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Minireview

Fluorescence lifetime, yield, energy transfer and spectrum in photosynthesis, 1950–1960*

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Abstract

The *fluorescence lifetime* of chlorophyll *a* gives information about the primary photo-physical events in photosynthesis. Most of the light energy absorbed by chlorophylls is utilized for photochemistry. There are two main additional pathways competing for the absorbed light energy: fluorescence and radiationless internal conversion (heat). Only a few percent of the absorbed energy proceeds along these two pathways. This historical minireview focuses on the first direct measurements of the lifetime of chlorophyll fluorescence, the time it takes to transfer energy from phycoerythrin to chlorophyll *a*, and the discovery of the fluorescence band at 720 nm (F720; then attributed to a dimer of chlorophyll). These works were carried out during the the late 1950s to the early 1960s in the laboratory of Professor Eugene Rabinowitch at the University of Illinois, Urbana-Champaign [Brody (1995) Photosynth Res 43: 67–74].

Introduction

The fluorescence lifetime, τ, and fluorescence yield, φ, are related by the expression $\tau = \varphi\ \tau_o$. The observed lifetime of the excited state of the molecule is τ and its natural lifetime is τ_o. Fluorescence yield is the fraction of absorbed energy emitted as fluorescence. The natural lifetime can be calculated from the absorption spectrum of the molecule. They are related by the following equation:

$$1/\tau_o = 8\pi n^2\ c\ \ln 10 \int \epsilon_m(2\nu_o - \nu)^2\ d\nu/\nu N 10^{-3}, \quad (1)$$

where ν_o is the wave number of the pure electronic transition (estimated to be midway between the absorption and fluorescence maxima), n is the index of refraction, c the speed of light, N the Avogadro's number, and ϵ_m is the molar absorption coefficient as a function of wavenumber (ν). The limits of integration cover the absorption spectrum of the first excited state of the molecule.

Before it was possible to directly measure fluorescence lifetimes, lifetimes were calculated from φ and τ_o (Latimer et al. 1955). Such calculation implicitly assumed that there was only a single lifetime for the fluorescence. The lifetime predicted from yield measurements did not agree with the directly measured fluorescence lifetime. This required a reinterpretation of the state of chlorophyll *in vivo*. When it was possible to directly observe the fluorescence decay, it was seen that it did not follow a simple exponential decay; rather, fluorescence is composed of two or more decay constants. Also, it was shown that the fluorescence yield increased as the temperature is lowered (Brody and Brody 1962). These two observations led to the discovery of aggregated states of chlorophyll *in vivo*.

* This minireview is dedicated to Professor Eugene Rabinowitch (1901–1973), mentor of the author (Steve Brody) as well as of the editor and author's classmate (Govindjee). The career and contributions of Eugene Rabinowitch are available in a dedication by Bannister (1972).

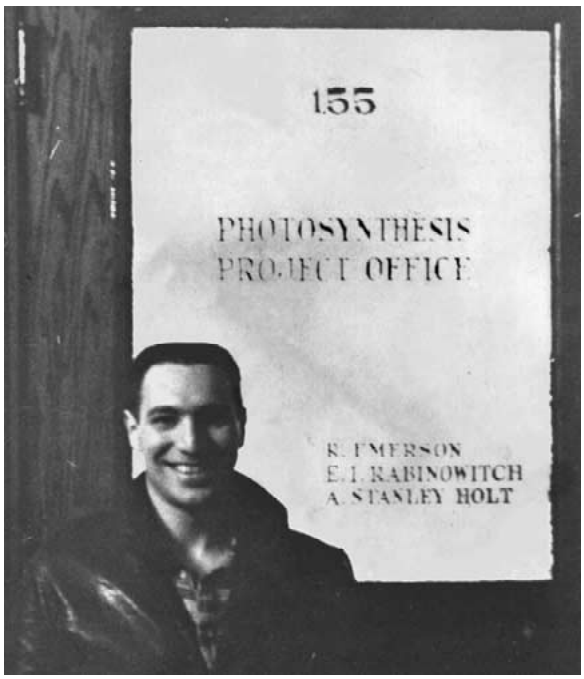

Figure 1. A 1955 photograph of the author standing before the entrance door to the laboratories of Eugene Rabinowitch and Robert Emerson at Urbana, Illinois, USA.

