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FLUOROMETRIC EVIDENCE FOR THE PARTICIPATION OF CHLOROPHYLL *a*-695 IN SYSTEM 2 OF PHOTOSYNTHESIS

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SUMMARY

1. The diatom *Phaeodactylum tricornutum*, grown in weak light is unusual in having a fluorescence emission band at 710 mμ in addition to the normal emission at 681 mμ at room temperature. At −187° this emission maximum is at 714 mμ and is much larger than the 681-mμ peak. The presence of this emission band is correlated with an absorption band near 700 mμ. The characteristics of the long-wavelength absorption band with respect to its fluorescence emission, formation only during light-limited growth, and lability are the same as those of a chlorophyll *a* form in Euglena called C_a695.

2. The time course of fluorescence during exposure to 436-mμ incident light, measured between 10 sec and 3 min, was the same for both the 681- and 710-mμ fluorescence bands. 3(3,4-Dichlorophenyl)-1,1-dimethylurea affected this time course at both wavelengths similarly.

3. When the temperature was lowered from 20° to −187°, the fluorescence yield decreased at 680 mμ and increased at 714 mμ. When the temperature was lowered after partial destruction of C_a695 by 10 % ethanol, the fluorescence at 680 mμ emitted by chlorophyll *a*-670 no longer decreased and the yield at 714 mμ increased much less.

4. These data from similar time courses and corresponding yield changes suggest that C_a695 receives excitation from C_a670 and could be a reaction center for System 2 of photosynthesis.

INTRODUCTION

Fluorescence emitted by chlorophyll *a* in illuminated leaves and algae can be a useful index of photosynthetic activity. GOEDHEER[1,2] has found that each of the major absorbing forms of chlorophyll *a in vivo*, C_a670, C_a680, and C_a695, has a corresponding fluorescence maximum at approx. 686, 696, and 717 mμ. The exact wavelength position of each of these bands may vary as much as ± 5 mμ depending upon the alga species. In 1952 DUYSENS[3] showed that most of the fluorescence is emitted at 685 mμ, and that the longer wavelength absorbing chlorophyll *a* (now called C_a680) is only weakly fluorescent at room temperature. More recently DUYSENS AND SWEERS[4]

Abbreviation: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

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observed that the major part of the fluorescence comes from Photosystem 2. This agrees with the generally accepted hypothesis that the shorter wavelength absorbing chlorophyll *a* (C_a670) functions primarily in Photosystem 2.

In the diatom, *Phaeodactylum tricornutum*, there is an emission band at 710 mμ (F-710) at room temperature. Except for a few species[5] such a long-wavelength fluorescence maximum has been observed only at −196°. Occurrence at room temperature makes it possible to compare the kinetics of 710 and 681 mμ fluorescence under physiological conditions. In Phaeodactylum the relative amount of F-710 can be controlled by adjusting the light intensity during growth. Evidence is presented which suggests that C_a695 which gives the 710-mμ fluorescence also reacts in Photosystem 2.

METHODS

Ph. tricornutum (formerly *Nitzschia closterium*, var. *minutissima*[6,7]), obtained from Woods Hole Oceanographic Institute, was cultured at 19° in artificial sea water[8] with 0.005 % Na_2SiO_3 either in test tubes bubbled with 3 % CO_2 in air or in flasks shaken in normal air. The cultures were illuminated with 40-W daylight fluorescent lamps. By varying the number and position of the lamps, the light intensity at the position of the cultures was varied from approx. 200 to 700 ft.-candles.

The apparatus for measuring fluorescence has been described[9,10]. The response of the apparatus as a function of wavelength was controlled automatically by a potentiometer adjusted by a photoelectric curve follower to give corrected emission spectra in terms of relative number of quanta per unit wavelength interval. The half-band width of the fluorescent light measured was 2.5 mμ.

Excitation was provided from two sources in different experiments described below. To compare excitation at different wavelengths, a xenon lamp was used in conjunction with a Bausch & Lomb High Intensity monochromator. For the kinetic and low temperature emission studies a General Electric H4 mercury lamp with Corning filter No. 9782 (4–96) and Balzer 435-mμ interference filter provided 436-mμ monochromatic light with an intensity of about 10^4 ergs/cm²/sec.

