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TRANSFER AND TRAPPING OF EXCITATION ENERGY IN PHOTOSYSTEM II AS STUDIED BY CHLOROPHYLL a_2 FLUORESCENCE QUENCHING BY DINITROBENZENE AND CAROTENOID TRIPLET

THE MATRIX MODEL

ARIE SONNEVELD, HENK RADEMAKER and LOUIS N.M. DUYSSENS

Department of Biophysics, Huygens Laboratory of the State University, P.O. Box 9504,
2300 RA Leiden (The Netherlands)

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Summary

1. The curves representing the reciprocal fluorescence yield of chlorophyll a of Photosystem II (PS II) in *Chlorella vulgaris* as a function of the concentration of m -dinitrobenzene in the states P Q and P Q⁻, are found to be straight parallel lines; P is the primary donor and Q the primary acceptor of PS II. In the weakly trapping state P Q⁻ the half-quenching of dinitrobenzene is about 0.2 mM, in vitro it is of the order of 10 mM. The fluorescence yield as a function of the concentration of a quencher is described for three models for the structure of pigment systems: the model of separate units, the model of limited energy transfer between the units, and the matrix model. If it is assumed that the rate constant of quenching by dinitrobenzene is high and thus the number of dinitrobenzene molecules per reaction center low, it can be concluded that the pigment system of PS II in *C. vulgaris* is a matrix of chlorophyll molecules in which the reaction centers are embedded. Theoretical and experimental evidence is consistent with such an assumption.

For *Cyanidium caldarium* the zero fluorescence yield Φ_0 and its quenching by dinitrobenzene were found to be much smaller than the corresponding quantities for *C. vulgaris*. Nevertheless, our measurements on *C. caldarium* could be interpreted by the assumption that the essential properties (rate constants, dinitrobenzene quenching) of PS II are the same for these two species belonging to such widely different groups.

2. The measured dinitrobenzene concentrations required for half-quenching *in vivo* and other observations are explained by (non-rate-limiting) energy transfer between the chlorophyll *a* molecules of PS II and by the assumptions that dinitrobenzene is approximately distributed at random in the membrane and does not diffuse during excitation.

3. The fluorescence kinetics of *C. vulgaris* during a 350 ns laser flash of variable intensity could be simulated on a computer using the matrix model. From the observed fluorescence quenching by the carotenoid triplet (C^T) and the measurement of the number of C^T per reaction center via difference absorption spectroscopy, the rate constant for quenching of C^T is calculated to be $k_T = 3.3 \cdot 10^{11} \text{ s}^{-1}$ which is almost equal to the rate constant of trapping by an open reaction center (Duysens, L.N.M. (1979) CIBA Foundation Symposium 61 (New Series), pp. 323–340).

4. The fluorescence quenching by C^T in non-treated spinach chloroplasts after a 500 ns laser flash (Breton, J., Geacintov, N.E. and Swenberg, C.E. (1979) Biochim. Biophys. Acta 548, 616–635) could be explained within the framework of the matrix model when the value for k_T is used as given in point 3.

5. The observations mentioned under point 1 indicate that the fluorescence yield Φ_0 for centers in trapping state P Q is probably for a fraction exceeding 0.8 emitted by PS II.

Introduction and Theoretical considerations

Chlorophyll a_2 fluorescence quenchers and aim of the measurements

In photosynthesis light is absorbed by the antenna pigments and the excitation energy is transferred from one antenna molecule to another and finally reaches a reaction center. The energy is trapped in this center and the primary photochemical reaction takes place. During this process of transfer and trapping, small losses occur, amongst other things in the form of fluorescence. There are several cell constituents which quench the fluorescence yield Φ of PS II: (i) the primary electron acceptor Q [1], (ii) the oxidized primary donor P^+ [2–9], and (iii) the carotenoid triplet C^T , which is formed during the lifetime of the excitation, especially in the state $P Q^-$ [4,6,9–12]. Amongst other things, the functional relationship between Φ and the concentration of one of these quenchers, $\Phi(\text{quencher})$, gives important information about the process of energy transfer and trapping of energy in PS II and the morphological and structural properties of the pigment system. The different functions of $\Phi(\text{quencher})$ may be determined experimentally by measuring both Φ and the concentrations of Q, P^+ and C^T under the same conditions. The relative fluorescence yield, Φ , can be precisely determined by measuring the ratio of the intensity of chlorophyll fluorescence and incident light. However, measurements of the concentrations of Q, P^+ and C^T require sensitive and rapid absorbance difference spectroscopy; because these concentrations are mostly less than 1% of that of chlorophyll, the signal-to-noise ratios are small.

(iv) Further evidence is obtained by using artificial fluorescence quenchers, which can be applied in a large range of concentrations, and which do not require ΔA measurements, in contrast to the *in vivo* fluorescence quenchers. In

the past numerous artificial quenchers of the chlorophyll *a* fluorescence in vitro and in vivo have been used [13–30]. We have chosen *m*-dinitrobenzene which is one of the most efficient quenchers and also has other advantages as described in the section Results and Interpretation.

In most earlier publications with experimental applications, such as Refs. 31 and 28, a variety of equations for the function $\Phi(\text{quencher})$ was used. These equations were not rigorously derived but rather postulated in a heuristic way without defining the conditions of validity. In the following section we will derive several, partly novel, equations which describe the function $\Phi(\text{quencher})$ for various models of the pigment system. In a number of papers the consistency of the evidence with one model was shown, but not the inconsistency with other models. We show that the mathematically simple matrix model appears to be the only model of the three models considered, that is consistent with the fluorescence experiments on PS II of different algae under aerobic conditions.

Theoretical considerations

In this section equations will be derived which will relate the fluorescence yield to the fraction of active traps and/or quenchers for various models of the pigment system. Moreover, the computer simulation of the time behaviour of the matrix model will be discussed.

(a) We start with the separate units model and assume that PS II consists of identical separate units, each containing N_0 ($N_0 \approx 200$) chlorophyll molecules and one reaction center, and that no transfer of energy between the units occurs. When de-excitation of an excited chlorophyll molecule can take place by fluorescence (k_f), internal conversion (k_{ic}), intersystem crossing to the triplet state (k_{isc}), trapping in the case of the reaction center chlorophyll molecule (k_t) in the open state P Q and k'_t in the weakly trapping state P Q⁻ and energy transfer to another molecule in the unit (k_h), we can write

$$P_F = \frac{N_0 k_f}{N_0 k_1 + k_t} \quad (1)$$

if $k_h > k_t \gg N_0 k_1$ [33]; P_F is the fluorescence yield of a (particular) unit in the open state P Q and $k_1 = k_f + k_{ic} + k_{isc}$. If k_t in this equation is replaced by k'_t one obtains P_F for a unit in the state P Q⁻.