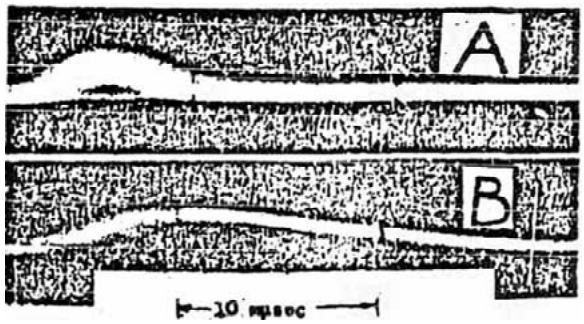

Figure 2. Oscilloscope display of the nanosecond (millimicrosecond) flash lamp and chlorophyll fluorescence. (A) Display of the response of the instrument to the lamp flash; (B) fluorescence of chlorophyll *a* in methanol (Brody 1957).

Figure 3. A 1957 photograph of Paul Latimer taken at Urbana, Illinois.

Lifetime of chlorophyll *a* fluorescence *in vitro* and *in vivo*: the first direct measurements

To directly observe and measure the decay of fluorescence from a molecule in the nanosecond region required the design and construction of a new instrument. The light source selected to excite the fluorescence was a small hydrogen lamp (Malmberg 1957). It had a flash duration of 2 ns. A photomultiplier tube was used to detect the fluorescence. Colored filters plus geometric arrangement of the hydrogen lamp, fluorescence sample, and photomultiplier detector were able to separate the exciting light from the fluorescence. A high-speed oscilloscope and special amplifiers clearly displayed the short-lived fluorescence.

The first directly measured fluorescence lifetimes of chlorophyll *a* and *b,* methyl chlorophyllide (*a* + *b*), phycoerythrin and phycocyanin, in solution, as well as from chlorophyll in the green alga *Chlorella pyrenoidosa,* red alga *Porphyridium cruentum*, and cyanobacterium *Anacystis nidulans* were reported by Brody (1957) and Brody and Rabinowitch (1957) (Figure 1 shows the author standing before the 155 Natural History Building, University of Illinois at Urbana; and Figure 2 shows the data). In a solution of ether, the lifetime of fluorescence of chlorophyll *a* is 5.1 ns and for chlorophyll *b* it is 3.9 ns. The fluorescence yields of chlorophyll *a* and *b* are 0.33 and 0.16, respectively (Latimer et al. 1956; see Figure 3 for a photograph of Paul Latimer; and Figure 4 for a group photograph that includes Tom Bannister, Eugene Rabinowitch and Marcia Brody, among others).

Figure 4. A group photograph taken by Govindjee in 1958. From left to right: Tom Bannister, Mary-Jeanne Bannister, Ruth Chalmers, Tita Emerson, Eugene Rabinowitch, Rajni (Varma) Govindjee, Robert Emerson, Marcia Brody, and Steve Brody.

Thus the natural lifetimes of chlorophyll *a* and *b* are 15 and 25 nanoseconds, respectively. These experimental values for the natural lifetime are in very good agreement with those calculated using Equation (1), when the limits of integration include the minor absorption bands of chlorophyll.

The fluorescence lifetimes of pigments in living cells are considerably shorter than those from the same pigment in solution. In *Chlorella, Porphyridium*, and *Anacystis*, the life times reported were 1.6, 1.5, and 1.2 nanoseconds, respectively (Brody and Rabinowitch 1956). The initial lifetimes reported in photosynthetic material were confirmed and the study expanded by Tomita and Rabinowitch (1962). Using a phase shift method to measure fluorescence lifetimes *in vivo*, it was, independently, observed in the former Soviet Union that the lifetime was dependent on light intensity (Dmitrievsky et al. 1957).

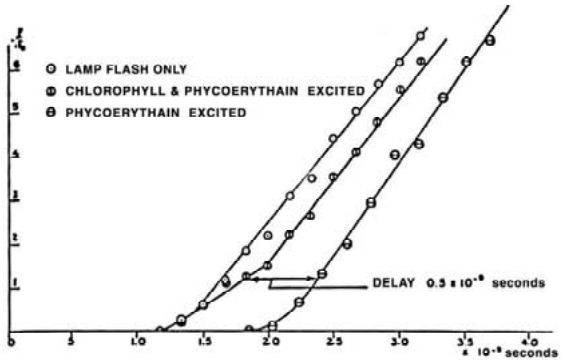

Figure 5. Delay in energy transfer, in *Porphyridium cruentum,* of light energy absorbed by phycoerythrin until emission as fluorescence by chlorophyll. The lamp flash is shown by open circles. Fluorescence of chlorophyll sensitized by light absorbed by phycoerythrin is shown by circles with horizontal line. In the latter case, a delay of 0.5 nanoseconds is observed. The fluorescence when both chlorophyll and phycoerythrin are irradiated, by the flash lamp, is shown by circles with a vertical line (Brody 1957).