Two types of sample holders were used. One type was a flat aluminum bar with a uniform depression containing a small amount of cell suspension covered by a glass microscope coverslip. Depressions in different holders were 0.1, 0.2, or 0.4 mm deep. By changing holders, a check could be made of the effect of self-absorption of fluorescence on the emission spectrum. In order to avoid distortion of the fluorescence spectra by reabsorption, all spectra presented here were from suspensions diluted to the point where further dilution or use of a thinner layer did not affect the relative heights of the two maxima.

For low temperature measurements the metal holder was placed in a metal block having a finger projecting downward into liquid N_2. A double layer of glass and dry air over the outer window retarded frosting. A thermocouple in the block monitored the temperature. A liquid sample could be inserted and cooled to −187° within 5 min.

The other type of holder was a lucite block with a capillary bore in which the fluorescence was measured. The capillary was connected by rubber tubing and a stopcock to a reservoir containing about 50 ml of a cell suspension, stirred in the dark. A dark-adapted sample was placed in the light beam by releasing fresh cells from

the reservoir. This was a convenient way in which to change samples for fluorescence induction measurements.

RESULTS

Effect of light intensity during growth on emission spectra

The relative heights of the fluorescence emission maxima at approx. 680 and 710 mμ from Phaeodactylum cells grown with high, intermediate and low light intensity are illustrated by Fig. 1. High-light cells were harvested from cultures in the logarithmic phase of growth before light became limiting by mutual shading of the cells. The rate of growth in cultures in low light was always light-limited and became lower as the cell density increased. The proportion of F-710 increased with the age or density of the culture. The low-light cells used for the experiments described here contained more or less F-710 depending on the age of the culture, but were still dividing slowly. These slowly dividing, low-light cells also contained more chlorophyll *a* and *c* per cell than did the high-light cells from the logarithmic phase of growth.

A comparison of derivative absorption spectra of high- and low-light grown cells indicated greater absorption near 700 mμ in the low-light grown cells[9]. Recently FRENCH has measured absorption spectra of these two types of cells cooled by liquid N_2. An absorption maximum near 700 mμ was clearly visible in the spectrum from the low-light cells, but not from the high-light cells.

Effect of excitation wavelength

Emission spectra were measured with low-light grown Phaeodactylum containing a high proportion of F-710 for 2 incident wavelengths: 485 and 435 mμ. The 485-mμ light should preferentially excite System 2 of photosynthesis through absorption by fucoxanthin or chlorophyll *c*, and the 435-mμ light should excite relatively more strongly System 1 through chlorophyll *a*. The ratio of fluorescence emission at 710 mμ and 681 mμ was 18 % higher when excitation was by 485-mμ than by 435-mμ light. The absorbance at 435 mμ was about 10 % greater than at 485 mμ. This indicates that F-710 may be a part of Photosystem 2.

Effect of temperature

Most algae and plants show a greatly enhanced long wavelength fluorescence emission when cooled. The effect of temperature on Phaeodactylum fluorescence is illustrated in Figs. 1 and 2. The spectra in Fig. 1 measured at −187° were from algae which had the least and the most F-710 detectable at room temperature. It is clear that a definite amount of a substance fluorescing between 710 and 715 mμ exists at room temperature, and that its fluorescence relative to that at 680 mμ increases considerably upon cooling. The small shift in peak position of F-710 toward longer wavelengths upon cooling probably results from the skewing effect of adjacent bands on each other.

The change in shape of the short wavelength emission band upon cooling is more complicated. An emission band near 690 mμ has been observed in numerous algae at low temperature[1]. Probably the short wavelength emission is composed of 2 components which change in proportion as the temperature decreases.

The spectra in Fig. 2 were measured from cells in their growth medium, first at room temperature then cooled to —187°, and allowed to warm slowly to room temperature again. It was not possible to make an accurate comparison of fluorescence yield in growth medium alone above and below 0° because the increase in light scattering when the ice formed caused an apparent increase in fluorescence.