If an artificial quencher of the chlorophyll fluorescence is present, then the following equation is valid for the fluorescence yield P_{Fi} for an open unit:

$$P_{Fi} = \frac{N_0 k_f}{N_0 k_1 + k_t + i k_d} \quad (2)$$

where it is assumed that one chlorophyll molecule is quenched by one quencher molecule. The number of quenchers per unit (or the number of chlorophyll molecules quenched) is i and k_d is the corresponding rate constant for quenching, $k_d < k_h$. Statistical fluctuations in the number of quencher molecules per unit may be expected to occur. The number of quenchers per unit may be described by a Poisson distribution. If D is the average number of

quenchers per unit, the fluorescence yield Φ for a system of PS II units can be expressed as:

$$\Phi = \sum_i P_i \cdot P_{Fi} \quad (3)$$

where P_i is the probability for a unit to have i quenchers. Because of the Poisson distribution we can write for P_i :

$$P_i = \frac{e^{-D} \cdot D^i}{i!} \quad (4)$$

(b) The model of limited energy transfer between the photosynthetic units. We use a model which is somewhat different from the model of Joliot and Joliot [32]. We have dealt extensively with this model in a previous paper [9]. Here we only mention that in this case there exists an additional rate constant for chlorophyll de-excitation in the form of k_{tr} ; k_{tr} , the rate constant for energy transfer to a chlorophyll molecule in another unit, 'averaged' over all chlorophyll molecules in a unit. For this model the fluorescence yield P_{Fi} for an open unit is given by:

$$P_{Fi} = \frac{N_0 k_f}{N_0(k_1 + k_{tr}) + k_t + i k_d} \quad (5a)$$

and the fluorescence yield Φ for a system of such units by:

$$\Phi = \frac{\sum_i P_i P_{Fi}}{1 - (k_{tr}/k_f) \sum_i P_i P_{Fi}} \quad (5b)$$

if $k_{tr} \gg k_1$ and $k_h \gg N_0 k_{tr}$ [9,34].

(c) In the matrix model (k_h is assumed to be larger than all other k values) the pigment system consists of a large number of antenna chlorophyll molecules in which the reaction centers are regularly distributed. There are no boundaries for energy transfer. Thus, we can write for a system with open reaction centers:

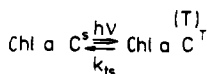
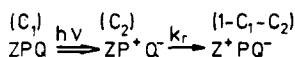
$$\Phi = \frac{N_0 k_f}{N_0 k_1 + k_t + D k_d} \quad (6)$$

The Poisson distribution does not play a role in Eqn. 6 because the excitation during its lifetime visits all chlorophyll molecules an equal number of times, including molecules where the energy is trapped or quenched.

By means of the matrix model the rather complex kinetics of fluorescence observed during a laser flash, in which the concentrations of P^* , Q and C^T are time-dependent, can easily be described. For this purpose Eqn. 6 is written:

$$\Phi = \frac{N_0 k_f}{N_0 k_1 + k_t(C_1 + C_2) + k_t'(1 - C_1 - C_2) + T k_T} \quad (7)$$

where T is the average number of C^T molecules per reaction center, which is formed during a flash, and k_T the rate constant for quenching by C^T ; C_1 is the



Scheme I. Scheme for photochemical and dark reactions occurring in Photosystem II. The (fractional) concentrations of the various states are given between brackets. C^S and C^T stand for a carotenoid molecule in the ground and triplet state. The kinetics according to the matrix model are described by Eqns. 7–12. For further explanation see text.

fraction of reaction centers in the state P^+Q^- , C_2 the fraction of centers in the state $P^+Q^{(-)}$, which presumably (see Refs. 2–9) have the same k_t as the P^+Q^- centers, and the complement $(1 - C_1 - C_2)$ the fraction in the state P^+Q^- . Assuming further that C^T is formed via the chlorophyll triplet and calling the denominator of Eqn. 7 X , it follows that:

$$\Phi_t = k_t C_1 / X \quad (8)$$

and

$$\Phi_{isc} = k_{isc} / X \quad (9)$$

where Φ_t means the trapping yield in a matrix of open centers ($C_1 = 1$) and Φ_{isc} the yield for the formation of the quencher C^T . Taking $I(t)$ as the time-dependence of the number of quanta/trap per s we can write simple differential equations for the time-dependence of the various states (see Scheme 1). The equations are:

$$dC_1/dt = -I(t)\Phi_t \quad (10)$$

$$dC_2/dt = I(t)\Phi_t - k_r C_2 \quad (11)$$

$$dT/dt = I(t)\Phi_{isc} - k_{ts} T \quad (12)$$

in which $C_1(0) = 1$, k_r is the rate constant for the reduction of P^+ by the secondary donor Z of PS II ($k_r \approx 30 \mu s^{-1}$ [9]) and k_{ts} the rate constant for the decay of C^T to the singlet ground state ($k_{ts} \approx 0.33 \mu s^{-1}$ [10]). The intensity-time course $I(t)$ is the time profile of the laser flash.

In Table I the values of the different parameters used for the computer calculations are given. These values are obtained from Ref. 33 and differ some-

TABLE I

VALUES OF THE DIFFERENT RATE CONSTANTS (s^{-1}) FOR DE-EXCITATION OF A (REACTION CENTER) CHLOROPHYLL MOLECULE IN THE LOWEST EXCITED SINGLET STATE WHICH ARE USED IN THE MODEL CALCULATIONS

N_0 is the number of chlorophyll molecules per reaction center or photosynthetic unit. Because of the lower ratio of Φ_{max} to Φ_0 for *C. vulgaris* in Fig. 4A $k_t' = 9 \cdot 10^{10}$ for Fig. 4B (see also Ref. 9).

$k_t = 6.7 \cdot 10^7$	$k_t = 4 \cdot 10^{11}$	$N_0 = 200$
$k_{ic} = 0$	$k_t' = 7.1 \cdot 10^{10}$	
$k_{isc} = 1.5 \cdot 10^8$	$k_{tr} = 30$ [9]	

what from the values mentioned in Ref. 9 because of the assumption made here and in Ref. 33 that the rate constant for energy transfer (k_h) is not rate-limiting.

