Chapter 1

Chlorophyll *a* Fluorescence: A Bit of Basics and History[1]

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[1]Portions of Govindjee (1995) 'Viewpoint: Sixty-three Years Since Kautsky: Chlorophyll *a* Fluorescence,' originally published in Australian Journal of Plant Physiology, Vol. 22: 131–160, <http://www.publish.csiro.au/journals/fpb>, are used in this chapter, with permission of CSIRO Publishing, Melbourne Australia.

George C. Papageorgiou and Govindjee (eds): Chlorophyll Fluorescence: A Signature of Photosynthesis, pp 1–42.

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Summary

Chlorophyll (Chl) fluorescence is a non-destructive intrinsic probe of several aspects of oxygenic photosynthesis. In this chapter, the goal is to bring to the readers the basics of Chl fluorescence, a bit of history, it's potential in understanding primary photophysical events (excitation energy transfer; charge separation), and secondary reactions (electron transport). This chapter is an extension of an earlier overview by the author (Govindjee, 1995). References are made to selected original and historical papers and reviews in order to lead the readers to earlier work often unavailable on the Internet. It is obvious now that in Chl *a* fluorescence measurements, we have come a long way since Kautsky's discovery 73 years ago. Chl *a* fluorescence has provided new and important information on the composition of the pigment systems, excitation energy transfer, physical changes in pigment-protein complexes, primary photochemistry, kinetics and rates of electron transfer reactions in Photosystem II (PS II), the sites of various inhibitors, and activators, and of lesions in newly constructed mutants. The purpose of this chapter is not to present the current and updated information on Chl fluorescence, but to guide the readers to the outstanding chapters written by international experts in the field. (Photographs of these authors are also included here.)

I. Introduction

Chlorophyll (Chl) *a* fluorescence is no longer in the purview of specialists alone as it has become a routine probe for information, sometimes misinformation, on the various aspects of photosynthesis. If used properly, it informs on the identity of the various pigments and pigment complexes, their organization, excitation energy transfer among them, and on the various electron-transfer reactions, specifically of Photosystem II (PS II). For the basics of photosynthesis, see Rabinowitch and Govindjee (1969), Ke

Abbreviations (also see the legend of Figure 4): Chl – chlorophyll; CP43, CP47 – minor antenna chlorophyll protein complexes in PS II core; Cyt – cytochrome; D1, D2 – polypeptide D1 and D2 of reaction center II; DTT – dithiothreotol; F685, F696, F720, F740 – fluorescence emission bands with peaks at 685 nm, 696 nm, 720 nm and 740 nm; F_o, F_m, F_v, F_t – fluorescence intensity at the minimal level, at the maximal level, $F_v = F_m - F_o$, and fluorescence level at time t; k_p, k_f, k_o, k_h, k_{tr} – rate constants of photochemistry, of fluorescence, of other losses, of heat loss, of energy transfer; LHCII, LHCI – Light-harvesting complex II, Light-harvesting complex I; NPQ – non photochemical quenching of Chl fluorescence; O, J. I. P, S, M, T – names for the various points in Chl fluorescence transient (see footnote[3]); OEC – oxygen evolving complex; **P**, p, P and **p** – degree of polarization of fluorescence, probability of exciton transfer among PS II units, Paillotin's connection parameter, and probability of effective collision; P680, P700 – reaction center Chls of PS II and PS I, respectively, with one of their absorption bands at 680 and 700 nm, respectively; PS II, PS I – Photosystem II, Photosystem I; Q_A – primary plastoquinone one-electron acceptor of PS II; Q_B – secondary plastoquinone two-electron acceptor of PS II; S_0, S_1, S_2, S_3, S_4 redox states of the oxygen-evolving (tetranuclear Mn) complex, the subscripts refer to the positive charges; Y_Z (or Z) – tyrosine-161 of D1 protein, electron donor to $P680^+$; ϕ_p, ϕ_f – quantum yield of photochemistry, quantum yield of fluorescence; τ, τ_o – lifetime of fluorescence (measured), intrinsic lifetime of fluorescence

There were several markings involving the P,p, etc. stuff that I could not decipher.

(2001) and Blankenship (2002).

Photosynthesis is initiated by light absorption. Chlorophyll *a* is one of the central molecules that absorbs sunlight and this energy is used to synthesize carbohydrates from CO_2 and water. Figure 1 shows light absorption by Chl *a*, Chl *b*, and other photosynthetic pigments (carotenoids, phycoerythrin and phycocyanin), as well as Chl fluorescence. (The cover of the book is a colored plate of Fig. 1, but, in addition, it includes the spectrum of the sunlight that falls on Earth's surface (courtesy of Nancy Kiang, NASA Goddard Institute of Space Studies). The *chloro* portion of the word chlorophyll is from the Greek *chloros* (χλωρος), which means yellowish green, and *phyllon* (φυλλον), which means leaf. The process of photosynthesis is the basis for sustaining the life processes of all plants. Since animals and humans ultimately obtain their food by eating plants, photosynthesis can be said to be the source of our life also.

A. Discovery of Chlorophyll Fluorescence

E. N. Harvey (1957) presented an early history of luminescence until 1900. Luminescence is a generic word for all types of light emission (delayed light emission; thermoluminescence; prompt fluorescence, and phosphorescence). A summary of the discovery of luminescence follows (Govindjee, 1995): 'Luminescence' was first observed, in 1565, by Nicolas Monardes, a Spanish medical doctor and botanist, in the extract of *Lignum nephritcum* (that was recommended for curing kidney ailments). (See Berlman, 1965, for a description of this observation.) However, Althanius Kircher (1646) was the first one to discuss, at length, its bichromatic appearance. It was yellow in transmitted light and blue in reflected light; perhaps, the blue light was fluorescence.

Sir David Brewster (1834), a Scottish preacher, first noted the red emission from Chl. While discussing his concept of the color of natural bodies, he remarked almost in passing 'In making a strong beam of the sun's light pass through the green fluid, I was surprised to observe that its color was a brilliant red, complementary to the green. By making the ray pass through greater thickness in succession, it became first orange and then…' The green fluid in Brewster's experiment was an alcohol extract of laurel leaves. It must have contained Chl, the green pigment of leaves, as named by Pelletier and Caventou (1818). Govindjee (1995) considered it likely that this was not only the discovery of Chl fluorescence, but also of the phenomenon of reabsorption of fluorescence in thick samples.

The clearest discovery of the phenomenon of fluorescence was that by Sir John Herschel (1845a,b) in a solution of quinine sulfate. He noted the 'celestial' blue color of this solution, but had unfortunately called it epipolic dispersion. The following year Brewster (1846) designated it as internal dispersion. It was left to Sir G.G. Stokes to call it fluorescence. Stokes (1852), professor of mathematics at Cam-

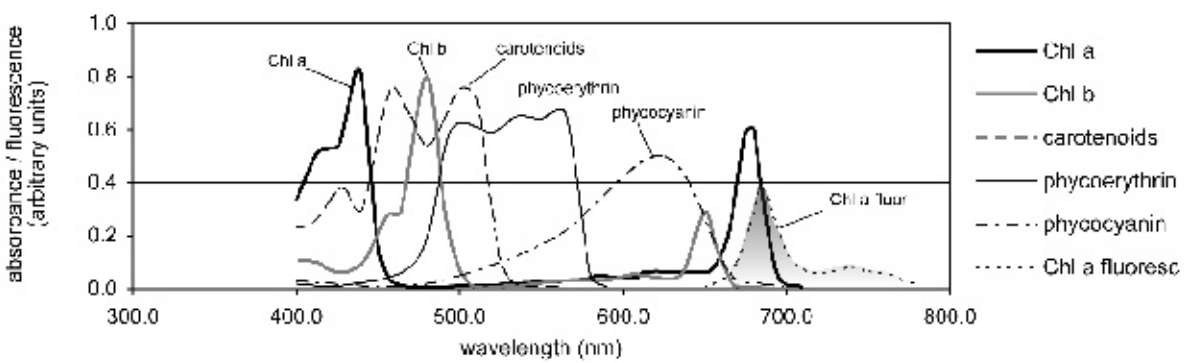


Fig. 1. In vivo absorption spectra of selected photosynthetic pigments from plants, algae and cyanobacteria, and fluorescence spectrum of Chl *a*. Chl *a* and Chl *b* absorption spectra, in diethyl ether (Du et al., 1998), were shifted in wavelengths to match the in vivo absorption peaks in PS II reaction centers and in light-harvesting Chl *a*/Chl *b* complex. Carotenoid absorption spectrum is an estimated (Govindjee, 1960) in vivo absorption spectrum in green algae. Phycoerythrin and phycocyanin absorption spectra are unpublished spectra from Govindjee's laboratory (also see Ke, 2001). Chl *a* fluorescence spectrum, from spinach chloroplasts, is from Fig. 5 (Govindjee and Yang). (See the cover of the book for a colored version of this figure that includes solar spectrum as well (Lean and Rind,1998). (N. Kiang and Govindjee, unpublished, 2004.)

bridge University, who is well known for the discovery that emission bands are shifted to wavelengths longer than the absorption bands (the Stokes shift), first used the term dispersive reflexion, but quickly added a footnote: 'I confess that I do not like this term. I am almost inclined to coin a word, and call the appearance fluorescence, from fluor-spar, as the analogous term opalescence is derived from the name of a mineral.'[Latin fluo = to flow + spar = a rock).] Stokes was the first one to recognize this phenomenon as light emission. E. Askenasy (1867) credited Stokes also for the discovery of both phycobilin and Chl *a* fluorescence in fresh red algae.

The term 'phosphorescence' dates back to the early 1500s and was so named after the Greek words for light (phos, φως) and to bear (phero, φερω). In fact, the element phosphorus was named from the same Greek word, since it was found to produce a bright light in the dark.

1. The Book and Its Authors

> *The physicist Leo Szilard once announced to his friend Hans Bethe that he was thinking of keeping a diary: 'I don't intend to publish it: I am really going to record the facts for the information of God.' Don't you think God knows the facts?' Bethe asked. 'Yes' said Szilard.' He knows the facts, but he does not this version of the facts' Freeman Dyson, Disturbing the Universe (Harper and Row, New York, 1979)*

George Papageorgiou and I agree with Leo Szilard; thus, the authors of this book have presented their versions of the facts. This, of course, has led to some contradictory views in this book. The readers' job is to decide which 'facts' they agree with. George Papageorgiou (Chapter 2) provides basic information on the fluorescence of photosynthetic pigments in vitro and in vivo, whereas Neil Baker and Kevin Oxborough (Chapter 3) discuss the use of Chl fluorescence as a probe of photosynthetic productivity. Esa Tyystjärvi and Imre Vass (Chapter 13) discuss the relationship of prompt fluorescence to delayed light emission and thermoluminescence. (For a historical perspective on thermoluminescence, see Vass, 2003.) Other chapters in this book are cited later in this chapter. Figure 2 shows a photograph of most of the authors in this book.

B. Relationship of Fluorescence to Photosynthesis

1. Pre-Kautsky Observations

It was N. J. C. Müller (1874), among others, who noticed that a green living leaf had a much weaker red Chl fluorescence than a dilute Chl solution. Although Müller had predicted an inverse relation between Chl fluorescence and photosynthesis, his experiments were not done with proper controls. Since both duration of experiment and temperature changed during his measurements, Govindjee (1995) found it difficult to credit him with the discovery of Chl fluorescence transient (or induction). Further, Müller's concepts on absorption bands cannot be accepted because he used acoustic analogy — vibrations of strings — he expected absorption at all the overtones. Transition dipoles are not strings.

2. Kautsky's Observations

On 19 October 1931, Hans Kautsky and A. Hirsch at the *Chemisches Institut der Universität* in Heidelberg, Germany, submitted a less-than-one page report (a '*Kurze Originalmitteilung*') whose title can be translated as 'New experiments on carbon dioxide assimilation.' Following illumination of dark-adapted leaves, the time course of Chl fluorescence, observed with the authors' eyes, was correlated, although qualitatively, with the time course of CO_2 assimilation, published earlier by Otto Warburg (1920). The main observations illustrated in Fig. 3 were (Govindjee, 1995): (a) Chl fluorescence rises rapidly to a maximum, then declines and finally reaches a steady level, all within a matter of minutes. (b) The rising portion of the curve was considered to reflect the primary photochemical reaction of photosynthesis, as it was unaffected by temperature (0 and 30 °C) and by a poison (authors say HCN, but it is likely that they used a KCN or NaCN solution). If the light was turned off at the maximum, the fluorescence transient recovered quickly. (c) The decline in the fluorescence curve was found to be inversely correlated with the increase in the rate of CO_2 assimilation; this suggested to the authors that more chemical energy is produced from photons when

Fig. 2, next four pages. **Photographs of the 56 of the 59 authors of the 31 chapters in this book. Photographs were provided by the authors.**

Regarding the Dyson quote: what I thought was odd was the spacing--I've never seen anything moved over like that out of the clear blue. So, now I move it back. If you want a different font you have to let me know what it is, I cannot guess what you want. However, it must be a Postscript Type 1 font to meet with Kluwer's specifications.

Chapter 1. Govindjee

Chapters 2 and 26. G. C. Papageorgiou

Chapter 3. N. R. Baker

Chapters 3 and 15. K. Oxborough

Chapter 4. R. M. Clegg

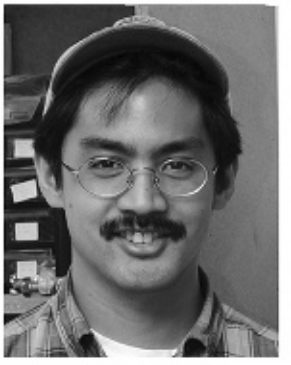
Chapter 5. R. Van Grondelle(Left) and B. Gobets(Right)

Chapter 6. W. J. Vredenberg

Chapter 7. M. Mimuro

Chapter 8. V. Shinkarev

Chapter 9. S. Itoh(Left) and K. Sugiura(Right)

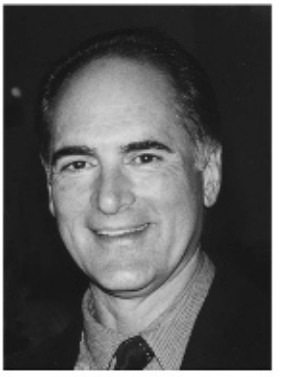


Chapter 10. (Left to Right) D. M. Kramer, A. Kanazawa, and J. A. Cruz

Chapter 11. U. Schreiber

Chapter 12. (Left to Right) R. J. Strasser, M. Tsimilli-Michael, and A. Srivastava

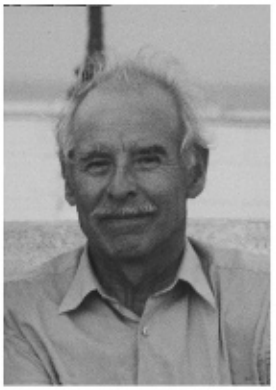
Chapter 13. E. Tyysjärvi(Left) and I. Vass(Right)



Chapter 14. L. Nedbal(Left) and J. Whitmarsh(Right)

Chapter 16. I. Moya(Left) and Z. G. Cerovic(Right)

Chapter 17.
J. F. Allen(Left) and C. W. Mullineaux(Right)



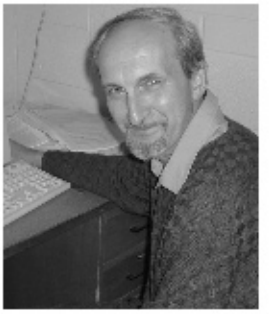
Chapter 18. G. H. Krause(Left) and P. Jahns(Right)

Chapter 19. D. Bruce(Left) and S. Vasil'ev(Right)




