LIFETIME OF THE EXCITED STATE IN VIVO

I. CHLOROPHYLL *a* IN ALGAE, AT ROOM AND AT LIQUID NITROGEN TEMPERATURES; RATE CONSTANTS OF RADIATIONLESS DEACTIVATION AND TRAPPING

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ABSTRACT Using a mode-locked laser (λ, 632.8 nm), fluorescence decay of chlorophyll (Chl) *a* in the green alga *Chlorella pyrenoidosa*, the red alga *Porphyridium cruentum*, and the blue-green alga *Anacystis nidulans* was measured by the phase-shift method under conditions when photosynthesis was not operative (3-(3,4-dichlorophenyl)-1,1-dimethylurea [DCMU] poisoning, or cooling to 77°K). In the presence of 10^{-5} M DCMU, the lifetime of Chl *a* fluorescence (τ) at room temperature is about 1.7 nsec in *Chlorella*, 1.0 nsec in *Porphyridium*, and 0.7 nsec in *Anacystis*. At 77°K, τ is 1.4 nsec (for fluorescence at about 685 nm, F-685) and 2.3 nsec (for F-730) in *Chlorella*, 0.9 nsec (F-685) and 1.2 nsec (F-730) in *Porphyridium*, and 0.8 nsec (F-685 and F-730) in *Anacystis*. From the above measurement, and the assumption that τ_0 (the intrinsic fluorescence lifetime) for Chl *a* in all three algae is 15.2 nsec, we have calculated the rate constants of radiationless transition (that includes energy transfer to weakly fluorescent system I) processes competing with fluorescence at room temperature to be about 5×10^8 sec^{-1} in *Chlorella*, 9×10^8 sec^{-1} in *Porphyridium*, and 13×10^8 sec^{-1} in *Anacystis*. At 77°K, this rate constant for Chl *a* that fluoresces at 685 nm remains, in the first approximation, the same as at room temperature. From the τ data, the rate constant for the trapping of excitation energy is calculated to be about 1.2×10^9 sec^{-1} for *Chlorella*, 2×10^9 sec^{-1} for *Porphyridium*, and 2×10^9 sec^{-1} for *Anacystis*. The efficiency of trapping is calculated to be about 66% (*Chlorella*), 68% (*Porphyridium*), and 60% (*Anacystis*). (It is recognized that variations in the above values are to be expected if algae grown under different conditions are used for experimentation.) The maximum quantum yield of Chl *a* fluorescence for system II (λ, 632.8 nm), calculated from τ measurements, is about 10% in *Chlorella*, 6–7% in *Porhyridium*, and 5% in *Anacystis* under conditions when photosynthesis is not operative; the values at 77°K appear to be very close to those with DCMU added at room temperature. ϕ for F-730 at 77°K, however, is somewhat higher than for F-685. The predicted quantum yields of fluorescence for Chl *a* in intact cells (both systems I and II) at low intensities of 632.8 nm light are about 2–3, 1–2, and 1% for *Chlorella*, *Porphyridium*, and *Anacystis*, respectively.

INTRODUCTION[1]