Materials and Methods

Materials

Chlorella vulgaris was grown as described previously [35]. After centrifugation the algae were suspended in a 5 mm cell in fresh growth medium and brought to an absorbance of 0.2 at 680 nm, after subtracting the absorbance at 750 nm from that of 680 nm as a correction for scattering. All measurements were done at room temperature (≈ 295 K) with a closed horizontal $30 \times 30 \times 5$ mm³ cuvette after a dark adaptation period of about 3 min in order to obtain the zero fluorescence level Φ_0 .

Cyanidium caldarium was grown in Allen medium [36] at 311 K and continuously bubbled with air containing 5% CO₂ at a flow rate of 100 l/h. The Allen medium was brought to a pH 3.5 by the addition of small amounts of Kratz' medium C [37]. The algae were illuminated with fluorescent light at an average light intensity of 3000 lux.

m-Dinitrobenzene from BDH was dissolved in alcohol to a concentration of 0.15 M. Small amounts of this solution were added to 6 ml of an algal suspension and the mixture was bubbled for about 20 min with air and 5% CO₂. The final alcohol concentration in the mixture was always below 2% and did not change the fluorescence yield of the algae.

Apparatus

The relative fluorescence yield of the algae was measured using the apparatus described in [38] and [39]. All the data were finally stored on disc and further processed by a Digital Equipment 11/45 computer. The state of maximal fluorescence, Φ_{\max} , was measured in a weak flash (< 100 nJ/cm²) 60 μ s after an oversaturating flash ($E \approx 600$ μ J/cm²) from a ruby laser [38,39]. In some experiments the relative fluorescence yield was determined with the experimental set-up used for the fluorescence induction measurements (see below). Also in this case the excitation was not actinic.

The apparatus and methods used for the measurements of the fluorescence yield kinetics in the time interval 0–650 ns are described in Ref. 9.

The concentration of the carotenoid triplet was measured using a single beam absorption spectrophotometer equipped with a Lambda Physik dye laser for actinic illumination; 32 traces were averaged in order to obtain a good signal-to-noise ratio. The triplet decay time was about 4 μ s.

Fluorescence induction in the presence of 28 μ M DCMU was measured by means of a Bausch and Lomb monochromator (100 cm² grating, 1200 lines per mm, 2 cm slit, slit-width of 2.5 nm) at 680 or 685 nm. Exciting light from a tungsten iodine lamp (24 V, 250 W) driven by a stabilized current power supply (Oltronix C28-10R) was filtered by the band filters Schott KG 3/2, Corning CS 4-96 and CS 5-61, and a Balzer interference filter B40. The maximum transmission of the filter set occurred at 417 nm and the half-width was about 7 nm. The monochromator was also used for the measurement of the fluorescence spectra.

Results and Interpretation

A. Quenching by *m*-dinitrobenzene

In the concentration range 0–2 mM no influence of dinitrobenzene on the absorption spectrum of *C. vulgaris*, measured with a Cary 14 spectrophotometer, could be detected within the limits of accuracy of the measurement ($\approx 1\%$). This also indicates that dinitrobenzene is not chemically changed by the algae. After washing with fresh medium the fluorescence yield was restored to the original level, measured before dinitrobenzene was added. This indicates that dinitrobenzene is removed by washing and shows that its quenching effect is reversible. Dinitrobenzene practically did not change the shape and position of the fluorescence emission spectrum of *C. vulgaris*.

In Fig. 1 typical plots of the reciprocal fluorescence yield, under Φ_0 and Φ_{\max} conditions, against the [dinitrobenzene] in the aqueous phase are shown for *C. vulgaris*. The quenching curves are straight and parallel. Using the Eqns. 3, 5b and 6 we have calculated the relationship between $1/\Phi$ and the concentration of quencher, D , for the three models of pigment systems, separate units, units with limited energy transfer and matrix, respectively. In Fig. 2 A and B the quenching curves for the different models are plotted for various values of D and k_d , taking for each calculation a constant product of k_d and the maximal concentration of D , because this product determines the extent of quenching. The absolute values of D and k_d are not known. The theoretical quenching curves for the matrix model presented in Fig. 2 A and B depend (see Eqn. 6) only on the product Dk_d and show a straight and parallel behaviour for Φ_0 and Φ_{\max} quenching, as was found experimentally. The quenching curves in the case of the separate units and limited transfer of energy between the units are not straight and parallel, as shown in Fig. 2A. For high numbers of quencher per trap, however, the statistical fluctuation of the number of quencher per unit is small and, therefore, Fig. 2B displays almost equal straight and parallel lines. If, however, the average number of quenchers per unit was small, only the matrix model would be consistent with the observations. We return to this subject in the Discussion.

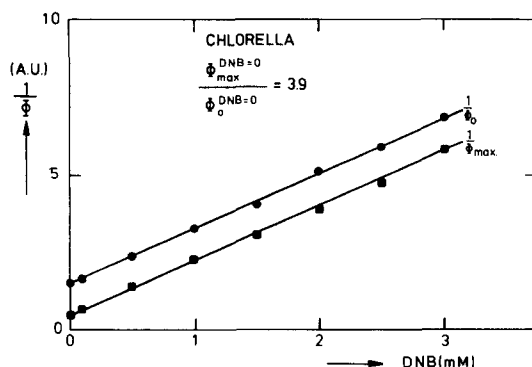


Fig. 1. Quenching of chlorophyll fluorescence of *Chlorella vulgaris* in states Φ_0 and Φ_{\max} by dinitrobenzene: the reciprocal fluorescence yield, $1/\Phi$, is plotted as function of the dinitrobenzene concentration in the aqueous phase. Excitation occurred at 433 nm and the emission was measured at 674 nm.

