FLUORESCENCE STUDIES ON ALGAE, CHLOROPLASTS AND CHLOROPLAST FRAGMENTS

BY

GOVINDJEE

Department of Botany, University of Illinois, Urbana, Illinois, U.S.A.

Special chlorophyll a molecules at the reaction centers (the "energy traps") are the only chemically active pigment molecules, i.e. they take part in the photochemical reaction; the bulk of the pigments simply absorb and transfer energy to the traps. If electron transfer is somehow blocked, the "traps" that continuously receive energy from the bulk pigments may lose it by increased fluorescence, delayed emission, or by internal conversion because they are unable to use this energy for photochemical reactions. If the"traps" are bleached upon blocking of the electron transfer, energy must be dissipated in the bulk molecules. In either case there is a possibility of obtaining information concerning the traps. Thus, we have studied the steady state fluorescence spectra by (a) saturating photosynthesis with excess light; (b) cooling samples to low temperatures; (c) poisoning with DCMU. These results are discussed in Part I of this paper.

A recording spectrofluorometer was used in this study [fig. 1].

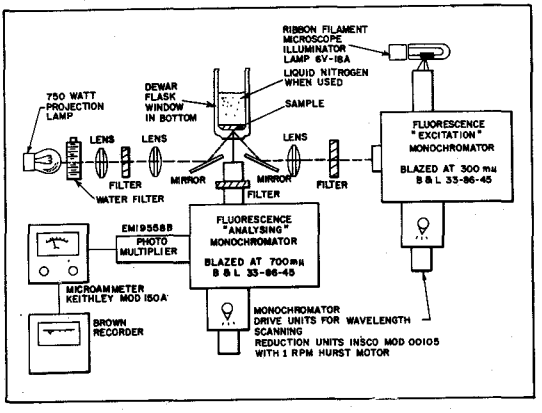


Figure 1: Diagram of spectrofluorometer (after Govindjee and Spencer).

PART I

1. Emission Spectra at High Light Intensities

A break in the curve of the "fluorescence intensity versus exciting light intensity" is observed only when system II is excited but not system I [fig. 2, lower graph].

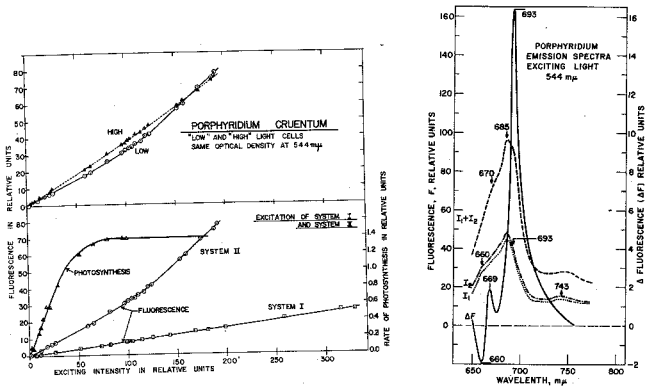


Figure 2: Fluorescence intensity (F) as function of exciting light intensity (I). Upper: F = f(I) for cells grown in "low" and "high" light intensities. Lower: F = f(I) for excitation in systems II and I. Rate of photosynthesis as function of green light intensity is also shown (after Krey and Govindjee).
Figure 3: Fluorescence spectra (of Porphyridium cruentum) obtained with light of different intensities (2).

The accepted view is that emission spectra at low and high intensities are identical. We have shown that this is not so in the red alga, Porphyridium. An emission band at 693 mμ appears when the emission spectrum at high light intensities is subtracted from the emission spectrum at low light intensities [fig. 3].

2. Emission Spectra at Low Temperatures

Several investigators (3,4) have shown that the emission spectra at -196°C have three bands--at 685 (F685), 696 (F696) and at 720 mμ (F720). The F720 is

usually a complex band [fig. 4] and is not always observed at 720 mμ. We believe that F696 and F720 originate in the energy traps. Of course, there are other possibilities which have to be considered before we can be sure about their origin. For example, the low temperature bands may arise from chlorophyll a molecules that can no longer effectively transfer energy to the "traps" because the latter may be partially bleached, or from different aggregates of chlorophyll a that are non-fluorescent at room temperatures but become fluorescent at -196°C; or from aggregates of chlorophyll that are "artifically" formed upon cooling and disappear upon warming.

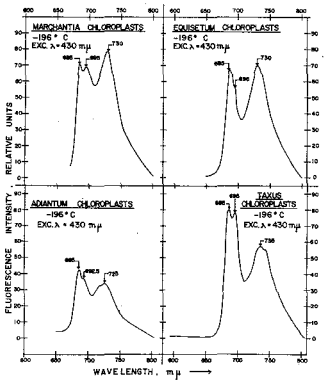


Figure 4: Fluorescence spectra of different organisms (at -196°C).