Brody and Rabinowitch (1) and Dmitrievsky et al. (2) independently measured the lifetime (τ) of the excited state of Chl *a* in vivo by the direct flash and the phase shift methods, respectively. Brody and Rabinowitch (1) reported a value of 1.6 $\pm$0.5 nsec for Chl *a* in the green alga *Chlorella*, 1.5 $\pm$0.4 nsec in the red alga *Porphyridium*, and 1.2 $\pm$0.4 nsec in the blue-green alga *Anacystis*. Dmitrievsky et al. (2) reported a τ of 0.6 nsec for Chl *a* in *Elodea*, 1.0 nsec in a leaf of *Cyperus*, and 1.6 nsec in chloroplasts from the leaves of *Aspidistra elatior*; these values were for low illumination and were found to increase (e.g., to 1.5 nsec in *Elodea*) when the light intensity was increased severalfold. Rabinowitch and coworkers (Tomita and Rabinowitch [3], Murty and Rabinowitch [4], Singhal and Rabinowitch [5]) have improved the techniques for τ measurements and extended the earlier τ measurements of Brody and Rabinowitch (1), including those of the times required for the energy transfer from accessory pigments to Chl *a* in various algae. Using phase shift techniques, Müller et al. (6, 7) provided new data on τ of *Chlorella* at different intensities and in the presence of DCMU. Butler and Norris (8) found τ to equal 3.1 nsec for the long wavelength Chl fluorescence (F-730) at 77°K in bean leaves. Using improved techniques, Nicholson and Fortoul (9) found τ of Chl *a* in *Porphyridium* and *Chlorella*, in weak light, to be 0.6 $\pm$0.2 nsec. Using a mode-locked He-Ne laser as a light source, Merkelo et al. (10) obtained a value of 1.40 $\pm$0.05 nsec by the phase method and of 1.6 $\pm$0.2 nsec by the direct fluorescence decay method for *Chlorella* excited with bright light. In the present investigation, using again the mode-locked He-Ne laser, we obtained new data on τ of Chl *a* in DCMU-poisoned *Chlorella*, *Anacystis*, and *Porphyridium* at room temperature, and on τ of Chl *a* in the same algae at 77°K. From these measurements, we calculated the rate constant of radiationless dissipation (that includes energy transfer to weakly fluorescent system I) and of energy-trapping processes and the absolute quantum yield of Chl *a* fluorescence in system II at 300 and 77°K.

MATERIALS AND METHODS

Fluorescence lifetime of Chl *a* in vivo was measured by the phase shift method using a He-Ne mode-locked laser as the source of illumination (Merkelo et al. [10]). The laser emits optical (632.8 nm) pulses at a rate of approximately 10^8 sec^{-1} or, more precisely, generates a fundamental beat note of 102.207 MHz; pulse spacing is derived from a standard frequency measurement with a high frequency counter. (The instrument was calibrated by measuring the velocity of light [the result was within 1% of the correct value] and by comparing the measured lifetime values with those obtained by flash excitation.) For the measurement of fluorescence lifetime at 77°K, the same Dewar flask and the same procedures of freezing the cells were used as described by Cho and Govindjee (11).

[1] This and the following paper are presented here to honor Eugene Rabinowitch in whose laboratory the first in vivo measurements (*a*) of the lifetime of excited states (S. S. Brody and E. Rabinowitch, 1957) and (*b*) of the quantum yield of chlorophyll *a* fluorescence (P. Latimer, T. T. Bannister, and E. Rabinowitch, 1957) in vivo were made.

The green alga *C. pyrenoidosa*, the blue-green alga *A. nidulans*, and the red alga *P. cruentum* were grown as previously described (Govindjee and Rabinowitch [12]). 3–5-day-old *Chlorella* and *Anacystis* and 7–10-day-old *Porphyridium* cultures were used. *Chlorella* and *Anacystis* were suspended in a carbonate-bicarbonate buffer (an 85:15 mixture of 0.1 M $NaHCO_3$ and 0.1 M K_2CO_3, pH 9.2). For *Porphyridium*, the buffer consisted of 16 g $NaHCO_3$, 1.0 g Na_2CO_3, and 15.2 g NaCl/liter of water (pH 8.5).

RESULTS AND DISCUSSION

After a photon is absorbed by a Chl *a* molecule in the "bulk" of a photosynthetic unit in green plants, the excited state is deactivated either by energy transfer to a "trap" at a reaction center, or by fluorescence, or by "radiationless dissipation," meaning internal conversion or energy transfer to quencher molecules not associated with reaction center II, or energy transfer to weakly fluorescent system I.[2] Since the three processes are competitive, the rates of fluorescence and of radiationless dissipation must affect the rate of trapping of energy in the reaction center. We will calculate the rate constants of radiationless dissipation in three algae, from new measurements of lifetime (τ) of Chl fluorescence in the presence of DCMU (section A), and at 77°K (section C); under both conditions, system II traps quickly close upon illumination. Calculations are also presented for the rates of trapping of excitation energy (section B) and the quantum yield of Chl fluorescence in vivo (section D).

