be referred to as E705, E686, and E675 on the basis of the position of their maxima. These same designations will be used for the intensities of fluorescence excited by these respective wavelengths.

The shortest-wavelength band which probably corresponds to the main (monomeric) absorption band of chlorophyll *a* in vivo is the first to appear during greening. In organisms which have been exposed to light for less than 30 hr, E705 and E686 are too small to be detected or resolved. After this time they are readily observed. E705 and E675 were followed systematically, and it was observed that the ratio E705/E675 increases during greening, going through a maximum at about 85 hr. This relationship is shown in Fig. 3, where R is plotted as a function of greening. R is a shorthand notation for $E705 \cdot I675/E675 \cdot I705$, where I675 and I705 represent number of quanta incident on the sample at 675 and 705 nm, respectively. After approximately 85 hr, R decreases, apparently reaching a plateau beginning

at about 98 hr; this plateau seems also characteristic of log-phase cells grown continuously in the light.

The single, main, red band in the F.E.S. narrows during the first 30 hr that *Euglena* is in the light. A quantitative measure of this change is obtained by determining the ratio of the E's at wavelengths 675 and 705 nm; these results are plotted as R in Fig. 3. The wavelength 705 nm was chosen simply as an experimental convenience; it does not imply that E705 is detectable or present before 30 hr.

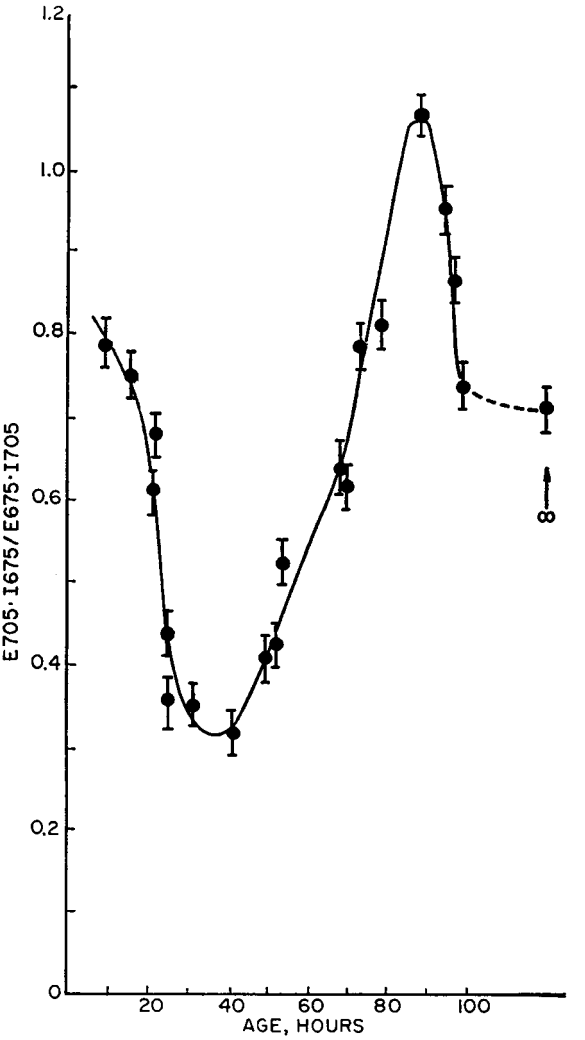


FIGURE 3 R as a function of hours that dark-grown *Euglena gracilis* strain Z have been exposed to light. R = E705·I675/E675·I705 represents a ratio of the fluorescence intensities obtained at 736 nm upon irradiation with light of wavelengths 705 and 675 nm, at 77°K. The fluorescence intensities have been corrected for quanta incident on the sample. The symbol ∞ is used to represent R measured for log-phase cells continuously cultured in the light.

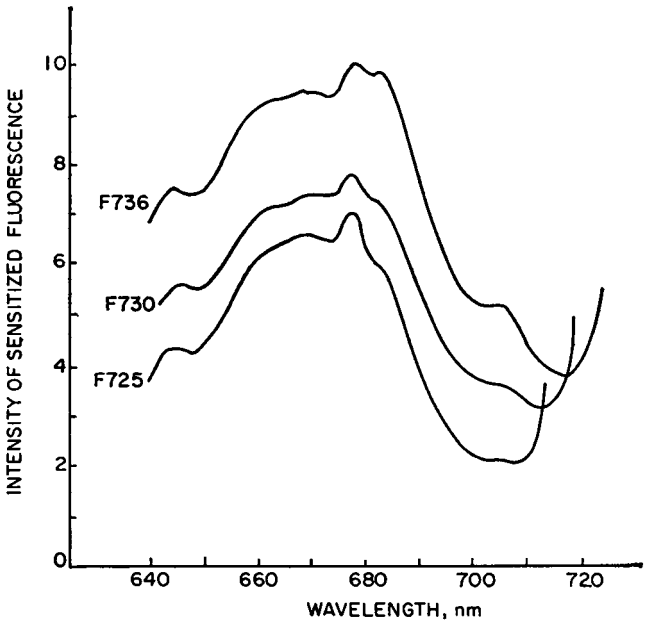


FIGURE 4 Low-temperature (77°K) fluorescence excitation spectra of 73-hr *Euglena gracilis* strain Z measured at 725, 730, and 736 nm, indicated as F725, F730, and F736, respectively. The spectra have been displaced upward 0.5, 2, and 3 units respectively, to facilitate comparison of data. Fluorescence intensity is given in arbitrary-units.

TABLE I

SUMMARY OF RED EXCITATION BANDS OBTAINED WITH 73-HOUR-OLD *EUGLENA GRACILIS*

Fluorescence maximum	Relative intensity of excitation bands			Ratio of excitation bands		
	E705	E686	E675	E686/E705	E675/E705	E675/E686
nm						
F736	2.40	8.20	8.40	3.41	3.50	1.02
F730	1.95	6.60	7.30	3.38	3.75	1.11
F725	2.05	6.95	8.30	3.39	4.05	1.19
				Maximum % change in ratio:		
				0.89	16.0	16.6

In order to determine whether the three maxima in the red region of the spectrum correspond to one, two, or three different molecular species, F.E.S. for 73-hr cells were monitored at three different wavelengths: 736, 730, and 725 nm. From Fig. 4 and Table I, in which these data are presented, it may be seen that although the ratio E686/E705 is fairly independent of the wavelength being monitored, the other ratios are not; also E705 and E686 seem to vary in about the same proportion relatively to E675. It may be, of course, that the ratio E686/E705 changes in organisms of different ages; the present work did not include such a study.

