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FLUORESCENCE STUDIES ON A RED ALGA, *PORPHYRIDIUM CRUENTUM*

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SUMMARY

Fluorescence intensities (F) as a function of exciting light intensities (I) were measured under steady-state conditions in the red alga *Porphyridium cruentum*. They show an increase in differential fluorescence yield (dF/dI) upon excitation with 544-mμ light (System II), but not with blue light (System I). On the basis of these results and the difference excitation spectra between excitation with and without green background light, it is shown that this change, showing extra fluorescence at "high" light intensities, is essentially due to light absorbed in System II.

Upon addition of 3(3,4-dichlorophenyl)-1,1-dimethylurea, a difference emission band at 692 mμ was observed (at low intensities) between the emission spectra of poisoned and unpoisoned cells in Porphyridium. This band is suggested to originate in the reaction center in System II.

Low-temperature (−196°) emission spectra were measured in Porphyridium upon excitation in Systems I and II. Three clearly distinguishable peaks at 685 mμ, 696 mμ and 712 mμ (and a shoulder at 740 mμ) were observed upon excitation with green light (System II). However, excitation with blue light (System I) produced a dominant peak at 712 mμ, whereas the 685-, 696- and 740-mμ bands appeared only as shoulders. Since the 696-mμ peak is more pronounced in the case of excitation in System II, and the 712-mμ peak for excitation in System I, it is suggested that the 696-mμ band originates in System II and the 712-mμ band in System I.

That the 696-mμ band and the 712-mμ band belong to different molecular species is also suggested by their different rate of decrease in fluorescence intensity with increasing temperature. In the −196° to −185° range, the rate of decrease of fluorescence intensity of the 696-mμ band is several times higher than that for the 712-mμ band.

INTRODUCTION

The discovery of the enhancement effect in photosynthesis led EMERSON and co-workers[1–3] to the conclusion that excitation of chlorophyll *a* alone is insufficient to sustain maximum efficiency of photosynthesis, and that the pigments having

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

absorption bands at wavelengths shorter than the red maximum of chlorophyll *a* play some essential part in photosynthesis. They suggested that photosynthesis requires two light reactions sensitized by two different pigments. HILL AND BENDALL[4] proposed that one of the two light reactions is responsible for the formation of a reducing agent necessary for the reduction of CO_2 and an oxidizing agent needed for the oxidation of a cytochrome; the other reaction leads to the oxidation of water and the reduction of a cytochrome. DUYSENS AND AMESZ[5] provided evidence for the suggested role of cytochromes and arbitrarily designated two pigment systems sensitizing the two light reactions: System I, containing non- or weakly fluorescent chlorophyll *a* and small amounts of the accessory pigments (for cytochrome oxidation) and System II, containing fluorescent chlorophyll and a higher proportion of accessory pigments (for cytochrome reduction).

The hypothesis of two light reactions and two pigment systems*, a summary of which is given by KOK AND JAGENDORF[6], has now been accepted by many workers in photosynthesis. The two light reactions are suggested by FRANCK AND ROSENBERG[7] to take place in a common reaction center and by DUYSENS AND SWEERS[8] in two distinct reaction centers. Difference spectroscopy has given evidence for the existence of one of the reaction centers, associated with System I, and designated as P700 by KOK[9]. On the basis of the existence of a new emission band at 693 mμ upon excitation by high-intensity light absorbed in System II, in *Porphyridium cruentum*, the existence of another trap, associated with photosystem II, was suggested[10].

The fact that curves** of fluorescence intensity (steady state) *versus* intensity of exciting light [$F = f(I)$] are not linear, was most clearly observed by FRANCK[11] and by BRUGGER[12]. FRANCK showed further that there is a definite relation between the photosynthesis and fluorescence curves. At "low" light intensities***, photosynthesis, as well as fluorescence, shows linear dependence on light intensity. When the "light curve" of photosynthesis§ [$P = f(I)$] starts bending towards light saturation, the fluorescence curve also departs from linearity; in the region of full saturation of photosynthesis ("high" light intensity§§), the fluorescence curve becomes linear again, with a slope of about 1.8 to 2.0 times that at the low intensities. We shall refer to the ratio of the two slopes as "R"§§§. FRANCK's experiments on the fluorescence intensity as function of light intensity were done with white excitation light, at a time when the concept of two pigment systems in photosynthesis had not been developed. In view of this new aspect of the picture, we investigated the dependence of the fluorescence yield on light intensity when either System I or System II is excited. Experiments suggesting that almost all the increase in differential fluorescence yield† at "high" exciting light intensities is caused by excitation in System II and not in System I, are described in the present paper.

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* Pigment system I in Porphyridium: Mainly chlorophyll a_1 (and carotenoids). Light of 440 mμ and 680 mμ is absorbed by System I. Pigment system II in Porphyridium: Mainly phycoerythrin, phycocyanin, chlorophyll a_2. Light of 544 mμ is absorbed mainly by System II.

** $F = f(I)$: Fluorescence intensity (F) as a function of incident light intensity (I).

*** "Low" light intensity: Light intensity for excitation of fluorescence in the linear part of the light curve of photosynthesis (first linear part of $F = f(I)$ curve)

§ $P = f(I)$: Rate of photosynthesis (P) as function of light intensity (I).

§§ "High" light intensity: Light intensity for excitation of fluorescence in the saturation region of photosynthesis (second linear part of $F = f(I)$ curve).

§§§ "R": Ratio of the slopes of $F = f(I)$ curve at "high" and "low" light intensity.

† Differential fluorescence yield (dF/dI): The slope of the $F = f(I)$ curve.

To identify the species of chlorophyll producing the "extra" fluorescence when photosynthesis is light-saturated, the emission spectra (for steady-state fluorescence) at different intensities of light absorbed in System II were measured in Porphyridium[10]. The emission spectra showed that the extra fluorescence at "high" intensity originates in a species with a fluorescence maximum at 693 mμ located closely on the long-wavelength side of the major chlorophyll *a* fluorescence band at 685 mμ. BISHOP[13] found that (3 μM) DCMU inhibits photosynthesis even at "low" light intensities. DUYSENS AND SWEERS[8], working with short periods of illumination, observed that upon excitation even with weak light absorbed in System II, chlorophyll *a* fluorescence is higher in DCMU-poisoned cells than in unpoisoned cells. If one postulates that the 693-mμ band in the "difference fluorescence spectrum", obtained by subtraction of the emission spectrum at high light intensities from the one at low light intensities is due to the "reaction center" in System II and if DCMU reacts with this reaction center, as suggested by DUYSENS AND SWEERS[8], or with some intermediate formed by Reaction II, it is not implausible that even at low light intensity, this poison may not only enhance the fluorescence band at 685 mμ, due to the bulk of chlorophyll *a* in System II, but will also produce an extra fluorescence band at 693 mμ from the "trap" associated with this system. The present paper reports this expected difference between the emission spectrum of poisoned cells compared to that of unpoisoned cells, both produced by excitation in System II with "low" light.