A. Rate Constant of Radiationless Dissipation at Room Temperature

Fluorescence lifetime τ was calculated from the measured phase shift by the equation (Bailey and Rollefson [13]):

$$\tan \theta = 2\pi f\tau, \tag{1}$$

where τ is the angle of phase shift and f is the modulating frequency. This equation is based on the assumption that fluorescence has only one exponential decay time.

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[2] For readers not familiar with the terminology used in this paper, we give the following scheme:

(*a*) Chl + $h\nu$ $\xrightarrow[\text{(light absorption)}]{}$ Chl* (rate constant, α_0);

(*b*) Chl* $\xrightarrow[\text{(fluorescence)}]{}$ Chl + $h\nu'$ (rate constant, α_f);

(*c*) Chl* $\xrightarrow[\text{(radiationless dissipation)}]{}$ Chl (rate constant, α_h);

(*d*) Chl* $\xrightarrow[\text{(trapping of energy)}]{}$ Chl (rate constant, α_t).

In step *d* the rate will depend upon the concentration of open traps. Now, $\tau_0 = 1/\alpha_f$; $\tau = 1/(\alpha_f + \alpha_h)$; $\tau' = 1/(\alpha_f + \alpha_h + \alpha_t)$. Therefore, $\alpha_f + \alpha_h = 1/\tau$, $\alpha_h = (1/\tau) - (1/\tau_0)$, and $\alpha_t = (1/\tau') - (1/\tau)$.

TABLE I

FLUORESCENCE LIFETIME (τ) OF CHL *a* IN ALGAE IN THE PRESENCE OF 10^{-5} M DCMU*

Chlorella		*Porphyridium*		*Anacystis*	
θ	τ	θ	τ	θ	τ
degrees	*nsec*	*degrees*	*nsec*	*degrees*	*nsec*
51.3	1.94	37.6	1.20	29.6	0.89
49.4	1.82	38.1	1.22	23.8	0.69
48.6	1.77	31.3	0.95	25.5	0.74
48.2	1.74	31.1	0.94	23.4	0.68
50.4	1.88	34.7	1.08	25.3	0.74
46.7	1.65	29.4	0.88		
47.0	1.67	30.8	0.93		
46.5	1.64				
44.6	1.54				
47.7	1.71				
Average	1.74		1.03		0.74

* θ is the measured phase shift, τ is the lifetime calculated from $\tan \theta/2\pi f$, exciting wavelength, 632.8 nm, frequency of modulation f, 102.207 MHz, filter used for fluorescence measurement, Corning CS 2-64; room temperature.

Tumerman and Sorokin (14) and Briantais et al. (15) have shown that fluorescence of Chl *a* in vivo in fact decays with one predominant lifetime;[3] the fluorescence yield ϕ of Chl *a* in vivo is linearly proportional to τ. Calculations (3, 14) indicated that the assumption of one predominant lifetime could best explain their data. Although there is no direct experimental proof that Chl *a* fluorescence decay is exponential, the assumption that it is exponential leads to curves which fit the experimental data obtained by flash excitation (16).

Table I shows the measured phase angles and the calculated lifetimes in several samples of three different algae with 10^{-5} M DCMU added. Differences in lifetime observed with cells of the same species are due not to instrumental variations, but to differences in the growth conditions of the algae. Taking an average of all experiments, we found that Chl *a* in *Chlorella* has a τ value of about 1.7 nsec, in *Anacystis*, 0.7 nsec, and in *Porphyridium*, 1.0 nsec. Our result with *Chlorella* (in the presence of DCMU) agrees well with that of Müller et al. (7), who found τ to equal 1.9 nsec. No other lifetime values exist for Chl *a* in *Anacystis* and in *Porphyridium* in the presence of DCMU.