It should be pointed out that the wavelengths cited as maxima for F.E.S. are only approximate; they were observed to shift about 3–4 nm to longer wavelengths as a function of greening and also as a function of increasing concentration of chlorophyll in solution (see below).

In Solution

A. *Ethanol.* F.E.S. of dilute solutions of chlorophyll *a* in ethanol have no peaks or shoulders at wavelengths longer than about 667 nm; however, at concentrations greater than 10^{-3} M, a band with maximum at about 705 nm is observed. See Fig. 5.

Both corrected and uncorrected F.E.S. of concentrated solutions of chlorophyll *a* in ethanol have bands with maxima at 667 and 676 nm, in addition to the maximum at 705 nm. In this section, in correspondence with the designations adopted above, fluorescence intensities measured at 736 nm, which result from irradiation at these wavelengths, will be referred to as E667, E676, and E705.

Again, in order to determine quantitatively the relationship between E705 and E667, R is calculated, but in this case, it is equal to E705·I667/E667·I705, where I667 and I705 stand for the number of quanta incident on the sample at 667 and

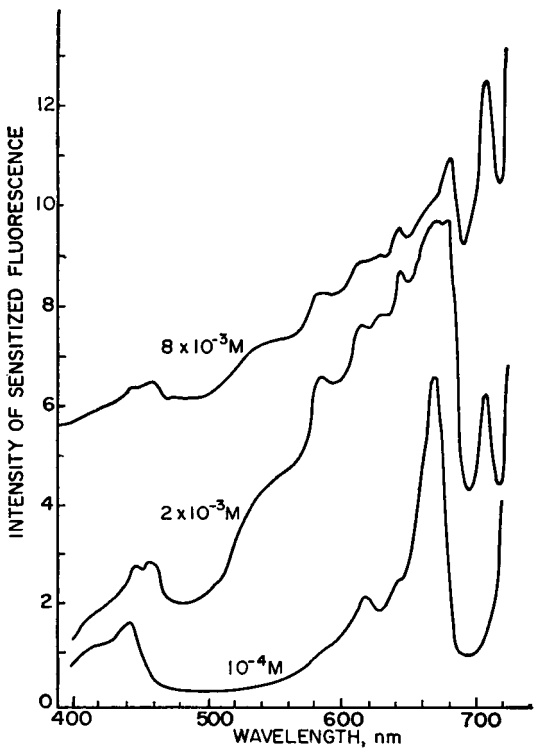


FIGURE 5 Low-temperature (77°K) fluorescence excitation spectra of chlorophyll *a* in ethanol, measured at 736 nm. The curves are for chlorophyll concentrations of 10^{-4}, 2×10^{-3}, and 8×10^{-3} M, as indicated. Spectrum of 8×10^{-3} M chlorophyll has been displaced upward 10 units to facilitate comparison of data. Intensity of sensitized fluorescence is given in arbitrary units.

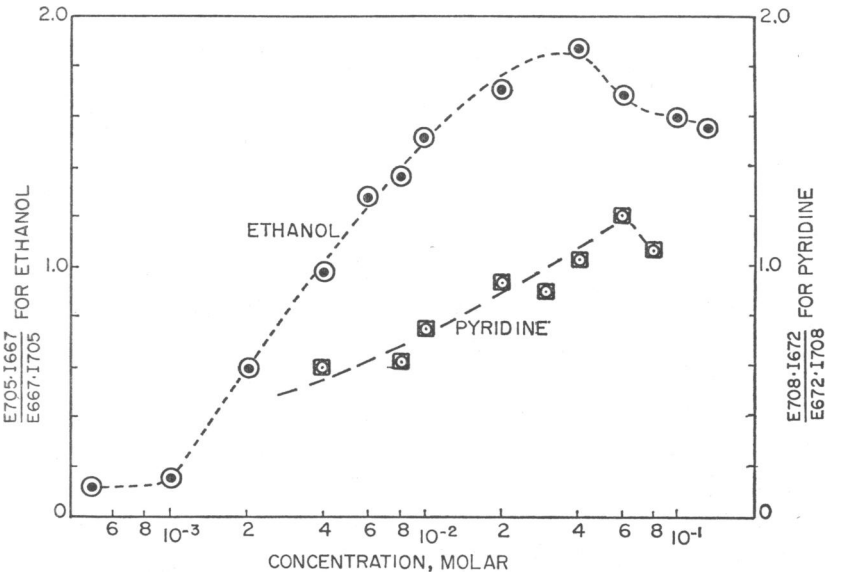


FIGURE 6 R as a function of chlorophyll *a* concentration in ethanol (circles) and in pyridine (squares). R equals E705·I667/E667·I705 for ethanol and E708·I672/E672·I708 for pyridine. It represents a ratio of the fluorescence intensities obtained at 736 nm upon irradiation with light of wavelengths 705 and 667 for ethanol and 708 and 672 for pyridine at 77°K. The fluorescence intensities have been corrected for quanta incident on the sample.

705 nm, respectively. In Fig. 6, R is plotted as a function of the logarithm of concentration over the range 10^{-4} to 10^{-1} M chlorophyll *a* in ethanol. At a concentration of about 3×10^{-2} M, R is seen to reach a maximum value.

B. *Pyridine.* F.E.S. of concentrated solutions of chlorophyll *a* in pyridine exhibit maxima at 708, 682, and 672 nm. (Again these positions are approximate.) In Fig. 7 are shown corrected F.E.S. for three different concentrations of chlorophyll. From Fig. 6 it may be seen that the ratio R = E708·I672/E672·I708 increases with concentration, reaching a peak at about 6×10^{-2} M.