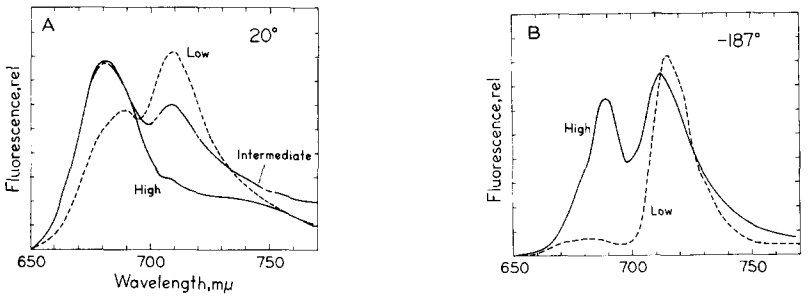


Fig. 1. Fluorescence emission spectra of Phaeodactylum grown with high (700 ft.-candles) (———), intermediate (——–) and low (200 ft.-candles) (– – – –) light intensity. Excitation at 435 mμ measured at 20° (A) and at —187° (B).

In order to make a more accurate comparison of fluorescence yield changes upon cooling, the cells were mixed with $BaSO_4$. The scattered light observed from this inert white powder was the same at room temperature and at —187° and varied little with wavelength. The changes in fluorescence yield at 680 and 715 mμ when Phaeodactylum from 5-day and 16-day low-light cultures were cooled from 20° to —187° are presented in Table I. At 680 mμ the fluorescence yield decreased from 2.5 to 3 times while at 715 mμ the yield increased by a factor of 7 to 10.

Lability

It is relatively easy to destroy the 710-mμ fluorescence band. Attempts to prepare chloroplasts with a high proportion of F-710 have been completely unsuccess-

TABLE I

FACTORS BY WHICH THE FLUORESCENCE YIELD CHANGED WHEN THE TEMPERATURE WAS LOWERED FROM 20° TO —187°

Phaeodactylum cells from 5-day and 16-day cultures were mixed in a thick paste of $BaSO_4$.

Sample	*Yield at —187° / Yield at + 20° ratio*	
	*680 mμ**	*715 mμ**
5-day cells		
Untreated	0.32	7.4
Poisoned (1 mM DCMU + 10% ethanol, 2 h)	1.1	1.9
16-day cells		
Untreated	0.40	9.9
Poisoned (10% ethanol, 22 h)	0.77	4.2

* Wavelength of fluorescence.

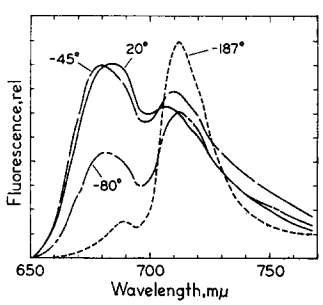
ful. Lyophilization or suspension of the algae in dehydrating media such as sorbitol–borate or cellulose powders caused a time-dependent destruction of F-710.

When the cells were suspended in a medium at pH 4.3 for an hour, the 710-mμ fluorescence maximum decreased and did not reappear when these cells were again brought to a higher pH.

A decrease in F-710 relative to F-680 was observed from cells which had been suspended in 1 mM 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 10 % ethanol for 1 h. 10 % ethanol alone in a 24-h period at 4° had about the same effect. This is illustrated in Fig. 3 and by the data in Table I. Although the difference in fluorescence at 710 mμ between the treated cells and controls is not great at room temperature, the difference at —187° is very large.

Emission spectra measured at room temperature from the same number of cells before and after 2 h in 1 mM DCMU and 10 % ethanol showed that this treatment decreased the fluorescence yield 2.7 times at 680 mμ and 6.3 times at 715 mμ. This experiment was performed with cells from the same 5-day culture used to measure the temperature effects shown in Table I.

A complicating factor in these fluorescence yield results is the possible contribution of the fluorescence band at about 690 mμ. Fluorescence yield changes at 680 mμ may include changes also at 690 mμ, but the data are insufficient for an analysis.