From the fluorescence lifetime (τ) measured when the reaction center is made

[3] The conclusion of Murty and Rabinowitch (4) that Chl *a* fluorescence in vivo decays with two periods, both in the nanosecond range, was found by Singhal and Rabinowitch (5) to be due to an artifact; however, the existence of two comparable lifetimes cannot be ruled out.

TABLE II

RATE CONSTANTS FOR RADIATIONLESS DISSIPATION (α_h) AND FOR TRAPPING (α_t) FOR CHL *a* IN VIVO*

Sample	α_h	α_t
	sec^{-1}	sec^{-1}
Chlorella	5.99×10^8	1.19×10^9
Porphyridium	9.09×10^8	1.97×10^9
Anacystis	12.86×10^8	1.98×10^9

* Where α_f, the rate constant of fluorescence, is assumed to have the value of 6.57×10^7 sec^{-1} for all three algae (reference 1).

inoperative by DCMU, the rate constant of radiationless dissipation (α_h) can be calculated from the formula (16, 17):

$$\alpha_h = \frac{1}{\tau} - \frac{1}{\tau_0}. \qquad (2)$$

We have assumed that τ_0 for Chl *a* in vivo in all three algae is 15.2 nsec, the value obtained by Brody and Rabinowitch (1) for Chl *a* in vitro. This is a valid approximation, since the absorption and fluorescence spectra of Chl *a* in vitro and in vivo are quite similar. Introducing the measured τ values into equation 2, we find that the rate constant of radiationless dissipation is about 5×10^8 sec^{-1}, for Chl *a* in *Chlorella*; in *Porphyridium*, it is 9×10^8 sec^{-1}, and in *Anacystis*, 13×10^8 sec^{-1} (Table II). Since fluorescence comes predominantly from photosystem II, the values calculated apply to the bulk Chl *a* of this photosystem.

B. Rate of Energy Trapping at Room Temperature

The rate constant of energy trapping (α_t) can be calculated from (reference 17):

$$\alpha_t = \frac{1}{\tau'} - \frac{1}{\tau}, \qquad (3)$$

where τ' is the fluorescence lifetime when all reaction centers are "open." This must be the case at the 0 level in the fluorescence induction curve, or at low intensity of excitation. (For the description of fluorescence induction, see references 18 and 19.) Substituting the measured value, 0.6 nsec (15), for τ' into equation 3, we find that the rate constant of trapping in *Chlorella* is about 1.2×10^9 sec^{-1}, and the efficiency of trapping, 66% (Tables II and III). For *Porphyridium*, the fluorescence intensity in point 0 is about a third of that when DCMU is added (16). Hence, the lifetime of Chl fluorescence in point 0 is ⅓ of 1.03, or 0.35 nsec. With this value for τ', the rate constant of trapping becomes 1.97×10^9 sec^{-1}, and the efficiency of trapping, 68%. In the case of *Anacystis*, the 0 level of fluorescence is about one-half

TABLE III

THE QUANTUM EFFICIENCY* OF EACH DECAY PROCESS OF THE EXCITED FIRST SINGLET STATE OF CHL *a* IN VIVO FOR PHOTOSYSTEM II

Sample	ϕ_f	ϕ_h	ϕ_t
Chlorella	0.04	0.30	0.66
Porphyridium	0.02	0.30	0.68
Anacystis	0.02	0.38	0.60

* $\phi_f = \frac{\alpha_f}{\alpha_f + \alpha_h + \alpha_t}$, $\phi_h = \frac{\alpha_h}{\alpha_f + \alpha_h + \alpha_t}$, $\phi_t = \frac{\alpha_t}{\alpha_f + \alpha_h + \alpha_t}$.

the fluorescence level with DCMU (7).[4] Hence, assuming τ' to equal 0.3 nsec, the trapping rate constant becomes 1.98×10^9 sec^{-1}, and the efficiency of the trapping process, 60%.