TABLE II

MAXIMA OF FLUORESCENCE EXCITATION BANDS (IN THE RED REGION OF THE SPECTRUM) FOR SENSITIZING EMISSION AT 736 NM (77°K)

In vivo	In vitro	
	Ethanol	Pyridine
nm	*nm*	*nm*
705–710	705	708
686	676	682
675	667	672

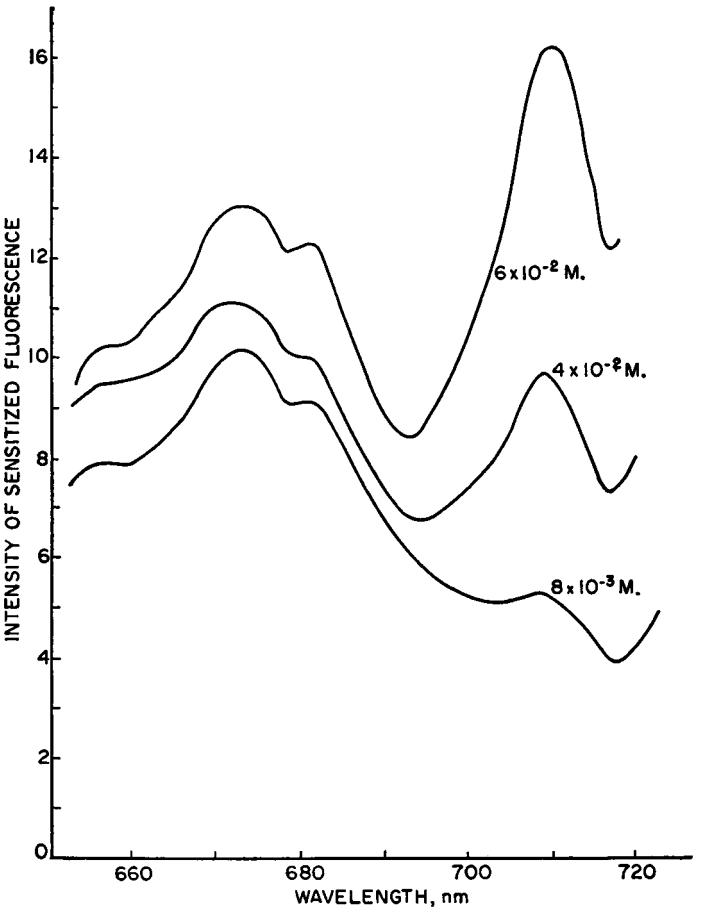


FIGURE 7 Low-temperature (77°K) fluorescence excitation spectra of chlorophyll *a* in pyridine measured at 736 nm. Data have been corrected for quanta incident upon the sample. The curves are for chlorophyll concentrations of 8×10^{-3}, 4×10^{-2}, and 6×10^{-2} M, as indicated. The spectra have been displaced upward to facilitate comparison of data. Intensity of sensitized fluorescence is given in arbitrary units.

The various F.E.S. maxima observed in vivo and in solution are summarized in Table II.

ANALYSES AND CONCLUSIONS

The data obtained above permit inquiry into the origin or nature of E705 in F.E.S. For some of the possibilities, mathematical models can be written and critically evaluated. Except in a few instances, specifically noted, this section deals exclusively with the in vitro ethanolic system.

1. The possibility that the 705-nm band arises as an artifact from the high-pressure xenon lamp was explored. The spectral output of this lamp (see Fig. 1)

reveals many maxima e.g. at 603, 615, 685, and 711 nm, and minima at 701, 663, and 608–592 nm. Though none of these maxima correspond exactly in position to the observed 705-nm band, to rule out the possibility that they contribute to F.E.S., a correction for the spectral distribution of the incident light was made by dividing $E(\lambda)$ by $I(\lambda)$, as described under Methods and Materials. Even after such correction, E705 is still evident. (See Fig. 7.)

In the case of cells which have light-adapted for more than 30 hr, a maximum at about 686 nm is apparent. Although this maximum coincides with one of the emission maxima of the xenon lamp, it seems to be present even after correction. Such a band was previously reported in vivo (6, 14, 15) and in solution (4, 14).

To further test the relationship between E705 and lamp spectral output, a tungsten iodide lamp which does not have multiple maxima in the red region of the spectrum (see Fig. 1) was used. From Fig. 8 it may be seen that although there are differences in the uncorrected F.E.S. obtained with xenon and tungsten lamps, arising from differences in their spectral output, with both lamps there is still a maximum at about 705 nm. Using the data in Fig. 1 to correct the data shown in Fig. 8, R is equal to 1.2 for both curves.

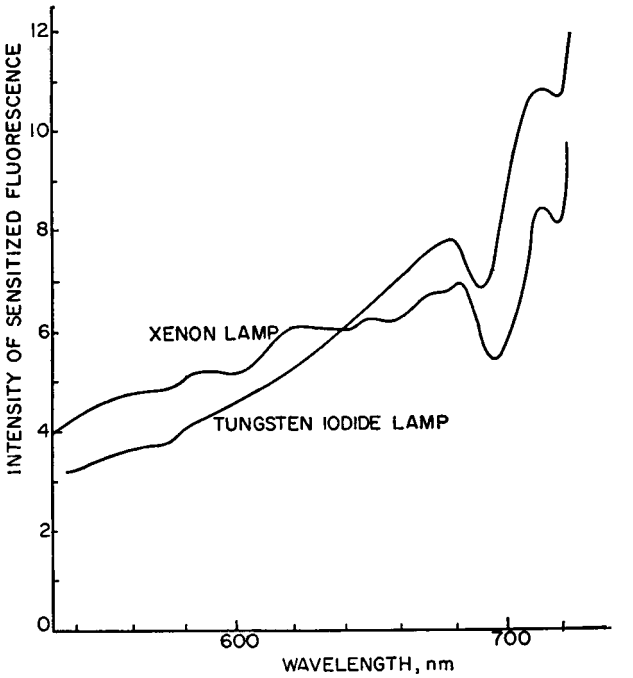


FIGURE 8 Low-temperature (77°K) fluorescence excitation spectra of 6×10^{-3} M solution of chlorophyll *a* in ethanol, measured at 736 nm. Two different light sources were used in conjunction with the excitation monochromator: a 1600-w high-pressure xenon and a 250-w tungsten iodide lamp, as indicated. Correcting for the incident light intensity, the value of E705·I667/E667·I705 is equal to 1.2 for both curves.