At low temperatures (−196°) an increased fluorescence yield and a large new band (at 720 mμ) on the long-wavelength side of the chlorophyll *a* fluorescence spectrum have been first observed by BRODY[14] in Chlorella. The species of chlorophyll *a* engaged at room temperature in the primary photochemical reaction in photosynthesis may be expected to show fluorescence when photosynthesis is suppressed. Emission spectra in *Porphyridium cruentum* measured at −196° show, besides the main chlorophyll *a* band (685 mμ, F685) present at room temperature, also new bands at the longer wavelengths, at 696 mμ (F696), 712 mμ (F720) and 740 mμ (F740). The new band at 696 mμ was first observed in bean leaves and its homogenates by LITVIN, KRASNOVSKY AND RIKHIREVA[15]. It has been observed by KOK[16] in Scenedesmus, BERGERON[17] (see also ref. 18) in Anacystis, BRODY AND BRODY[19] in Chlorella, GOEDHEER[20] (see also ref. 21) in several different organisms. It is shown here that the ratio of intensity of the different bands depends on the spectral composition of exciting light, *i.e.*, on whether it is absorbed mostly in System I or II. These experiments show clearly that the 696-mμ band belongs to System II and the 712-mμ band to System I. Different species are responsible for the fluorescence maxima at −196°; this was suggested by the different effect on the fluorescence intensity of F685, F696 and F720 with increasing temperature (between −196° and room temperature) in isolated spinach chloroplasts[22]. We have confirmed these findings in *Porphyridium cruentum*.

MATERIALS AND METHODS

Growth and preparation of Porphyridium cruentum for fluorescence measurements

Bacteria-free *Porphyridium cruentum* was obtained from the algal collection of Professor R. C. STARR (Botany Department, Indiana University). The culture medium was the one used by BRODY AND EMERSON[23] with slight modifications. A description of the culturing set-up may be found in an earlier publication[24]. The

cells were grown at about 15° for 1 week. A mixture of 5 % CO_2 in air was bubbled through the culture flasks. Two different intensities of white light were obtained with 12 15-W, cool-white fluorescent lamps, which were so arranged as to give an illumination intensity of about $1.10 \cdot 10^4$ erg·cm^{-2}·sec^{-1} at the bottom of the culture flasks. Algae grown in that intensity are referred to as "high-light cells"*. "Low-intensity" white light was provided by two such lamps ($6.8 \cdot 10^2$ erg·cm^{-2}·sec^{-1}) and the algae grown in such an intensity are referred to as "low-light cells"**.

"Low-light cells" show a higher total amount, and different ratios of the various pigments than "high-light cells". Absorption spectra for both types of cells were measured with a Bausch and Lomb spectrophotometer (Spectronic 505) equipped with an integrating sphere. The measurements were made with 1 cm path length; the absorbance values were converted to 0.1 cm path length, which was the optical path length for fluorescence measurements. Fig. 1 shows the absorption spectra "normalized" at 676 mμ for "high" and "low-light cells". The ratio of absorption maxima of phycoerythrin to that of chlorophyll *a* is 2.03 in the "low-light cells" and 1.47 in the "high-light cells", in agreement with the results of BRODY AND EMERSON[23].

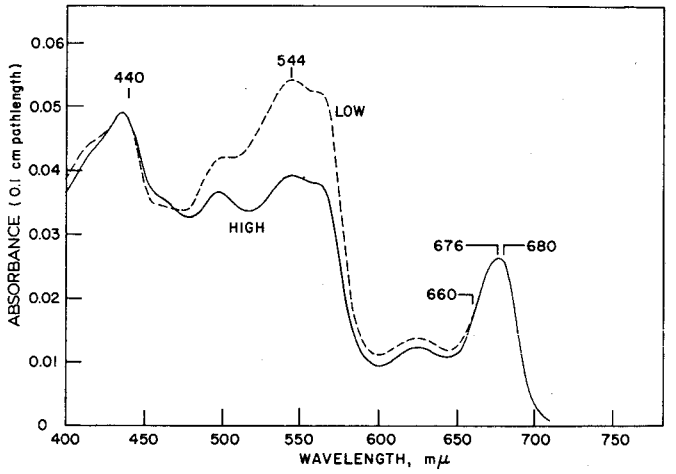


Fig. 1. Absorption spectra of suspensions of *Porphyridium cruentum* grown in "low" and "high" intensity of white light. Measurements with automatic Bausch and Lomb spectrophotometer (band width, 5 mμ). The two curves are adjusted at 676 mμ; both curves overlap perfectly in the 660–700-mμ range. The numbers on the graph refer to the wavelength of light, in mμ.

The algae were kept in the illumination bath until the experiment was started, then centrifuged out of the culture medium, washed and suspended in Warburg's carbonate–bicarbonate buffer No. 11 with added NaCl (16 g $NaHCO_3$ + 1.0 g Na_2CO_3 + 15.2 g NaCl per l of distilled water). In buffer No. 11, the photosynthetic efficiency is known to be very high (see ref. 25). In order to eliminate reabsorption of fluorescence, rather dilute samples with absorbance around 0.03 at 685 mμ (0.1 cm path lengh) were used for room temperature measurements. For low-temperature measurements, a thin film of algal suspension was frozen between a microscope cover slip and the window of the Dewar flask, by slowly pouring liquid N_2 on the cover slip.

* "High-light cells": Cells grown in an incident light intensity of about $1.1 \cdot 10^4$ erg·cm^{-2}·sec^{-1}.
** "Low-light cells": Cells grown in an incident light intensity of $6.8 \cdot 10^2$ erg·cm^{-2}·sec^{-1}.

Description of the equipment

The instrument used in the present study to measure fluorescence was the same as used earlier[21,22,26]. Two light sources were used to excite fluorescence, in order to be able to work in the range of saturation of photosynthesis, and to observe the two-light effect. One light beam was obtained from a ribbon filament (6 V, 18 A) incandescent lamp through a Bausch and Lomb monochromator (grating size, 100 mm × 100 mm). The second beam was also provided by an incandescent lamp equipped with the appropriate interference filter and additional glass filters. A 544-mμ Bausch and Lomb interference filter (half band width 10 mμ) combined with a green glass filter (Schott VG-5) was used for excitation in System II and a C.S. 5-60 (or C.S. 5-61) Corning glass filter (for fluorescence light curves) or a 440-mμ interference filter *plus* a C.S. 4-76 Corning glass filter (for the quenching effect) for excitation in System I.

