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THE EXTENT OF ENERGY MIGRATION AND CHLOROPHYLL *A* ORIENTATION IN CHLORELLA

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SUMMARY

1. A method is described by which $\bar{n}$, the average number of energy transfers between different chlorophyll *a* molecules during the mean lifetime of the excited state, can be measured *in vivo*. The effect produced by added quencher molecules on the fluorescence excitation spectrum and yield enables $\bar{n}$ to be calculated without assumptions regarding the mutual orientation of the pigment molecules.

2. The calculated mean value of $\bar{n}$ was 275. Although subject to considerable errors, this value is comparable with $\bar{n}_r$ measured in Chlorella and in concentrated chlorophyll solutions by fluorescence depolarization. Because $\bar{n}_r$ is calculated on a basis of random molecular orientation, the chlorophyll *a* molecules *in vivo* cannot possess a high degree of preferred orientation.

3. The time interval occupied by each transfer process is calculated, together with the trapping efficiencies to be expected *in vivo*.

INTRODUCTION

In photosynthesis, resonance energy transfer appears to play an essential part in conveying energy derived from light absorption by pigment molecules to photochemically active centres present in relatively small concentration. This energy transfer is marked by the sensitization of both photosynthesis and chlorophyll *a* fluorescence by accessory pigments[1,2], and the low values of fluorescence quantum yield[3] and polarization[4] shown by chloroplasts.

The extent of energy migration among chlorophyll molecules in solution can be calculated from the observed fluorescence polarization of concentrated solutions in viscous media which prevent molecular rotations during the lifetime of the singlet excited state. The relationship

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3}{2}\overline{\mathrm{Sin}^2\theta}\cdot\bar{n}\right) \tag{1}$$

derived by WEBER[5], relates the observed polarization p to that in the absence of energy transfer p_0, the angle between the emission oscillators θ, and the average number of energy transfers $\bar{n}$ between different molecules before emission. For randomly oriented molecules, the case in solution, $\mathrm{Sin}^2\theta = 0.404$, so that the equation (1) becomes

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3}{5}\cdot n_r\right) \tag{2}$$

In systems which may not be randomly oriented, such as chlorophyll *in vivo*, $\bar{n}_r$ represents the minimum number of random transfers required to produce the observed depolarization. Since $\bar{n}$ represents an average value, in systems quenched by a process of energy migration to trapping centres the observed fluorescence will result from the shorter transfer sequences, and equation (2) becomes

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{\phi}{\phi_0}\frac{3}{5}\bar{n}_r\right) \tag{3}$$

where ϕ_0 is the fluorescence yield of the unquenched system, and ϕ the reduced yield produced by transfer quenching. Application of equation (3) in the case of chloroplast fluorescence, providing the appropriate value for ϕ/ϕ_0 is known, permits the calculation of $\bar{n}_r$, a minimal value which may be equivalent to more extensive energy migration in a system having some preferred orientation.

On the other hand, the fluorescence quenching produced by energy transfer to trapping centres is given by the relationship

$$\frac{\phi_0}{\phi} = \frac{1 + K\bar{n}T}{1 - T} \tag{4}$$

where T is that fraction of all the molecules which act as trapping centres having a relative probability of transfer K from the fluorescent species, compared with unit probability of transfer between the fluorescent species. K is related to the integrated overlap between the fluorescence spectrum of the donor molecule and the molecular absorption spectrum of the trapping molecule. $\bar{n}$ is the average number of transfers between different molecules without assumptions regarding mutual orientation.

Providing the quantities T and K are known in a system quenched by transfer to trapping centres, the value of $\bar{n}$ can be calculated, and if $\bar{n}_r$ is known the average angle between molecules is given by

$$\overline{\mathrm{Sin}^2\theta} = \frac{2\,\bar{n}_r}{5\,\bar{n}} \tag{5}$$

When additional trapping centres T_2 are added to a partly quenched system, the equation (4) can be written

$$\frac{\phi_0}{\phi_2} - \frac{\phi_0}{\phi_1} = \bar{n}K_2T_2 \tag{6}$$

where ϕ_1 and ϕ_2 are the fluorescence yields before and after the addition of trapping centres T_2 with transfer probability K_2, and where the total fraction of trapping molecules is small compared with unity.