2. The possibility that E705 might arise from undissolved chlorophyll in concentrated solutions was considered next. In the first place, it was noted that E705 is present in 8×10^{-3} M solutions—a concentration within the published "limit of solubility" of chlorophyll (16). Secondly, that there is no appreciable undissolved material even at concentrations up to 5×10^{-2} M is shown by the absence of precipitate after centrifugation of the solution at 150,000 *g* for 45 min. Thirdly, even at concentrations of about 10^{-1} M there was no evidence of light scattering or turbidity, phenomena usually associated with colloidal suspensions. Fourthly, if E705 did arise from undissolved material, one would expect R to increase steadily with chlorophyll concentration, rather than go through a maximum, as was seen in Fig. 6. In the fifth place, it should be kept in mind that chlorophyll crystals are not fluorescent; therefore any light absorbed by undissolved chlorophyll would be incapable of giving rise to emission at 736 nm.

If, on the other hand, light were absorbed in an optically black solution by nonfluorescent material having an absorption band at about 695–700 nm, it is conceivable that there would arise in the F.E.S. both a minimum at these wavelengths and a false maximum at about 705 nm. However, it can be shown that under such conditions, R would never reach or exceed unity, in contradiction to experimental findings (see Fig. 6).

3. If the F.E.S. band in question were to arise solely through absorption by a highly fluorescent (chlorophyll-like or non-chlorophyll-like) trace impurity, having an absorption maximum at about 705 nm, it would contribute to fluorescence at 736 nm in the following way: $E(\lambda_1) = G \cdot I(\lambda_1)\phi^i(1-10^{-zc})$, where G is a constant characteristic of the geometry of the system, ϕ^i is the relative contribution of the impurity to fluorescence at 736 nm, C is the concentration of chlorophyll, λ_1 equals 705 nm, and Z is a term including optical path length as well as the extinction coefficient of the impurity and its relative proportion to that of chlorophyll; all other symbols are as previously defined.

If all the light absorbed at 667 nm is by monomeric chlorophyll, and there is essentially total absorption at this wavelength, then the ratio

$$\frac{E(\lambda_1)}{E(\lambda_2)} = \frac{I(\lambda_1)\phi^i(1 - 10^{-zc})}{I(\lambda_2) \cdot \phi'},$$

where ϕ' is the relative fluorescent contribution of monomeric chlorophyll at 736 nm and λ_2 equals 667 nm. Solving for concentration, one finds

$$ZC = \log\left[\frac{1}{1 - \frac{E(\lambda_1) \cdot I(\lambda_2) \cdot \phi'}{E(\lambda_2) \cdot I(\lambda_1) \cdot \phi^i}}\right],$$

from which it can be seen that, under these conditions, a linear relationship is to be expected between C and the log term. However, from Fig. 9 (in which data obtained

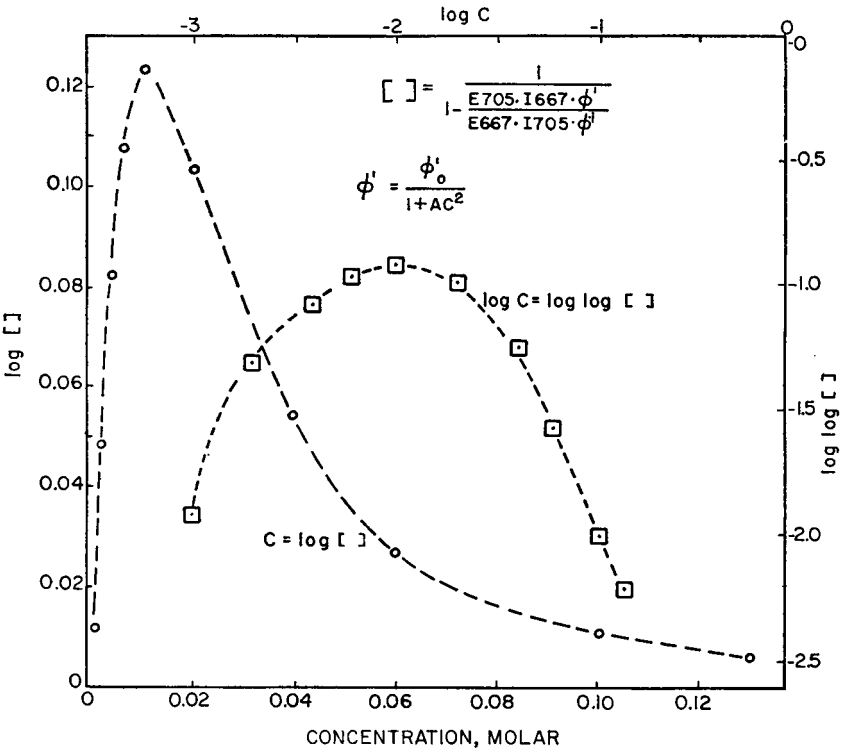


FIGURE 9 The data given in Fig. 6, for chlorophyll in ethanol, are used to test two mathematical models for the system. In the first model it is assumed that a fluorescent impurity gives rise to the band at 705 nm. The resulting equation is

$$ZC = \log\left[\frac{1}{1 - \frac{E705 \cdot I667 \cdot \phi'}{E667 \cdot I705 \cdot \phi^i}}\right] = \log [\quad],$$

where $\phi' = \phi'_0/1 + AC^2)$; see text for definition of terms. The curve obtained with this equation is depicted by circles. In the second model it is assumed that aggregates of chlorophyll are formed as a function of concentration and have an absorption maximum at 705 nm. Energy transfer between monomer and aggregate is neglected. The resulting equation is X log C = log log [ ]. The curve obtained with this equation is depicted by squares. Neither model yields the required linear relationship.

in the present work have been used to calculate the log term), it may be seen that a linear relationship does *not* obtain, and therefore the possibility that E705 is an impurity may be eliminated.