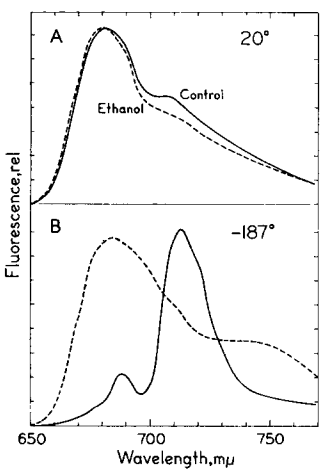


Fig. 2. Fluorescence emission spectra from low-light grown Phaeodactylum, measured consecutively at 20° (———), —187° (– – – –), —80° (— — —), and —45° (— — —). Excitation at 435 mμ.

Fig. 3. Fluorescence emission spectra of Phaeodactylum before (———) and after (– – – –) treatment with 10 % ethanol for 22 h at 4°. Excitation at 435 mμ. Measured at 20° (A) and at —187° (B).

Another observation correlated with the destruction of F-710 by 10 % ethanol was the disintegration of the chloroplast structure within the cells. Normally a circular brownish–green chloroplast was in the center of each cell, but after treatment with ethanol the chloroplast was spread through the long cell to give a green beaded appearance. Also the whole cell suspension changed in color from brown to green. The cells did not break under this treatment.

Loss of 710-mμ fluorescence was correlated with a decrease in absorption at 695–700 mμ as shown by derivative absorption spectrophotometry[9].

Leaf chloroplast suspensions exhibit a fluorescence maximum near 735 mμ which may be as high or higher than the 685-mμ maximum when the spectra are measured at low temperature. To compare the stability of this 735-mμ band with the 714-mμ band in Phaeodactylum cells at low temperature, chloroplasts were heated to 75° for 5 min, and algae were heated to 70° for 3 min before the spectra were measured. The chloroplasts still retained considerable long wavelength fluorescence although the maximum had shifted to about 730 mμ. The algae, on the other hand, lost all of the 714-mμ emission band.

Kinetics

The fluorescence kinetics at 681 and 715 mμ after exposing dark adapted cells to blue light are shown in Fig. 4. The slow response time of the signal-smoothing system precluded comparison of the initial rise (10-sec period).

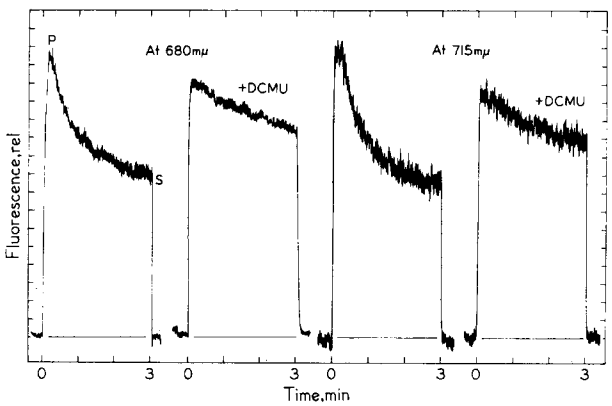


Fig. 4. Fluorescence kinetics of dark-adapted, high-light grown Phaeodactylum, measured at 680 and 715 mμ, before and after addition of 0.1 mM DCMU. Excitation from Hg lamp in conjunction with Corning filter No. 9782.

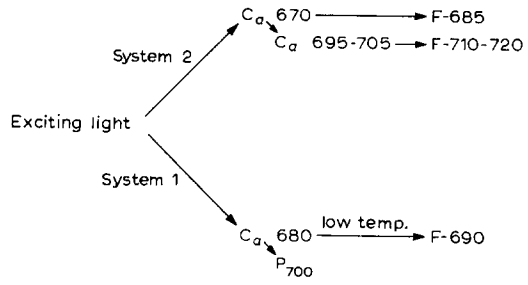
The variable fluorescence, $V = (P-S)/S$, where P is the maximum level of fluorescence and S the level after 3 min in the light, was measured in several experiments with high- and low-light grown cells. The average value of V was 1.5 at 681 as well as at 715 mμ from cells from a 5-day culture having a low proportion of F-710 and 1.9 at both wavelengths from cells from a 16-day culture with a high proportion of F-710.