Chapter 20. (Left to Right) T. Golan, X. P. Li, P. Müller-Moulé, and K. K. Niyogi

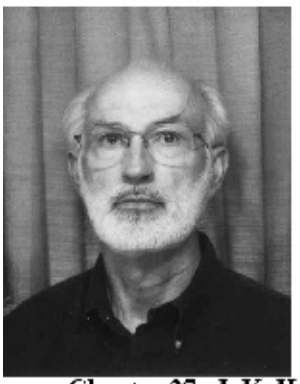

Chapter 21. A. M. Gilmore Chapter 22 W. W. AdamsIII (Left) and B. Demmig-Adams(Right)


Chapter 23. M. Tevini Chapter 24. N. G. Bukhov(Left) and R. Carpentier(Right)



Chapter 25. M. K. Joshi(Left) and P. Mohanty(Right) Chapter 26. K. Stamatakis

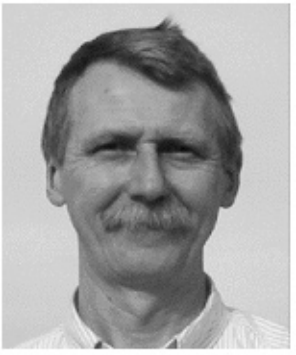



Chapter 27. J. K. Hoober(Left) and J. H. Argyroudi-Akoyunoglou(Right)

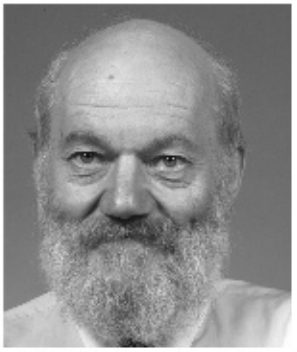

Chapter 28. H. K. Lichtenthaler(Left) and F. Babani(Right)


Chapter 29. J. Cavender-Bares(Left) and F. A. Bazzaz(Right)



Chapter 30. (Left to Right) P. G. Falkowski, M. Koblizek, M. Gorbunov, and Z. Kolber




Chapter 31. J. A. Raven(Left) and S. C. Maberly(Right)

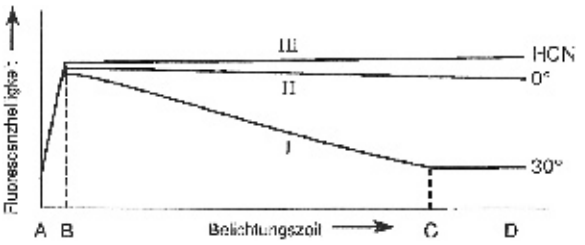


Fig. 3. Schematic representation of the Kautsky curve (Kautsky and Hirsch, 1931): Chl *a* fluorescence changes in the leaves, as observed by eyes (reproduced from Govindjee,1995).

less Chl fluorescence is seen. (d) The long lag in the carbon assimilation was considered rather strange—it seems that 'light-dependent' processes are required for the full development of the carbon assimilation process; also unexplained was the long time needed for the recovery of fluorescence transient if the light was turned off after the transient was completed. I consider these observations to be a landmark in the history of photosynthesis. Lichtenthaler (1992) has provided further details about Kautsky and his work on Chl fluorescence induction kinetics. Reto Strasser, Merope Tsimili-Michael and Alaka Srivastava (Chapter 12) provide a quantitative view for the understanding of the intricacies of Chl fluorescence induction or transient.

C. Basic Equations: Relationship of Photochemistry to Chlorophyll a Fluorescence

Since the relationship of Chl fluorescence to photochemistry is paramount to its use as a probe of photosynthesis, the basic algebra behind this relation will be presented below. Upon absorption of light, Chl *a* molecules, in the Chl-protein complexes of Photosystem (PS) II, that contain many Chl molecules, go to their excited singlet states (Chl *a**); they then decay to the ground state by several *pathways*. The quantum yield (ϕ) of a process 'i' of the ensemble of Chl molecules is related to the rate constants (k's) of the various de-excitation pathways (subscripts: f for fluorescence, p for photochemistry, o for all others that include mainly heat (h) losses; here, the rate constant of the excitation energy transfer (k_{tr}) is included in k_p as it leads to photochemistry:

$$\phi_i = k_i / (k_f + k_p + k_o) \quad (1)$$

As a reminder, the k, that is assumed to be a first order rate constant, represents the number of transitions per second, or the number of events per second. The inverse of k is nothing else but τ (lifetime), i.e., the time needed for one transition or event.

The quantum yield of photochemistry (ϕ_p) is written, therefore, as,

$$\phi_p = k_p / (k_p + k_f + k_o) \quad (2)$$

The quantum yield of minimal Chl *a* fluorescence (ϕ_{fo}), i.e., when photochemistry is maximal (k_p approaches a value close to 1):

$$\phi_{fo} = k_f / (k_p + k_f + k_o) \quad (3)$$

The quantum yield of maximal Chl *a* fluorescence (ϕ_{fm} (m for maximal)), i.e., when photochemistry is minimal (k_p approaches zero, i.e., it can be neglected; this is achieved either at saturating exciting light, or in the presence of diuron (DCMU, (3-(3,4- dichlorophenyl)-1.1´-dimethyl urea) that blocks electron flow beyond the plastoquinone acceptor Q_A of PS II) is:

$$\phi_{fm} = k_f / (k_f + k_o) \quad (4)$$

$(\phi_{fm} - \phi_{fo}) / \phi_{fm}$ can be equated to the maximal variable fluorescence $(F_v = F_m - F_o)$[2] divided by F_m (the assumption being that there are no changes in absorption cross section of the fluorescent Chl species, and that there are no changes in the incident light intensities):

$$(\phi_{fm} - \phi_{fo}) / \phi_{fm} = (F_m - F_o)/F_m = F_v / F_m \quad (5)$$

Rearranging Eqs. (3) and (4), for the values of ϕ_{fo} and ϕ_{fm}, respectively, we can write for equation (5):

[2]The F_o measurement: It is essential to mention that one of the common mistakes most first time users of Chl fluorescence make is not recognizing that measurement of F_v/F_m requires that F_o be measured precisely. When fluorescence is measured by low intensity exciting light, one must do the experiment at different low light intensities and choose the intensity where F_t does not change with time, i.e., the exciting light does not have an actinic (actinic means activates photosynthesis) effect. This becomes important when DCMU (3-(3,4 dichlorophenyl)-1,1´ dimethylurea) is present because then the fluorescence rise is fast and one can easily miss the true F_o. Further, DCMU must be added in total darkness and the sample should not be exposed to any light before measurements are made: Since DCMU functions by displacing Q_B, even low light can cause quick net formation of Q_A^- raising artificially the measured F_o as Chl fluorescence is high when Q_A^- is present.

$$\{k_f/(k_f + k_o)\} - \{k_f/(k_p + k_f + k_o)\}/\{k_f/(k_f + k_o)\} \quad (6)$$

Dividing the numerators by the denominator, we have:

$$1 - \{(k_f + k_o)/(k_p + k_f + k_o)\} = k_p/(k_p + k_f + k_o) = \phi_p \quad (7)$$

[Hint: 1 – a/b = (b – a)/b.]

Thus, F_v/F_m is a measure of ϕ_p, quantum yield of PS II photochemistry since most Chl *a* fluorescence at room temperature is from PS II (Warren Butler, 1978).

The quantum yield of Chl *a* fluorescence (ϕ_f) is related to the rate constants of various pathways of de-excitation; k_o in most cases is composed mainly of k_h for heat dissipation, as noted above, and k_q for quenching by quenchers (e.g. carotenoids, O_2 triplets, etc.). Here, we separate energy transfer (k_{tr}) from photochemistry (k_p). Thus, Eq. (1) can be expanded to:

$$\phi_f = k_f/(k_f + k_h + k_{tr} + k_q + k'_p) = k_f/\Sigma k_i \quad (8)$$

where, $k'_p = k_p + k_{tr}$.

One of the most highly cited papers about relationship between the quantum yield of photosynthetic electron transport and the quenching of Chl fluorescence has been that of Genty et al. (1989; Chapter 3, Baker and Oxborough).

Vladimir Shinkarev (Chapter 8) discusses the quantitative relationships between Chl *a* fluorescence in multiple flashes with PS II reactions. Several authors discuss qualitative and quantitative relations between fluorescence and photosynthesis: Heinrich Krause and Peter Jahns (Chapter 18), Ulrich Schreiber (Chapter 11), Strasser et al. (Chapter 12) and William Vredenberg (Chapter 6). Interestingly, the views expressed by different authors are often unique and have different features. Future experiments are needed to substantiate or refute some of the newer ideas presented.

D. Basic Fluorescence Measurements

For a complete description of the various methods used in fluorescence spectroscopy, see Lakowicz (1999).

1. Intensity, Quantum Yield, and Lifetime

By definition, the absolute quantum yield of fluorescence (ϕ_f) is obtained by dividing the total number of photons emitted (F; integrated over space and time) by the total number of photons absorbed by the fluorescent molecules (I_a). When the only de-excitation pathway is fluorescence emission, ϕ_f can be calculated from the expression $\tau = \tau_o\,\phi_f$ that relates the actual lifetime of fluorescence (τ) to the theoretical intrinsic lifetime of fluorescence (τ_o). τ_o, when the only pathway of deexcitation is fluorescence, is inversely proportional to the probability of absorption; it is measured by the area under the absorption band plotted on a wave number scale ($1/\lambda$, or ν/c):

$$1/\tau_o = [3 \times 10^{-9}\,(\nu/c)^2]\,\Delta(\nu/c)\,\varepsilon_m \quad (9)$$

where, $\Delta(\nu/c)$ is the half-band width of the absorption band, ε_m is the extinction coefficient of the molecule, and the quantity within the squared brackets takes care of the proportionality between emission and absorption (see Clayton, 1970). For a more detailed equation, see Brody (1956), as modified from Theodor Förster (1951). The precise value of τ_o of Chl *a* in ether is 15.2 ns (Brody, 1956).

Measurements of F/I_a will not agree with ϕ_f from τ measurements if there is a change in absorption cross-section of the fluorescent pigment bed, such as when non-fluorescent complexes are formed. Further, in view of the homogeneous emission of fluorescence in all directions only a portion of fluorescence is measured with constant intensity of incident light, and thus, only relative ϕ_f values are usually reported. A major advantage of estimating ϕ_f through measurements of τ is that it is independent of the concentration of Chl in the sample allowing quantitative comparisons of ϕ_f values between different samples.

2. Polarization, Excitation Spectra, Emission Spectra, and Kinetics

a. Polarization

The polarization of Chl *a* fluorescence is useful for assessing the ordered nature of the pigment molecules, their shape and excitation migration in a homogeneous pigment assembly. When polarized light is used to excite an assemblage of photosynthetic pigments, and the observed Chl fluorescence is exten-

sively depolarized, the *depolarization* is usually due to the excitation energy migration among randomly oriented Chl molecules. The degree of polarization **P** is defined as:

$$\mathbf{P} = (F_{par} - F_{perp}) / (F_{par} + F_{perp}) \quad (10)$$

where, F_{par} and F_{perp} are the fluorescence intensities of the vertically and horizontally polarized emissions when the sample is excited with vertically polarized light. Often, anisotropy, **r**, is the preferred measurement. It is simply:

$$\mathbf{r} = (F_{par} - F_{perp})/(F_{par} + 2F_{perp}) \quad (11)$$

b. Excitation Spectra of Acceptor Fluorescence

Excitation spectra of Chl *a* fluorescence in a photosynthetic organism is a plot of the number of photons emitted by Chl *a* molecules as a function of wavelength of exciting light of equal number of incident photons; it is also called action spectra of fluorescence. If there is 100% excitation energy transfer from the donor (e.g. Chl *b*) to the acceptor (Chl *a*) molecules, the action spectrum follows the sum of the percent absorption spectra of both the pigments. However, if the action spectrum is lower than the percent absorption spectrum, it indicates a lowered efficiency of excitation transfer from the donor to the acceptor molecules whose magnitude can be precisely calculated from this difference. This is the case for several accessory pigments. Robert Clegg (Chapter 4) provides the basics of the mechanism of energy (exciton) migration and transfer, whereas Rienk van Grondelle and Bas Gobets (Chapter 5) provide an overview of transfer and trapping of excitation in plant photosystems, while Mamoru Mimuro (Chapter 7) focuses on exciton migration and trapping and fluorescence in cyanobacteria and red algae.

c. Emission Spectra of Fluorescence

Emission spectra of fluorophores reflect the energy states of the fluorophores that emit light; it provides information on the composition of the fluorescent pigments in the system. The emission spectrum is usually the mirror image of the absorption spectrum of first excited state of the fluorophore. It is essential that the measured emission spectrum be corrected for the spectral distribution of the monochromator and photodetector used for the measurement. Most of the room temperature Chl *a* fluorescence in vivo is from PS II, but there is also a small but nonnegligible amount of fluorescence from PS I (Pfündel, 1998; Gilmore et al., 2000). At low temperature (e.g., 77 K), however, PS I fluorescence increases dramatically. Shigeru Itoh and Kana Sugiura (Chapter 9) focus on this PS I fluorescence.

d. Kinetics of Chlorophyll a Fluorescence

The fluorescence kinetics, which reflect various partial reactions in PS II occurring at different time scales (Chapter 8, Shinkarev), can be measured after a single excitation flash or after multiple periodically applied excitation flashes (Doug Bruce and Sergej Vasiel'ev (Chapter 19), Paul Falkowski, Michal Koblizek, Maxim Gurbanov and Zbignew Kolber (Chapter 30), and Schreiber (Chapter 11); see Section II.A (and the legend of Fig. 4) for a background on the names of the different intermediates): (1) Decay kinetics, in the ps to ns time scale, of Chl *a* fluorescence after short (ps to ns) light flashes measure the lifetime of this fluorescence. (2) Decay of Chl *a* fluorescence in the ns, the μs and the s (or even minutes) time scales measures electron flow events from (a) the electron donor 'Y_z' of PS II to the oxidized reaction center Chl *a* of PS II, $P680^+$ (as fluorescence rises due to removal of the quencher of Chl fluorescence $P680^+$; ns to μs); (b) electron transfer from the primary bound quinone acceptor Q_A to the mobile quinone acceptor Q_B (as Chl *a* fluorescence declines, 100μs to 400 μs); (c) back flow of electrons from reduced Q_A (of PS II) or reduced Q_B to the donor side of PS II, i.e., the oxygen evolving complex (s to min). (3) Increase in the quantum yield of fluorescence, in continuous exciting light, measures both the electron flow from P680 to Q_A and then to the plastoquinone (PQ) pool (about 1 second); the subsequent decrease is related to events involving protonation, among other reactions (seconds to minutes). Further elaboration of the above events will follow in Section V.

E. A List of the Past Books and Reviews on Chlorophyll a Fluorescence

The following books are useful for research on Chl *a* fluorescence: Lakowicz (1999) for all aspects of fluorescence; van Amerongen et al. (2000) for photosynthetic excitons; Govindjee et al. (1986) for

basic aspects of Chl *a* fluorescence; Lichtenthaler (1988) and DeEll and Toivonen (2003) for practical applications of Chl *a* fluorescence; and Muttiah (2002) for remote sensing applications.

History of Chl fluorescence has been reviewed only in a limited number of publications: Duysens (1986) presented a historical perspective; Govindjee (1995) reviewed Chl *a* fluorescence measurements since its discovery; Dutton (1997) reviewed the first experiments on energy transfer from fucoxanthin to Chl *a*; Govindjee (1999) presented a historical perspective of the role of carotenoids including excitation spectra of Chl *a* fluorescence and Brody (2002) the first measurements of the lifetime of fluorescence; Mimuro (2002) discussed the visualization of energy transfer in phycobilin-containing organisms through spectral and time resolved picosecond spectroscopy; Delosme and Joliot (2002) discussed the first measurements on the flash-number dependent period 4 oscillations in Chl fluorescence; and P. Joliot and A. Joliot (2003) presented the history of the measurements on the probability of energy transfer among PS II units.