For these calculations ϕ^i was set equal to unity. Since ϕ' varies with concentration, it was necessary to determine the function. In Fig. 10 is shown concentration quenching of fluorescence from chlorophyll in ethanol at room temperature; this

relationship can be represented by

$$\phi' = \phi'_0/(1 + AC^2), \tag{1}$$

where ϕ'_0 is relative fluorescence contribution of monomeric chlorophyll in infinitely dilute solution and A is calculated to give the best fit to the experimental data. The values used for ϕ'_0 and A are 0.15 (see Methods and Materials) and 1100, respectively. It is assumed that equation (1), which obtains for concentration quenching at room temperature, also obtains at 77°K.

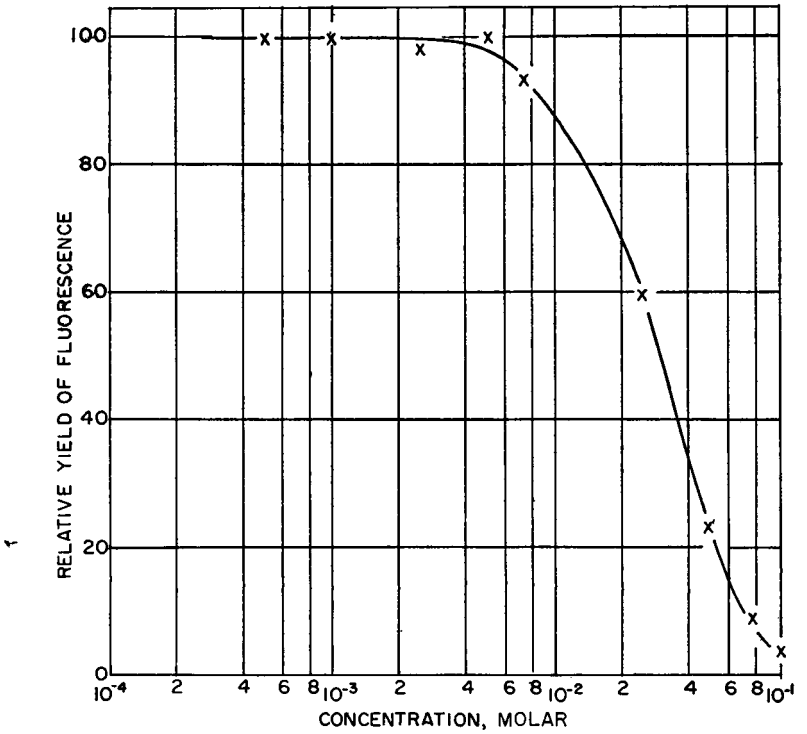


FIGURE 10 Relative fluorescence yield of chlorophyll *a* in ethanol as a function of concentration at room temperature. Fluorescence was excited with light of 436 nm and emission monitored at 675 nm (half-band width of 3 nm). Data can be described by the equation $\phi'/\phi'_0 = 1/(1 + AC^2)$ with A equal to 1100.

4. The possibility that E705 arises *solely* from absorption by fluorescent aggregates[1] of chlorophyll which form in highly concentrated solutions was next considered. If this were the sole source, the following approximation would obtain:

[1] The possibility that aggregates form on cooling is neglected, since the F.E.S. does not change appreciably upon cooling (35). The increase in the fluorescence yield of monomeric chlorophyll upon cooling is the same in dilute and concentrated solutions (34).

$$WC^X = \log\left[\frac{1}{1 - \frac{E(\lambda_1)\cdot I(\lambda_2)\cdot \phi'}{E(\lambda_2)\cdot I(\lambda_1)\cdot \phi''}}\right] = \log [\quad],$$

where X is the number of monomer units forming the aggregate, W is a term which includes the optical path length as well as the extinction coefficient and equilibrium constant for the aggregate; other terms are as defined previously. A plot of the equation log log [ ] = X log C log W would be expected to yield an approximately straight line with a slope of X. As can be seen from Fig. 9, such a linear relationship does *not* hold for the concentration range studied.

5. Possibilities 4 and 3 will now be reconsidered as items 5 and 6, respectively. In this reconsideration, it is assumed that fluorescence emission at 736 nm cannot be attributed solely to direct light absorption by materials other than monomeric chlorophyll; an additional factor for energy transfer is introduced.

An expression can be derived which describes both quantitatively and qualitatively the observed dependence of F.E.S. on concentration by including such a factor for energy transfer. Dimers are assumed to be formed according to the equation $M^2K = D$, where M and D are concentrations of monomer and dimer, respectively, and K is their equilibrium constant. Furthermore, the total chlorophyll concentration is given by

$$C = M + 2D, \tag{2}$$

where

$$M = \sqrt{1 + 8KC/4K}. \tag{3}$$

A calculation of R, which defines the relationship between the corrected F.E.S. at λ_1 and that at λ_2, is made using the following equation:

$$R = \frac{E(\lambda_1)}{E(\lambda_2)} \cdot \frac{I(\lambda_2)}{I(\lambda_1)} = \frac{A(\lambda_1)\phi(\lambda_1)}{A(\lambda_2)\phi(\lambda_2)}. \tag{4}$$

The amount of light absorbed at λ_1 or λ_2 is specifically represented by $A(\lambda_1)$ and $A(\lambda_2)$. The generalized notation, $A(\lambda)$, is given by

$$A(\lambda) = 1\text{–}10^{-OD(\lambda)}, \tag{5}$$

where $OD(\lambda)$ is the total optical density at λ. This total optical density is given by

$$OD(\lambda) = \epsilon'(\lambda)Md + \epsilon''(\lambda)\,Dd, \tag{6}$$

where $\epsilon'(\lambda)$ and $\epsilon''(\lambda)$ are, respectively, the extinction coefficients of M and D at λ, and d is the optical path length. In all the present experiments, $OD(\lambda_2) \geq 2$, so

that $A(\lambda_2)$ may be set equal to unity. The "averaged fluorescence efficiency," $\phi(\lambda)$, obtained by irradiation at λ is given by the following equation:

$$\phi(\lambda) = \frac{\phi' \cdot OD'(\lambda) + \phi'' \cdot OD''(\lambda) + \phi'' \cdot \phi_T \cdot OD'(\lambda)}{OD(\lambda)}, \tag{7}$$

where ϕ' and ϕ'' are as defined previously, and $OD'(\lambda)$ and $OD''(\lambda)$ are the optical densities of M and D, respectively. The last term in the numerator is the correction necessary for energy transfer considerations. Energy absorbed by M is transferred to D with an efficiency ϕ_T and is then emitted by D with an efficiency ϕ''. It is assumed that ϕ'' is constant and independent of C, and that ϕ' varies with C as described under possibility 3 above.