3. Fluorescence Intensity as a Function of Temperature

Fluorescence spectra as a function of temperature (-196°C to 20°C) have been measured in Porphyridium, Anacystis, Chlorella and chloroplast fragments from spinach. We observe that F685 remains fairly constant [fig. 5] in the -196°C to -30°C range; the rate of decrease of F696 is more than twice as great as that of F720 when the temperature rises from -196°C to -130°C. This suggests that these bands arise from different molecules. (This assumes that the vibrational band behaves similarly to the electronic band.) The F685 may originate in the bulk pigments (5) and the F696 and F720 (or F738) --which increase upon decreasing temperature--may arise from the "traps". The

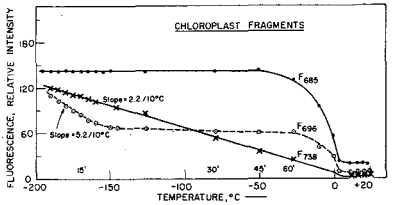


Figure 5: Fluorescence intensity at 685 mμ (F685), 696 mμ (F696) and at 760 mμ (F738) as function of temperature in chloroplast fragments from spinach (after Govindjee and Yang).

F696 is observed only below -130°C. As a hypothesis, we suggest that F696 is due to trap II and F720 to trap I.

4. Emission Spectra with DCMU

A difference emission band at 692 mμ was observed between the emission spectra of DCMU-poisoned and normal cells of Porphyridium [fig. 6, curve on extreme right]. If one postulates that the 693 mμ band in the difference fluorescence spectrum (section 1) is due to the system II trap and if DCMU reacts with this trap [as suggested by Duysens and Sweers (6)], then it is plausible that DCMU can lead to the production of a 693 mμ band from the system II trap.

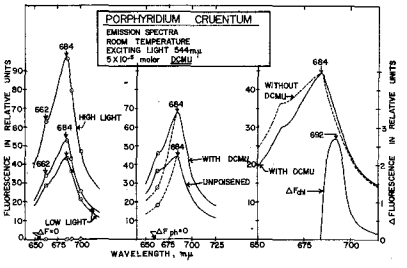


Figure 6: Left: Fluorescence spectra of Porphyridium (poisoned with DCMU) excited with light of different intensities. Middle: Fluorescence spectra for poisoned and normal cells. Dashed lines are for extrapolation of chlorophyll fluorescence. ΔF ph = change in phycocyanin fluorescence. Right: Normal and poisoned spectra normalized at 684 mμ. ΔF Chl = change in chlorophyll a fluorescence (after Krey and Govindjee).

In Part I of our presentation, we have been concerned with the possibility of obtaining information regarding the "energy traps" from fluorescence studies after the electron transfer had been suppressed or blocked. Now, we would present experiments concerning our attempts to discriminate between the fluorescence characteristics of the two pigment systems, either by exciting with different wavelengths or by physically separating them.

PART II

Fluorescence spectra excited by different wavelengths have different shapes (7). Fig. 7 shows emission spectra of spinach chloroplasts. We observe a 30% higher fluorescence around 730 mμ when chlorophyll a rather than when chlorophyll b is excited.

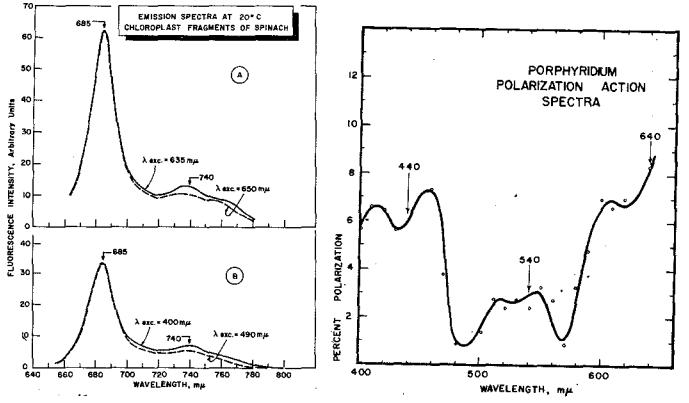
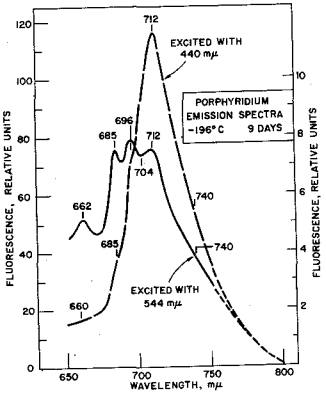


Figure 7: Fluorescence spectra with thin (2% absorption at 680 mμ) suspensions of chloroplast fragments excited by different wavelengths of light (after Govindjee and Yang).
Figure 8: Action spectrum of polarization of fluorescence in Porphyridium (measured in Dr. Weber's laboratory).