The two light beams were focused at the same spot on the flat window at the bottom of the Dewar flask containing the algal suspension. Fluorescence was collected from the illuminated surface. A sharp cut-off red glass filter (Corning C.S. 2-63) was placed in front of the entrance slit of the analyzing monochromator. The band width of the slits of the analyzing monochromator was 2.0 mμ for emission and 3.5 mμ for excitation spectra. The wavelength drum of this monochromator was turned automatically by a synchronous motor. Fluorescence was detected by an EMI 9558B photomultiplier. The signal amplified by a Keithley 150 A microvoltammeter was recorded on a Brown recorder.

The emission spectra thus obtained were evaluated by correcting the average (with respect to the noise) of each curve for the wavelength dependence of the photomultiplier sensitivity and the spectral efficiency of the monochromator. These corrections were made every 2.5 mμ. When difference emission spectra were determined, the differences were calculated every mμ, using the uncorrected emission spectra, and the correction factors were then applied to them.

The rate of photosynthesis was measured by EMERSON's manometric technique (see ref. 24 for details). The light intensities were measured by a thermopile connected to a calibrated Keithley microvoltmeter.

The temperature was measured by a thermocouple with the reference end in ice-cold water or liquid N_2.

EXPERIMENTAL RESULTS

Fluorescence intensity (F) as a function of incident intensity (I) of monochromatic light

"Low-light" and "high-light cells". Fig. 2 (top) shows the $F = f(I)$ curve of fluorescence (measured at 688 mμ) for "low-light" and for "high-light cells" when excited by light absorbed primarily in System II (544 mμ). The intensity of incident light at which the $F = f(I)$ curve departs from linearity (see Table I) is about $0.7 \cdot 10^{15}$ quanta$\cdot$cm$^{-2}\cdot$sec^{-1}. The increase in the differential fluorescence yield was found to be 80–100 % for "low-light cells", *i.e.* the "R"-value was 1.8–2.0. With "high-light cells", the corresponding increase is between 10 % and 38 %, *i.e.* the "R" value is between 1.1 and 1.38. Since "low-light cells" showed a greater effect, all the following experiments were made with such cells.

Excitation with different monochromatic light. Fig. 2 (bottom) shows the curve $F = f(I)$ (measured at 688 mμ), obtained upon excitation with light mainly absorbed

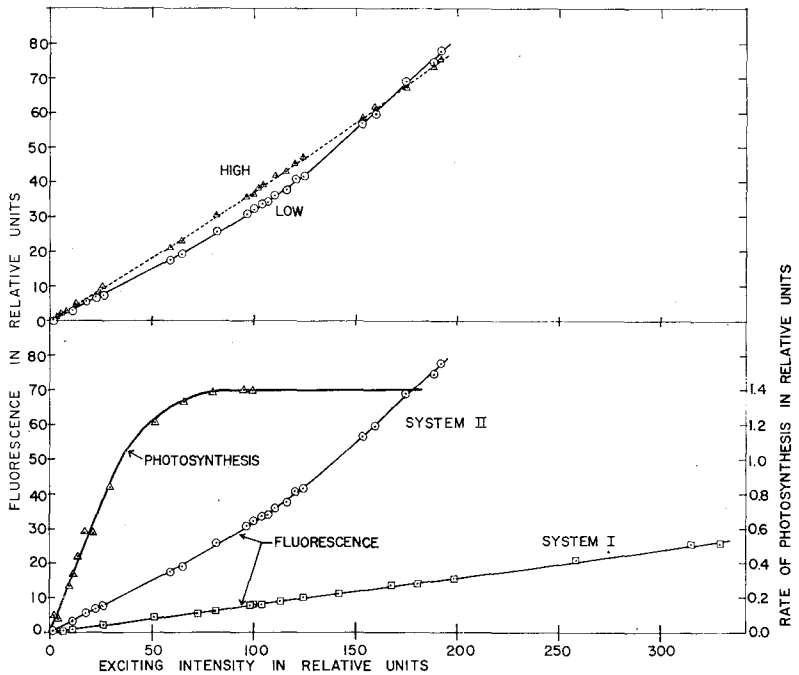


Fig. 2. Fluorescence intensity at 688 mμ as a function of intensity of incident exciting light. 100 units on the abscissa correspond to $1.00 \cdot 10^{15}$ quanta·cm^{-2}·sec^{-1}. Top: Light curves [$F = f(I)$] for "high-light cells" (triangles) and "low-light cells" (circles) upon excitation with 544-mμ light at room temperature. The absorbance for both types of cells was adjusted to be the same at 544 mμ. Bottom: Light curves upon excitation in System I (blue light, squares) and System II (green light, circles) at room temperature. The curve with triangles is for the rate of photosynthesis as a function of intensity of green light obtained with only one green filter, Schott VG-5. (Photosynthesis data cannot be too accurately compared with the fluorescence data because of somewhat different spectral characteristics of exciting light used in the two cases.)

in Systems I or II (100 units on the abscissa are equivalent to $1 \cdot 10^{15}$ quanta·cm^{-2}·sec^{-1} for both blue and green light). The wavelengths of excitation for System I were 350–520 mμ, obtained by filtering white light with C.S. 5-60. (This filter transmits maximally at 440 mμ (66 %) and has a 33 % transmission at 350 and 480 mμ.) No increase in the slope of the $F = f(I)$ was observed. The wavelength of excitation for System II was 544 mμ and a break in the $F = f(I)$ curve was observed.

Since we did not see any increase in the differential fluorescence yield for light absorbed in System I, we have to find out whether the absorbed light intensity was sufficient to reach the region where the fluorescence curve departs from linearity. From Table I, we see that for excitation in System II, about one-third of the available intensity of 544-mμ light was enough to reach the non-linear part of the light curve of photosynthesis; half of it was sufficient to reach into the saturation range of photosynthesis. The available intensities of light absorbed in System I are of the same order of magnitude as those used for excitation of System II. However, for the excitation of System I, the departure from linearity of both the photosynthesis and the fluorescence light curves should be, if there is such a bending of the fluorescence and photosynthesis curve, at a higher intensity than with excitation of System II, since

TABLE I

(a) Available incident and absorbed quanta·cm^{2-}·sec^{-1} at 544 mμ and 440 mμ in our experiments:

Wavelength (*mμ*)	*A* (*1.0 cm*)	*Per cent absorption*	*Incident* (*quanta·cm^{-2}·sec^{-1}*)	*Absorbed* (*quanta·cm^{-2}·sec^{-1}*)
approx. 440	0.0477	10.7	$3.30 \cdot 10^{15}$	$35.3 \cdot 10^{13}$
544	0.0540	11.7	$1.95 \cdot 10^{15}$	$22.8 \cdot 10^{13}$