To evaluate ϕ_T, it is assumed that concentration quenching of chlorophyll fluorescence (as observed at room temperature) arises from increasing efficiency of energy transfer from fluorescent to weakly fluorescent (aggregated) chlorophyll, as well as from an increasing concentration of these aggregates. It is also assumed that values of ϕ_T and concentration of aggregate determined at room temperature apply also at low temperature. Experimental evidence based upon fluorescence spectroscopy supports this assumption (34).

An expression for ϕ_T in terms of the relative fluorescence contribution is readily obtained, i.e. $\phi_T = (\phi'_0 - \phi')/\phi'_0$. Substituting for ϕ', using equation (1), yields

$$\phi_T = AC^2/(1 + AC^2). \tag{8}$$

In Fig. 11 is plotted equation (4);[2] by comparison with Fig. 6 it may be seen that the mathematical model duplicates the observed in vitro relationship of R in ethanol as a function of C; to this extent it is "correct."

The two most significant similarities between the curves in these figures are the existence of a similar maximum value for R and of a similar value of C for which $dR/dC = 0$.

To get a reasonable agreement between the model and the experimental data, the two sets of values given in Table III were used for the parameters in equation (4). The resulting R's are shown as curves A and B in Fig. 11. There are many possible sets of values which give similar agreement. The initial estimates for $\epsilon''(\lambda)$ were based on difference spectroscopy measurements at room temperature (17) and absorption spectra at the temperature of liquid nitrogen.[3] The above values of K, A, ϵ', and ϵ'' which are satisfactory for chlorophyll at 77°K are similar to those

[2] When equations (5), (6), and (7) are substituted into equation (4), the following equation results:

$$R = (1 - 10^{-[\epsilon'(\lambda_1) \cdot M \cdot d + \epsilon''(\lambda_1) \cdot D \cdot d]}) \left\{ \frac{\phi''\epsilon'(\lambda_1) \cdot M + \phi''\epsilon''(\lambda_1) \cdot D + \phi''\phi_T\epsilon'(\lambda_1) \cdot M}{\phi'\epsilon'(\lambda_2) \cdot M + \phi''\epsilon''(\lambda_2) \cdot D + \phi''\epsilon'(\lambda_2) \cdot M} \right\}.$$

Equations (1), (2), (3), and (8) are used to evaluate ϕ', D, M, and ϕ_T, respectively.

[3] S. S. Brody and S. B. Broyde. In preparation.

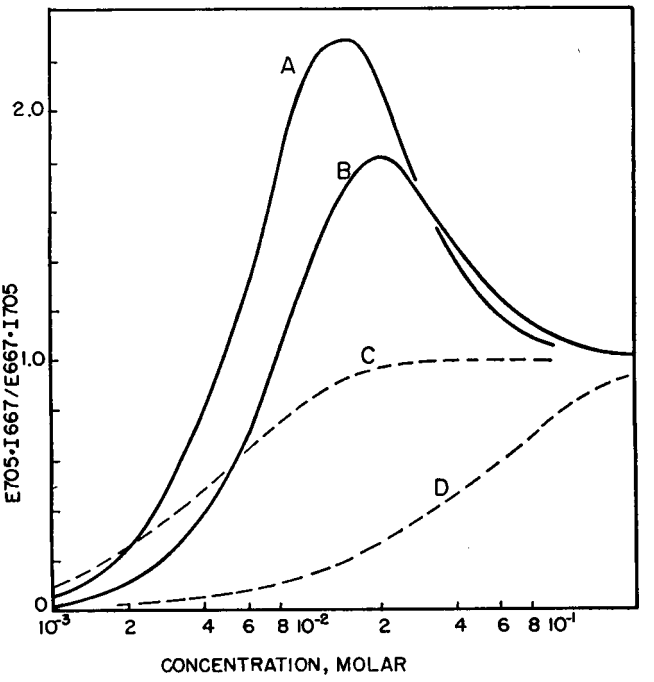


FIGURE 11 Theoretical values of R (equal to E705·I667/E667·I705) as a function of chlorophyll *a* concentration in ethanol. For curves A and B, equation (4) is used (see text); it is assumed that dimers are forming with increasing concentration according to $M^2K = D$. For curves C and D, it is assumed that a "pigmented impurity" is present as a constant proportion of the total chlorophyll concentration. In both cases a term is included to account for energy transfer from monomeric to dimeric chlorophyll in the first case, and to the "impurity" in the second case; the transfer efficiency is a function of concentration (see equation 8). The values of the parameters used for curves A, B, C, and D are listed in Table III.

TABLE III

VALUES OF PARAMETERS TO OBTAIN CURVES IN FIGURE 11

Parameter	Curve			
	A	B	C	D
X	—	—	0.01	0.01
K	2	2	—	—
ϕ'' or ϕ^i	0.8	0.8	0.9	0.8
ϕ'_0	0.1	0.1	0.1	0.1
$\epsilon''(667)$	130,000	30,000	73,000	30,000
$\epsilon'(667)$	70,000	70,000	67,000	70,000
$\epsilon'(705)$	100	100	1,500	100
$\epsilon''(705)$	40,000	10,000	3,500	10,000
A	1,200	800	1,000	800
d	0.04	0.065	0.05	0.065

determined at room temperature (17). This may be indicative of the similarity of the state of chlorophyll in the solution at both temperatures.

No correction is made here for the increase in concentration which results from the contraction of ethanol upon cooling to 77°K.

In summary, this model, which gives the most probable representation of the chlorophyll F.E.S. in solution (of all the possibilities considered in the present work), is based on the following assumptions: first, that energy absorbed by the monomer can contribute to fluorescence at 736 nm (F736) directly as well as by transfer to the aggregate; second, that energy received by the aggregate, either by direct absorption or by transfer from the monomer, can also contribute to F736.