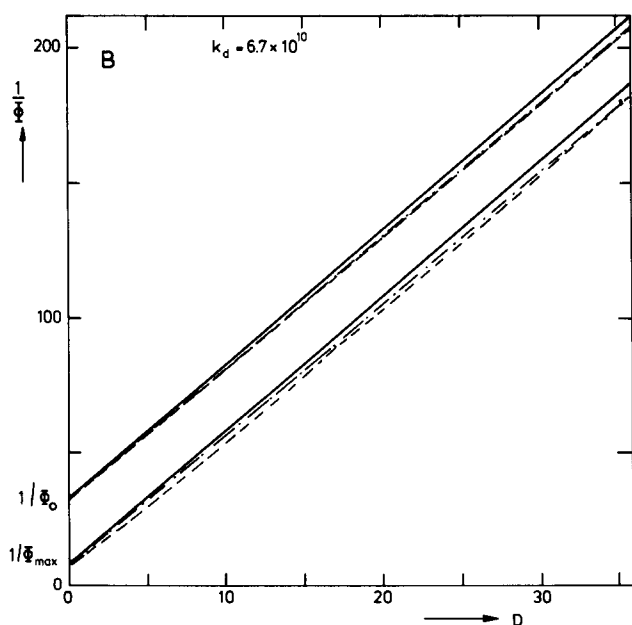
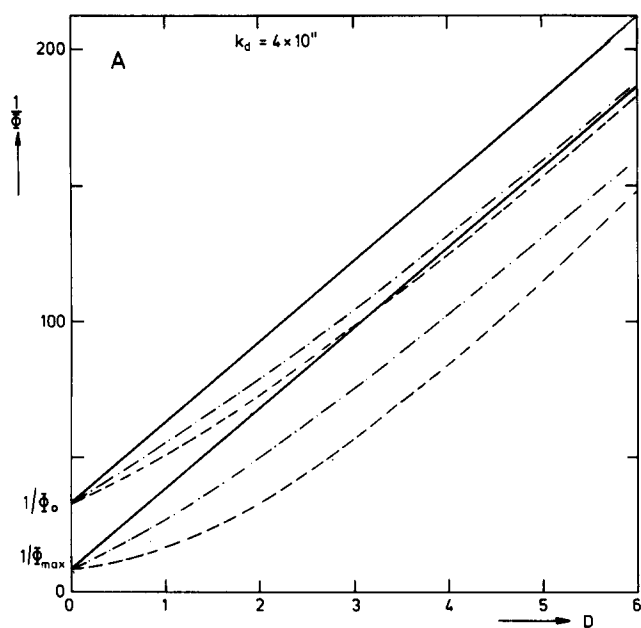


Fig. 2. A and B Simulation of Fig. 1 using three different models of pigment systems as presented in the Introduction: the matrix model (—), the model of limited energy transfer between units (· — · — ·), and that of separate units (-----). The reciprocal fluorescence yield, $1/\Phi$, is plotted as function of the average number of artificial quencher molecules per unit or reaction center, D ; $k_d(\text{s}^{-1})$ is the rate constant of quenching and is different for each figure. In (B) the curves (· — · — ·) and (-----) practically coincide.

Similar experiments have been performed with the phycocyanin-containing alga *Cyanidium caldarium*. Fig. 3 shows a plot of the reciprocal fluorescence yield as a function of the dinitrobenzene concentration for *C. caldarium* in the state Φ_0 . One observes that dinitrobenzene quenches the fluorescence of *C. caldarium* much less than that of *C. vulgaris* in the state Φ_0 (See Fig. 1). This marked difference can be explained as follows. *C. vulgaris* contains about 200 chlorophyll molecules per reaction center, in contrast to *C. caldarium* which has a much smaller unit, about 5 reaction centers per 200 chlorophyll molecules [40]. Let us add, for instance, one molecule of dinitrobenzene per 200 chlorophyll molecules and assume that this molecule quenches the fluorescence as strongly as a reaction center. This situation would mean for *C. vulgaris* a 2-fold increase of the quenching as the number of quenchers is doubled. On the other hand, the fluorescence yield of *C. caldarium* will only decrease with about 20% because the relative increase of the number of trapping centers is much smaller.

If the PS II systems in two species A and B only differ by the number of chlorophyll *a* molecules per reaction center, and if Φ_0 is emitted by an active part of the pigment system, and if $k_t \gg N_0 k_1$ and Dk_d , one would expect the fluorescence yield to be proportional to the number of chlorophyll molecules per reaction center (see Eqn. 6). Using Eqn. 6, if no artificial quencher is present, it follows that $\Phi_0^A/\Phi_0^B = N_0^A/N_0^B$, where Φ_0^A is the zero fluorescence yield of a system with units with N_0^A chlorophyll molecules per reaction center and Φ_0^B the corresponding yield for a system with N_0^B chlorophyll molecules per trap. In the presence of artificial quenchers it follows from Eqn. 6 that the ratio of the slopes of the 'normalized' quenching curves ($\Phi_0/\Phi_0^{\text{DNB}}$ vs. the concentration of dinitrobenzene; Φ_0^{DNB} is the fluorescence yield in the presence of dinitrobenzene (DNB)) of A and B will be N_0^A/N_0^B .

For the algae *C. vulgaris* and *C. caldarium* we took as a measure of N_0^A and N_0^B the half-times of the fluorescence rise in the presence of DCMU. The excitation occurred at 417 nm, where for *C. caldarium* the energy is mainly absorbed by chlorophyll *a* and not by phycocyanin. The difference in contributions of carotenoids, which presumably is not appreciable, is not considered. The results obtained are given in Table II. The experimental data of Φ_0 (*C. vulgaris*)/

TABLE II

LOWEST FLUORESCENCE YIELD (Φ_0) OF *CHLORELLA VULGARIS* AND *CYANIDIUM CALDARIUM*, AND THE HALFTIME ($t_{1/2}$) OF THE FLUORESCENCE RISE OF DARK ADAPTED ALGAE IN THE PRESENCE OF 28 μM DCMU AS MEASURED WITH CONTINUOUS LIGHT

Excitation occurred at 417 nm where the absorbance of the algae was 0.1 A unit. Since for this wavelength of excitation the shapes of the fluorescence emission spectra are almost the same, Φ_0 has been measured at the fluorescence maxima of 685 and 680 nm, respectively; the slitwidth of the monochromator was 2.5 nm. a.u., arbitrary units.