(b) Maximum intensity of light used in photosynthesis measurements at 544 mμ:

Incident: $0.98 \cdot 10^{15}$ quanta·cm^{-2}·sec^{-1}
Absorbed: $11.5 \cdot 10^{13}$ quanta·cm^{-2}·sec^{-1}

(c) Intensity for departure from linearity in fluorescence and photosynthesis curves for excitation with System II:

	Incident (*quanta·cm^{-2}·sec^{-1}*)	*Absorbed* (*quanta·cm^{-2}·sec^{-1}*)
Photosynthesis	$0.3 \cdot 10^{15}$	$3.5 \cdot 10^{13}$
Fluorescence	$0.70 \cdot 10^{15}$	$8.2 \cdot 10^{13}$

(d) Intensity of saturation of photosynthesis and for the onset of the second linear part of the $F = f(I)$ curve for excitation in System II:

	Incident (*quanta·cm^{-2}·sec^{-1}*)	*Absorbed* (*quanta·cm^{-2}·sec^{-1}*)
Photosynthesis	$0.8 \cdot 10^{15}$	$9.4 \cdot 10^{13}$
Fluorescence	$1.25 \cdot 10^{15}$	$14.6 \cdot 10^{13}$

the maximum quantum yield of photosynthesis at 440 mμ is, according to BRODY AND EMERSON[27], about half of that at 544 mμ. The possible departure from linearity of photosynthesis light curves is roughly estimated on the basis of the known quantum yield of photosynthesis at different wavelengths and the shape of the "light curves" of photosynthesis (see ref. 28). The departure may begin at about $0.6 \cdot 10^{15}$ quanta·cm^{-2}·sec^{-1} (absorbed intensity). The maximum available blue light in our experiment exceeds these intensities, which means that the differential fluorescence yield for excitation in System I does not change with intensity.

The quenching effect: the difference emission band

EMERSON and co-workers[1-3] discovered that simultaneous excitation of the two pigment systems results in an enhanced rate of photosynthesis, compared to the sum of the rates of photosynthesis obtained upon excitation of each system separately. The enhancement effect "translated" into fluorescence measurements could mean a quenching of fluorescence when the two systems are excited together, as was shown in the green alga Chlorella[29] and in several organisms[8].

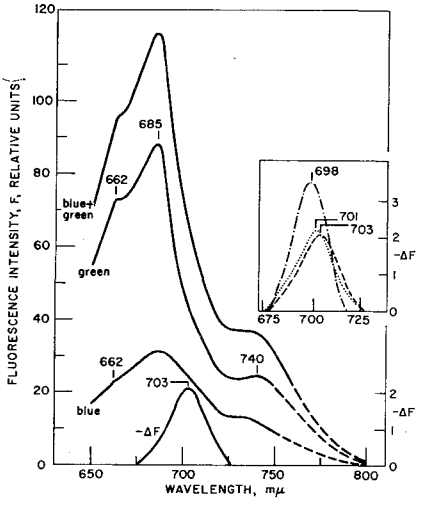
Fig. 3 shows the emission spectra (steady-state fluorescence) for excitation of System I (blue) and System II (green), and of both systems simultaneously. The incident intensity of the green excitation beam was $0.15 \cdot 10^{15}$ quanta·cm^{-2}·sec^{-1}, that is, about one-fifth of the intensity at which the fluorescence curve deviates from

linearity. The incident intensity for blue excitation was $0.1 \cdot 10^{15}$ quanta$\cdot$cm$^{-2}\cdot$sec^{-1}.

The difference emission curve ($-\Delta F$) is shown in the same figure, but on a ten times enlarged scale. It is obtained by subtracting the sum of the emission spectra upon excitation with blue (440 mμ) and green (544 mμ) light separately, from the emission spectrum produced by excitation with both lights simultaneously.

The difference emission curve shows a peak between 690 mμ and 705 mμ depending on the sample (see insert, Fig. 3). This variation might be due to different relative amounts of pigments in the different algal samples, or to slightly different intensities of excitation. We should note that during the so-called steady state the quenching effect is very small (2 %) and often absent. Because of the smallness of the effect obtained in our experiments, the exact location of the difference band is uncertain.

Excitation spectra with different background light. Since the quenching effect was so small when System I was excited with 440-mμ light and System II with 544-mμ light, we attempted to find more appropriate wavelengths by measuring excitation spectra upon illumination with different background light. Fig. 4 shows excitation spectra (measured at 700 mμ) with no background, with green and with blue background light. The intensities were chosen in such a way that an increased rate of fluorescence was observed when both light beams were exciting System II and a quenching of fluorescence was observed when Systems I and II were excited simultaneously.



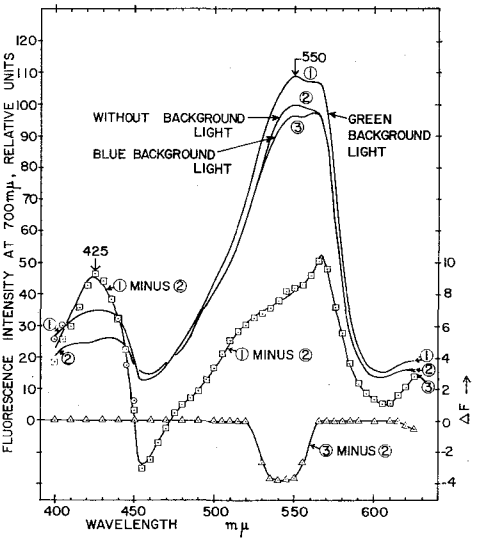


Fig. 3. Emission spectra obtained upon excitation by "low" intensity light of 440 mμ (blue), 544 mμ (green) and blue and green given together (uppermost curve). Measurements with automatic spectrofluorimeter. The ΔF curves obtained in three different experiments are shown in the insert. Measurements beyond 750 mμ were not made and the curves have been extrapolated to zero fluorescence at 800 mμ.

Fig. 4. Excitation spectra measured at 700 mμ at room temperature with different background light. 1, green background light; 2, without background light; 3, blue background light. Increase in fluorescence in the phycocyanin absorption region and around 425 mμ is shown in the curve 1 *minus* 2; a quenching of fluorescence is observed around 455 mμ. The curve 3 *minus* 2 shows a quenching of fluorescence in the phycocyanin region and no change in the 400–500-mμ region.