Since there may be more chlorophyll a in system I, the 730 mμ fluorescence may be associated with system I. Olson,et. al. (8) have shown that the long-wave emission is polarized. We have measured the action spectrum of polarized fluorescence in Porphyridium; it corresponds to pigment system I [fig. 8]. The same has been shown in Chlorella (9).

1. Excitation in System II and System I (at -196°C)

Fig. 9 shows that excitation in system I (by 440 mμ light) leads to a dominant band at 712 mμ, whereas excitation in system II (by 544 mμ light) leads to almost equal fluorescence intensities at the three peaks (685, 696 and 712 mμ). We can thus assign F696 to system II and F720 to system I.



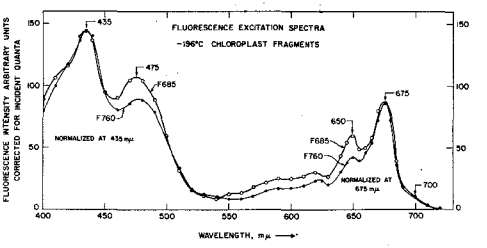


Figure 9: Fluorescence spectra of a 9-day-old culture of Porphyridium (at -196°C) upon excitation in systems II and I (after Krey and Govindjee).
Figure 10: Action spectra of fluorescence in spinach chloroplasts (at -196°C) measured at 685 mμ (F685) and at 760 mμ (F760). The two curves have been normalized at 675 mμ(after Govindjee and Yang).

2. Excitation Spectra

Excitation spectra of fluorescence of spinach chloroplast fragments, measured at 685 and 760 mμ (at -196°C) show differences in the ratio of chlorophyll a to b peaks [fig. 10]. The a to b ratio is 2.06 for F760 and 1.43 for F685, suggesting that the long-wave fluorescence is preferentially excited by system I and short-wave fluorescence by system II.

3. Partial Separation of Two Pigment Systems and Their Fluorescence Characteristics (at -196°C)

We have partially separated the two pigment systems by the following method (10). The chloroplasts from spinach were incubated in a digitonin medium and then fractions of different sizes were separated by differential centrifugation. The larger (4000 to 7000 Å) particles (fractions 1 and 2), having lower degree of polarization (3%) and more of system II, showed a 696 mμ band, whereas the smaller (200 to 1000 Å) particles (fractions 3 and 4), having higher degree of polarization (6%) and more of system I, did not show the 696 mμ

band. This again suggests that F696 belongs to system II and F720 to system I [fig. 11]. Fractions enriched

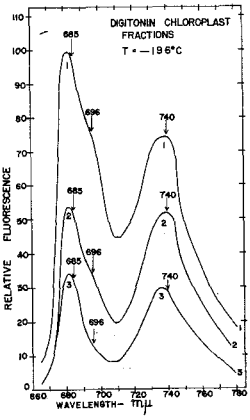


Figure 11: Fluorescence spectra (at -196°C) of several fractions of spinach chloroplasts prepared by digitonin solubilization method (after Cederstrand and Govindjee).

in system II showed measurable quenching of fluorescence upon addition of oxidized cytochrome c, whereas fractions enriched in system I did not.

SUMMARY

Fluorescence characteristics of algae, chloroplasts and chloroplast fragments investigated by the author and co-workers are briefly reviewed here. These results are consistent with the two systems-two traps hypothesis.

The main findings presented in Part I of this paper are: (a) the appearance of an emission band at 693 mμ in Porphyridium upon excitation with high intensity system II light but not with system I light; (b) the appearance of 696 mμ and 720 mμ bands at -196°C in Marchantia, Taxus, Adiantum and Equisetum; (c) F696 decreases at more than twice the rate the F720 decreases when the organisms are warmed from -196°C to -130°C, whereas the F685 remains constant; (d) the appearance of a difference emission band at 692 mμ between the DCMU-poisoned and normal Porphyridium cells. It is suggested that the emission bands (at 692, 693, 696 and 720 mμ) obtained when electron transfer is suppressed or blocked, may arise from "energy traps".

Our attempts to characterize and separate the two pigment systems (presented in Part II of this paper) lead to the following findings: (a) the emission spectra in pigment systems partially separated by digitonin solubilization and the emission spectra, obtained by differential excitation in the two pigment systems, show that F696 belongs to system II and F720 to system I; (b) the chloroplast fractions enriched with system II show measurable quenching of fluorescence when oxidized cytochrome c is added, whereas those with system I do not; (c) the emission spectra excited by 430 and 635 mμ light show 30% higher fluorescence at 730 mμ than those excited by 480 and 650 mμ light, suggesting that long-wave fluorescence is preferentially excited by chlorophyll a (system I) and short-wave fluorescence by chlorophyll b (system II).