6. Another possible model (based on item 3, above) to interpret E705 in the F.E.S. is that energy is transferred to a fluorescent impurity IM, whose concentration is assumed to represent a constant proportion, X, of the total chlorophyll concentration, i.e. $IM = X \cdot C$ and $M = C - IM$. All other assumptions and relationships used for its formulation are the same as for the dimerization model, given in item 5, above. The two sets of values given in Table III were used in the equation assuming an impurity. The resulting R's are shown as curves C and D in Fig. 11. As can be seen in the figure, this model does not result in a function exhibiting a maximum value of R at a particular concentration, nor does it give values of R greater than 1, but rather R approaches unity asymptotically. Therefore, we may eliminate this possibility as a model for describing the F.E.S. of chlorophyll in ethanol.

GENERAL DISCUSSION

From Fig. 6 it was seen that the value of R in vitro, which in general increases with concentration, drops at concentrations greater than 3×10^{-2} M and 6×10^{-2} M, respectively, for ethanol and pyridine. One interpretation is the formation of larger, less fluorescent aggregates at higher concentrations (18). A decrease in R may also be explained in terms of increased efficiency of energy transfer between monomer and dimer; this latter possibility was the one adopted in the present work, because it makes unnecessary the involvement of higher aggregates, about which little is known. (However, it was suggested earlier (4, 19) that higher aggregates do exist in vivo.)

From the mathematical analysis given above for the F.E.S., we may conclude that in solution the value of R is determined by the relative concentration of aggregated and total chlorophyll, as well as ϕ_T between monomer and aggregate. Though the model derived for the in vitro F.E.S. situation may apply to the in vivo one as well, it is doubtful that ϕ_T varies with total chlorophyll concentration in the same fashion in vivo as it does in vitro (since, for example, it has been shown that aggregated chlorophyll in vivo has specific orientations (20), and it is likely that additional considerations come into play in the disposition of aggregates in photosynthetic organisms).

One should expect, therefore, that for any given ratio of aggregate to monomer (or to total chlorophyll concentration) ϕ_T might be smaller or larger than in the corresponding in vitro case; these two situations in vivo would result in smaller and larger values of R, respectively.

If however, in spite of these limitations, one takes the value of R, observed in vivo, as indicator of the concentration of aggregated chlorophyll, it may be concluded that in *Euglena* the aggregate does not represent a constant fraction of the total chlorophyll, but, instead, an increasing fraction. (Of course, even in cells continuously cultured in the light, concentration of aggregate is always less than that of monomer.) The relative concentration of aggregate in greening *Euglena* seems to depend on the "age" of the pigment system in the way that concentration of aggregates in solution depends on total chlorophyll concentration. Furthermore, one may also conclude that the apparent concentration of aggregate grows larger with age, as evidenced by the increasingly greater efficiency with which monomeric chlorophyll sensitizes emission by the aggregate (as compared with its own emission). These observations and the conclusion drawn from them are in agreement with one reached previously on the basis of a study of fluorescence emission as function of greening in *Euglena* (9).

Since the maximum value of R occurs, in vivo, at 85 hr of light adaptation (Fig. 3), and, in solution, at a chlorophyll concentration of 3×10^{-2} M (Fig. 6), one might propose that the latter concentration of chlorophyll represents the one in 85-hr cells. However, on the basis of emission spectroscopy, it was earlier suggested (9) that such cells have an "effective" chlorophyll concentration of 10^{-1} M. The origin of this inconsistency may well be that ϕ_T is greater in cells than in solution; under such a condition the ratio of emissions (F_A/F_M) from the aggregate, F_A, and monomer, F_M, will increase at a faster rate in vivo than in solution for the same concentration of chlorophyll. This ratio was indeed the one used by Brody, Brody, and Levine (9) for estimating "effective" chlorophyll concentration; however, in their study, no attempt was made to correct for energy transfer. Because of this and as seen from the present work, their estimates of chlorophyll concentration in vivo are probably too high, and, as a consequence, their calculations of lamellar area are too low by about a factor of 2.

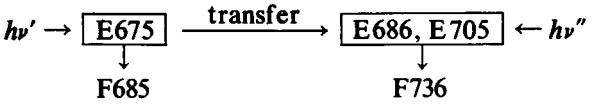
Though all the variables which contribute to the observed dependence of R on greening during the first 30 hr (Fig. 3) are not known, the presence of chlorophyll precursors is probably involved. Relatively high concentrations of pigments, such as protochlorophyll and chlorophyllide, are contributing to absorption during this period (21), and it has been reported that fluorescence spectra of such cells are quite different from those seen in later stages (22, 23).

As noted above, the position of the "E705 maximum" is not constant, but is found to shift from 703 to 708 nm with increasing age, and also with increasing concentration of chlorophyll in solution. This variation in location probably cor-

responds to those noted for absorption maxima. The latter have sometimes been interpreted as evidence for the existence of several different forms of chlorophyll (24), presumably arising from the same state of chromophore attached to different proteins. However, on the basis of the present in vivo and in vitro work (as well as earlier work (4, 18)), these shifts may be attributed to increasing concentrations of chromophore, a condition which is to be expected in cells of different ages or in cells having different amounts of chlorophyll.

It was seen (Table II) that even after correction, both in vivo and in vitro excitation spectra exhibit three bands in the red end of the spectrum. The shortest-wavelength band has generally been associated with monomeric chlorophyll. From Fig. 4 and Table I, it appears that the middle- and the long-wavelength band arise either from the same molecular species or from two different species between which there is a high and constant efficiency of energy transfer. The former alternative seems most attractive on the basis of the finding that the ratio E686/E705 is independent of the wavelength at which fluorescence is being monitored. The conclusion that these bands arise from the same molecular species, the aggregate, was proposed earlier (4, 14, 18, 25, 28); it was further suggested (14, 25, 28) that both (C_a683 and C_a705) are associated with system I. That aggregation does give rise to long-wavelength absorbing forms at about 700 nm was further demonstrated in the chlorophyll monolayer work of Sperling and Ke (27).