Chlorophyll *a* fluorescence and its relationship to photosynthesis has been consistently reviewed since a very long time. Two of the earliest discussions on this topic were by Franck (1949) and by E. Katz (1949). However, the reviews beginning in the 1950s by Franck (1951), Rabinowitch (1951, 1956) and Wassink (1951) included detailed discussions of the relationships between Chl fluorescence and photosynthesis.

In the 1960s, Butler (1966) and Govindjee et al. (1967; see an updated version in 1973) presented comprehensive reviews; Robinson (1967) proposed the use of terms such as 'lake model' for unrestricted excitation energy transfer among great many PS II units; and Fork and Amesz (1969) wrote a review on the action spectra and energy transfer.

In the 1970s, Govindjee and Papageorgiou (1971), Goedheer (1972), Papageorgiou (1975a), Butler (1977, 1978, 1979), Harnischfeger (1977), Lavorel and Etienne (1977), Duysens (1979) and Govindjee and Jursinic (1979) were the major reviewers of different aspects of Chl *a* fluorescence. Knox (1975) presented theoretical considerations, and Strasser (1978) reviewed his so-called 'grouping model' of PS II units.

In the 1980s, Bose (1982), Schreiber (1983), Krause and Weis (1984), van Grondelle (1985), Briantais et al. (1986), Fork and Mohanty (1986), Govindjee and Satoh (1986), Lavorel et al. (1986), Lichtenthaler et al. (1986), Moya et al. (1986), Murata and Satoh (1986), Renger and Schreiber (1986), Schreiber et al. (1986), Seely and Connolly (1986), van Gorkom (1986), van Grondelle and Amesz (1986), Holzwarth (1987), Lichtenthaler and Rinderle (1988) and Bolhar-Nordenkampf et al. (1989) reviewed various aspects of Chl *a* fluorescence. Fork and Satoh (1986) reviewed the status of the so-called 'State Changes'.

In the 1990s, several reviews were published by: Horton and Bowyer (1990), van Kooten and Snel (1990), Holzwarth (1991, 1996), Karukstis (1991), Krause and Weis (1991), Lichtenthaler (1992), Renger (1992), Gaevskii and Morgon (1993), Schreiber and Bilger (1993), Vyhnalek et al. (1993), Dau (1994a,b), Evans and Brown (1994), Govindjee (1995), Joshi and Mohanty (1995), Mohammed et al. (1995), Kramer and Crofts (1996), Owens (1996), Papageorgiou (1996), Sauer and Debreczeny (1996), Campbell et al. (1998), Schreiber et al. (1998) and Lazár (1999). In 1995, Wydrzynski et al. (1995) edited a special volume on Chl *a* fluorescence.

In the 2000s, Maxwell and Johnson (2000) and Strasser et al. (2000) reviewed basic and quantitative aspects of Chl *a* fluorescence. Rohacek (2002) discussed various Chl *a* fluorescence parameters; and Saito et al. (2002) discussed remote sensing of Chl *a* fluorescence. Krause and Jahns (2003) discussed the application of Pulse Amplitude Modulation (PAM) to the physiology of plants; Mimuro and Akimoto (2003) reviewed energy transfer from carotenoids to Chl in brown algae and diatoms, whereas Mimuro and Kikuchi (2003) discussed energy transfer from phycobilins to Chl *a* in cyanophyta and rhodophyta. Kromkamp and Forster (2003) reviewed the use of variable Chl fluorescence in aquatic systems. Lazár (2003) and Trissl (2003) discussed various models of Chl fluorescence.

The chapters in this volume provide extensive citations to original papers and reviews on almost all aspects of Chl *a* fluorescence. In spite of this extensive literature, many questions remain unanswered.

II. The Two-Light Reaction and Two-Pigment System Concept

A. The 'Z'-scheme of Oxygenic Photosynthesis

Any discussion of Chl fluorescence requires that we have the basic understanding of at least the steps involved in the electron flow in photosynthesis. These

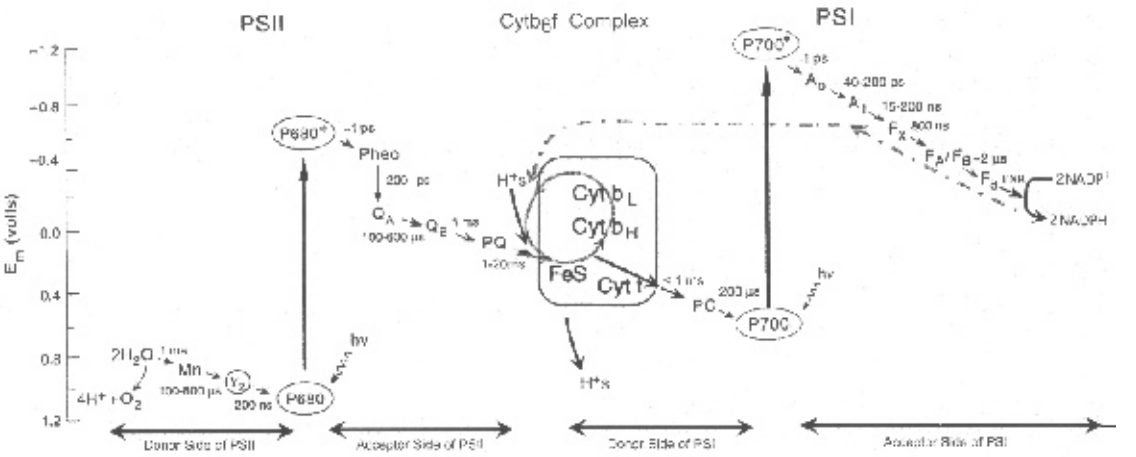


Fig. 4. The Z-scheme of oxygenic photosynthesis for electron transfer from water to oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$). The symbols are: Mn for Mn cluster; Y_z for tyrosine–161 on D1 protein; P680 for a pair of chlorophylls (Chls), the reaction center (RC) Chls of Photosystem II (PS II), having one of its absorption bands at 680 nm; P680* for the excited P680; Pheo for the primary electron acceptor of PS II; Q_A for the primary plastoquinone electron acceptor of PS II; Q_B for the secondary plastoquinone electron acceptor of PS II; PQ for plastoquinone pool; FeS for Rieske iron sulfur protein; Cyt f for cytochrome *f*; $Cytb_H$ for high potential cytochrome b_6; $Cytb_L$ for low potential cytochrome b_6; PC for plastocyanin; P700 for a pair of Chl *a* and Chl *a′*, the RC Chls of PS I; P700* for excited P700; A_0 for primary electron acceptor of PS I, a Chl monomer; A_1 for secondary electron acceptor of PS I, vitamin K; F_X, F_A and F_B for 3 different iron sulfur centers; Fd for ferredoxin; and FNR for ferredoxin-NADP reductase. Approximate estimated times for various steps are also noted on the figure (modified from Whitmarsh and Govindjee, 2001). A circular path (shown in light grey) in the Cyt b_6f complex symbolizes the existence of a Q-cycle; and a dotted (light grey) line from the electron acceptor side of PS I to the PQ/Cyt b_6f region symbolizes the existence of a cyclic flow around PS I under certain conditions. (Modified from Govindjee, 2000; Whitmarsh and Govindjee, 2001; and Paul Falkowski, personal communication.)

steps were first described by Robert Hill and Fay Bendall (1960) in a 'Z'-scheme. Figure 4 shows a current version; it includes the approximate times needed for the various steps in the scheme. It represents the steps in the pathway of electron transport from water to $NADP^+$ (nicotinamide adenine dinucleotide phosphate) leading to the release of oxygen, the 'reduction' of $NADP^+$ to NADPH (by the addition of two electrons and one proton), and the building-up of a high concentration of hydrogen ions inside the thylakoid (in the lumen; needed for ATP production). (For references and history, see Govindjee and Krogmann, 2004.) This scheme is called the Z-scheme simply because the diagram was initially drawn in the form of the letter 'Z' (Govindjee and Govindjee, 1975; Demeter and Govindjee, 1989). (The letter Z also represents the zigzag nature of the scheme.) Usually, however, it is drawn to emphasize the redox potentials (energy levels) of the electron carriers. Thus, it is turned 90 degrees counterclockwise. It, therefore, may be called the 'N'-scheme. For references and discussion of the various aspects of the Z-scheme, see Ke (2001) and Blankenship (2002).

The Z-scheme owes its origin to several investigators. First, it was Robert Emerson and his co-workers, at the University of Illinois (at Urbana-Champaign) who discovered the 'enhancement effect' in oxygen evolution, which occurred when light absorbed in one photosystem (now called PS I) was added to light absorbed in another photosystem (now called PS II) (Emerson et al., 1957). Experiments with chloroplasts, and those using a mass spectrometer, absorption spectrometer, a fluorometer and electron spin resonance spectrometer were crucial to the establishment of the 'two-light reaction and two-photosystem' concept (see Govindjee, 2000; Govindjee and Krogman, 2004 for references and the time line of discoveries in oxygenic photosynthesis). It was Bessel Kok and co-workers at Baltimore, Maryland, and Louis N.M. Duysens, Jan Amesz and co-workers in Leiden, The Netherlands, who discovered the crucial antagonistic effect of light absorbed in PS I and PS II on the oxidation-reduction state of the reaction center Chl, P700 (Kok, 1959), and of cytochrome *f* (Cyt *f*, the electron carrier in the middle of the intersystem chain of intermediates; Duysens et al., 1961). Duysens' experiments established the 'series' nature of the present scheme (Duysens, 1989). Light captured by PS I leads to oxidation of Cyt *f* (i.e., takes an electron away from it and places

it on, say, 'NADP$^+$'), whereas when light is captured by PS II, oxidized Cyt *f* is reduced by an electron coming from PS II. The theoretical concepts of Hill and Bendall (1960) and the work of Horst T. Witt et al. (1961; see Witt, 2004) in Berlin, Germany, played important and crucial roles in substantiating the 'Z-scheme.' Hints of such a scheme were available in the books by Eugene Rabinowitch (1945, 1951,1956). The final evidence of its validity came from state-of-the-art detailed biophysical, biochemical, molecular biology, and genetic research in about 20 laboratories around the world.

I describe below the basic steps as a background for the various chapters in this book (Fig. 4) The left side of the diagram shows an energy scale in terms of oxidation-reduction potential (E_m) at pH 7. (At pH 7, the standard hydrogen electrode has an E_m of –0.4 volts.) Intermediates that are higher up in the diagram have a lower (more negative) E_m and can add an electron to any intermediate below them. This occurs in electron transfer: from reduced pheophytin (Pheo$^-$) to P700$^+$ (middle of the diagram), from A_o^- (a special chlorophyll monomer) to NADP$^+$ (top right end of diagram), and from H_2O to the oxidized form of PS II reaction center Chl, P680$^+$ (lower left of diagram). Energy input is needed to transfer electrons from P680 to Pheo and from P700 to A_o, and this is where light energy is required.

1. The First Step

Photosynthesis starts with the excitation (see vertical arrows in Fig. 4) of special reaction center Chl *a* molecules (labeled as P680 in PS II; Zouni et al. (2001) and Ferreira et al. (2004) for its structure), and P700 in PS I; Jordan et al. (2001) and Ben-Shem et al. (2003) for its structure). The excitation energy comes either directly from absorbed photons but, most often, by excitation energy (also called exciton) transfer from adjacent pigment molecules in assemblies of pigment-protein complexes called antennas. (See Kühlbrandt et al. (1994) and Liu et al. (2004) for crystal structure of light-harvesting complex.) These 'antenna' pigment molecules (Chls and carotenoids) absorb photons and then transfer electronic excitation energy by a process called resonance excitation energy transfer from one molecule to the next, and finally to the reaction center (Chapter 4, Clegg).

The first chemical step happens within only a few picoseconds (10^{-12} s) when excited P680* transfers an electron to Pheo, producing oxidized P680 (P680$^+$) and reduced Pheo (Pheo$^-$) in PS II (Greenfield et al., 1997; for a historical account, see Seibert and Wasielewski, 2003), and excited P700* transfers an electron to A_o, producing oxidized P700 (P700$^+$) and reduced A_o (A_o^-) (Ke, 2002; Chapter 9, Itoh and Sugiura). These are the only steps where light energy is converted to chemical energy, precisely oxidation-reduction energy. The rest of the steps are *downhill* energy-wise, i.e. spontaneous or exergonic.

2. The Electron Transfer Steps

The recovery (reduction) of P680$^+$ to P680 and of P700$^+$ to P700 takes place in a time scale of several ns to μs. P700$^+$ receives an electron that was passed down from reduced Pheo to Q_A (which is bound to the reaction center II protein complex), then to Q_B (another bound plastoquinone molecule). Q_B, that has accepted two electrons from Q_A, takes on also two protons from the stroma, and then it detaches from its protein binding site and diffuses through the hydrophobic core of the thylakoid membrane to the cytochrome b_6f (Cyt b_6f) complex (see below), where the electrons are passed on to an iron-sulfur protein (FeS, the Rieske protein) and to Cyt *f*; the electron is then transferred to a mobile copper protein PC (plastocyanin) that shuttles between Cyt b_6f complex and the PS I complex; the reduced PC carries a single electron to the oxidized P700$^+$. Thus the electron is passed in a 'bucket fire brigade' manner through the 'intersystem chain of electron (or H-atom) carriers'.

The protein complex Cyt b_6f (see Kurisu et al., 2003, for its structure in *Mastigocladus laminosus*; and Stroebel et al., 2003, in *Chlamydomonas reinhardtii*) contains FeS, Cyt *f*, and two Cyt b_6 molecules. It is generally assumed that the 'bottleneck', or the slowest step of the entire sequence, is the passage of an electron from reduced Q_B (now in the form of plastoquinol, PQH_2) to the Cyt b_6f complex. This step involves not only diffusion of PQH_2, but the oxidation of PQH_2 by FeS, and the consequent release of two protons into the lumen. The combined event takes several ms (10^{-3} s). On the other hand, several other steps may compete to be the bottleneck. (Cyt b_6 plays a key role in the Q-cycle; see e.g., Crofts, 2004, for its history; and for details, see David Kramer, Thomas Averson, Atsuko Kanazawa, Jeffrey Cruz, Borisov Ivanov and Gerald Edwards (Chapter 10) In brief, the Q-cycle involves the following steps: One of the two electrons in PQH_2 goes toward Rieske FeS center,

whereas the other goes toward one of the two Cyt b_6 molecules (situated on the lumen side), and then to the other Cyt b_6 molecule (situated on the stromal side); this is followed by a second molecule of PQH_2 repeating the process. This process results in another PQ molecule (located on the stromal side) receiving two electrons; the doubly reduced PQ molecule then picks up two protons from the stromal side. It diffuses to the lumen side to oxidize the Cyt b_6f again. The end result is that for a net oxidation of one PQH_2 molecule four protons are released to the lumen side doubling the proton to electron transferred (to PS I) ratio.

In PS I, the electron on A_o^- is passed ultimately to $NADP^+$ via several intermediates: A_1, a phylloquinone (vitamin K); F_X, F_A, and F_B which are bound iron-sulfur proteins; ferredoxin, which is a somewhat mobile iron-sulfur protein; and the enzyme ferredoxin-NADP reductase (FNR) which is actually an oxido-reductase and whose active group is FAD (flavin adenine dinucleotide).