REFERENCES

(1) W.L.Butler and N.I.Bishop, In: Photosynthetic Mechanisms of Green Plants, B.Kok and A.T.Jagendorf, Eds. Natl. Acad. Sci.-Natl. Res. Council Publ.(1963) pp. 91-100.

(2) A.Krey and Govindjee, Proc. Natl. Acad. Sci.(USA) 52, 1568-1572 (1964).

(3) Govindjee (pp. 318-334), J.A.Bergeron (pp. 527-536), B. Kok (pp.45-55), and S.S.Brody and M. Brody (pp. 455-478), In: Photosynthetic Mechanisms of Green Plants (1963).

(4) J.C.Goedheer, Biochim. Biophys. Acta, 88, 304-317 (1965).

(5) W.Robinson, Annl. Rev. Phys. Chem. 15, 311-348 (1965).

(6) L.N.M.Duysens and H.E. Sweers, In: Studies on Microalgae and Photosynthetic Bacteria, S. Miyachi, Ed. Univ. Tokyo Press (1963) pp. 353-372.

(7) L.N.M. Duysens, Doctoral Thesis, U. Utrecht (1952).

(8) R.A.Olson, W.H.Jennings and W.L.Butler, Biochim. Biophys. Acta 88, 331-337 (1964).

(9) J.Lavorel, Biochim. Biophys. Acta, 88, 20-36 (1964)

(10) N.K.Boardman and J.M.Anderson, Nature, 203, 166-167 (1964).

DISCUSSION

Arnon: Were your fluorescence measurements, particularly in the long wavelengths, corrected for the phototube characteristics?

Govinjee: Yes, the emission spectra were corrected for the sensitivity of the photomultiplier and the spectral efficiency of the monochromator. I may add that the excitation spectra were corrected for the number of incident quanta.

Oster: A single pigment could exhibit a fluorescence spectrum (and, indeed its photochemistry) which depends upon the wavelength of exciting light, Consider the exaggerated case of malachite green (Oster and Oster in Luminescence in Organic and Anorganic Materials- Wiley, New York, 1962). This pigment which has absorption peaks in the blue and the red region is fluorescent when bound to high polymers. The emission is red when excited by red light and green (and some red) when excited by blue light. In other words, there are two band systems (presumably associated with the two dichroic axes of the molecule) which are only loosely coupled. Such a two-band system could exist for chlorophyll but with strong coupling.

Govindjee: Thank you for your remark, Dr. Oster.

Thomas: I may remark that, in our experiments, we did not observe marked differences in the a/b ratio. However we did not establish pertaining difference spectra. It may well be that the use of digitonin yields different effects.

Govindjee: Yes, I agree with Professor Thomas. Our results may be different because of the use of digitonin.

Sironval: How much digitonin was used per mg total chlorophyll in your experiments?

Govindjee: Saturated solution-the precipate was filtered out. There might be some artifacts due to digitonin.

Teale: The graph showing the influence of decreasing temperature on chloroplast fluorescence indicated that, compared with room-temperature, the lowest temperatures increased the fluorescence intensity by a factor of twenty or so. Why is this ratio so much larger than the yield increase normally observed on lowering the temperature?

Govindjee: The fluorescence intensities were measured at 685 mμ (F.685), 696 mμ (F 696) and at 760 mμ (F 720); the areas under the curves were not measured. There are several problems:

1. spectra at lower temperatures are sharper
2. there is perhaps some contribution due to changes in scattering
3. the fluorescence yield in these chloroplast preparations measured at room-temperature was about 0.5 to 1.0%;

these measurements were obtained by comparing fluorescence yields of chloroplast to that of chlorophyll a in solution which was assumed to be about 33% (as found by Weber and Teale and Latimer et al). In the present experiments, we have been primarily interested in changes in the temperature range -196^{0} C to -100^{0} C where the interesting differences were observed.

Arnon: (To Oster, dicussing the paper by Govindjee).
Did I understand correctly your comment about different fluorescence spectra obtained from one chemically pure pigment (illuminated by different wavelengths of light) to imply that similar evidence for the chlorophyll pigments does not necessarily establish that one is dealing with two different pigment complexes?

Oster: Two different chlorophyll complexes may give the same absorption spectra but different fluorescence spectra. You may wish to

call them two different pigments but when clearly separated from the substrate they may well turn out to be a single chemical substance. It is conceivable that you could also have two kinds of photochemistry for a single pigment depending upon the wavelength of exciting light (the case of bound malachite green is a clear example of this).