Time required to transfer excitation energy in red algae

In 1952 there was already experimental evidence showing that there is transfer of excitation energy in photosynthetic systems (French and Young 1952; Duysens 1952, and see a historical paper by Dutton 1997). The transfer efficiency was reported to be 90% from phycobilins to chlorophyll (Duysens 1952). Using the instrumentation to measure lifetime of fluorescence, it was possible, for the first time, to directly measure the time required to transfer excitation energy from phycoerythrin to chlorophyll in the red alga *Porphyridium cruentum* (Brody 1957, 1960). The phycoerythrin was irradiated with a nanosecond burst of green light. The excitation energy absorbed by phycoerythrin is transferred to phycocyanin and subsequently to chlorophyll. Some of the excitation energy transferred to chlorophyll is emitted as fluorescence. The time between the nanosecond burst of green light and the appearance of the red fluorescence from chlorophyll is the time required to transfer excitation energy in the red alga. Figure 5 shows the data obtained for the lamp flash and the fluorescence from chlorophyll when sensitized by light absorbed by phycoerythrin. The measured time for energy transfer is 0.5 ns (Figure 5).

Figure 6. George Porter, a 1967 Nobel-laureate in Chemistry. Photograph has been taken from http://www.nobel.se/chemistry/laureates/1967/porter-bio.html.

The expected delay in energy transfer from phycoerythrin to chlorophyll, *in vivo*, was calculated by considering the kinetics of energy transfer as a series of first order processes (Brody 1960). This analysis used the fluorescence lifetime of phycoerythrin in solution (7.1 ns), the transfer efficiency to phycocyanin (95%), the lifetime of phycocyanin (1.8 ns) and assumed that resonance energy transfer was the mechanism by which energy was transferred. This analysis yielded a value of 0.58 ns for the delay in energy transfer. This value was in very good agreement with that experimentally measured value, lending support to the hypothesis that resonance energy transfer was the major mechanisms in photosynthesis. Many years later the measurements of the time required to transfer excitation energy from phycoerythrin to chlorophyll in *Porphyridium cruentum* were confirmed using a streak camera (Porter et al. 1978; see Figure 6 for a photograph of George Porter).

The number of chromophores, *R*, on each chroprotein

Fluorescence lifetime measurements were then used to assay the number of chromophore groups, R, associated with each molecule of phycoerythrin and phycocyanin and their molar absorption coefficient. The technique previously employed for this analysis required hydrolysis of the material with hot hydrochloric acid or alkali which may result in more or less destruction of the chromophore (Lemberg and Legge 1949). It is possible to use a nondestructive method to assay the number of chromophores, R, associated with a chromoprotein as well as the molar extinction coefficient, ϵ_m. To carry out this analysis, it was necessary to know the particle weight of the chromoprotein, m (Brody and Brody 1961b). The method is based upon the quantitative relationship that exists between lifetime of the excited state of a molecule and its absorption spectrum (Equation (1)) plus the information on fluorescence lifetime, fluorescence yield, specific extinction coefficient, ϵ_s, and chromoproteid particle weight, m.

The number of chromophore groups, R, on each chromoprotein molecule is equal to the ratio of the weight of a chromoprotein particle, m, to the weight of chromoprotein material that contains 1 mole of chromophore, M, i.e., $R = m/M$. The value of M is determined from absorption and fluorescence data. The specific absorption coefficient ϵ_s is related to the molar absorption coefficient ϵ_m by the relationship $\epsilon_m = M\epsilon_s$. Now ϵ_s is readily determined by simply measuring the absorption of a weighted amount of material. So, M can be determined by substituting $M\epsilon_s$ for ϵ_m in the above equation , containing the lifetime.

The preliminary results for ϵ_m and R employing this nondestructive analysis yielded for phycoerythrin the values 2.38×10^4l/mole cm and 33, for ϵ_m and R, respectively, and for phycocyanin the values 9.9×10^4l/mole cm and 22. Previously Lemberg and Legge (1949) reported a chromophore number of 16 for phycocyanin. All these values were subject to the uncertainties of the early estimates of both the particle weights and specific absorption coefficients.