The missing electron on $P680^+$ is replaced, ultimately, from water molecules (see the left bottom of Fig. 4) via an amino acid tyrosine (a specific one in D1 protein of PS II, also referred to as Y_z in the literature) and a cluster of four mangenese (Mn) ions. (For the role of another tyrosine on D2 protein, see Rutherford et al., 2004.) These reactions also require a few ms. A minimum of eight quanta (photons) of light (four in PS II and four in PS I) are required to transfer four electrons from two molecules of water to two molecules of $NADP^+$. This produces two molecules of NADPH and one molecule of O_2. However, the measured minimum number of required photons is usually 10–12 per O_2 molecule (Emerson and Lewis, 1943); this is partly due to a possible cyclic reaction around PS I.

3. ATP Synthesis

The light reactions provide not only the reducing power in NADPH but also the energy for making and/or release of ATP (from its binding site), both essential for producing sugars from CO_2. ATP is produced through an enzyme called ATP synthase, from ADP (adenosine diphosphate), inorganic phosphate (P_i) and the proton motive force (pmf) across the thylakoid membrane. The pmf is composed of two components: an electrical potential and a proton gradient. The proton gradient comes from the storage of protons (hydrogen ions) inside the lumen, giving a pH of 6 inside the lumen and pH of 8 outside, in the stroma. Then, basically, protons escaping from the thylakoid lumen through a central core of the enzyme ATP synthase (embedded in the membrane) cause conformational (rotational) changes in the enzyme, which catalyzes the phosphorylation of ADP and the release of ATP on the stromal side. (For historical discussions, see Jagendorf, 2002; and Junge, 2004; and for further information, see Chapter 10, Kramer et al.

To recapitulate, protons are concentrated into the lumen in several ways: Oxidation of water not only releases O_2 and 'sends' electrons to $P680^+$, but it also releases protons (H^+) into the lumen. When Q_B is reduced in PS II, it not only receives two electrons from Q_A but it also picks up two protons from the stroma matrix and becomes PQH_2. It is able to 'carry' both electrons and protons and thus it is a H-atom carrier. At the Cyt b_6f complex, it is then oxidized, but FeS and Cyt b_6 can only accept electrons (not protons). So the two protons are released into the lumen. The Q-cycle of the Cyt *bf* complex provides *extra* protons into the lumen. As discussed above, two electrons travel through the two hemes of Cyt b_6 and then reduce PQ on the stroma side of the membrane. The reduced PQ takes on two protons from the stroma, becoming PQH_2, which migrates to the lumen side of the Cyt b_6f complex where it is again oxidized, releasing two more protons into the lumen. Thus the Q-cycle allows the formation of *more* ATP. When $NADP^+$ is reduced by two electrons, it also picks up one proton, in effect removing it from the stroma and further increasing the gradient across the membrane.

B. The 1952 Observations of L. N. M. Duysens: Active and Inactive Chlorophylls

Duysens (1952), based on his own studies on Chl *a* fluorescence excited by phycoerythrin, phycocyanin and Chl *a* and those of French and Young (1952), concluded that in cyanobacteria and red algae, there are two forms of Chl *a*: (1) Chl *a* that is 'active' in photosynthesis and receives efficient excitation energy transfer from the phycobilins, and is fluorescent; and (2) Chl *a* that is 'inactive' in photosynthesis and is either non-fluorescent or weakly fluorescent. As became known much later, it was this 'inactive' Chl *a* that turned out to be the Chl *a* of PS I!

C. Photosystem II and Photosystem I Fluorescence: Background

Different spectral forms of Chl *a* (see French, 1971) are present in different pigment-protein complexes of both PS I and PS II. Most of the Chl *a* fluorescence (approx. 90%) at room temperature originates in PS II complexes, PS I complexes being weakly fluorescent. Further, it is only PS II fluorescence that varies with changes in photochemistry, i.e. the variable Chl fluorescence belongs strictly to PS II. Why is PS I weakly fluorescent, and why there is no variable fluorescence in it are important questions that have not been systematically dealt with yet. Among several more, the following hypothesis can be made (see Govindjee, 1995): (a) The reaction center Chl *a* of PS I, the P700, is a deeper energy trap than the reaction center Chl of PS II, the P680, and, thus, PS I photochemistry may not be 'trap-limited,' i.e. energy trapping in P700 is more irreversible than in P680. As a result, the antenna fluorescence of PS I does not compete with PS I chemistry. (b) The physico-chemical nature of antenna Chl *a* of PS I, that absorb, on the average, at longer wavelength of light is such that k_h predominates over k_f. We know that the lifetime of PS I Chl *a* fluorescence is shorter than that of PS II Chl *a* fluorescence, i.e. in PS I, excitation energy is trapped faster than in PS II (Holzwarth, 1991; also see Gilmore et al., 2000). Of course, this means a low quantum yield of Chl *a* fluorescence in PS I, as $\phi_f = \tau/\tau_o$. One of the most interesting suggestions about the weaker fluorescence in PS I has been provided by Borisov (2000): (1) a 'new' (sub-ps) state of PS I RC, that precedes primary charge separation, exists; (2) this state forms 5–10 times faster than the charge separation, and, thus, the yield of fluorescence and other losses decrease 5–10 fold; and (3) dielectric relaxation of hydrogen atoms in nearby water molecules prevents the excitation to return to the antenna Chls, and, thus lowering fluorescence. PS II is different because the formation rate of the 'new' state in PS II is close to that of the primary charge separation, leading to a back flow of excitation and to higher fluorescence (also see Itoh and Sugiura (Chapter 9) for further discussions).

D. The Two-Light Effect in Fluorescence

The concept of two light reactions through Chl *a* fluorescence studies was first considered by Hans Kautsky and U. Franck (1943). They attributed the observed rise and fall of fluorescence to two light reactions succeeding one another almost immediately, one responsible for the rise and the other for the fall. E. C. Wassink (1951), however, pointed out that the quenching of fluorescence might have been caused by a side reaction. Kautsky et al. (1960), based on newer experiments on Chl fluorescence in vivo, reiterated the suggestion that two consecutive light reactions worked in photosynthesis.

Kautsky et al. (1960) discussed the concept that the oxidized state of a compound, A, a member of the electron transport chain, determined the quenching of fluorescence: when A was oxidized, Chl fluorescence was quenched, but when A was reduced, it was not. During the Chl fluorescence transient, the rise was due to the reduction of A, whereas the successive decline was due to its oxidation by the next member of the chain, B — the latter was formed from the reduced B by another light reaction. The absence of fluorescence decline when the inhibitor phenylurethane was present was explained to be due to a block of reoxidation of reduced A. In their model, A was closer to the O_2-evolving process, and B to the CO_2-fixation reactions; it now seems that A could be equated to Q_A (see section II.E). Although the above model is quite revealing, it lacked impact because, as stated by Govindjee (1995): (a) it ignored the existence of the two-pigment-system concept already evolved from the work of Emerson et al.(1957); (b) it was not the correct explanation of the observed fluorescence decline; and (c) it was published in a journal that many scientists may not have read. As noted above, Hill and Bendall (1960) had proposed a scheme of two light reactions that included a step for providing energy for ATP synthesis during a downhill process between the two light reactions (see Duysens, 1989, for the historical perspective of the discovery of the two-light reaction scheme). For further discussions on history, see Wild and Ball (1997), Govindjee (2000) and Govindjee and Krogmann (2004).

Govindjee et al. (1960) discovered the two-light effect in Chl fluorescence in *Chlorella* cells: far-red light (absorbed in the long-wavelength pigment system, later known as PS I, Duysens et al., 1961) quenched the high Chl *a* fluorescence (excited by blue or 670 nm light, the short-wavelength pigment system, PS II) in *Chlorella* cells. This antagonistic effect of light I and II on Chl *a* fluorescence yield was considered fluorescence evidence for the two-light-reaction two-pigment-system concept of photosynthesis. Butler (1962) demonstrated, in a

more impressive manner, the same phenomenon in anaerobic leaf with red (650 nm, PS II) and far-red (720 nm, PS I) light.

E. Introduction of 'Q' (Q_A), the 'Quencher of Chlorophyll Fluorescence'

Duysens and Sweers (1963) provided the current explanation of the experiments discussed above: light II, absorbed in PS II, reduces a quencher of Chl *a* fluorescence, labeled as Q, and light I, absorbed predominantly by PS I, oxidizes Q^- back to Q. The herbicide DCMU blocks the reoxidation of Q^-, but not the reduction of Q. Today, Q is known as Q_A and was shown to be a PQ molecule (van Gorkom et al., 1978). The antagonistic effect of light I and II on Chl *a* fluorescence yield is a useful tool to investigate the site of an inhibitor between Q_A and P700, the reaction center Chl *a* of PS I, as shown, e.g. for bicarbonate-reversible formate inhibition (Govindjee et al., 1993a).

F. Separation of Photosystem II and Photosystem I Fluorescence

1. Physical Separation

Boardman and Anderson (1964), using the detergent digitonin, physically separated suspensions of thylakoid membranes in two fractions: a heavier fraction, that was enriched in PS II activity, and a lighter fraction, that was enriched in PS I activity. This was followed by observations of fluorescence characteristics of these two fractions by Boardman et al. (1966) and Cederstrand and Govindjee (1966). Compared to the PS II-enriched samples, the PS I-enriched samples had a higher ratio of F735 to F696 (at 77 K); and the 696 nm band was present mostly in PS II-enriched fractions. (Figure 5 shows the emission bands in unfractionated thylakoids.) At room temperature, the PS I-enriched fraction, whose peak absorption was at a longer wavelength than the peak absorption of the PS II-enriched fraction, had also a higher degree of polarization of Chl *a* fluorescence.

2. Fluorescence of the Pigment Systems

We focus here on *emission spectra* as they are what characterize Chls from other photosynthetic pigments. Although Chl *a* fluorescence is heterogeneous at room temperature because of the existence of two photosystems (PS I and PS II)), the major fluorescence band at 683–685 nm and its vibrational satellite at 720–735 nm originate mostly in the PS II antenna complexes (Fig. 5A). I am unable to state the exact proportion of fluorescence that comes from each of the PS II pigment protein complexes. I suggest that most of the variable Chl *a* fluorescence originates in the CP 43 and the CP 47 Chl *a* protein complexes, with CP 47 being responsible for a weak 693–695

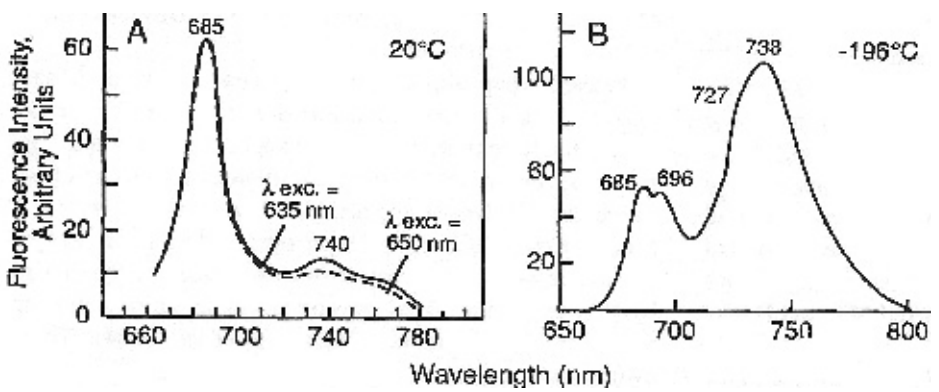


Fig. 5. Emission spectra of spinach thylakoids. (A) Room temperature emission spectra. Chl *a* fluorescence as excited by 635 nm (having slightly more light going to PS I than to PS II) and by 650 nm (having slightly more light going to PS II than to PS I). (B) Low temperature (77K, labeled as –196 °C) emission spectrum excited by 635 nm light. At room temperature, fluorescence band at 685 nm originates in PS II antenna; and a very small amount in the 710–760 nm region from PS I antenna. At 77 K, the emission bands at 685 and 696 nm are suggested to originate mostly from PS II antenna, and those around 727 nm and 738 nm mostly from PS I antenna. (Data of Govindjee and Yang, 1966; redrawn from Govindjee, 1995) (For further discussion on emission bands, see Papageorgiou (Chapter 2), Mimuro (Chapter 7), Itoh and Sugiura (Chapter 9), and Van Grondelle and Gobets (Chapter 5).)

nm emission when PS II reaction centers are closed either by strong light or by the addition of DCMU that blocks electron flow. The existence of the weak 693–695 nm emission at room temperature was shown by Krey and Govindjee (1964, 1966), Papageorgiou and Govindjee (1967, 1968a,b) and Govindjee and Briantais (1972). On the other hand, a PS I emission, that may be from an 'ordered' set of Chl *a* molecules, is centered around 705–715 nm (Lavorel 1963; Wong and Govindjee 1979; Goedheer 1981). (For further discussion of additional or different PS I fluorescence band(s), see Chapter 9, Itoh and Sugiura.)

At 77K, however, Chl *a* in vivo produces, at least, four emission bands: F685, F695, F720 and F740 in addition to the long wavelength shoulders due to the various vibrational satellite bands (Fig. 5B). Brody (1958) discovered that cooling the cells of green alga *Chlorella* to 77K leads to the formation of a new broad emission band at about 725 nm. It was shown by Govindjee and Yang (1966) and Cho and Govindjee (1970a) to be composed of, at least, two bands. Mar et al. (1972) showed that it could also be distinguished from F685 as it had a longer lifetime of fluorescence. Although Litvin and Krasnovsky (1958) had observed the existence of a band at 695 nm in etiolated leaves (originating in a Chl precursor), it was in 1963 that the existence of F695 was discovered and recognized to originate in PS II (Bergeron, 1963; S. S. Brody and M. Brody, 1963; Govindjee, 1963; Kok, 1963). Although it was recognized independently in three laboratories that F685 and F695 belong to PS II and F720 and F740 to PS I (Boardman et al., 1966; Cederstrand and Govindjee, 1966; Govindjee and Yang, 1966; Murata et al., 1966a), earlier assignments to particular protein complexes were in error. Contrary to earlier beliefs, F685 cannot belong simply to light-harvesting complex IIb (LHCIIb) since it is present in LHCIIb-lacking organisms (e.g., *Gonyaulux polyedra*, see Govindjee et al., 1979). (For a discussion of the assembly of LHCIIb, see Chapter 27, Hoober and Akoyunoglou.) Although their complete assignment is still not fully established, most of F685 and F695 belong to Chl *a* in core PS II complexes (Gasanov et al., 1979; Rijgersberg et al., 1979), and F720 and F740 to PS I reaction center I, containing intrinsic antenna Chls, and light harvesting complex I (LHCI), respectively (Mullet et al., 1980a,b). Nakatani et al. (1984) correctly assigned F685 to originate in CP43 Chl *a* and F695 to Chl *a* in CP47. The F720 band originates in a Chl *a* complex absorbing at 695 nm (Das and Govindjee, 1967) and F740 in a Chl *a* complex absorbing at 705 nm (Butler 1961). On the other hand, a band at 680 nm (F680) appears at 4K only when LHCIIb is present (Rijgersberg et al., 1979). Thus, F680 belongs to Chl *a* from LHCIIb; it cannot be normally observed due to highly efficient transfer from it to other complexes. In addition, Shubin et al. (1991) have observed a new emission band in a cyanobacterium *Spirulina platensis* at 758 nm (F758), at 77K, which originates in a Chl complex with an absorption band at 735 nm (Chl_{735}^{758}). Interestingly, this complex transfers its excitation energy to the oxidized form of the reaction center of PS I, $P700^+$, and thus, quenching of F758 is observed during the photo-oxidation of P700.

For the spectral properties and the biological significance of dimeric and trimeric Chl *a* in PS I that absorb light at longer wavelengths ('red Chl *a*') than P700, the primary electron donor of PS I, see van Grondelle and Gobets (Chapter 5) and Itoh and Sugiura (Chapter 9).