The excitation spectrum in the presence of green background light, compared to the one without background light, showed the characteristic difference, an increased fluorescence rate, in the region where mainly System II absorbs. The difference spectrum between the two excitation spectra is plotted on a ten times enlarged scale. The two spectra differ also in the blue region, where a quenching of fluorescence was expected. Depending on the sample, sometimes an increased fluorescence was observed instead of quenching, and sometimes a quenching effect at certain wavelengths and an increase in fluorescence at other wavelengths was observed. An example of the latter case is shown in Fig. 4, where in the blue region an increased fluorescence is observed between 400 and 450-mμ range, and a quenching between 450 and 470-mμ range. The net result can be understood if the effect is due to two opposing effects—a quenching and a stimulation of fluorescence—leading to variable results at different wavelengths.

The excitation spectrum in the presence of blue background compared to the one without background shows no difference in the blue region and a quenching in the region where mainly System II absorbs.

From this difference spectrum we see that the maximum quenching is on fluorescence excited by 544 mμ.

It may be noted that the difference excitation spectrum shows a different shape of the phycocyanin band than excitation spectrum without background light. Perhaps, phycocyanin present in Systems II and I have different absorption spectra, or else the net effect is the result of two opposing effects, as suggested above.

Effect of DCMU on emission spectra

Increase of chlorophyll a fluorescence. The presence of DCMU is presumed by DUYSENS AND SWEERS[8] to inhibit the reduction of cytochrome by System II, but not the oxidation of cytochrome by System I. Upon addition of DCMU, the fluorescence efficiency of the cells is increased both at "low" and "high" 544-mμ light. Fig. 5 (left) shows that there is no difference in the shape of the emission spectra obtained by excitation with "low" and "high" light intensities. Fig. 5 (middle) shows the increase in fluorescence intensity for "low" light excitation in DCMU-poisoned cells. The height of the 685-mμ band increased 1.6 fold.

The emission spectra of poisoned and unpoisoned cells were "normalized" at the chlorophyll *a* peak. For "low" intensities, there was a difference (Fig. 5, right) between the emission spectra of poisoned and unpoisoned cells, which resembled that found in unpoisoned cells between the emission spectra of "high" and "low" excitation intensity. The difference curve is plotted on a ten times expanded scale. A peak at 692 mμ was observed. There was no difference between the emission spectra of the poisoned and the unpoisoned cells for "high" exciting intensities in the same wavelength region.

Constancy of the phycocyanin fluorescence. Fluorescence from System II, measured around the main chlorophyll *a* fluorescence band, increases upon addition of DCMU. Whether or not the phycocyanin fluorescence also increases together with the chlorophyll *a* fluorescence must be determined by analysis of the short-wavelength side of the main emission band. For this analysis, the chlorophyll *a* band was approximated by a Gaussian distribution curve. The result is shown in Fig. 5 (middle curve). Around the phycocyanin maximum for the poisoned and the unpoisoned cells, the

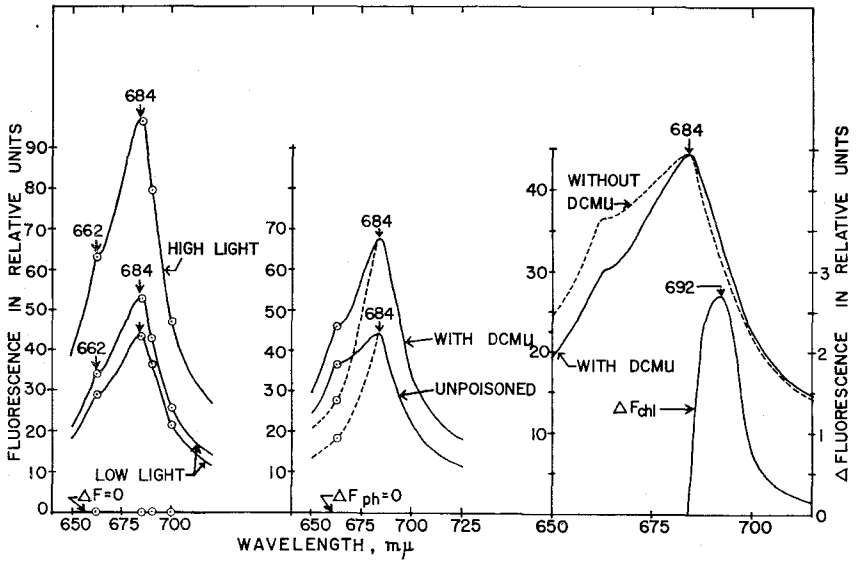


Fig. 5. Effect of DCMU on the emission spectra of Porphyridium. (a) left: Emission spectra obtained upon excitation with 544-mμ light after addition of 50 μM DCMU for "low" (lower curves) and "high" (upper curve) intensity of exciting light. The ΔF curve shows no difference in the shape of the emission spectra at "low" and "high" light intensities. (b) middle: Emission spectra for poisoned and unpoisoned cells. $\Delta F_{Ph} = 0$; no change in phycocyanin fluorescence (see text). (c) right: The emission spectra for poisoned (50 μM DCMU; solid curve) and unpoisoned (dashes) cells (both at "low" intensity) are normalized at the chlorophyll *a* peak. Exciting wavelength: 544 mμ. The difference emission (solid curve) between poisoned and unpoisoned cells is shown on a ten times enlarged scale. ΔF_{Chl} = change in chlorophyll fluorescence. Note the peak at 692 mμ.

difference between the measured and the Gaussian curve is the same. This indicates that the phycocyanin fluorescence remains the same after poisoning with DCMU.

Fluorescence measurements at lower temperatures

Emission spectra at liquid-N_2 temperature (*—196°*). Instead of one single band with a peak at 685 mμ, observed at room temperature, four emission bands (at 685, 696, 712 and 740 mμ) are present in the emission spectrum of Porphyridium at liquid-N_2 temperature. Fig. 6 shows emission spectra upon excitation in System I (long dashes) and System II (solid curve), for a 3-day-old culture. The emission spectrum obtained upon "blue" excitation is plotted on a ten times expanded scale, compared to the one for "green" excitation. Excitation of System II yields an emission spectrum which has three clearly separated bands of almost equal height, located at 685 mμ, 696 mμ, and 712 mμ; whereas upon excitation of System I, the 712-mμ band is dominant, and the 696-mμ and 685-mμ bands appear as shoulders on the short-wavelength side of this dominant band. The phycocyanin fluorescence is seen as a separate peak at 661 mμ in the emission spectrum obtained upon excitation with green, but not with blue light.

The effect of aging of Porphyridium is demonstrated in Figs. 6 and 7: 3-day-old and 9-day-old cultures were used. As the cultures age, the ratios of the fluorescence intensities at 712 mμ to 685 mμ and to 696 mμ increase (*cf.* Table II). The peak at around 740 mμ was not clearly observable in all Porphyridium samples used.