	Φ_0 (a.u.)	$t_{1/2}$ (ms)
<i>C. vulgaris</i>	1088	39
	1070	43.5
<i>C. caldarium</i>	143	280
	146	290

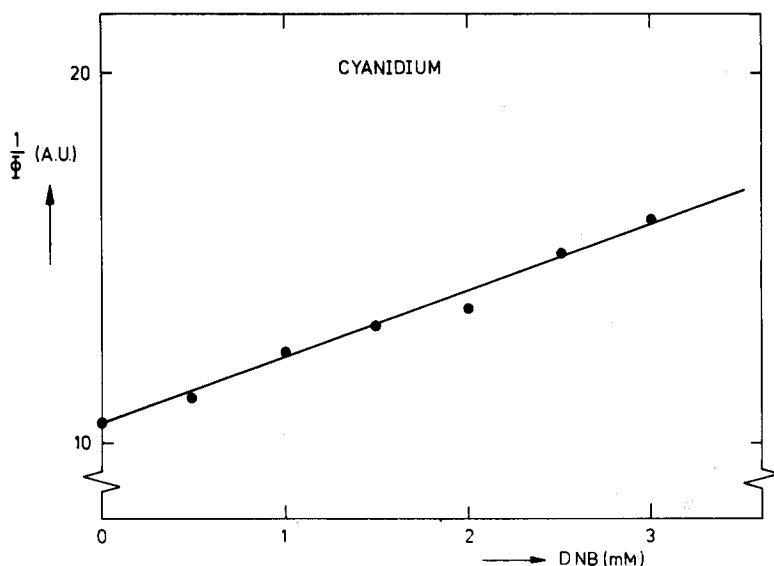
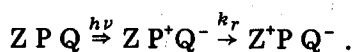


Fig. 3. The quenching of the chlorophyll fluorescence of *Cyanidium caldarium* in state Φ_0 by dinitrobenzene: the relative reciprocal fluorescence yield, $1/\Phi$, is plotted as function of the dinitrobenzene concentration in the aqueous phase. Excitation occurred at 433 nm and the emission was measured at 674 nm. Note that for $1/\Phi$ the same units as for Fig. 1 are used.

Φ_0 (*C. caldarium*) = 7.5 and $t_{1/2}$ (*C. caldarium*)/ $t_{1/2}$ (*C. vulgaris*) = 6.9, which means N_0 (*C. vulgaris*)/ N_0 (*C. caldarium*) = 6.9, are in reasonable agreement with the assumptions made before. Furthermore, these values also agree with the ratio of the slopes of the 'normalized' Φ_0 -quenching curves of *C. vulgaris* and *C. caldarium* which was found to have a value of 7.1. This follows from the non-normalized curves of Figs. 1 and 3, which have equal slopes.

B. Laser-induced fluorescence induction simulated with the matrix model

Fig. 4A shows the fluorescence kinetics of *C. vulgaris* during a 350 ns laser flash (see Fig. 4C) of variable intensity. Because of some fluctuation along the time ordinate the curves are uncertain and therefore dotted at times < 50 ns. At time zero all curves start at the fluorescence level Φ_0 . Laser flashes with intensities up to just saturating cause the reactions



This leads to a fluorescence increase due to the formation of the only weakly quenching state $P Q^-$. If the intensity of the flash is not rate-limiting the rate of the fluorescence rise is determined by the rate of reduction of the fluorescence quencher P^+ . This occurs with a rate constant k_r by the secondary donor Z of PS II. At intensities higher than saturating ($\geq 2\%$), one observes a fluorescence decrease after the initial fluorescence rise. This decrease even below Φ_0 can be attributed to the formation of the fluorescence quencher C^T which is populated rather efficiently after the reaction centers are 'closed'. In a previous paper [9] we showed a computer simulation of the fluorescence curves of Fig. 4A using a

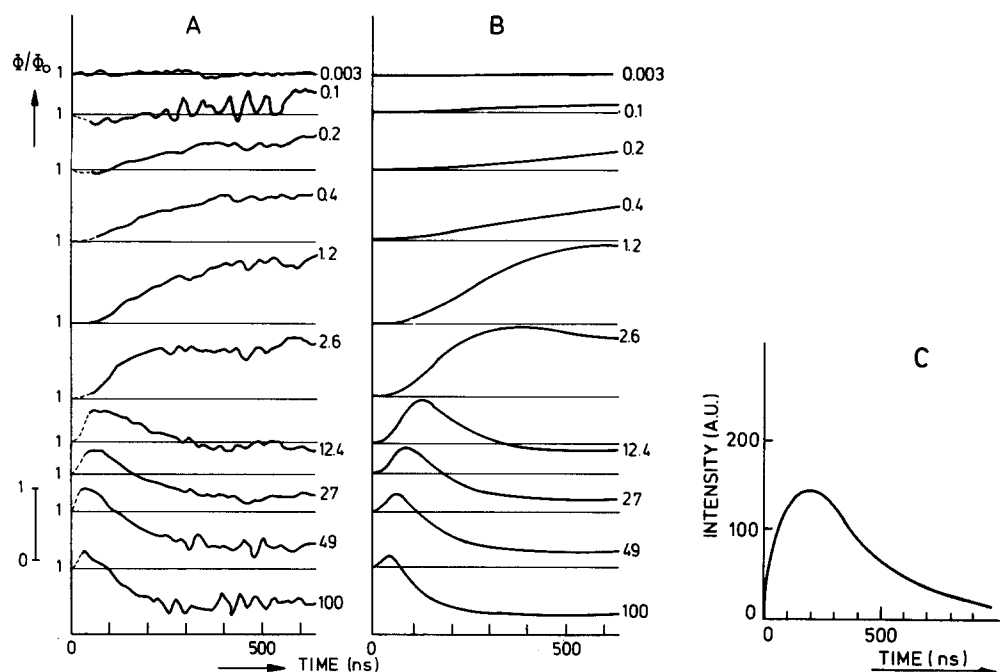


Fig. 4. (A) Relative fluorescence yield Φ of dark adapted *Chlorella vulgaris* during a 350 ns dye laser flash (see Fig. 4C) of intensity as indicated in percents of the maximum intensity on the right. Three measurements are averaged. (B) Simulation of Fig. 4A using the matrix model (Eqns. 7–12) as presented in the Introduction; for the rate constant of quenching by C^T the best fit value $k_T = 3.3 \cdot 10^{11} \text{ s}^{-1}$ has been used. (C) The intensity time course of the 350 ns flash of the dye laser.

model of the pigment system with limited transfer between the photosynthetic units (model b). In Ref. 9 the formation of C^T was assumed to be limited to one triplet per unit (not necessarily connected to the reaction center but randomly formed via k_{isc}). In the matrix model the formation of C^T is also assumed to be at random but not limited to one triplet per unit, in agreement with experimental evidence that the number of C^T triplets per unit or reaction center can be much larger than one [41–43]. Since the results of the dinitrobenzene fluorescence quenching strongly indicate that the matrix model is the correct description of the pigment system (see Discussion), it appears necessary to repeat the computer simulation of Fig. 4A using the matrix model. Fig. 4B shows the result of the simulation for a reduction time of the oxidized primary donor P^+ by the secondary donor Z of 35 ns [9] and a value of $3.3 \cdot 10^{11} \text{ s}^{-1}$ for the rate constant of quenching by C^T , k_T . This value of k_T was found to give the best fit. Over the whole intensity range the calculated fluorescence kinetics show a good correspondence with the experimental kinetics. For the intensities 12.4 and 27 the correspondence between experimental and simulated kinetics is better for the matrix model than for the model in which limited energy transfer between the units was assumed. It is concluded that the matrix model is consistent with the carotenoid triplet quenching.