III. Photosynthetic Unit and Excitation Energy Transfer

A. Photosynthetic Unit

Hans Gaffron and K. Wohl (1936a,b) interpreted the results of Emerson and Arnold (1932a, 1932b) on O_2 evolution, in brief saturating repetitive light flashes, as follows. A collection of 2400 Chl molecules somehow cooperates to evolve, with high quantum efficiency, one molecule of O_2: light energy, absorbed anywhere in this *unit*, the *photosynthetic unit*, migrates by 'radiationless excitation energy transfer' to the *photoenzyme* where several excitons (in today's language) cooperate to initiate photosynthesis. For a glimpse of one of the classical papers on energy transfer by Förster (1946), see the box on the next page. (For a detailed discussion of excitons and their fate in photosynthesis, see van Amerongen et al., 2000.) This is in contrast to diffusible chemicals being formed at each site, and then diffusing to the *photoenzyme*. This concept of a photosynthetic unit composed of many pigments serving a photoenzyme has been conceptually supported by the discovery of excitation energy transfer and of the reaction center Chls labeled as P700 (Kok, 1956) and P680 (Döring et al., 1967) and the many pigment-protein complexes that contain only antenna or bulk pigments.

G. Wilse Robinson (1967) coined the terms *lake*

Margitta and Robert Clegg (Department of Physics, University of Illinois at Urbana, Illinois, Urbana, IL 61801, USA) provided me with the following translation of the beginning paragraphs of the classical paper by Theodor Förster (1946) Energiewanderung und Fluorescenz, Die Naturwissenschaften 33 (6): 166–175. Robert Clegg wrote, 'This is a jewel of a paper—too bad that most people have not read it, and usually people do not know of its existence. It is almost never referenced. But it came before all the other articles that Förster published on the topic, and has essentially the major parts of his Fluorescence Resonance Energy Theory (FRET). Förster already remarked in this paper that this process was important for photosynthesis. This paper was written right after the World War II, interestingly from his home (Niedernjesa, Kr. Göttingen, a village in the area of Göttingen. Germany).'

'Recently, the so-called process of energy transfer has been discussed in connection with photobiological events (26). It seems that for various biological systems a quantum of energy that has been absorbed by a particular molecule does not evoke a change at the particular molecular location where the quantum has been absorbed, but the chemical change transpires with a second molecule that is spatially removed from the initially absorbing molecule. Thereby the energy is transferred over distances that are large relative to the contact distances between adjacent molecules, and the energy transfer process extends beyond the influence of chemical valence and other chemical intermolecular interactions.

Such a process of energy transfer provides a rationalization for certain observations of carbon dioxide assimilation in plants. According to measurements of EMERSON and ARNOLD (6) on algae Chlorella, short-term high intensity light pulses (sparks) bring about a saturation phenomenon whereby a certain threshold of carbon dioxide assimilation cannot be exceeded, no matter how intense the light pulse is. This saturation level is attained when between 2 and 4 light quanta, which are required for the reduction of one carbon dioxide molecule, are absorbed per 1000 chlorophyll molecules. GAFFRON and WOHL (10, 45) conclude from this result that this number of molecules act collectively to accomplish the reduction of one carbon dioxide molecule. Since it must be assumed that this chemical reduction process takes place at distinct localities, this interpretation requires that the energy is propagated from the location of individual absorbing chlorophyll molecules to the location where the reduction takes place. This interpretation also explains the saturation level of assimilation for continuous light illumination, as well as the lack of an induction period that would be necessary if a single chlorophyll molecule were obliged to gather the multiple photons required for reducing one carbon dioxide molecule. All these processes involving carbon dioxide assimilation are accounted for by the assumption of distinct localities for carbon dioxide assimilation, and they all give similar quantitative estimates of participating molecules.' …

'In order to understand these [energy transfer] processes, it is prudent to observe similar processes with non-biological material.' …

'Fluorescence processes with solutions of dye molecules have been known for a longer time that can be interpreted in terms of such energy transfer models.' …

[References cited above were: (6) Emerson R and Arnold WA (1932) J Gen Physiol 15: 391–420; J Gen Physiol 16:191–205; (10) Gaffron H and Wohl K (1936) Naturwiss 24: 81–90; Naturwiss 24: 103-107; (26) Möglish von F, Rompe R and Timoféeff-Ressvosky NW (1942) Naturwiss 30: 409–419 (45) Wohl K (1937) Z Physikal Chem 37: 105–121]

I changed the font from Berhart Light Italic to Warnock Light Italic. If you have something else in mind you have to let me know.

versus *puddles* for the organization of antenna and reaction center chromophores. In the lake model, also called the statistical or the matrix model, the exciton may freely visit all reaction centers. In contrast, in the isolated puddles, the separated units, or the restricted model, the exciton can visit only its own reaction center. However, the situation is 'in-between', i.e. there is some probability of energy exchange between the different puddles. Looking at the existence of various pigment-protein complexes, it is quite likely that a 'pebble-mosaic' model (Sauer, 1975) may be the real picture. (For further literature citations and discussion, see Kramer et al.,2004.) It still remains a challenge to provide a complete mathematical and physical model for exciton migration in oxygenic photosynthesis.

Whether there is a directed or a random exciton migration must depend on many factors including the relative energy levels of the donors and the acceptors. The directed model (the funnel model) seems to be appropriate for heterogeneous energy transfer in phycobilisomes, or even when one deals with transfer from short wavelength to long wavelength forms of Chl *a* (Govindjee et al., 1967; Seely, 1973). However, a random migration is more appropriate for homogeneous energy transfer among isoenergetic pigment molecules (see discussion in Pearlstein, 1982).

Butler and Strasser (1977), Strasser and Butler (1977, 1978) and Strasser (1978) have discussed various *bipartite* or *tripartite* and *grouping* models of organization of pigments. These concepts have been extensively used in the literature, and discussed at length in Strasser et al. (Chapter 12).

B. Excitation Energy Transfer and Migration

A detailed and mechanistic picture of excitation energy (exciton) transfer is only possible when the distances and orientations of the chromophores are known accurately. A major breakthrough in this direction has been the visualization of the structure of major light-harvesting complex of higher plants (LHCII) on the basis of electron diffraction (Kühlbrandt et al., 1994), and X-ray crystallography studies (Liu et al., 2004). The derived models show the detailed arrangement of individual Chl *a* and Chl *b* molecules, and their orientations and distances. From Förster's resonance theory (Förster, 1946, 1948), one can calculate excitation energy transfer from one molecule to another—the rate of this transfer is dependent upon three crucial parameters: (a) $1/R^6$, where R is the distance between the donor and the acceptor molecules; (b) $(\kappa)^2$, where κ (orientation factor) = $\cos\alpha - 3\cos\beta_1 \cos\beta_2$; here, α is the angle between the dipoles of the acceptor and donor molecules, whereas β_1 (or β_2) is the angle that the line that joins the two dipoles (the vector) makes with the dipole of the donor (or the acceptor); and (c) the overlap of energy levels, as calculated by the overlap integral between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor molecule (For a complete description and methods, see van Grondelle and Amesz, 1986; Clegg (Chapter 4), and Yang et al.,2003.)

In Förster's theory, excitation energy transfer from a donor (at a higher energy state) to an acceptor (usually at a lower energy state) occurs after the excited donor molecule looses some energy as 'heat'. The overlap integral between donor and acceptor molecules is temperature dependent. Thus, temperature dependence of energy transfer had been predicted. Cho et al. (1966) and Cho and Govindjee (1970a) observed changes in emission spectra of *Chlorella* cells as they decreased temperatures down to 4K; similar changes were observed in cyanobacterium *Anacystis* (Cho and Govindjee, 1970b). Although other interpretations are possible, these results were taken to support the Förster theory for energy transfer from phycobilins to Chl *a* and for transfer from Chl *a* fluorescing at 685 nm to that fluorescing at 695 nm (see a review in Govindjee, 1999).

Excitation energy migration (homogeneous energy transfer) studies among Chl *a* molecules were pioneered by William Arnold and E. S. Meek (1956) through the observation of depolarization of Chl fluorescence. Similar investigations were later pursued in my laboratory by Ted Mar and Daniel Wong (Mar and Govindjee, 1972; Wong and Govindjee, 1981) and by Whitmarsh and Levine (1974). A decrease in the polarization of Chl fluorescence by closure of PS II reaction centers was taken as evidence of increased energy migration. However, due to a lack of detailed knowledge of the orientation of dipoles, and due to a possible lack of coherence of excitons even after one or two transfers, conclusions from such studies have been rather limited, and extraction of quantitative information about energy migration rather difficult (Knox, 1975).

The existence of excitation energy transfer (heterogeneous energy transfer), however, has been convincingly shown by the technique of steady-state sensitized fluorescence, from fucoxanthol to Chl *a*

(Dutton et al., 1943; see Dutton (1997) for a historical article), from phycobilins to Chl *a* (Duysens, 1952; French and Young, 1952) and from Chl *b* to Chl *a* (Duysens 1952). Excitation in the absorption band of the donor molecule shows a quenching of the donor fluorescence and a stimulation of the acceptor fluorescence.

Müller (1874) had already commented on the lower fluorescence intensity of leaves over that in solution, implying the use of the absorbed energy in a leaf for photosynthesis. This concept was emphasized when it was noted that the quantum yield of Chl *a* fluorescence in vivo is 0·03–0·06 in contrast to 0·25–0·30 in vitro (Latimer et al., 1956)—the majority of the absorbed photons in vivo must be used in photosynthesis Since the quantum yield of fluorescence (ϕ_f) is directly proportional to the lifetime of fluorescence (τ), and since the latter can also provide unique information on the primary photochemical events of photosynthesis, a major advancement was made when Brody and Rabinowitch (1957) and Dmetrievsky et al. (1957), independently, and by independent methods (direct flash and phase shift), measured the lifetime of Chl *a* fluorescence in vivo. Even in the very first paper, Brody and Rabinowitch (1957) showed that there was a delay in observing Chl *a* fluorescence when phycoerythrin was excited, showing that energy transfer takes a finite time when it moves from phycobilins to Chl *a*. (See Brody, 2002 for a historical perspective.) Tomita and Rabinowitch (1962) calculated this time to be about 300 ps and the efficiency of the energy transfer to be 80–90%. The time of energy transfer from Chl *b* to Chl *a* was too fast to be resolved, but the efficiency of transfer was confirmed to be 100%, as found earlier by Duysens (1952) in steady-state measurements.

Indeed, when ultrashort (femtoseconds to picoseconds) flashes of light are used to excite donor molecules, one can measure precise times for the transfer of excitation energy from the donor to the acceptor molecule: as the donor fluorescence subsides, the acceptor fluorescence appears. A beautiful cascade has been observed in the red algae where one can follow precisely the excitation energy transfer by this technique, from phycoerythrin to phycocyanin to allophycocyanin (Yamazaki et al., 1984; also see Mimuro, 2002, for a historical article). These events occur in picosecond time scale (see Fig. 6). For a current discussion of excitation energy transfer, see chapters by Clegg (Chapter 4), van Grondelle and Gobets (Chapter 5), Mimuro (Chapter 7) and Itoh and Suguira (Chapter 9).

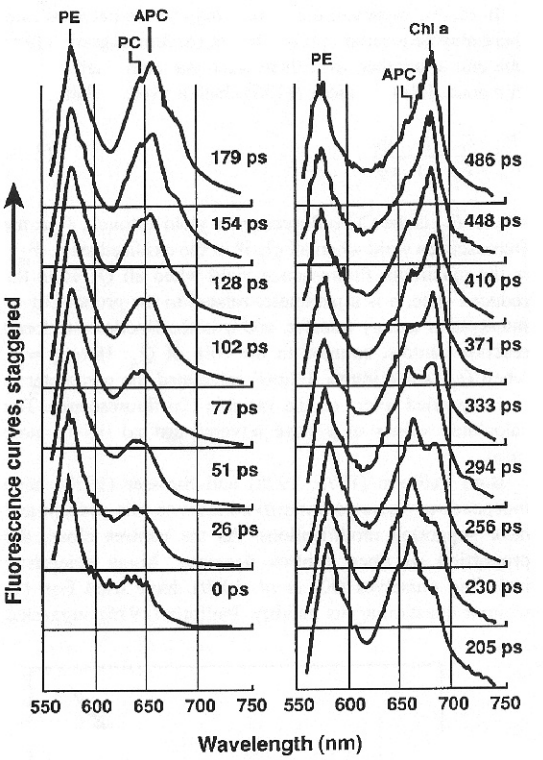


Fig. 6. Excitation energy transfer in the red alga *Porphyridium cruentum* as deduced from time (0 to 486 ps) -dependent emission spectra. PE refers to phycoerythrin; PC to phycocyanin; APC to allophycocyanin; and Chl *a* for chlorophyll *a*. Excitation was with a 6 ps 540 nm flash, absorbed mostly in PE. PE fluorescence is at ~575 nm. By about 100 ps, PC and APC fluorescence bands are clearly observed, and Chl fluorescence overtakes them as time progresses from 179 ps to 486 ps. These experiments clearly show that the path of excitation energy transfer is PE to PC to APC and then to Chl *a* (Data of Yamazaki et al., 1984; reproduced from Govindjee, 1995). (For further information, see Chapter 7, Mimuro.)

C. Chlorophylls in Crystal Structures of Light-harvesting Chlorophyll Complex, Photosystem II, Photosystem I and in Cytochrome b_6f complex

We have come a long way since the discovery of Chl fluorescence in vitro and in pigment protein complexes. Distances and orientations of specific Chl molecules are known; thus, we can begin to think about the mechanisms of energy transfer. For example, most

Chl *a* molecules in LHC I are separated by more than 18 Å from the Chl molecules of the nearest reaction center (Ben-Shem et al., 2003). However, there are three contact regions where the distances are reduced to 10–15 Å. Due to the $1/R^6$ dependence of energy transfer, they must play a significant role in increasing the rate of energy transfer to the reaction center. On the other hand, specific Chl molecules have been identified in CP-47 (one of the inner antennae of PS II) that form a stack in the middle of the protein leading to the suggestion that they may aid in fast energy transfer processes (Ferreira et al., 2004). Liu et al. (2004) have gone a step further in their discussion of the arrangement of Chls in LHCII: they suggest that a specific Chl *a* numbered 612 may be the putative terminal fluorescence emitter. The most intriguing observation is the existence of a single Chl *a* molecule in Cyt b_6f complex (Kurisu et al., 2003; Stroebel et al., 2003). It is located between subunits F and G of the subunit IV, with its 20-carbon phytyl chain threading through the p-side redox chamber into the central cavity; unfortunately, the bound 9-cis β-carotene is too far (at least 14 Å) to quench the Chl triplet! It may be just a 'filler' of space. Perhaps, it is simply a vestige of evolution (Xiong et al., 2000). I wonder if its fluorescence can be used to probe the function and the reactions in the complex.

IV. The Fluorescence Transient

Figure 7 shows a characteristic fluorescence transient in a pea leaf. A dark-adapted leaf (or a chloroplast suspension from higher plants, algal or cyanobacterial cells) shows characteristic changes in Chl *a* fluorescence intensity with time when illuminated with continuous light. These changes have been called fluorescence induction, fluorescence transient or simply the Kautsky effect. They are classified as *fast* (up to 1 s; labeled as OJIP; see section IV.B) and as *slow* (up to several minutes; labeled as PSMT; see footnote [3]) changes. During the O to P phase, fluorescence rises and during the P to T decline fluorescence declines to a steady state. These transients have been the subject of a vast number of studies and continue to be used as qualitative and even quantitative probes of photosynthesis. The fast changes have been a bit easier to interpret than the slower changes. For further details, see Schreiber (Chapter 11) and Strasser et al. (Chapter 12, this volume).