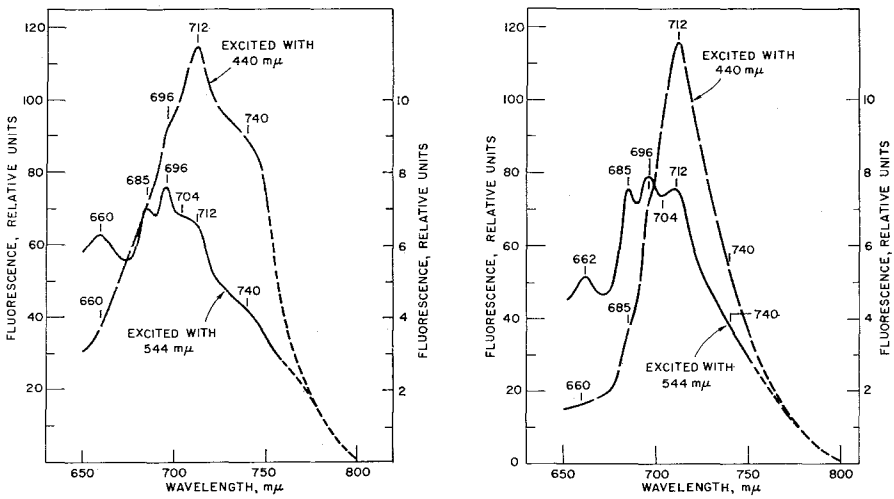


Fig. 6. Emission spectra at liquid-N_2 temperature for a 3-day-old culture of Porphyridium. Exciting wavelength: 544 mμ (solid curve, ordinate on left) and 440 mμ (long dashes, ordinate on right). Measurements beyond 750 mμ were not made and the curves have been extrapolated to zero fluorescence at 800 mμ.

Fig. 7. Emission spectra at −196° for a 9-day-old culture of Porphyridium (for legend, see Fig. 6).

TABLE II

RATIOS OF THE FLUORESCENCE INTENSITIES AT 685, 696 AND 712 mμ

	3 days	*9 days*
(*a*) *Excitation of System I*		
$\frac{F712}{F685}$ §	1.60	3.20
$\frac{F712}{F696}$	1.25	1.60
(*b*) *Excitation of System II*		
$\frac{F712}{F685}$	0.94	1.00
$\frac{F712}{F696}$	0.86	0.96

§ F712 is fluorescence intensity at 712 mμ referred to as F720 in the text.

The emission spectra at −196° were obtained with "low" exciting light, but the intensity of fluorescence measured at 685 mμ (F685), 696 mμ (F696) and 712 mμ (F720)* is linearly dependent on incident intensity, *i.e.* there is no change in the differential fluorescence yield.

Fluorescence intensity as a function of temperature. The behavior of the different fluorescent species—recognizable in the emission spectra at −196° upon excitation

* The use of F720 for emission (at 712 mμ) is consistent with the usage of these terms outlined in an earlier paper[22].

in Systems I and II—was studied as a function of temperature. The fluorescence intensities at 661 mμ (F660), 685 mμ (F685), 696 mμ (F696) and 712 mμ (F720) change at different rates, when a Porphyridium suspension, cooled to −196°, is allowed to warm up gradually.

Unfortunately, there is a strong overlap between the 685-mμ band, the 696-mμ band, and the 712-mμ band. At no wavelength in this region there is only one band participating in fluorescence, but for qualitative results, fluorescence intensity was observed at 704 mμ for F696, since at this wavelength there is a lesser overlap between F696 and F685. Similarly, 740 mμ was chosen to observe F720, since at 712 mμ there is too much overlapping with the 696-mμ band. This position may appear somewhat too far out, but the results obtained were quite similar to those at 720 and 730 mμ. The F685 and F660 were observed at their peaks.

Figs. 8 and 9 show the fluorescence at 661 mμ, 685 mμ, 704 mμ, and 740 mμ as a function of temperature, for excitation in System I and in System II, respectively.

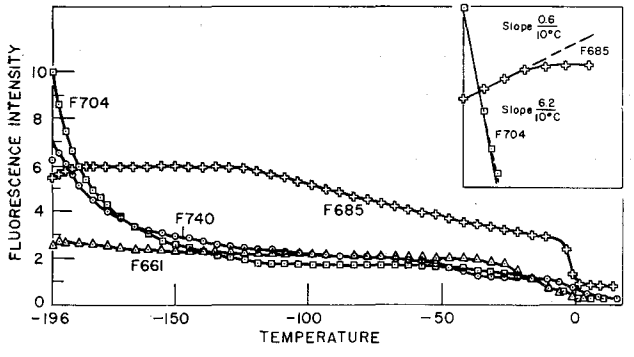


Fig. 8. Fluorescence intensity at 685 mμ (F685), at 704 mμ (referred to in the figure as F704; as F696 in the text), at 740 mμ (F740; as F720 in the text) and at 661 mμ (F661; as F660 in the text) as a function of temperature [$F = f(T)$]. Exciting light: 440 mμ. Insert shows rate of change of fluorescence intensity at 685 mμ and at 704 mμ between −196° and −185° (both scales for the insert are on a three times expanded scale).

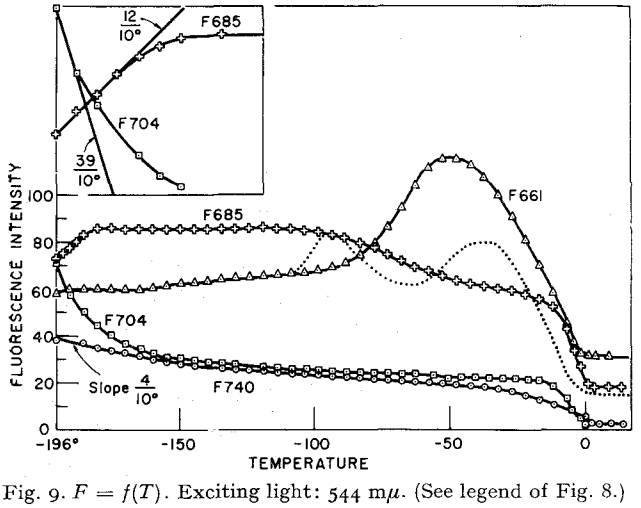


Fig. 9. $F = f(T)$. Exciting light: 544 mμ. (See legend of Fig. 8.)

The 685-mμ band remains constant up to around $-100°$ (except for a small increase in the temperature range between $-196°$ and about $-185°$) and then drops gradually. The 704-mμ fluorescence declines very fast in the $-196°$ to $-185°$ range; the decline then slows down and the curve $F = f$ (temperature) becomes approximately constant. The fluorescence intensity at 740 mμ decreases much more slowly than at 704 mμ in the temperature range $-196°$ to $-150°$; the ratio of the initial changes of the 704-mμ to 740-mμ fluorescence is about 10 (both decreasing). Above $-150°$, the curves for the fluorescence at 704 and 740 mμ run parallel. All species show, for both types of excitation, a sudden change in fluorescence around 0°, where the sample melts. There are no strong changes with further rise in temperature to 20°.