For *Euglena*, the present work is in agreement with the assignments made between fluorescence excitation and emission bands, as shown below:



The substance emitting at 698 nm, F698 (4, 12), was correlated by Goedheer (7) with the absorption band C_a680; for two reasons this assignment is questioned. (The difference in peak positions between C_a680 and E686 is not considered significant, for reasons given above.) First, E686 is always observed in our concentrated solution studies (see Table II), even though F698 is not present. Second, in mature *Euglena*, in which C_a683 constitutes about 50% of the total chlorophyll (26), F698 can still be seen only under special circumstances (28). Murata et al. (29) have also argued against this assignment of Goedheer's.

It was shown above that concentrated solutions of chlorophyll give rise to F.E.S. which are qualitatively similar to those observed in vivo, and that the single mathematical model presented above (equation 4) may therefore be used to describe both systems. These similarities between F.E.S. in vivo and in concentrated solutions of polar solvents further support the interpretation proposed earlier (1, 4) that long-wavelength emission in vivo arises from chlorophyll aggregates. Though it also has

been shown (30–33) that chlorophyll dimerizes readily at room temperature in nonpolar solvents, the fluorescence properties of these dimers are not similar to those observed for chlorophyll aggregates in vivo (34). Not only does fluorescence yield of chlorophyll *a* dimers in nonpolar solvents decrease upon cooling to 77°K, but, additionally, the yield of fluorescence is quite low in nonpolar solvents. On the other hand, the spectral properties of the type of aggregate formed in polar solvents are very similar to those observed in vivo, and therefore serve as a better model. At this point, it might be well to recall that Brody and Brody (14) suggested that the spectral properties of P700 (2), which absorbs maximally at about 705 nm (1, 5, 14), may be attributed to its aggregated state. In summary, it is concluded from the present work that the fluorescent properties of chlorophyll in vivo are consistent with their originating from monomeric and aggregated forms within a highly polar environment.

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REFERENCES

1. BRODY, S. S. 1958. *Science.* **128**:838.
2. KOK, B. 1961. *Biochim. Biophys. Acta.* **48**:527.
3. BUTLER, W. L. 1961. *Arch. Biochem. Biophys.* **93**:413.
4. BRODY, S. S., and M. BRODY. 1963. *Natl. Acad. Sci.—Natl. Res. Councils, Publ.* **1145**:454.
5. BUTLER, W. L., and J. E. BAKER. 1963. *Biochim. Biophys. Acta.* **66**:206.
6. GOEDHEER, J. C. 1965. *Biochim. Biophys. Acta.* **102**:73.
7. GOEDHEER, J. C. 1964. *Biochim. Biophys. Acta.* **88**:304.
8. BUTLER, W. L. 1965. *Biochim. Biophys. Acta.* **102**:1.
9. BRODY, S. S., M. BRODY and J. LEVINE. 1965. *J. Protozool.* **12**:465.
10. JACOBS, E. E., A. E. VATTER, and A. S. HOLT. 1954. *Arch. Biochem. Biophys.* **53**:228.
11. ANDERSON, A. F. H., and M. CALVIN. 1963. *Nature.* **194**:1097.
12. BROYDE, S. D., and S. S. BRODY. 1966. *Biophys. J.* **6**:353.
13. BRODY, S. S., and M. BRODY. 1962. *Trans. Faraday Soc.* **58**:416.
14. BRODY, S. S., and M. BRODY. 1965. *Arch. Biochem. Biophys.* **110**:583.
15. GOVINDJEE and L. YANG. 1966. *J. Gen. Physiol.* **49**:772.
16. STENSBY, P. S., and J. L. ROSENBERG. 1961. *J. Phys. Chem.* **65**:906.
17. BROYDE, S. B., and S. S. BRODY. *Biochim. Biophys. Acta.* In press.
18. BRODY, S. S. 1964. *J. Theoret. Biol.* **7**:352.
19. FRANCK, J., and J. L. ROSENBERG. 1963. *Natl. Acad. Sci.—Natl. Res. Council, Publ.* **1145**:101.
20. OLSON, R. A. W. H. JENNINGS, and W. BUTLER. 1964. *Biochim. Biophys. Acta.* **88**:331.
21. BUTLER, W. L., and W. R. BRIGGS. 1966. *Biochim. Biophys. Acta.* **112**:45.
22. LITVIN, F. F., A. A.KRASNOVSKY, and G. T. RIKHIREVA. 1960. *Dokl. Akad. Nauk SSSR.* **135**: 1528.
23. LITVIN, F. F., G. T. RIKHIREVA, and A. A. KRASNOVSKY. 1962. *Biofizika.* **7**:578.
24. FRENCH, C. S. 1959. *Biol. Colloq. Oregon State Chapter Phi Kappa Phi.* **20**:52; *Brookhaven Symp. Biol.* **11**:65.
25. BRODY, S. S., and M. BRODY. 1961. *Nature.* **189**:547.

26. BROWN, J. S. 1962. *In* La Photosynthese. Centre National de la Recherche Scientifique. 371.
27. SPERLING, W., and B. KE. 1966. *Photochem. Photobiol.* **5**:865.
28. BRODY, S. S., C. A. ZEIGELMAIR, A. SAMUELS, and M. BRODY. 1966. *Plant Physiol.* **41**:1709.
29. MURATA, M., M. NISHIMURA, and A. TAKAMIYA. 1966. *Biochim. Biophys. Acta.* **126**:234.
30. KATZ, J. J., G. CLOSS, F. PENNINGTON, M. THOMAS, and H. STRAIN. 1963. *J. Am. Chem. Soc.* **85**: 3801, 3809.
31. ANDESRON, A. F., and M. CALVIN. 1964. *Arch. Biochem. Biophys.* **107**:251.
32. SAUER, K., J. R. L. SMITH, and A. J. SCHULTZ. 1966. *J. Am. Chem. Soc.* **88**:2681.
33. ARNOFF, S. 1962. *Arch. Biochem. Biophys.* **98**:344.
34. BROYDE, S. B., and S. S. BRODY. 1967. *J. Chem. Phys.* **46**:3334.
35. BRODY, M., and S. S. BRODY. 1966. *Biochim. Biophys. Acta.* **112**:54.