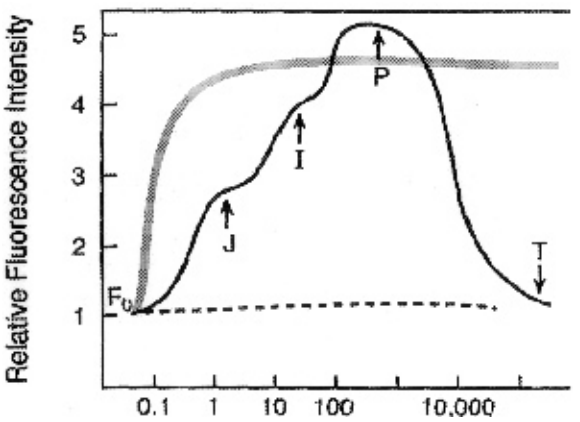


Fig. 7. The O-J-I-P Chl *a* transient (solid line) from pea leaf (Strasser and Govindjee, 1992; reproduced from Govindjee, 1995). The light grey curve is an idealized curve for the transient in the presence of DCMU (3-(3,4)-1, 1′ dichlorophenyl dimethyl urea). Excitation, 650 nm; ~ 2,000 μmol photons m^{-2} s^{-1} (For further information, see Schreiber (Chapter 11) and Strasser et al.(Chapter 12).)

A. Some Correlations

1. Complementarity

Kautsky and Hirsch (1931) had already mentioned the antiparallel (complementary) relation between Chl *a* fluorescence and photosynthesis. Complementarity was quantitatively established by MacAlister and Myers (1940) during the *DPS*[3] transient. Delosme et al. (1959) confirmed it but they showed also that during the *OI* phase photosynthetic O_2 evolution and Chl *a* fluorescence increase in parallel. Thus, the *OID* phase of the fluorescence transient is actually an 'activation' phase before O_2evolution begins. Papageorgiou and Govindjee (1968a,b) and Mohanty et al. (1971a) showed the parallel increase in fluorescence during a later phase, the SM[3] phase, and constancy of O_2 evolution during the MT[3] decline. Thus, it is clear that the antiparallel relation between fluorescence and photosynthesis is observed only under certain experimental conditions, namely when $k_p + k_f =$

[3]The terms 'O', 'I', 'D', 'P', 'S', 'M' and 'T', in the chlorophyll fluorescence transient, refer to the initial fluorescence levels, the 'origin' (O), the 'intermediate' (I), 'dip' (D), 'peak' (P), 'semi steady state' (S), 'a maximum' (M), and a 'terminal steady state' T' (see Lavorel, 1959; Bannister and Rice, 1968; Munday and Govindjee, 1969a,b; Govindjee and Papageorgiou, 1971; Papageorgiou,1975a; Yamagishi et al., 1978; and Govindjee, 1995). Quite often 'M' is just a shoulder and one refers to 'T' simply as 'S' (for steady state).

constant and k_o = constant (see Eq. (2)). When these conditions are not satisfied, the antiparallel relation between Chl *a* fluorescence and photosynthetic O_2 evolution breaks down. Kautsky and Hirsch (1931) have mentioned that it took a long dark time to restore the transient if the light was turned off after a long period of illumination. Duysens and Sweers (1963) showed that the OPS transient was not restored if light was turned off at the 'S' level and turned back on immediately. The hypothesis of Q (now called Q_A) was that Chl fluorescence increased when Q_A was reduced and decreased when Q_A^- was oxidized. If this was the *only* factor controlling OPS transient, the transient should have been restored right away. Mohanty and Govindjee (1974) and Briantais et al. (1986) discussed the dual nature of this phase extensively: one related to Q_A and the other to some 'high energy state.' Papageorgiou and Govindjee (1971) showed a relationship of Chl fluorescence to the suspension pH, whereas Briantais et al. (1979) showed a relation of proton gradient changes with the P to S decay. In terms of Eq. (8), this implies that another rate constant (perhaps, k_h), besides k_p, is affected by pH changes.

2. Plastoquinone Pool Size

The *O (I D) P* rise is mostly due to the decrease in the concentration of Q_A and, thus, to the accumulation of Q_A^-. The area over the fast phase of Chl *a* fluorescence transient (OIDP) measures the size of the electron acceptor pool of PS II, the plastoquinone (PQ) pool size, provided, e.g. the same area can be measured, under similar experimental conditions, when the PQ pool cannot be reduced, and only Q_A can be reduced; this condition is obtained when an inhibitor, such as DCMU, is added to the sample. The earliest calculations of the acceptor pool were made by Malkin and Kok (1966) and by Murata et al. (1966b). The area that is bound by the Chl *a* fluorescence transient measured in the presence of DCMU and the asymptotes that are parallel to the time axis (abscissa) and the fluorescence intensity axis (ordinate) corresponds to one electron equivalent (on Q_A^-). The same area without DCMU corresponds to the total number of electron equivalents of the electron acceptor pool downstream of PS II. Such experiments have, in general, provided estimates of 9–10 PQ molecules for the PQ pool size. However, see Trissl et al. (1993) and Trissl and Lavergne (1995) for a discussion of potential problems. Further, Vredenberg (Chapter 6) challenges these interpretations in light of his 'three-state' hypothesis, which considers the PS II reaction centers fully closed only when both pheophytin and Q_A are reduced. Further research is needed to make estimates of PQ pool more precise.

3. Sites of Inhibition

A simple and effective use of the Chl *a* fluorescence transient is for identifying lesions, caused by mutations or inhibitors either on the electron donor side of PS II, or on the electron acceptor side. A block in the electron flow beyond PS II, e.g. after the electron acceptor Q_A (Duysens and Sweers 1963), causes a faster fluorescence rise (OP) to a high steady level. In the course of such experiments, Vernotte et al. (1979) discovered that Chl fluorescence was often about 10–20% higher when the PQ pool was fully reduced (saturating light, no DCMU). (Also see discussions by Kramer et al.(Chapter 10), Schreiber (Chapter 11) and Falkowski et al. (Chapter 30).) This was interpreted as a direct quenching of Chl fluorescence by the oxidized PQ pool. If, however, the block is in the electron flow on the donor side of PS II, e.g. between H_20 and P680 (the reaction center Chl *a* of PS II), a slower Chl *a* fluorescence rise occurs and the fluorescence remains low. This condition is, however, restored to normal if the cause of the block is removed (Mohanty et al., 1971b; Critchley et al., 1982; Metz et al., 1989). (For fluorescence induction measurements with repetitive light pulses, see Bruce and Vasiel'ev (Chapter 19) and Falkowski et al.(Chapter 30).)

B. The Fast Transient of Chlorophyll a Fluorescence(OJIP)

When a dark-adapted photosynthetic organism is exposed to light, Chl fluorescence rises from a low level (F_o) to a high level (F_p), as discussed in Sections I.B and IV.A. This is the fast phase of the fluorescence induction or transient, and reflects PS II activity. Most of the literature on fluorescence transient had used the term OIDP for the fast fluorescence transient, and it had been tacitly assumed that the OI phase, measured during transients by all investigators (Munday and Govindjee, 1969a,b), is equivalent to the photochemical phase OI, recorded at high intensity excitation, and with fast measuring instruments, where a gun was used to open the shutter rapidly (Morin, 1964; Delosme, 1967). However, using a Walz LED

fluorometer, originally developed by Schreiber et al. (1986), and at extremely high intensity excitation light, Neubauer and Schreiber (1987) and Schreiber and Neubauer (1987) discovered that the OIDP should be represented as OI_1 (D_1) I_2 (D_2) P transients since there were two, instead of one, inflection(s) between O and P. Using a commercial Hansatech LED instrument PEA (Plant Efficiency Analyzer), Strasser and Govindjee (1991, 1992) observed two inflections between O and P, and labeled them as J and I, not I and J, or I_1 and I_2. (See Fig. 7 for a OJIP transient curve; also shown is the transient curve with DCMU addition.) Measurements of Strasser et al. (1995) on the intensity dependence of the quantum yield of fluorescence at O, J, I and P revealed that the J is equivalent to I of Delosme (1967). Further, J and I are equivalent to I_1 and I_2 of Neubauer and Schreiber (1987) (see Strasser et al., 1995). (For further details, see Schreiber (Chapter 11) and Strasser et al.(Chapter 12).)

The current understanding of OJIP transient rise is that it reflects, in the first approximation, the successive reduction of the electron acceptor pool of PS II (Q_A, the one-electron acceptor-bound PQ, Q_B, the two-electron acceptor-bound PQ, and the mobile PQ molecules). The hypothesis of Duysens and Sweers (1963) that Q_A is the determining factor governing the increase in Chl *a* fluorescence is implicitly accepted by most researchers (see, however, Chapter 6, Vredenberg). The inflections represent the heterogeneity of the process. The OJ rise is the photochemical phase, the inflection J represents the momentary maximum of Q_A^-, $Q_A^- Q_B$ and $Q_A^- Q_B^-$; 'I' may reflect the concentration of $Q_A^- Q_B^{2-}$ and P may reflect the peak concentration of Q_A^-, Q_B^{2-} and PQH_2 (Stirbet et al., 1998; X-G. Zhu, Govindjee and Steve Long, personal communication). The OJIP transient can be used as a quick monitor of the electron acceptor side reactions, the pool heterogeneity and the pool sizes, and the effects of inhibitors and mutations on these processes, as well as on the donor side. Hsu (1993) has confirmed the earlier conclusion from the P. Joliot-R. Delosme laboratory that the fast fluorescence rise is influenced by the S-states of the oxygen evolving complex (OEC). At this moment, we may not be able to easily obtain any quantitative information on the individual rate constants since the secondary reactions of both PS I and PS II are slow compared with the single-turnover of the PS II reaction centre leading to the overlapping and complex effects (also see Trissl et al., 1993). Thus, we need to wait for more sophisticated measurements of parallel transients of individual reactions and components, as well as for the evolution of more sophisticated deconvolution procedures.

V. The Photosystem II Reactions and Chlorophyll Fluorescence

Most of the Chl *a* fluorescence in PS II preparations and in thylakoids that we measure, at room temperature, is from antenna Chl *a* molecules (mostly from the minor antenna complexes CP-43 and CP-47) not reaction center Chl *a* molecules. The variable Chl *a* fluorescence is created either from exciton equilibration between the antenna and the reaction center Chl *a*, or from exciton/radical pair equilibration (see Renger, 1992, for the earlier literature and discussion of PS II chemistry). It had been generally believed that all the PS II fluorescence was prompt fluorescence. Klimov et al. (1977) suggested that all of the variable Chl *a* fluorescence of PS II was recombinational luminescence from the back reaction of $P680^+$ with $Pheo^-$. Although there hasn't been a general acceptance of this concept (Van Gorkom, 1986), the exciton/radical pair equilibration recombination model (Holzwarth, 1991) seems capable of accommodating it. Further research and discussion is required to reach a consensus.

A good part of fluorescence from the isolated PS II reaction center, however, originates in the recombination of $P680^+$ with $Pheo^-$ (see e.g. Govindjee et al., 1990a). Van Mieghem et al. (1992) and Govindjee et al. (1993b) concluded that PS II charge separation is decreased if Q_A^- is present, but is increased if doubly reduced Q_A, Q_A^{2-}, is present. The prior redox state of the donor side may also affect the reactions presented above. The known distances between CP43/CP47 Chls *a* and RCII Chls *a* may be suggestive of prevention of fast equilibration (ouni et al., 2001; Vasil'ev et al., 2001). With newer data, the above views, however, may be in need of revision (Ferreira et al., 2004, and van Grondelle and Gobets (Chapter 5)).

A. Electron Transport on the Donor Side of Photosystem II

The donor side of PS II involves electron transfer from Y_z (a specific tyrosine161 in the D-1 protein) to the oxidized reaction center Chl $P680^+$ (Chapter 8, Shinkarev). The Y_z^+ recovers its lost electron from

water via a four Mn (or a three Mn-one Ca and one Mn) cluster. After four such reactions, four positive charges accumulate on a Mn cation cluster that reacts with two molecules of water to evolve one molecule of O_2, releasing four protons into the lumen of the thylakoid. Kok et al. (1970) explained the period 4 oscillations, discovered by Joliot et al. (1969), in O_2 evolution per flash as a function of the number of light flashes, spaced ~1 s apart; they represented the redox states of the Oxygen evolving complex (OEC) as S_0, S_1, S_2, S_3 and S_4, where the subscripts represent the number of positive charges on OEC, and each transition ($S_n \rightarrow S_{n+1}$) takes place as an electron is transferred from OEC to the $P680^+$, formed in light. In dark, the system starts mostly in the S_1 state and the maximum O_2 is released after the 3rd flash followed by a periodicity of 4 in flash number dependence of O_2 release. (For a basic description, see Govindjee and Coleman, 1990; for a historical minireview, see Joliot, 2003, and for the detection of an intermediate in O_2 evolution, see Clausen and Junge, 2004.)

1. Oxidized Reaction Center Chlorophyll, $P680^+$, as a Quencher of Chlorophyll a Fluorescence in Photosystem II; Donation of Electron from Tyrosine Y_Z (or Z) to $P680^+$

In the ns to sub-μs time scale, the Chl *a* fluorescence rise, after a brief (~ ns) actinic flash, measures the electron flow from Y_Z (or Z) to $P680^+$.This rise was discovered by Mauzerall (1972) and explained by Butler (1972) to be due to the removal (reduction) of the quencher $P680^+$. Sonneveld et al. (1979) elegantly measured this reaction, after correcting for quenching by Chl *a* triplets, and showed that it was faster (approx. $t_{½}$ ~ 20 ns) during transition of S_o and S_1, and slower and more complex during transitions of S_2 and S_3. This fluorescence rise can be observed even at longer times due to the equilibrium reactions between $S_o \leftrightarrow Y_Z$(or Z) $\leftrightarrow$ P680 (Kramer et al., 1990; Shinkarev and Govindjee, 1993).

2. Water to Y_Z (or Z) reaction

As mentioned above, electron transfer from Y_Z to $P680^+$ can be measured through Chl *a* fluorescence rise in the nanosecond to sub-microsecond range after an actinic flash. However, this does not take into account the equilibria between the S-states and Z, and between Z and P680. There are two possibilities of how S-states (i.e., the valence states through which the manganese cluster of the OEC recycles in order to oxidize water) can control Chl *a* fluorescence yield: (a) a more positively charged S-state can slow electron transfer from that state to Y_Z and, in turn, the electron transfer from Y_Z to $P680^+$, leading to an accumulation of $P680^+$ which acts as a natural quencher of Chl *a* fluorescence; and (b) a direct influence of S-states on the Chl *a* fluorescence yield. There also exists the possibility of O_2, per se, released during S_4 to S_o transition to cause quenching of Chl *a* fluorescence. Shinkarev et al. (1997) measured the kinetics of the difference between the inverse of the fluorescence yield after the first flash (S_1 to S_2 transition, no O_2 evolution) and that after the third flash ($S_3 \rightarrow S_4 \rightarrow S_o$ transition, O_2 evolution; see Kok et al., 1970; Renger, 2003). Analysis of this data shows that a quencher is produced with a lag of approximately 1 ms and a rise half time of about 2 ms (Chapter 8, Shinkarev). The amplitude of this quencher oscillates with a period of 4 in synchrony with O_2 evolution, but there are serious quantitative differences. In the same way, there may be inconsistencies with the H^+ release patterns (Lavergne and Junge, 1993). It is still tantalizing to consider the possibility that this phase is a monitor of the kinetics of the $S_4 \rightarrow S_o$ O_2-evolving step. Whether it could be O_2 itself (for arguments regarding O_2 as a quencher of Chl fluorescence, see Papageorgiou, et al., 1972; and Papageorgiou, 1975b) is a valid question to ask. Since fluorescence can be measured in intact leaves, Chl fluorescence kinetics could become an excellent probe for monitoring crucial functional steps of PS II in situ.