The very fast decline of the 696-mμ band (observed at 704 mμ) can be also seen in the emission spectra taken upon excitation by green light at different temperatures (Fig. 10). At very low temperatures ($-196°$ to $-185°$) the 696-mμ band was clearly separable from the other bands, but at $-175°$, there is only a shoulder at

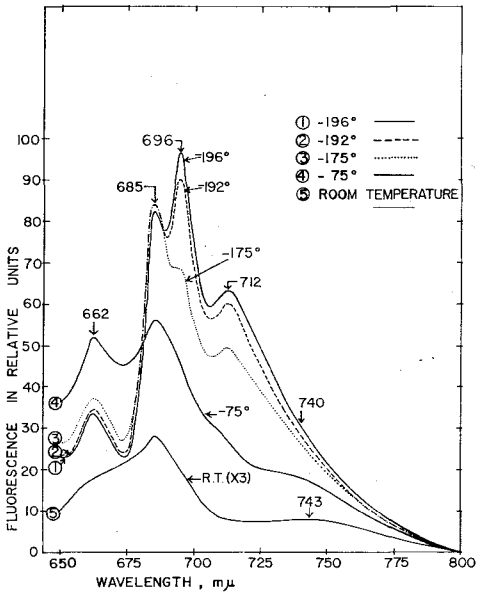


Fig. 10. Emission spectra obtained at several different temperatures upon excitation with 544-mμ light. The room temperature (R.T.) spectrum (multiplied by three) is also shown.

696 mμ and in the emission spectrum at $-75°$ there is no indication of F696. The 712-mμ band still has a clearly separable peak at $-175°$, and a recognizable shoulder at $-75°$, since its decline is slower. For comparison, a room temperature emission spectrum is given whose actual values were multiplied by three.

The phycocyanin fluorescence shows a quite different behavior upon excitation with green light. There is no significant change between $-196°$ and $-100°$. At about $-100°$, at a temperature where the chlorophyll *a* fluorescence starts decreasing, the phycocyanin fluorescence increases as if the efficiency of energy transfer from phycocyanin to chlorophyll *a* is affected. In some samples, a single maximum was found at about $-50°$; in others, two maxima, in the region between $-100°$ and $-30°$. The

emission spectrum (for green excitation) at $-75°$ shows a high phycocyanin fluorescence—being almost as high as the fluorescence intensity of the 685-mμ species. Above $-50°$, the phycocyanin fluorescence started decreasing, becoming almost constant above 0°. Excitation with blue light did not show such temperature changes in fluorescence at 661 mμ; it decreased steadily. (Of course, excitation with blue light produces very little phycocyanin fluorescence.)

DISCUSSION

The present paper reports measurements of changes in the fluorescence yield during steady-state conditions. These changes may be very different from the changes occurring during short periods of illumination which have been extensively studied by DUYSENS AND SWEERS[8]. LAVOREL[30] and ROSENBERG, BIGAT AND DEJAEGERE[31] have shown spectral differences between the fluorescence of the induction and the steady-state periods.

The excess fluorescence at high light intensities from System II

The "light curves" [$F = f(I)$] of fluorescence presented here show an increase in differential fluorescence yield at higher excitation intensities in System II, but not in System I (Fig. 2) although the available light intensities were high enough for saturation of photosynthesis.

The ratio ("R") of the differential fluorescence yields (dF/dI) at "high" to "low" intensity ranged from 1.8 to 2.0 in experiments with Porphyridium (grown in "low" intensity white light) in which System II was excited. FRANCK[11] obtained the same change in green algae upon excitation with white light. This also suggests that in these algae (as well as in the red ones), the larger part of the excess fluorescence observed at "high" light intensities (at room temperature), is excited by light absorbed in System II.

BUTLER AND BISHOP[32], working on a green alga, Scenedesmus, and TEALE[33], also working with green algae and chloroplasts, came to the same conclusion on the basis of difference excitation spectra measured in "high" and "low" excitation light intensities. In the present paper (Fig. 4), we have shown the difference excitation spectra in *Porphyridium cruentum* and it may be noted that it resembles the action spectrum of the EMERSON enhancement effect (ref. 3). The difference excitation spectra obtained with a background of green light shows participation by phycoerythrin and not by chlorophyll *a*—as if light absorbed only in System II were responsible for the excess fluorescence.

Emission spectra (measured under steady-state conditions) show a difference fluorescence band at 693 mμ, when the spectrum obtained upon excitation with "high" intensity is compared to the one obtained upon excitation with "low" intensity of incident green light (absorbed in System II). The difference emission spectrum induced by short periods of illumination, however, has a maximum at about 685 mμ (ref. 8; also see Fig. 2, Curve 2, s–f of ref. 34). It is not yet quite clear why different results are obtained under the two conditions. As suggested earlier by us[10], the excess fluorescence at high light intensities may originate in System II—perhaps in the energy trap of System II—when photosynthesis is saturated. Of course, alternate explanations cannot be excluded at this time. For example, the changes in the spectral fluorescence in the steady state may be due to an indirect effect on the chloro-

phyll *a* fluorescence, due to the accumulation of some intermediate that causes an increase in the fluorescence yield and a broadening of the emission spectrum of the chlorophyll *a* of System II.

DCMU is known to inhibit Reaction II and it is likely that it reacts with the "energy trap" of System II (ref. 8). We have observed (Fig. 5) a small difference between the emission spectra of DCMU-poisoned and unpoisoned cells at "low" light intensity. The two curves "normalized" at the peak of chlorophyll *a* fluorescence show a band at 692 mμ. This may also suggest that a part of the excess fluorescence in the poisoned cells originates in the trap II. If the 692-mμ emission band—observed by poisoning Reaction II and by saturating photosynthesis—is from trap II, the absorption by this species may be associated with a pigment molecule absorbing around 680 mμ. This estimate is based on the assumption that the energy difference between the fluorescence band at 693 mμ and the location of the absorption band of the pigment responsible for it, is the same as the energy difference between 670 and 685 mμ, the absorption and fluorescence peaks of the bulk of the chlorophylls. We shall call the hypothetical energy trap of System II, pigment 680 or P680 in symmetry with P700 of System I.