In a rough approximation, the efficiency of trapping (60–70%) calculated here, compares well with the values (66–82%) obtained by other investigators (see Hoch and Knox [20], Weber [17], and Müller et al. [7]). The differences in trapping efficiency are, in all likelihood, caused by different physiological conditions of the cells used. We recall, for example, that the quantum yield of oxygen evolution (at 680 nm) is as high as 0.12 in young, but as low as 0.07 in "mature" *Chlorella* cells (21).

A comparison of the three algae shows that the efficiency of energy trapping in system II of *Anacystis* is significantly lower than in that of *Chlorella* and *Porphyridium*. In *Anacystis*, the efficiency of radiationless dissipation (that includes energy transfer to weakly fluorescent system I) is as high as 38%; this high value explains why the lifetime of Chl *a* fluorescence of normal *Anacystis* cells is almost the same as that of poisoned cells, an observation confirmed by the fact that the quantum yield of Chl *a* fluorescence under steady-state conditions is the same in normal and in DCMU-poisoned cells.[4] The reason for rapid, radiationless dissipation may be either efficient energy transfer to only weakly fluorescent photosystem I or to rapid loss by "internal conversion," or to both causes.

The rate of radiationless dissipation is higher in *Porphyridium* than in *Chlorella*. As the trapping rate constant in the former also is larger than in the latter, the trapping efficiency is about the same in both of them.

C. Rate Constant of Radiationless Dissipation at 77°K

Table IV shows the phase shifts θ obtained at 77°K with the three algae, using Corning CS 2-64 filter or 7-59 sharp cutoff filter (in front of the photomultiplier) (Corning Glass Works, Corning, N. Y.). The former has zero transmittance up to

[4] Mohanty, P. K. Personal communication.

TABLE IV

PHASE SHIFT MEASUREMENTS OF ALGAE AND METHYLENE BLUE AT 77°K*

	Chlorella		*Porphyridium*		*Anacystis*		Methylene blue (23°C)	
	θ(2-64)	θ(7-59)	θ(2-64)	θ(7-59)	θ(2-64)	θ(7-59)	θ(2-64)	θ(7-59)
	54.7	60.0	37.1	40.9	33.7	36.1	14.3	16.4
	51.1	56.6	34.5	39.0	24.3	26.4		
	52.0	58.0	33.7	38.8	25.6	27.8		
	52.0	56.9	34.9	39.9	24.2	27.7		
	51.1	55.4	31.5	36.4	28.9	30.5		
	54.8	59.5			22.4	24.7		
	51.1	58.2						
	49.6	55.2						
	49.1	54.5						
Average θ	51.7	57.1	34.3	39.0	26.5	28.8		
"Corrected" θ	51.7	55.0	34.3	36.9	26.5	26.7	14.3‡	14.3

* θ is the phase shift in degrees; 2-64 and 7-59 are the Corning cutoff filters; exciting wavelength, 632.8 nm; frequency of modulation, 102.207 MHz.

‡ Merkelo and coworkers (personal communication) have made some experiments in which they were unable to demonstrate consistently the needed corrections. The corrections used here are only valid if methylene blue fluoresces with constant τ vs. λ. We assume that it does; however, we cannot rule out the possibility that the 2.1° difference for methylene blue may, instead of an error, be also a real property of fluorescence emission of methylene blue.

640 nm, 50% at 670 nm, and 85% at 720 nm and longer wavelengths; the latter, zero transmittance up to 685 nm, 50% at 710 nm, and 85% at 750 nm and longer wavelengths. The results obtained with all three algae show that the phase shift, and thus τ, is higher when the CS 7-59 filter is used. This filter cuts off most of the fluorescence band at 685 nm (F-685), and allows mainly the F-730 band to go through. Part of the increase in phase shift may be due to differences in transit times of lights of different wavelengths in the photomultiplier (6) (see footnote ‡ in Table IV). The phase shift may be corrected for this error by assuming that the difference in phase shift, measured with methylene blue with the same two cutoff filters, is entirely an artifact. (This assumption is correct if methylene blue has a single τ.) The corrected phase shift still shows a higher θ (and thus τ) value with the CS 7-59 than with the CS 2-64 filter.