We are able to compare the number of C^T per reaction center calculated in the simulation of the fluorescence kinetics and the concentration we measured

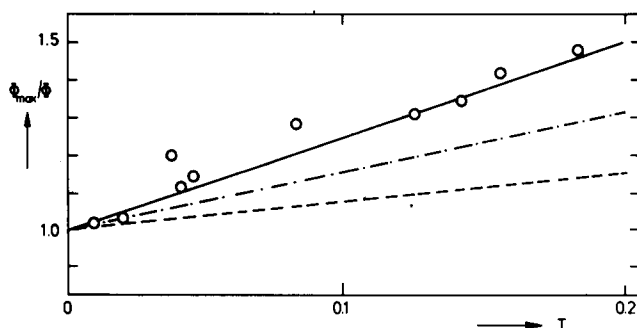


Fig. 5. The reciprocal of the chlorophyll fluorescence yield (Φ) plotted as a function of T ; T is the average number of C^T per unit or reaction center and $\Phi(T=0) = \Phi_{\max}$. The different curves are calculated using three different models of pigment systems as discussed in the Introduction: the matrix model (—), the model of limited energy transfer between units (- · - · -), and that of separate units (- - - -). The rate constant of quenching by C^T , $k_T = 3.3 \cdot 10^{11} \text{ s}^{-1}$, is obtained from Fig. 4B. The experimental points (\circ) are obtained from Ref. 42. For further explanation see text.

via the 520 nm absorbance change which is due to C^T . At 520 nm a typical absorbance change of $9 \cdot 10^{-4}$ was found at the laser intensity of about $2 \cdot 10^{15} \text{ photons} \cdot \text{cm}^{-2}$ (\approx intensity of 5% in Fig. 4). Using a molar absorption coefficient for C^T of $1.5 \cdot 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [41] we obtain a value for C^T of about 0.5 per PS II reaction center, if 200 chlorophyll molecules per reaction center are present. This agrees well with the number of 0.6 C^T per reaction center which is calculated by the simulation model for the same intensity of the laser flash.

Very recently Breton et al. [42] reported a 'Stern-Volmer' relationship for spinach chloroplasts between the relative fluorescence yield and the concentration of C^T after a 500 ns laser flash of $3 \cdot 10^{14} \text{ photons} \cdot \text{cm}^{-2}$. We used the Eqns. 3, 5b and 6 for the simulation of the results of Breton et al. This was done by replacing k_d by k_T and D by T in these equations. We used a value of $3.3 \cdot 10^{11} \text{ s}^{-1}$ for k_T . This value for k_T is obtained from the results shown in Fig. 4 A and B. The maximal value of C^T per PS II reaction center measured by Breton et al. was 2, at a flash energy of $3 \cdot 10^{16} \text{ photons} \cdot \text{cm}^{-2}$. For the energy of $3 \cdot 10^{14} \text{ photons}$ we estimate (on basis of the model used for Fig. 4) this value to be about 0.2, using the above mentioned value of 0.5 C^T per reaction center for $2 \cdot 10^{15} \text{ photons} \cdot \text{cm}^{-2}$. The relation between the concentration of C^T and the flash energy is far from linear according to our model, because the first triplet quenches the formation of the second triplet. Fig. 5 indicates that the matrix model is the only model of the three models considered, that is consistent with the results obtained by Breton and coworkers [42]. In Fig. 5 Φ_{\max}/Φ is plotted as a function of T , because a flash with an energy of $3 \cdot 10^{14} \text{ photons} \cdot \text{cm}^{-2}$ will convert almost all reaction centers to the weakly quenching state $P Q^-$ (Φ_{\max}).

Discussion

A. *m*-Dinitrobenzene quenching and the matrix model

First the large differences are discussed between the experimentally observed half-quenching concentrations of dinitrobenzene in vitro (11 mM) and in vivo (0.22 mM for Φ_{\max}). The observed half-quenching concentration of 11 mM in

an organic solution [13] can be explained by the assumption that no complexes are formed between chlorophyll *a* and dinitrobenzene, and that the quenching is determined by the diffusion of dinitrobenzene and excited chlorophyll towards each other (cf. Refs. 18 and 19). If dinitrobenzene is dissolved in the membrane its diffusion in the extremely viscous membrane can be considered as negligible during the lifetime of the excited chlorophyll (≤ 2.5 ns). A chlorophyll molecule then will be quenched directly only if it is 'in touch' with a dinitrobenzene molecule. Because of extensive transfer of excitation energy between chlorophyll *a* molecules [25,26,28,33,44], the excitation of chlorophyll molecules not adjacent to a dinitrobenzene molecule will be quenched in an indirect way. So the process of (non-rate-limiting) energy transfer will considerably decrease the concentration of dinitrobenzene needed for half-quenching. This picture is consistent with our observation that lowering the temperature to liquid nitrogen temperature does not markedly decrease quenching, which would happen if diffusion of dinitrobenzene were a prerequisite for quenching.

The following model and calculation yields dinitrobenzene concentration of the right order of magnitude for half-quenching of the Φ_{\max} of chlorophyll *a* in vivo.

1. The dinitrobenzene concentration in the membrane is about the same for membrane lipid as in the aqueous medium. We found a partition coefficient of 2.5 for dinitrobenzene between water and hexane. Unfortunately, we were not able to determine the partition coefficient between water and membrane directly, but if we make the assumption that the partition coefficient is of the order of 2, then the rate of trapping of dinitrobenzene, k_d , must be equal to that assumed under point 2 and vice versa, because $k_d D$ follows from the experiment.