B. Electron Transport on the Acceptor Side of Photosystem II

The acceptor side of PS II involves electron transfer from excited P680, P680*, to pheophytin (Pheo) and then to a one-electron acceptor Q_A, a bound plastoquinone. From reduced Q_A, Q_A^-, electrons are transferred to Q_B. After two such reactions, the doubly reduced Q_B^{2-} 'picks up' two protons becoming Q_BH_2 (or simply PQH_2). (For a detailed description, see Crofts and Wraight, 1983.) Since there are 8–10 PQ molecules in the thylakoid membrane pool (see above), it takes some time (~500 ms) to reduce the entire PQ pool.

1. Q_A^- to Plastoquinone Reactions

In the μs to ms time scale, the Chl *a* fluorescence

I was only kidding about the length of the title!!!

decay, after a brief flash, measures the electron transfer from Q_A^- to Q_B. These measurements were first made by Forbush and Kok (1968) who used a ~ 1 ms saturating flash to induce a single turnover of PS II reaction centers; they observed a fast decay phase ($t_{½}$ ~ 0.6 ms), which they correctly attributed to re-oxidation of Q_A^-; about 18 flashes were needed to reduce the secondary acceptor PQ pool (then called the *A* pool). They also remarked at the heterogeneity of this PQ pool. Although Mauzerall (1972) reported the microsecond to millisecond fluorescence decay, the first detailed and reliable measurements on this decay were those by Zankel (1973) who observed a phase of $t_{½}$ ~ 200 μs and another of 1 ms, and related them to the equilibria between what we now call Q_A, Q_B, and the PQ pool, the fast and the slow reducing pool.

2. The Two-electron Gate: Discovery of Q_B

The existence of a 'two-electron gate,' through which electrons pass only in pairs, somewhere between PS II and electron acceptance by methyl viologen from PS I, was shown by Bouges-Bocquet (1973) in a paper that was submitted within a week or so of that by Velthuys and Amesz (1974). Bouges-Bocquet had called the carrier B, and shares the credit of independent discovery of the two- electron gate. The concept of the two-electron gate was elegantly demonstrated in an experiment, that I consider to be a major breakthrough, by Velthuys and Amesz (1974). In these experiments, the possible oscillations due to the donor side (the S-state cycling related to O_2-evolution steps) were eliminated by alkaline Tris-washing, and an external electron donor was provided for the functioning of PS II. A series of preflashes were given and then the herbicide DCMU was injected and Chl *a* fluorescence yield monitored. There was an obvious binary oscillation in the Chl *a* fluorescence yield: high after the first and all odd preflashes, and low after the second and all even preflashes (Fig. 8). This work provided, for the first time, information on how one electron acceptor, Q_A (then called Q), communicates with the two-electron-acceptor PQ molecule. The authors interpreted their results in terms of an electron carrier *R* (now known as Q_B) which exchanges electrons one by one with Q_A, but two by two with PQ. This is the essence of, what we call today, the *two-electron gate*. Bowes and Crofts (1980) explained their results, in which Chl *a* fluorescence yield decays faster after the first than after the second flash, in terms

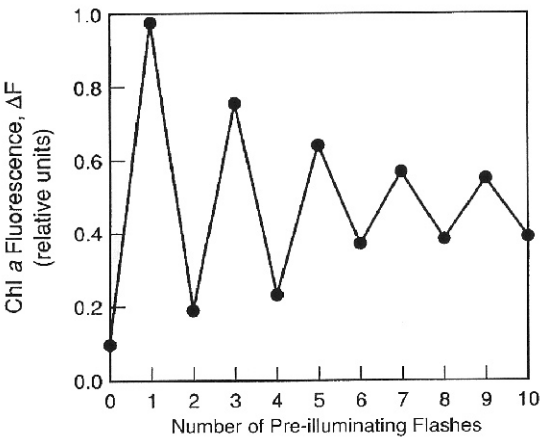


Fig. 8. Binary oscillations in Chl *a* fluorescence that led to the concept of two-electron gate on the acceptor side of PS II. Chl *a* fluorescence yield changes (ΔF) observed after a series of pre-illumination flashes, followed by DCMU (or dithionite) addition. Alkaline Tris-washed chloroplasts were used to block the water to P680 reaction, and thus the period 4 oscillations on the electron donor side of PS II, and *p*-phenylenediamine was added as an artificial electron donor in order to run the PS II. (Data of Velthuys and Amesz, 1974; figure reproduced from Govindjee, 1995; also see Shinkarev (Chapter 8).)

of a slower electron flow from Q_A^- to Q_B^- than from Q_A^- to Q_B possibly because of electrostatic repulsion from Q_B. It was Velthuys (1982) who first realized that Q_B is not a permanent cofactor of PS II but merely a molecule of the pool that remains tightly bound only when it is present in the one-electron reduced, semiquinone form. For a historical perspective of the 2-electron gate in photosynthetic bacteria, see Verméglio (2002).

C. Role of Bicarbonate

In addition to the crucial role of CO_2 in carbon fixation (Benson, 2002; Bassham, 2003), it is also required, as HCO_3^-, for the functioning of PS II (van Rensen et al., 1999). There are two major roles of HCO_3^- in PS II: one is on the donor side and the other is on the acceptor side. Neither PS I nor the reaction centers of photosynthetic bacteria require HCO_3^- for their functioning (Govindjee, 1991).

The history of the role of HCO_3^- on the electron donor side of PS II has been discussed by Stemler (2002). In a large number of experiments, bicarbonate is displaced from its binding site by formate or NO; this leads to an inhibition of PS II reactions which is reversed by the addition of HCO_3^-. The role of HCO_3^- in

stabilization and functioning of the donor side of PS II has been reviewed by Klimov et al. (1995). The role of HCO_3^- in electron and proton transfers in the Q_A^- to the PQ pool steps has been reviewed by Govindjee and Van Rensen (1978, 1993); Blubaugh and Govindjee (1988); Diner et al. (1991); Van Rensen et al. (1999) and van Rensen (2002). Using Chl *a* fluorescence transient measurements, Wydrzynski and Govindjee (1975) were the first to demonstrate that the inhibition of electron flow from Q_A^- to the PQ pool, by formate, was uniquely reversed by the addition of HCO_3^-; this stimulation reveals a major role of HCO_3^- on the electron acceptor side of PS II: the effect of bicarbonate depletion resembled more like the DCMU block on the acceptor side of PS II than by a block on the donor side of PS II.

Jursinic et al. (1976) concluded that electron flow out of Q_A^- to the PQ pool is faster in the presence than in the absence of HCO_3^-. Tracking Chl *a* fluorescence yield changes, after each excitation flash in a series of flashes, Govindjee et al. (1976) showed that the binary oscillations, due to the existence of the two-electron gate were abolished in the absence of HCO_3^-. These results, obtained with thylakoids thoroughly depleted of bicarbonate, suggested that the protonation and the exchange of Q_B^{2-} by the PQ pool is drastically, but reversibly slowed down since the addition of HCO_3^- restored these reactions to the level seen in the untreated (non- HCO_3^- depleted) samples.

Under the experimental conditions of Jursinic et al. (1976), Y_z to $P680^+$ reaction was shown to be normal even in the bicarbonate-depleted samples. Govindjee et al. (1989) confirmed this result and showed that it was independent of the S-states. However, this does not contradict (or disprove) the existence of a role of HCO_3^- on the donor side of PS II (Jursinic and Dennenberg, 1990; Stemler and Jursinic, 1993) under other experimental conditions. In fact, under low pH, bicarbonate-depletion caused inhibition prior to Q_A reduction (El-Shintinawy and Govindjee, 1989, and El-Shintinawy et al., 1990). Klimov and co-workers have now established an important role of HCO_3^- on the donor side of PS II (see e.g., Klimov et al., 1995) On the other hand, there is indeed a clear stimulatory role of HCO_3^- in the reactions from Q_A^- to PQ, as discussed above. Eaton-Rye and Govindjee (1988a, 1988b) and Xu et al. (1991) showed a drastic formate-induced and bicarbonate-reversible slowing down of electron transfer from Q_A^- to Q_B after the second and subsequent flashes, but not after the first flash. This has been interpreted to suggest that it is protonation of the site near Q_B^-, rather than electron transfer per se, that is inhibited by bicarbonate-reversible formate. A similar result was obtained by Diner and Petrouleas (1990) for the bicarbonate-reversible NO effect. A role of HCO_3^- in protonation reactions has also been suggested from proton measurements by Van Rensen et al. (1988).

The atomic level model of the PS II reaction center presented by Ferreira et al. (2004) suggests that bicarbonate may be bound on both the acceptor and donor sides of PS II reaction centers giving credence to the concept that bicarbonate may play roles on both the donor and acceptor sides of PS II.

Blubaugh and Govindjee (1988) hypothesized that one of the functions of bicarbonate is to stabilize the negative charge on Q_B^- formed after the flash by delivering a H^+ to a particular histidine. Here, bicarbonate was suggested to be H-bonded to a particular arginine (D1-R269 and/or D1-R257) and, perhaps, stabilized by other arginines, placing it in an optimal region for such a role. In the absence of HCO_3^-, this is much slowed and, thus, electron transfer after the second and succeeding flashes is slowed. In addition, the importance of D2-R251 and D2-R233, but not D2-R139, for stabilization of HCO_3^- was shown by Cao et al. (1991) (also see Govindjee, 1993) through the use of site-directed *Synechocystis* sp. PCC 6803 mutants (D2-R251S, D2-R233Q and D2-R139H). However, we consider it likely that D1-R269 and/or D1-R257 is involved in the binding of HCO_3^- in addition to the non-heme iron (Diner and Petrouleas, 1990). We suggest that both D1- R269 and D1-R257 may be of importance for the HCO_3^- in the functioning of the two-electron gate on the acceptor side of PS II (Xiong et al., 1997, 1998a,b). Chlorophyll *a* fluorescence measurements on bicarbonate-depleted herbicide-resistant Dl mutants, mutated at different amino acids near the Q_B-binding niche (between helices IV and V of the D1 protein) suggest a role of a broad binding niche for bicarbonate ions (Govindjee et al. 1990b, 1991, 1992; Cao et al. 1992; Vernotte et al., 1995).

The (bi)carbonate binding niche in human lactoferrin (Anderson et al., 1989), the only other Fe-(bi)carbonate protein known to us, may serve as a partial model for further investigations. Here (bi)carbonate is not only liganded to Fe, but is H-bonded to an arginine and several other amino acids. Mäenpaa et al. (1995) have demonstrated that a mutant (CAI) of *Synechocystis* sp. PCC 6803, that lacks certain glutamic acids in the loop between helix IV

and V of its D1 protein, shows a high resistance to bicarbonate-reversible formate treatment. Since this mutation is not in the $Q_A Fe Q_B$ niche, this result may suggest the importance of conformational changes.

We are, obviously, far from understanding the bicarbonate binding and its function on both the donor and acceptor sides of PS II. Chl fluorescence measurements still hold promise for obtaining answers to these questions.

D. Connectivity Between Photosystem II Units

In the lake model (see earlier discussion), excitons migrate freely (random walk). If they encounter a closed reaction center Chl *a,* they can just go to another center (Knox, 1975; Pearlstein, 1982). Such a model predicts a linear relationship between lifetime of fluorescence, τ, and quantum yield of fluorescence, ϕ_f, as the traps are progressively closed, by increasing the intensity of excitation or by raising the level of an inhibitor. Briantais et al. (1972) introduced a τ versus ϕ_f diagram, and showed a proportionality between the two quantities throughout the entire range of excitation intensities in *Chlorella* cells. This result and the earlier results of Tumerman and Sorokin (1967) were taken to support the *lake* model. They did not support the strictly 'isolated puddles' model, where exciton can visit *only* one reaction center, because fluorescence would have to be dealt with as a sum of fluorescence from open and closed units, leading to a significant nonlinearity in the τ versus ϕ_f curve.

In reality, however, the picture may be 'in-between', i.e. there may be a certain probability of exciton migration from one unit to another, as if there were interconnected puddles or a pond. A. Joliot and P. Joliot (1964) had derived a relationship (see P. Joliot and A. Joliot (2003) for a historical perspective):

$$(F_{(t)} - F_o)/ (F_{max} - F_o) = (1 - p)q / (1 - pq) \qquad (12)$$

where, $F_{(t)}$ is the Chl *a* fluorescence yield at time t, F_o is the fluorescence yield when all Q_A is in the oxidized state, F_{max} is the maximum fluorescence yield when all Q_A is in the reduced state, p is a parameter related to the probability of interunit energy transfer, and q is the fraction of closed reaction centers. Here $q = 1$, when Q_A^- is maximum. Joliot and Joliot (1964) calculated the parameter p, which depended solely on the variable Chl fluorescence. The calculated values of p have hovered around 0·5 in most cases.

Both Paillotin (1976, 1978) and Strasser (1978) pointed out difficulties with this concept and suggested modifications. As the centers close, the proportion of open centers decreases. Paillotin (1976) suggested using a physical connection parameter P that depends only upon exciton migration from a closed to an open reaction center; he relates it to Joliots' *p* as follows:

$$P = p\,(1 - F_o/F_{max}) = p \times F_{variable}/F_{max}, \qquad (13)$$

On the other hand, Strasser (1978) proposed that the probability of exciton migration in Joliots' equation be corrected by the ratio of $F_{variable}/F_o$. For a relationship between the three equations, see Strasser et al. (1992). Trissl et al. (1993) and Trissl and Lavergne (1995) have challenged some of these concepts and provided reasons for further caution in making quantitative calculations. (For other views on this subject, see Strasser et al.(Chapter 12), and Vredenberg (Chapter 6).)

VI. Non-photochemical Quenching of Chl Fluorescence

High light (beyond what is needed for maximum photosynthesis) is a major plant stress. Under extreme high-light conditions, the photosynthesis apparatus can be damaged irreversibly (see Adir et al., 2003, for a historical minireview on 'photoinhibition'). Plants and algae have devised various strategies to protect themselves (photoprotection) (Björkman and Demmig-Adams, 1994; Gilmore and Govindjee, 1999, Horton et al., 1999, and Niyogi, 1999; Holt et al., 2004). Strategies adopted by cyanobacteria for photoprotection are discussed by Bruce and Vasil'ev (Chapter 19), George Papageorgiou and Kostas Stamatakis (Chapter 26) and John Allen and Conrad Mullineaux (Chapter 17). One of the strategies for survival in high light is to eliminate the excess absorbed energy as heat (thermal dissipation), which can be measured as non-photochemical quenching (NPQ) of Chl fluorescence. The process of NPQ in higher plants involves acidification of the thylakoid lumen, operation of the xanthophyll cycle, and specific components of the antenna of PS II (see a quantitative description in Gilmore et al., 1998). These components include the *psbS* gene product, some other minor antenna complexes and even certain portions of LHCIIb (Li et al., 2000; Crimi et al., 2001; Chow et al., 2000; Frank et al., 2001; Elrad et

al., 2002; Govindjee, 2002).