In practice the number of trapping centres in chloroplasts can be increased by suspending the material in aqueous solutions of aromatic nitrocompounds[6] which quench chlorophyll fluorescence. In most cases no changes in chlorophyll absorption take place, so that $K_2 = 1$. The value of T_2, the fraction of quenched chlorophyll molecules produced by the nitrocompound cannot be calculated from the concentration of the aqueous solution employed to produce quenching. Fortunately in Chlorella the occurrence of chlorophylls *a* and *b*, which are both quenched by nitrocompounds, enables T_2 to be measured by the following considerations. The appearance of the fluorescence spectrum characteristic of chlorophyll *a* when Chlorella is excited by radiation absorbed predominantly by chlorophyll *b* indicates efficient energy transfer between these pigments, which has also been demonstrated *in vitro*. Providing the probability of transfer between chlorophyll *b* molecules is negligible compared with that for transfer to chlorophyll *a*, then we can represent the chlorophyll transfers *in vivo* by the scheme

$$\begin{array}{ccccc} b & \longrightarrow & [a] & \longrightarrow & \text{Fluorescence or trapping} \\ \uparrow{\scriptstyle \lambda_b} & & \uparrow{\scriptstyle \lambda_a}\,\bar{n} & & \end{array}$$

where λ_b and λ_a are radiation wavelengths exciting chlorophyll *b* and *a* respectively. The relative fluorescence yields ϕ_a and ϕ_b of the two chlorophylls can be written

$$\phi_a = \frac{1 - T_2}{1 + \bar{n}T_2} \qquad\qquad \phi_b = \frac{(1 - T_2)^2}{1 + \bar{n}T_2}$$

assuming that the nitrocompound is distributed uniformly and quenches a similar fraction of both chlorophylls. The ratio of the relative yields is $\phi_b/\phi_a = (1 - T_2)$, so that T_2 can be measured in terms of the change produced by quenching in the relative yields of chlorophylls *a* and *b*, together with change in ϕ_a. The value of $\bar{n}$ is given by the relationship

$$\bar{n} = \frac{(\phi_0/\phi_a')\,(\phi_b/\phi_a) - 1}{1 - \phi_b/\phi_a} \tag{7}$$

where ϕ_0 is the chlorophyll *a* yield in the absence of any quenching, and ϕ_a' the yield after the addition of the quenched fraction T_2.

EXPERIMENTAL

The most suitable quencher for use with Chlorella was found to be *m*-dinitrobenzene, which penetrated the chloroplasts rapidly and reversibly without producing detectable absorption or chemical changes. The polarization of the fluorescence of the chlorophylls in organic solvents was unaltered by quenching, suggesting that quenching results from the formation of a weak complex in the ground state, undetectable in absorption measurements. This conclusion was supported by the decreased quenching efficiency observed at higher temperatures.

Fluorescence measurements were made using a xenon arc lamp together with a grating monochromator to select exciting radiation of the desired wavelengths. The red fluorescence from the specimen cell was observed normally to the excitation direction through a glass filter (Corning 5850) which transmitted wavelengths longer than 680 mμ, and was detected with a red-sensitive photomultiplier (RCA 6217). In a series of readings with graded concentrations of quencher, equal amounts of Chlorella were used, so that the optical geometry was similar for all readings. At each excitation wavelength, the cell blank was eliminated by measuring the fluorescence intensity at progressive dilutions of the original cell suspension, and extrapolating to zero concentration. The wavelengths λ_b and λ_a were chosen as 480 mμ and 670 mμ respectively, and at each quencher concentration the relative yield ϕ_a was recorded, together with the ratio ϕ_b/ϕ_a.

Fluorescence polarization measurements were made with the polarization photometer described by WEBER[7]. Solutions of chromatographically pure pigments in cyclohexanol, having a maximum concentration of $8 \cdot 10^{-3}$ *M*, were examined in very thin layers[8] to avoid reabsorption effects.

RESULTS

In Fig. 1 typical plots of $\phi_a/\phi_{a'}$ and ϕ_b/ϕ_a against quencher concentration in the aqueous phase are shown for a specimen of Chlorella having an absolute quantum yield of chlorophyll ϕ_a of 2.7 % before quenching. Similar plots of the results of many experiments showed that the ratio ϕ_b/ϕ_a decreased by an average of 11 % for a four-fold decrease in ϕ_a. The degree of quenching produced by constant concentrations of *m*-dinitrobenzene was independent of the amount of Chlorella employed, showing that the amount taken up from solution is very small.

In order to calculate the relative transfer probabilities of the chlorophylls, the fluorescence excitation and fluorescence spectra were measured and are shown in Fig. 2. The excitation spectrum of chlorophyll *a* was derived from that of Chlorella by considerations of the band width and band maximum of the red fluorescence, and the excitation spectrum of the chlorophyll *b* component obtained by difference. The position assigned to the fluorescence spectrum of the latter pigment is tentative, but has been related to its excitation spectrum in the same manner as for chlorophyll *a*.

The fluorescence polarization of Chlorella excited with radiation of 660 mμ wavelength was 0.06 $\pm$ 0.01. In Fig. 3 the effect of concentration on the fluorescence polarization of chlorophylls *a* and *b* in cyclohexanol is shown. Cd 644 mμ radiation was used to excite fluorescence.