A low concentration of DCMU (0.1 mM) had no effect on the shape of the emission spectrum, but it inhibited the decrease in fluorescence at both wavelengths.

DISCUSSION

A comparison of the kinetics, effects of inhibitors, and fluorescence yield changes upon cooling at 681 and 715 mμ, all indicate that the pigments which emit these fluorescence bands function in the same photochemical system.

An energy transfer scheme recently proposed by GOEDHEER[11] and diagrammed below may be used to explain the above results. C_a is chlorophyll *a* absorbing at the designated wavelength, F is fluorescence emission maximum, and P_{700} (ref. 12) is the reaction center for System 1 of photosynthesis.



The pigment, C_a695, was first observed by FRENCH[13,14] in Euglena and Ochromonas. When growing at low light intensity, Euglena forms a high proportion of C_a695 (ref. 15). It fluoresces near 710 mμ at room temperature (720 mμ at low temp.) and is much more labile than the rest of the chlorophyll *in vivo*. BUTLER[16] studied a pigment in greening leaves, which he called C-705, with properties very similar to C_a695.

Phaeodactylum with relatively high fluorescence at 710–720 mμ also has a small absorption maximum near 700 mμ.

If C_a695–705 is the reaction center for Photosystem 2 as indicated above, it must be assumed that in most photosynthesizing cells it gets much more energy by transfer from C_a670 than from its own absorption at physiological temperatures, because Emerson's studies of the red drop and enhancement showed that Photosystem 2 does not function efficiently when irradiated with red light beyond about 680 mμ. If enhancement were observed in Ochromonas, low-light grown Euglena or Phaeodactylum, our hypothesis would have to be altered because C_a695 has an appreciable absorption in these organisms.

In terms of the above scheme, the results with Phaeodactylum may be interpreted as follows: Excitation energy transferred to C_a670 is partially emitted as fluorescence at 681 mμ and partially transferred to C_a695. In high-light grown cells the energy from C_a695 is used nearly exclusively for photosynthetic reactions, but in low-light grown cells which contain a larger amount of C_a695, a part of the energy is emitted as fluorescence at 710 mμ. At low temperature C_a695 receives relatively more energy from C_a670, the fluorescence yield at 680 mμ is less and the yield at 715 mμ is greater. Although treatment with ethanol causes a decrease in the fluorescence yield at both 680 and 715 mμ, proportionally more of the longer wavelength fluorescence from C_a695 than of the shorter wavelength emission from C_a670 is diminished. When the temperature is lowered after the ethanol treatment, the fluorescence yield at 680 mμ no longer decreases as much as before the treatment because most of the C_a695 has been destroyed, and the yield at 715 mμ no longer increases as much as before for the same reason.

The observation that the proportion of variable fluorescence and the inhibition by DCMU were the same at 680 and 715 mμ is strong support for placing C_a670 and

C_a695 in the same photosystem. BUTLER[17], LAVOREL[18], VREDENBERG AND DUYSENS[19] compared the amount of variable fluorescence at 685 mμ and at wavelengths longer than 720 mμ in green and in blue-green algae. They found that the variable fluorescence was less at the longer wavelengths and concluded that the long wavelength fluorescence contains a larger contribution from Photosystem I than the short wavelength fluorescence. Recent experiments by BOARDMAN, THORNE AND ANDERSON[20], and KOK AND RURAINSKI[21] also showed that the chloroplast fractions which had greater System-I activity had relatively more long-wavelength fluorescence. These results do not conflict with those reported here because the long-wavelength fluorescence from isolated chloroplasts does not arise from C_a695. C_a695 is very labile, whereas the long-wavelength fluorescence is resistant to chloroplast disintegration as well as to heating. The source of this fluorescence beyond 730 mμ is unknown.

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