An alternative method of estimating the number of chromophores is based on the time required for excitation energy absorbed by the protein to be emitted as fluorescence by the chromophore (Brody 1960). Energy absorbed by the protein migrates among the certain amino acids (tyrosine, tryptophan, and phenylalanine). Assuming the maximum time that energy resides with an amino acid residue is 10^{-12} second and the lifetime of the excited state or delay in energy transfer is τ_d, the maximum number of possible transfers, or jumps, $N = \tau_d/10^{-12}$. A one-dimensional random walk will result in $\sqrt{N}$ different amino acid molecules being visited. The average weight of the protein fraction, M^*, associated with each chromophore can be estimated by simply taking 120 as an average weight of an amino acid and mul-

tiplying by $2\sqrt{N}$. (The $\sqrt{N}$ is multiplied by 2 since energy may be absorbed by the protein fraction on either side of the chromophore.) Dividing the particle weight of a chromoprotein by M^* gives the number of chromophores. The particle weights of phycoerythrin and phycocyanin given by Eriksson-Quensel (1938) are 290 000 and 27 300, respectively. This procedure yields a chromophore number of 30 for phycoerythrin and 32 for phycocyanin.

The methods used in those early days had utilized, by necessity, molecular weight determinations which proved to be somewhat inaccurate and some of the basic assumptions had to be modified. Further, the number and type of bilin chromophores depends on the organism used to obtain the protein. The variation is particulatly pronounced among phycoerythrins (see a review by Glazer 1994). Accurate information is now available for a number of the complete complement of bilin peptides. For example, *Synechococcus* sp. PCC 6301 C-phycocyanin carries one phycocyanobilin (PCB) on the α subunit and two PCBs on the β subunit. Thus, the C-phycocyanin hexamer ($\alpha_6\beta_6$) contains 18PCBs. Some of the other phycoerthrin hexamers carry 34 bilins.

Fluorescence spectrum at low temperature

At low temperature (77 K), the major pathways for light energy absorbed by a molecule are fluorescence, transitions to lower energy states, primary photochemistry, and loss as thermal energy. All biochemical reactions are stopped. Using low temperature techniques to investigate fluorescence properties of chlorophyll *in vivo*, it was possible to observe long-lived excited states and other forms of chlorophyll, whose fluorescence is not readily observed at room temperatures. The interesting and important forms of chlorophyll readily observed at low (and room temperature were what was then called the 'chlorophyll dimer,' also referred to as F720 (Brody 1958) (Figure 7) and an unidentified band at 695–698 nm (Broyde and Brody 1964, 1965a, b, 1966; this band was discovered independently in 1963 in several laboratories: see Govindjee 1995 for a historical minireview on chlorophyll fluorescence). A quantitative analysis of the fluorescence band, F720, as a function of chlorophyll concentration in solution led to F720 *in vitro* being identified as a molecular dimer (aggregate) of chlorophyll (Brody 1964). F720 was observed both *in vivo* and in concentrated solutions of chlorophyll (Brody

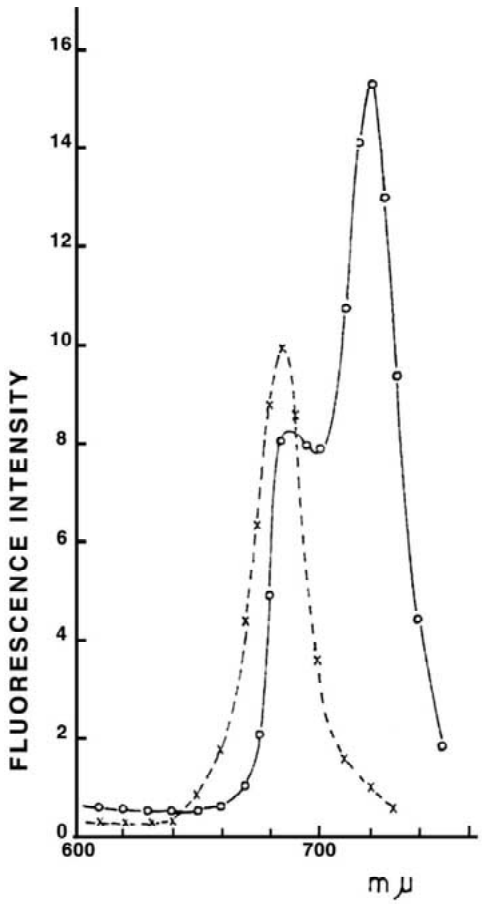

Figure 7. Fluorescence spectrum of *Chlorella* at room temperature (crosses) and at 77 K (circles). At 77 K, the new long wavelength emission band has a maximum at 720 nm (Brody 1958).

Figure 8. Eugene Rabinowitch. Photograph by Govindjee.

1958, 1964). The significance and possible role of the chlorophyll dimer in photosynthesis was reported by Brody and Brody (1961c). F720 *in vivo* is now attributed to a specific chlorophyll–protein complex of Photosystem I (Govindjee et al. 1986).

Acknowledgments

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