The fluorescence lifetimes of the F-685 and the F-730 bands can only be approximately estimated from these data. First, a rough estimate of the percentage of total fluorescence contributed by each band was made from fluorescence emission spectra of the algae at 77°K (11, 22, 23). These estimates are shown in Table V. F-695 is assumed to have the same fluorescence lifetime as F-685. In *Chlorella*, the lifetimes

TABLE V

FLUORESCENCE LIFETIME OF CHL *a* IN ALGAE AT 77°K*

	Chlorella		*Porphyridium*		*Anacystis*	
	F-685	F-730	F-685	F-730	F-685	F-730
Corning filter						
2-64	0.25	0.75	0.45	0.55	0.50	0.50
7-59	0.06	0.94	0.13	0.87	0.16	0.84
θ, *degrees*	42.0	56.0	30.2	38.0	26.2	26.8
τ, *nsec*	1.40	2.31	0.91	1.22	0.77	0.79

* The first two horizontal lines in the table show the calculated percentage of the total Chl fluorescence in each band as transmitted by the cutoff filter; the third line is the phase angle θ, and the last line, the lifetime (τ) for each band; F-685 and F-730 are the two main fluorescence bands at 77°K.

of F-685 and F-730 were estimated by solving the simultaneous equations (for derivation, see the Appendix):

$$0.25 \cos \theta_1 \sin (51.7 - \theta_1) + 0.75 \cos \theta_2 \sin (51.7 - \theta_2) = 0,$$

$$0.06 \cos \theta_1 \sin (55.0 - \theta_1) + 0.94 \cos \theta_2 \sin (55.0 - \theta_2) = 0. \quad (4)$$

The approximate solution for θ_1 is 42.0° and for θ_2, 56.0°. Corresponding τ values are 1.4 nsec for F-685 and 2.31 nsec for F-730. Similarly, in *Anacystis*, τ (F-685) is 0.77 nsec and τ (F-730), 0.79 nsec; in *Porphyridium*, τ (F-685) is 0.91 nsec and τ (F-730), 1.22 nsec. In each case τ (F-730) $>$ τ (F-685). This result was expected because the yield of F-730 increases at 77°K (see references 11, 22, 23, and literature cited there). Our τ values (0.7–2.3 nsec) are smaller than the 3.1 nsec that Butler and Norris (8) found for the lifetime of F-730 in bean leaves at 77°K. This may be attributed to the use of different biological specimens; note that in *Anacystis* τ (F-730) is much smaller than in *Chlorella*.

In all three algae, the fluorescence lifetime of F-685 at 77°K is similar to that at room temperature with DCMU added. If one assumes that the rate constant of fluorescence (α_f) does not significantly change from room temperature to 77°K, then α_h must be temperature independent. This result agrees with the fact that α_h for Chl *a* in vitro also is temperature independent. Butler and Norris (8) found that 10^{-5} M Chl *a* dissolved in ethanol has the same lifetime at room temperature and at liquid nitrogen temperature. Since the mechanism of radiationless dissipation (that may include intersystem crossing from singlet to triplet) is not known, we will not speculate on the significance of this result.

The experimental results obtained at 77°K point to three facts. First, the fluorescence at F-730 does not have the same lifetime in all species, but varies from 0.79 to 2.3 nsec. Second, the lifetime of F-685 at 77°K (0.77–1.40 nsec) is similar to that

at room temperature with DCMU added (0.74–1.74 nsec). Third, one of the characteristics of radiationless energy dissipation in Chl *a* in vivo appears to be that its rate is independent of temperature down to 77°K.