A. Early Ideas on Non-Photochemical Quenching

For earlier thoughts and literature on the effects of strong light on photosynthesis by J. Myers, B. Kok, E. Rabinowitch and L. N.M. Duysens, prior to 1965, see discussion in Govindjee and Seufferheld (2002). Papageorgiou and Govindjee (1967, 1968a, 1968b) began looking at the effects of uncouplers of photophosphorylation, even in the presence of DCMU, on Chl *a* fluorescence of intact green and blue-green photosynthetic cells. They observed complex changes in both fluorescence kinetics and fluorescence emission spectra; since DCMU was present, it was evident that these changes were unrelated to 'Q_A-dependent quenching.' In the absence of DCMU, the un-relatedness of the slow Chl *a* fluorescence changes to photosynthesis was supported by the observation that the rate of O_2 evolution paralleled the SM (see footnote[3]) fluorescence rise, and remained constant during the MT fluorescence(see footnote[3]) decline (Papageorgiou and Govindjee 1968a, 1968b; Mohanty et al. 1971a).

Murata and Sugahara (1969) observed an uncoupler sensitive lowering of Chl *a* fluorescence yield when they added reduced phenazine methosulfate (PMS) to DCMU-treated spinach chloroplasts. Wraight and Crofts (1970) showed a correlation between the protonation of the interior of the thylakoid, and the lowering of the Chl *a* fluorescence yield. However, Papageorgiou (1975b) showed dual quenching by the lipophilic PMS cation, direct collisional quenching of excited Chl *a* in situ, and indirect quenching, via cyclic electron transport and acidification of thylakoid lumen. While fluorescence quenching by Q_A was optimal at pH 6.5, the 'high energy state' (protonation) quenching was optimal at pH 8.5. Briantais et al. (1979, 1980) showed that the slow decline phase of Chl fluorescence is correlated with the lumen $[H^+]$ in isolated chloroplasts. This fluorescence lowering cannot be due to direct quenching by protons as they cannot accept electronic excitation energy.

Papageorgiou (1975a) considered the possibility that some of the 'non-Q_A related' or 'high-energy-state, or X_E' quenching may occur through changes in structure that allow diffusion of quenchers (such as O_2) to the pigment site. Fixation of cells by glutaraldehyde did eliminate quenching of Chl *a* fluorescence by PMS (Mohanty et al., 1973). In view of the absence of PMS-induced effects on excitation energy transfer from PS II to PS I, and in view of the fact that fluorescence intensity changes paralleled lifetime of fluorescence changes, Mohanty et al. (1973) concluded that these changes were due to increases in rate constant of heat loss, k_h, not of excitation energy transfer k_{tr}. These were the beginnings of the observations on non- photochemical quenching of Chl *a* fluorescence of PS II.

Since the conclusions of Murata and Sugahara (1969), Wraight and Crofts (1970), Mohanty et al. (1973) and Briantais et al. (1979, 1980) on thylakoids and chloroplasts were more understandable than those obtained earlier on algal cells, Mohanty and Govindjee (1973) investigated the effects of salicylanalides, uncouplers of photophosphorylation, on DCMU-treated cyanobacterial cells They observed that these uncouplers abolished the time-dependent Chl *a* fluorescence increase, a sort of opposite effect to that observed with the PMS-system in thylakoids. In both cases, uncouplers of photophosphorylation caused drastic changes in 'non-Q_A-related' Chl *a* fluorescence changes. I hope that with the new theoretical and experimental framework available now, these early observations in intact cells can be reinvestigated and finally understood at a molecular level.

B. Xanthophyll Cycle and the Non-photochemical Quenching

Seven years before the observations of N. Murata and K. Sugahara, Yamamoto et al. (1962) had discovered the reversible de-epoxidation of violaxanthin to antheraxanthin and then to zeaxanthin, a process that came to be known as the *xanthophyll cycle*:

violaxanthin (V) ↔ antheraxanthin(A) ↔ zeaxanthin (Z)

As V is converted to A and then to Z, $½O_2$ is removed at each step, and in the reverse process (epoxidation) $½O_2$ is added at each step.

Harry Yamamoto, who has invested years of research characterizing this cycle biochemically, concluded that it played an unknown but important regulatory role in photosynthesis (see Yamamoto, 1979; Yamamoto et al., 1999). (A photograph of Yamamoto appears in Govindjee and Seufferheld, 2002.) It was later that B. Demmig-Adams and her coworkers suggested that the pigments of the Xantho-

phyll cycle play a role in NPQ of Chl *a* fluorescence by increasing k_h (Demmig- Adams et al. 1990; for a personal historical minireview, see Demmig-Adams, 2003; also Williams Adams and Barbara Demmig-Adams (Chapter 22)).

Non-photochemical quenching of Chl *a* fluorescence simply implies enhanced dissipation of electronic excitation via pathways other than those involved in photochemistry (k_p) and fluorescence (k_f). The most obvious alternate pathways are direct heat losses (k_h) and (excitation) transfer to other molecules (k_q), such as for example carotenoids, and intersystem crossings to Chl *a* triplets (k_{trip}).

Thus, NPQ includes what we may call 'non-Q_A related' changes. It is measured as follows (see Baker and Oxborough (Chapter 3), Krause and Jahns (Chapter 18) and Schreiber (Chapter 11)):

$$NPQ = (F_m - F'_m)/ F'_m \quad (14a)$$

$$NPQ= (F_m/ F'_m) -1 \quad (14b)$$

$$NPQ +1 = (F_m/ F'_m) \quad (14c)$$

$$1+ K_{SV} [Q] = (F_m/ F'_m) \quad (14d)$$

where, F_m is maximal Chl fluorescence in dark-adapted samples, F'_m is maximal Chl fluorescence in light-adapted samples, K_{SV} is Stern-Volmer constant and [Q] is the concentration of the quencher of fluorescence.

There is a general consensus among several researchers that lumen acidity may not only activate the enzyme violaxanthin de-epoxidase to convert violaxanthin to antheraxanthin and zeaxanthin, but may also cause conformational changes of antenna pigment protein complexes such that the quenching of Chl *a* fluorescence by zeaxanthin and antheraxanthin (Gilmore and Yamamoto, 1993) is favored. Quenching processes in fluorescence studies are best analyzed by the well-known Stern-Volmer[4] relationships (Stern and Volmer, 1919; Papageorgiou, 1975a,b; Demmig-Adams et al., 1990; see Eqs. 14d and 14e):

$$F \text{ (control)}/F \text{ (with quencher)} - 1 = k\mathbf{p}\tau [\text{Quencher}] \quad (14e)$$

where, F = fluorescence intensity, k = collision rate constant, **p** = probability of effective collisions and τ = lifetime of fluorescence in the absence of the quencher.

Using the Stern-Volmer relationship, Gilmore and Yamamoto (1993) obtained a correlation between the Chl *a* fluorescence yield and the combined [H^+] and [zeaxanthin (Z) + antheraxanthin (A)]. Thus, the k_h, proposed earlier, may be equated most simply to k_q [H^+] [Z + A]. This does not *preclude* the existence of other quenching mechanisms. It becomes a matter of knowledge of which mechanism dominates and when (Kramer and Crofts, 1996). However, the role of zeaxanthin in photoprotection in vivo has been emphasized by several, including B. Osmond and coworkers (Casper et al., 1993).

A decrease in fluorescence intensity in a photosynthetic system, even when the number of total absorbed quanta is kept constant, need not necessarily mean a decrease in quantum yield of fluorescence of PS II if the absorption cross-section of the fluorescent pigment bed (PS II) decreases and that of the weakly fluorescent bed (PS I) increases. Such a change would not reflect changes in rate constants of de-excitation pathways. However, if fluorescence intensity changes are strictly proportional to lifetime of fluorescence changes, we can be sure that these reflect quantum yield changes and, thus, changes in the rate constants of de-excitation. Gilmore et al. (1995, 1998) observed an almost linear relationship between Chl *a* fluorescence intensity changes (measured by a PAM (Pulse Amplitude Modulated fluorometer) and the fraction of a short (approximately 0.5 ns) lifetime component of Chl *a* fluorescence (measured by a multifrequency phase fluorometer) during quenching of Chl *a* fluorescence that was dependent upon [H^+] and [zeaxanthin + antheraxanthin]. Gilmore et al. (1995, 1998) observed that as more zeaxanthin (or antheraxanthin) was formed, even when electron transport was blocked, the amplitude of the higher lifetime (1.7 ns) of the fluorescence component decreased linearly in proportion to the increase in the amplitude of the lower (0.5 ns) lifetime of the fluorescence component. This meant that the complex that contained both Chl and zeaxanthin (or antheraxanthin), formed upon the increase in concentration of

[4] We note that the Stern-Volmer equation was originally derived considering collisional quenching in homogeneous solutions (i.e., diffusional limited reactions; see Förster, 1951). In NPQ it is applied, however, in a quasi solid-state system, in which only excitation energy moves, but not molecules. Thus, there is only a formal similarity between these two processes (expressed by Eq. 14e).

these xanthophylls, had a lowered quantum yield of fluorescence and, thus, increased rates of heat losses within it. This is like having a 'dimmer' switch, where light (fluorescence) is dimmed as the concentrations of zeaxanthin and antheraxanthin increase (Gilmore at al., 1998; Fig. 9). For a further discussion of the mechanism of non-photochemical quenching, and of how carotenoids may quench Chl *a* fluorescence, see Crofts and Yerkes (1994) and Frank et al. (1994), respectively. Further, Vasiel'ev et al. (1998) discuss quenching by quinones as a model for quenching of fluorescence in antenna molecules. An interesting suggestion for NPQ of Chl fluorescence involving charge transfer state of zeaxanthin and Chl has been presented by Dreuw et al. (2003).

One of the major mechanisms by which plants protect themselves against excess light is by dissipating energy as heat, as noted above; this is an important strategy for the survival of plants. Non-photochemical Chl quenching, when plants are exposed to excess light, is a theme that is covered in several chapters: Krause and Jahns (Chapter 18); Bruce and Vasil'ev (Chapter 19), Golan et al. (Chapter 20), Gilmore (Chapter 21); Adams and Demmig-Adams (Chapter 22) and Kramer et al. (Chapter 10). Schreiber (Chapter 11) has provided an overview of the application of the Pulse Amplitude Modulation (PAM) fluorometry for measurements of quantum yield of photochemistry of PS II in low light and in excess light (when non-photochemical quenching occurs, i.e., energy is lost as heat).

VII. Concluding Remarks

Chlorophyll *a* fluorescence has been a wonderful tool not only to understand how plants cope with excess light, but with UV light (Manfred Tevini, Chapter 23), water stress (Nikolai Bukhov and Robert Carpentier, Chapter 24), and heavy metal ion stress (Manoj Joshi and Prasanna Mohanty, Chapter 25). Plants regulate the distribution of excitation energy between PS I and PS II by a phenomenon labeled as 'State Changes'. Allen and Mullineaux (Chapter 17) show how Chl fluorescence is used to understand the mechanism of this regulatory phenomenon. Papageorgiou and Stamatakis (Chapter 26) provide a novel application of Chl fluorescence as a monitor of osmotic volume changes and of water and solute transport in cyanobacterial cells On the other hand, Hoober and Akoyunoglou (Chapter 27) show how

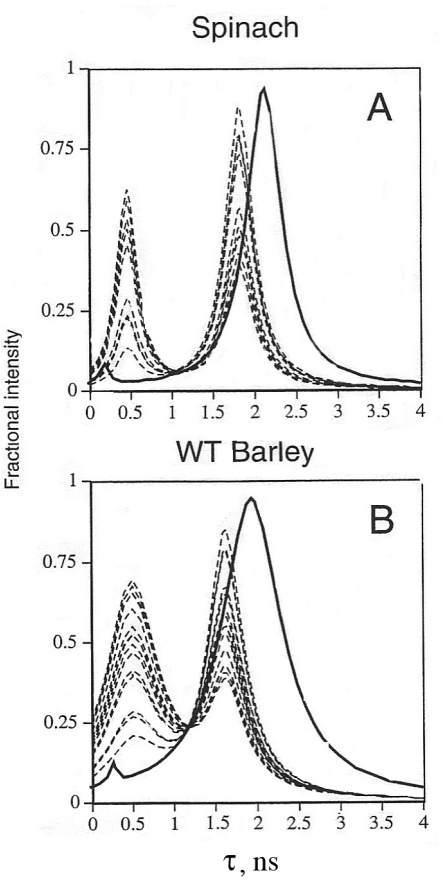


Fig. 9. A dimmer switch in photoprotection. As light intensity increases, more zeaxanthin and antheraxanthin are formed dimming the Chl fluorescence yield, provided the transthylakoid proton gradient is not eliminated. Gilmore et al. (1998) could deduce this from the distribution of lifetime of Chl fluorescence in spinach (panel A) and Wild type (WT) barley thylakoids. To eliminate the effect of changes in electron transport, 10 μM DCMU was added to block all electron transport. The solid curve shows the lifetime of distribution pattern, with most fluorescence with a lifetime of 2 ns for Chl fluorescence, in the presence of 2 μM nigericin (a protonophore): increasing DTT (dithiothreotol, that reduces the concentration of zeaxanthin) did not cause any changes in the lifetime of Chl fluorescence components. However, in excess light, in the absence of nigericin, when fluorescence yield is quenched (0.5 ns component is formed at the expense of 1.7 ns component), addition of increasing concentrations of DTT that produces increasing amounts of violaxanthin at the expense of zeaxanthin reverses the dimmer switch changing the 0.5 ns lifetime of Chl fluorescence component to 1.7 ns component. Note that in the absence of a proton gradient (solid curve), τ is higher (2 ns) than in its presence (~1.7 ns) showing the effect of protonation alone. (Reproduced from Gilmore et al., 1998; also see Gilmore (Chapter 21).)

Chl fluorescence measurements have been applied to the problem of the assembly of light harvesting complexes of PS II.

One of the most useful applications of Chl fluorescence has been in studies of regulation of photosynthetic electron transport (Kramer et al., Chapter 10); light adaptation and senescence of plants (Hartmut Lichtenthaler and Babani, Chapter 28), of terrestrial plants in various ecological niches (Jeannine Cavender-Bares and Fakhri Bazzaz, Chapter 29) and of photosynthesis in our vast oceans (Falkowski et al., Chapter 30), and in inland waters (John Raven and Steven Maberly, Chapter 31).

To me, one of the most fascinating areas has been imaging of Chl fluorescence (Lichtenthaler and Mihe, 1997; Buschmann et al., 2000). Lichtenthaler and Babani (Chapter 28), Ladislav Nedbal and John Whitmarsh (Chapter 14) and Kevin Oxborough (Chapter 15) have presented the state-of the-art fluorescence intensity images.

A very important application has been in the area of remote sensing of photosynthesis via remote sensing of Chl fluorescence since it has the promise of measuring land and ocean productivity from satellites, airplanes, and helicopters; this has been covered by Ismael Moya and Zoran Cerovic (Chapter 16).

The wide areas of photosynthesis, as studied over the years, has been elegantly covered in this book. Some of the authors have presented views that are not yet accepted by others who have written their chapters in this book. Vredenberg (Chapter 6) has challenged the current accepted views on Chl fluorescence; it remains to be seen if he is right.

My *viewpoint* presented here is only a drop in the *lake* of Chl fluorescence research. My current research interest is in fluorescence lifetime imaging microscopy and in the use of sinusoidal light (forced oscillations) to study the regulation of excitation energy transfer from phycobilins to PS II, as published in Holub et al. (2000), and Nedbal et al. (2003), respectively.

Acknowledgments

I thank George Papageorgiou for editing this manuscript. I also thank Rajni Govindjee, Bacon Ke and David Kramer for suggestions and corrections in this chapter. I am thankful to Rumana Tayyab for preparing figures 2, 4 and 7, and for checking the references. During the preparation of this chapter, I have been supported by the Department of Plant Biology, University of Illinois at Urbana-Champaign and by the School of Life Sciences, Jawaharlal Nehru University, New Delhi.

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Comment: "Březina V, Adamec F, Štys": an "S" is wider than an "r" so the little mark is wider on the "S".

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