2. The rate constant of quenching of a chlorophyll molecule by an adjacent dinitrobenzene molecule, k_d , is the same as that for trapping in a reaction center in the state P Q ($k_t = 4 \cdot 10^{11} \text{ s}^{-1}$). This is consistent with rates which can be estimated from in vitro dinitrobenzene quenching [18].

3. The dinitrobenzene molecules are distributed at random in the membrane.

4. The chlorophyll molecules in the membrane are accessible to dinitrobenzene from all sides in a similar way as in a solution.

The calculation is as follows: From Eqn. 6 (with k_t replaced by k'_t), using the rate constants and N_0 of Table I and the above-mentioned value of k_d , $4 \cdot 10^{11} \text{ s}^{-1}$, we calculate the average number of dinitrobenzene molecules that produces half-quenching in vivo to be one per 700 chlorophyll molecules (other models (Eqns. 3 and 5b) would give somewhat larger values). This requires, in a medium in which diffusion can be neglected, a concentration of about 0.5 mM dinitrobenzene. This can be calculated as follows. From the molecular weights of chlorophyll *a* and dinitrobenzene and assuming a density of $1.25 \text{ g} \cdot \text{cm}^{-3}$ (see also Refs. 18 and 45) for both components it follows that the radii of the spheres, in which one molecule is present, are roughly 6.8 and 3.8 Å, respectively. Consider an arbitrary chlorophyll *a* molecule. This would be quenched by dinitrobenzene, if the center of one or more dinitrobenzene molecules would be within a sphere of radius $6.8 + 3.8 = 10.6$ Å with the same center as the chlorophyll *a* molecule. This sphere has a volume of 5 nm^3 . As calculated

we need only one dinitrobenzene molecule per 700 chlorophyll *a* molecules or on the average 1/700 dinitrobenzene molecule per 5 nm³, which corresponds to a concentration of $(6 \times 10^{23} \times 700 \times 5 \times 10^{-24})^{-1} = 0.5$ mM. If the partition coefficient for dinitrobenzene between membranes and water is 2.5, then the concentration for half-quenching would be 0.2 mM in water as found experimentally. It seems likely that only part of the chlorophyll molecules is accessible to dinitrobenzene. Then a somewhat higher concentration of dinitrobenzene would be required, which might indicate that dinitrobenzene is slightly attracted to places where the chlorophyll is accessible, or that the partition coefficient is somewhat higher, or some other parameter is different from that estimated.

The foregoing discussion shows that half-quenching of the chlorophyll *a* fluorescence in vivo is obtained for a number of less than one dinitrobenzene molecule per reaction center if we assume that $N_0 = 200$ chlorophyll molecules per center are present. This corresponds with the model simulations displayed in Fig. 2A and not with those in Fig. 2B for which much higher numbers of dinitrobenzene molecules per reaction center are needed. The theoretical curves for the matrix model presented in Fig. 2A correspond with the experimental results displayed in Fig. 1, in contrast to the quenching curves for the models of limited transfer and separate units, which are somewhat bent and non-parallel curves. Therefore we conclude that the pigment system of PS II in *C. vulgaris* can for a large part be considered as a matrix of chlorophyll molecules in which the reaction centers are embedded. This conclusion is consistent with results obtained with *C. caldarium* (see Fig. 3 and Table II). This alga has much smaller units than *C. vulgaris*. As already mentioned in the section Results and Interpretation the matrix model was found to be consistent with the observed differences between the two species.

In summary, the quenching by dinitrobenzene can be consistently described by assuming the matrix model, random distribution, a low partition coefficient (2.5), a high rate of quenching ($4 \cdot 10^{11} \text{ s}^{-1}$) and no diffusion. Different assumptions seem to be rather unplausible, e.g. if the separate unit model is assumed for *C. caldarium*, which has units of about 30 chlorophylls, the observed linear relationship between the reciprocal fluorescence and dinitrobenzene concentration (see Fig. 3) can only be simulated with a partition coefficient of about 100 or more and thus a k_d of about 10^{10} s^{-1} or less, a value at least ten times lower than that estimated from in vitro experiments [18].

It would be desirable to check our conclusions by determining k_d directly by means of picosecond absorption difference spectrophotometry. Another check would be a direct determination of the partition coefficient, which is not easy either because of the low concentration of membranes. A preliminary experiment on *C. vulgaris* yielded a value between 0 and 50.

From the effect of dinitrobenzene on oxygen pulses in subsequent flashes and other evidence, it was concluded by Etienne et al. [28] that dinitrobenzene at a concentration of 0.5 mM eliminated photochemical activity and fluorescence dequenching in a non-negligible fraction of the reaction centers. Such an effect would make the slope of the Φ_{\max} curve in Fig. 1 steeper than that of the Φ_0 curve, in contrast to the parallelism reported by us. Possibly the effect of dinitrobenzene on the oxygen pulses is caused by the use of actinic flashes

close to that of saturation, combined with the somewhat increased initial value of the ratio S_1/S_0 of the S-states in the presence of dinitrobenzene.

B. Heterogeneity of Photosystem II and the origin of Φ_0

Experiments by Melis and coworkers [46–48] indicated that there are two types of PS II in spinach chloroplasts, α and β , of which the largest, α , is a matrix system, and β a smaller system consisting of separate units. It has been shown by Melis and Schreiber [48] that the presence of the β centers, which are responsible for about 15% of the variable fluorescence yield emission, causes a deviation of the hyperbolic relationship between the fluorescence and the concentration of the primary acceptor in the reduced state, Q^- , that was to be expected for a pure homogeneous matrix system. This corresponds to a non-linear relationship between the reciprocal fluorescence Φ^{-1} and the concentration of Q^- ($= Q_{\alpha}^- + Q_{\beta}^-$). Van Gorkom and coworkers, however, reported for Tris-washed chloroplasts a linear plot of Φ^{-1} vs. the concentration of Q^- [49]. These results have been discussed in terms of the matrix model [33]. In Tris-washed preparations the fluorescence changes caused by the minor subsystem β are probably suppressed and the Φ_0 of the subsystem is small (cf. Ref. 33).