D. Maximum Quantum Yield of Chlorophyll Fluorescence

Quantum yield of Chl fluorescence (ϕ) can be determined directly by measuring the ratio of the number of emitted quanta to that of absorbed quanta as was first done by Latimer et al. (24) (also see Weber and Teale [26]); however, it can also be calculated from the relationship

$$\phi = \frac{\tau}{\tau_0}, \tag{5}$$

where τ_0 is assumed to be 15.2 nsec (1). It was found by Brody and Rabinowitch (1) that the two methods of calculating ϕ do not agree closely, because in the direct method we include absorption by weakly fluorescent (or nonfluorescent) Chl molecules, whereas calculation from τ values gives ϕ for the fluorescent Chl molecules only. In the present-day theory of photosynthesis (26) this probably means that ϕ from equation 5 will give the ϕ values of Chl *a* in the fluorescent system II, while direct determination averages it for systems I and II. Equation 5 permits calculation of ϕ values at 77°K, where it is difficult to measure the number of absorbed quanta and thus determine ϕ by the direct method.

Table VI shows the ϕ values calculated from equation 5 for room temperature with DCMU added, and for 77°K. The quantum yield of Chl fluorescence (F-685) at 77°K is approximately the same as at room temperature with DCMU

TABLE VI

QUANTUM YIELD OF (STEADY-STATE) CHLOROPHYLL *a* FLUORESCENCE AS CALCULATED FROM FLUORESCENCE LIFETIME MEASUREMENTS*

Conditions	*Chlorella*	*Porphyridium*	*Anacystis*
With DCMU (mainly system II, at room temperature)	0.11	0.07	0.05
At low light intensity (at room temperature)	0.04	0.022	0.019
F-685 at 77°K (mostly system II)	0.09	0.06	0.05
F-730 at 77°K (mostly system I)	0.15	0.08	0.052
Predicted average yield for intact cells (combined systems I and II) at room temperature‡	(*a*) 0.025 (*b*) 0.02	0.0175 0.011	0.0125 0.009

* λ excitation, 632.8 nm.

‡ For explanation of *a* and *b* rows, see text.

added: about 10% in *Chorella*, 6–7% in *Porphyridium*, and 5% in *Anacystis*. We note that ϕ of Chl fluorescence at 77°K is only twice that at room temperature and not 10- or 20-fold as in references 27–29. The error in the latter values may have been caused by difficulties in measuring the true path length and, thus, determining the true number of absorbed quanta.

The ϕ values for F-730 at 77°K are somewhat larger than for F-685. Assuming $\tau_0 = 15.2$ nsec, they are 15, 8, and 5% for *Chlorella*, *Porphyridium*, and *Anacystis*, respectively. There is, however, a discrepancy if we calculate ϕ of F-730 as follows. In the case of *Chlorella* we have assumed in Table V that F-685 fluorescence is 25% and F-730 fluorescence, 75%, of the total fluorescence. If ϕ is calculated as 9% for F-685, then ϕ for F-730 is $0.75/0.25 \times 9\%$, or 27%, assuming, of course, that the size of system I is the same as that of system II. Thus, there are two choices for ϕ of F-730:

(*a*) 15%. τ_0 is assumed to be 15.2 nsec. This assumption may be challenged because the F-730 band is broader than the F-685 band, and thus the Chl *a* form responsible for it may have a lower τ_0. It has been shown, however, that the F-730 band is composed of several bands including the vibrational satellite bands of F-685 and F-695 (11, 22, 24). Also, analyses of excitation spectra of long wave fluorescence indicate that the long wave absorption bands of Chl *a*, responsible for the F-730 complex band, are similar in shape to those of Chl *a*-670 and Chl *a*-680. Thus, we retain the assumption that τ_0 for the F-730 band is the same as that for F-685. To explain why the F-730 fluorescence yield is three times that of F-685, while the calculated yield increases only by a factor of two (from 0.09 to 0.15) one has to make a further assumption that system I is larger than system II; that is, either system I absorbs more light at 632.8 nm, or more energy is transferred to system I than to system II (as suggested by Murata et al. [29]).

(*b*) 27%. If we accept this value, we have to suggest that our basic assumption that τ_0 remains 15.2 nsec for the Chl *a* form that fluoresces at 77°K is wrong. The absorption spectrum at 77°K is not drastically different from that at room temperature. For this reason, and for the reasons given under *a* above, we favor the first explanation. We cannot make a final choice between the two possibilities; however, whichever assumption proves correct, the order of magnitude of the yield (15–27%) remains far below the 60–80% suggested on the basis of direct measurements.