The quenching of System II fluorescence by light absorbed in System I

GOVINDJEE *et al.*[29] noted a decrease of the fluorescence yield of chlorophyll *a* in Chlorella (excited by System II), when an additional beam of far-red light (absorbed in System I) was superimposed. At that time, these authors tried to invoke a physical mechanism for this effect. It may be remarked here that these measurements were made with a galvanometer which had some time lag; readings were taken when the signal remained steady for 4–5 sec. These readings may have been averages of the induction and the steady-state periods. DUYSENS AND SWEERS[8] measured changes in a short period of illumination and discovered a quenching effect. They suggested[8] that a chemical oxidant (Q) is produced by light I, which causes quenching of chlorophyll *a* of System II and that this Q is reduced to QH by light absorbed in System II. J. MYERS (private communication) has shown that the quenching effect is observable only during the induction period; MUNDAY (unpublished) finds the same result in his Chlorella samples. MUNDAY has further shown that far-red preillumination causes a quenching of the induction peak in several cultures of Chlorella. (Several other cultures of Chlorella, however, failed to show this effect clearly.) A systematic re-investigation of the quenching effet is planned. In this paper we have shown that a small quenching effect can be measured in certain cultures of Porphyridium during so-called steady-state fluorescence; this quenching is also on fluorescence excited by System II. Excitation spectra with System I background light showed a decrease in fluorescence yield in the region where System II absorbs (Fig. 4).

The difference between emission spectra excited by light absorbed in System I (blue) and light absorbed in System II (green) separately and given together (Fig. 3) show peaks at variable positions (between 690 and 704 mμ). Because of the smallness of the difference emission band, we cannot be sure about the exact location of the emission band of the chlorophyll *a* molecule whose fluorescence is quenched. Furthermore, in several Porphyridium suspensions, MUNDAY (unpublished) finds an enhancement of the induction peak of chlorophyll *a* fluorescence with far-red (705 mμ) preillumination; this calls for further investigation.

The nature of the emission bands at low temperature

The existence of emission bands at 685 mμ (F685), 696 mμ (F696) and 712 mμ (F720) at −196° is confirmed in the red alga, *Porphyridium cruentum* (Figs. 6, 7 and 10). As far as the chemical of these bands is concerned, BRODY AND BRODY[19] (*cf.* ref. 15) suggested that the 720-mμ band originates from chlorophyll aggregates present but non-fluorescent at room temperature, which become fluorescent at −196°, and that the 696-mμ band is due to an aggregate of chlorophyll *b* (in Chlorella) or another aggregate of chlorophyll *a* (discovered by them in a specially prepared chlorophyll *a* solution (in acetone) also becoming fluorescent only at low temperatures.

The question: What is the photosynthetic function of the various fluorescent species observed at −196° remains unsolved.

KOK[16] believes that out of the two fluorescent species on the long-wavelength side of chlorophyll *a* one fluorescence band (F700) originates in the chlorophyll *a* molecules transferring energy to the final trap (P700); and the other from an "overflow" pigment (F730). Both belong to System I. (No suggestion was given for a long-wavelength side fluorescence band belonging to System II.) Different assignments regarding the fluorescence bands at the long-wavelength side of the bulk of chlorophyll *a* fluorescence peak at 685 mμ were made by us[22]. The 696-mμ band is suggested to originate from System II and not from System I. On the other hand, the 720-mμ band is suggested to arise from System I. (The 696-mμ band is likely to originate from chlorophyll *a* molecules absorbing significantly below 696 mμ than in chlorophyll molecules which absorb at 700 mμ.)

The following discussion favors the suggestion that F696 at liquid-N_2 temperature originates in System II and F720 in System I.

(1) Emission spectra at −196°, upon excitation of the different pigment systems, have characteristically different shapes (Figs. 6 and 7). Excitation of System II (with 544 mμ) in Porphyridium yields a clearly separated band at 696 mμ, besides the one at 685 mμ and a shoulder at 712 mμ. In the emission spectrum obtained upon excitation in System I, however, the 712-mμ band is dominant, and the 696-mμ band and also the 685-mμ band appear only as shoulders. This suggests that the 696-mμ band belongs to System II, while the 712-mμ band belongs to System I.

(2) CEDERSTRAND[35] has shown that chloroplast fragments (prepared by the method of BOARDMAN AND ANDERSON[36]), composed mainly of System II, show at −196° a higher ratio of F696 to F720 than those fractions composed mainly of System I. These results also suggest that 696-mμ fluorescence belongs to System II, and the 712-mμ fluorescence to System I.

There are several possibilities as to the function of the species fluorescing at 696 mμ (at −196°). The fluorescence band may either be due to the trap of the second photochemical system[17,18,22] or the fluorescence might come from the chlorophyll *a* molecules transferring energy (in System II) to the corresponding final trap. These bands cannot be due to the bulk chlorophylls because the latter have an emission band at 685 mμ. Unless we assume that the pigments feeding energy to the trap absorb at longer wavelengths than the bulk chlorophylls, we cannot conclude that the 696-mμ band is due to this "collector" system. Thus, we favor its attribution to the energy trap in System II. However, we cannot exclude, at this time, the possibility that low temperatures cause the formation of "species" that do not exist at room temperatures.

The 712-mμ band, belonging to System I, may be the fluorescence of P700, trap I, or to the pigments feeding energy to this "trap". Here also, trivial causes cannot be excluded.

The 740-mμ band may be partly due to the first vibrational band and partly to an additional pigment. Further quantitative investigation is required because all the spectra so far presented at —196° (by us and also by all other investigators) have not been corrected for reabsorption and reemission of fluorescence within single cells. The use of very thin suspensions (and the geometry of our instrument) has allowed us to reduce the problem of reabsorption and reemission of fluorescence by the neighboring cells.

Change in fluorescence intensity for F685, F696 and F720 with temperature

When the cooled sample is allowed to warm up gradually, the fluorescence intensity from the different species decreases at different rates in Porphyridium (Figs. 8 and 9). This confirms our earlier finding on spinach chloroplasts[22]. The striking differences in the rate of change in the fluorescence intensity of the bands at 661 mμ (F660, referred to as F661 in the figures), 685 mμ (F685), 696 mμ (F696, referred to as F704 in the figures) and at 712 mμ (F720, referred to as F740 in the figures) indicate that they belong to different species. These data also suggest different functional nature of these species. A quantitative separation of the bands and its analysis is, however, made impossible due to the extensive overlap of the bands.

Following the arguments presented by ROBINSON[37], one may suggest that the fluorescence at room temperature is from the bulk of the chlorophylls (F685) in System II; upon cooling, the value of kT (k, Boltzman's constant and T, temperature) becomes smaller and smaller in relation to the "trap depth" and the energy trap becomes fluorescent. The F720 rises with decreasing temperature (0° to —196°) (Figs. 8 and 9) and the F696 becomes fluorescent only below —130° and its fluorescence intensity increases sharply with further decrease in temperature to —196°. If one assumes that F720 and F696 are due to energy traps, the above-mentioned differences may be taken to suggest that the two traps have different "depths". These, of course, are speculations.

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