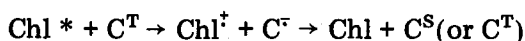
The analysis of the analogous quenching by dinitrobenzene for *C. vulgaris* and *C. caldarium* (Figs. 1 and 3) given in the preceding section did not reveal heterogeneity of PS II: the quenching curves (Φ^{-1} vs. concentration of dinitrobenzene) were found to be linear. In *C. vulgaris*, in contrast to spinach chloroplasts, the contribution of the β subsystem to the fluorescence may well be smaller than 15%. So far measurements of β centers have not been reported for *C. vulgaris*. From preliminary experiments we conclude that the percentage of β centers in *C. vulgaris* is very low.

In the past it was often suggested that Φ_0 consists of 'dead' fluorescence not caused by PS II [32] or contains an appreciable contribution of Photosystem I fluorescence [26,28,43]. If, as is generally accepted, Photosystem I does not possess an appreciable variable fluorescence, these suggestions are not in agreement with the linear and parallel quenching curves of Fig. 1 which indicate that Φ_0 is largely emitted by one type of chlorophyll *a* of PS II, which is also responsible for the variable fluorescence. Simple calculations based on matrix systems show that Φ_0 is probably emitted by PS II for a fraction exceeding 0.8, even if D and k_d would be equal for Photosystems I and II. Moya [50] concludes, just like ourselves, from the proportionality between Φ and τ_f (fluorescence lifetime) that the dead fluorescence in *C. vulgaris* at 685 nm is negligible. The linearity of the curve is not discussed by Moya but is consistent with the matrix model as proposed by us. From the fluorescence quenching and the inhibition of primary photochemistry by dibromothymoquinone in chloroplasts at 77 K, Kitajima and Butler [20] concluded that the major part of Φ_0 is emitted by chlorophyll *a* of PS II. This conclusion is based indirectly on the validity of their Eqns. 1 and 3. However, if the data of their Fig. 3, which gives Φ_{\max} and Φ_0 as function of the concentration of dibromothymoquinone, are plotted as in our Fig. 1, strongly curved lines are obtained not in agreement with the Eqns. 1 and 3 of their model. This curvature may be caused by removal of the quencher by photoreduction [51] or by the occurrence of separate units at liquid nitrogen temperature (our Eqn. 3 and Refs. 52 and 53).

Fluorescence quenching by the carotenoid triplet C^T

As already mentioned the matrix model is consistent with the experimental results shown in Figs. 4A and 5. Figs. 4B and 5 have been simulated using a value for the rate constant of quenching by C^T that was almost equal to the rate constants for quenching by Q and P^+ . Further it was assumed that C^T is rapidly formed via the chlorophyll triplet and for the formation of the latter the rate constants of chlorophyll *a* in solution (in vitro) were supposed to be valid. This means that in the state $P Q^-$ of all reaction centers with a fluorescence yield $\Phi_{\max} \approx 12\%$, about 28% of the energy is lost to the triplet state of chlorophyll from which C^T arises. This corresponds well with results of Den Haan [10] who estimated, assuming that C^T had the same rate constant for quenching as Q, that in the state $P Q^-$ a carotenoid triplet was formed with an efficiency of about 24%. These efficiencies are consistent with the measurement of a concentration of $0.5 C^T$ per reaction center at a light intensity of $2 \cdot 10^{15}$ photons \cdot cm $^{-2}$, because with the help of the matrix model a number of $0.6 C^T$ was calculated for the same intensity. Apparently the in vitro parameters used in the model are valid in vivo as well.

One might ask what kind of quenching occurs between a chlorophyll *a* molecule in the lowest excited singlet state, Chl *, and C^T . Because there does not seem to be an appreciable $T_0 \rightarrow T_n$ absorption around 685 nm, de-excitation of Chl * may not be due to energy transfer from Chl * to C^T . Therefore, analogously to the fluorescence quenching of Chl * by colourless quenchers, which takes place by means of an electron transfer reaction, the following reaction for quenching of Chl * by C^T is considered:



in which the overall reaction has an efficient rate constant k_T ; C^S is a carotenoid molecule in the singlet ground state. The rate constant $k_T = 3.3 \cdot 10^{11}$ s $^{-1}$ used in the model calculations is of the same order as rate constants estimated for certain electron transfer reactions in vitro [18] and in vivo [54].

The above mentioned reaction presumably has a higher probability than the corresponding reaction between Chl * and C^S , as in the former reaction extra energy (≈ 0.9 eV) from the triplet of C^T is available for charge separation. Because the reduction potentials of C^T and C^S are not known, we cannot estimate the standard free energy changes of the redox reactions. By means of rapid absorbance difference spectroscopy the mechanism of these reactions may be established.

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List of symbols

Symbols used for one equation are defined in situ.

Q	the 'primary' electron acceptor of Photosystem II which is a plastoquinone molecule; Q^- is the reduced state, the plastoquinone anion [55,56]
P	$P-680$ = reaction center chlorophyll <i>a</i> dimer of Photosystem II [51]; P^+ is the oxidized state of P
C^T	carotenoid molecule in the lowest triplet state
T	average number of carotenoid triplets per unit or reaction center
i	number of artificial fluorescence quenchers per unit or reaction center
D	the average of i for a system of units or reaction centers
N_0	number of chlorophyll <i>a</i> molecules per unit or reaction center
k_f	rate constant for de-excitation of a chlorophyll <i>a</i> molecule in the lowest excited singlet state by fluorescence emission
k_{ic}	rate constant for de-excitation via internal conversion
k_{isc}	rate constant for de-excitation via intersystem crossing
k_t	rate constant for energy trapping by the reaction center in the state P Q
k_t'	rate constant for energy trapping by the reaction center in the state $P Q^-$
k_h	rate constant for energy transfer from an excited chlorophyll molecule to another chlorophyll molecule (in the same unit)
k_{tr}	rate constant, 'averaged' over all chlorophyll molecules in a unit for energy transfer to a chlorophyll molecule in another unit; this rate constant only plays a role in the model of limited energy transfer between photosynthetic units
k_l	$k_f + k_{ic} + k_{isc}$, rate constant for energy loss
k_d	rate constant for quenching of an excited chlorophyll molecule by an artificial quencher molecule
k_T	rate constant for quenching by C^T
Φ	(relative) fluorescence yield of a system of units or chlorophyll molecules in vivo. Φ_0 is the zero fluorescence yield in the state P Q and Φ_{max} is the maximal yield in the state P Q^-
P_{Fi}	Φ for one (particular) unit; the subscript i stands for the number of artificial quenchers in that unit

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