At room temperature, the fluorescence yield (ϕ) of Chl *a* in vivo is *roughly* doubled when the intensity of incident light is increased from a low value (in the linear portion of the light curve of photosynthesis) to a high value (in the saturating portion of the light curve of photosynthesis [18, 30–32]). If we assume that the quantum yield of Chl *a* fluorescence in saturating light has the same value as when DCMU is added, we can make some predictions for the yield under other conditions. Since ϕ for system II in the presence of DCMU is 10, 7, and 5% for *Chlorella*, *Porphyridium*, and *Anacystis*, respectively, the ϕ for system II at low light intensities

would be 5, 3.5, and 2.5%. Assuming that light energy is almost equally distributed between pigment system I (almost nonfluorescent or very weakly fluorescent, at room temperature) and pigment system II (strongly fluorescent), at 632 nm (wavelength of excitation in our experiments), the average for both systems in weak light can be predicted to be 2.5, 1.75, and 1.25% for *Chlorella*, *Porphyridium*, and *Anacystis*, respectively (see *a* in the last row of Table VI). These predicted values agree well with most of the earlier measurements (24, 25, 33).

Independent calculations of ϕ from τ values at low light intensity (0.6 nsec for *Chlorella*, 0.34 nsec for *Porphyridium*, and 0.3 nsec for *Anacystis*) lead to values of 4, 2.2, and 1.9% for *Chlorella*, *Porphyridium*, and *Anacystis*, respectively. Assuming that the size of system I equals that of system II the predicted yields for intact cells (combined system I and II at room temperature) are 2, 1.1, and 0.9% for *Chlorella*, *Porphyridium*, and *Anacystis* (see *b* in the last row of Table VI). These values are in approximate agreement with those predicted above.

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APPENDIX

Assume that in response to a pulse excitation the fluorescence of a material has two exponential decay components characterized by decay constants τ_1 and τ_2. Under sinusoidal excitation, these fluorescence signals will produce, individually, corresponding phase shifts θ_1 and θ_2 which are related to the fluorescence lifetimes by the well-known expression:

$$\tan \theta_{1,2} = \omega\tau_{1,2}, \qquad (A\ 1)$$

where ω is the radial frequency of excitation. The intensity of the combined fluorescence, $I(t)$, in response to a sinusoidal excitation can be written in the form:

$$I(t) = A \cos \theta_1 \sin (\omega t - \theta_1) + B \cos \theta_2 \sin (\omega t - \theta_2), \qquad (A\ 2)$$

where A and B are constants. This expression can also be written as:

$$I(t) = Cf(\theta_1, \theta_2) \sin (\omega t - \phi), \qquad (A\ 3)$$

where C is a constant, f is a function of θ_1 and θ_2 only, and ϕ is the composite phase shift measured with a fluorimeter; ϕ can be expressed in terms of θ_1 and θ_2 only as a transcendental function. Another approach to relate ϕ to θ_1 and θ_2 is to identify the maxima and minima of $I(t)$.

Clearly, from expression A 3, these are at $t_f = (\phi/\omega) + (n\Pi/\omega) + (\Pi/2\omega)$ where $n = 0, 1, 2 \cdots$. The first derivative of A 2 will be zero for these values of time:

$$A \cos \theta_1 \cos (\omega t_f - \theta_1) + B \cos \theta_2 \cos (\omega t_f - \theta_2) = 0, \quad (A\,4)$$

or

$$A \cos \theta_1 \sin (\phi - \theta_1) + B \cos \theta_2 \sin (\phi - \theta_2) = 0. \quad (A\,5)$$

Knowing the relative amplitudes of the two fluorescence components A and B, under at least two conditions, and the composite phase shift ϕ, the fluorescence lifetime of each component can be determined.

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