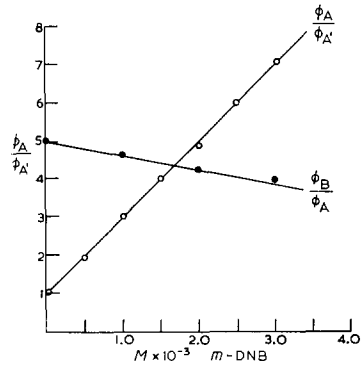

Fig. 1.

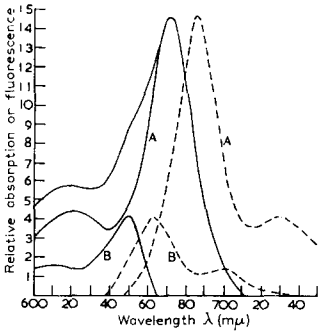

Fig. 2.

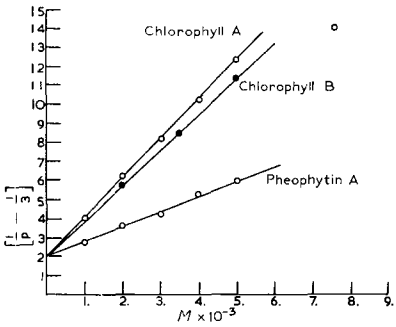

Fig. 3.

Fig. 1. The change in chlorophyll *a* yield expressed as the ratio $\phi_a/\phi_{a'}$ and the chlorophyll *b* yield expressed as the ratio ϕ_b/ϕ_a produced by *m*-dinitrobenzene quenching of Chlorella.

Fig. 2. The fluorescence and fluorescence excitation spectra of Chlorella, and the component chlorophylls. ----, fluorescence spectra; ——, fluorescence excitation spectra.

Fig. 3. The effect of concentration on the fluorescence polarization of concentrated solutions of chlorophylls *a* and *b* and pheophytin *a* in cyclohexanol.

DISCUSSION

According to the theory developed by FÖRSTER[9], the relative probability of transfer between chlorophyll *b* molecules compared with the transfer to chlorophyll *a* is given by the ratio

$$\frac{Pb \longrightarrow b}{b \longrightarrow a} = \frac{R^6_{ba}}{R^6_{bb}} \cdot \frac{\int_0^\infty I_\lambda \cdot b_\lambda}{\int_0^\infty I_\lambda \cdot a_\lambda}$$

where R_{b-a} and R_{b-b} are the average intermolecular separations of the respective chlorophyll molecules, and the integrals represent the integrated overlaps between the chlorophyll *b* fluorescence spectrum and the molecular absorption spectra of chlorophylls *a* and *b* respectively. This latter quantity was estimated from Fig. 2 as approximately 6. Substituting this value, and the relative concentration of the two pigments in the Chlorella, which had a molecular ratio $a:b = 3:1$, we have

$$\frac{Pb \longrightarrow b}{b \longrightarrow a} = \frac{1}{(3+1)^2} \times \frac{1}{6} = 0.01$$

This low relative probability of transfer means that excited chlorophyll *b* molecules transfer directly to neighbouring chlorophyll *a* molecules, and the assumptions made in the derivation of equation (7) appear justified.

A correction which must be applied to the observed change in the ratio ϕ_b/ϕ_a arises from the fluorescence sensitization by carotenoids at 480 mμ wavelength, whereas radiation of 670 mμ wavelength is absorbed almost entirely by chlorophyll *a*. The contribution due to carotenoid transfer, which from measurements made with brown algae does not appear to be affected by quenching, was estimated to be about 23 % at wavelength 480 mμ. The required correction factor is $1/(1-0.23)$ and the corrected value of $\phi_b/\phi_a = 0.143$. Substituting this value in equation (7), and setting $\phi_0 = 0.32$, the absolute quantum yield of chlorophyll *a in vitro*[10], we obtain

$$n = \frac{\left(\frac{0.32}{0.027} \times 4\right) - 1}{0.143} = 275$$

The errors involved in the measurement of $\bar{n}$ depend on many factors, some of which cannot be evaluated, such as the relative quenching efficiency of *m*-dinitrobenzene for the two chlorophylls *in vivo*, the distribution of the two pigments, the carotenoid contribution, and the uncertainty of the value of ϕ_0 *in vivo*. Nevertheless it is instructive to compare this value with $\bar{n}_r$, calculated both for Chlorella and for chlorophyll solutions. The interpretation of the observed polarization $p = 0.06$ in Chlorella depends on the value assigned to the ratio ϕ/ϕ_0 in equation (3). Provided the observed fluorescence originates from regions in the chloroplast which are not subject to competitive transfer quenching, we can set $\phi/\phi_0 = 1$ and $p_0 = 0.43$, and obtain $\bar{n}_r = 12$. This small value, compared with $\bar{n} = 275$, would suggest a high degree of molecular orientation, and applying equation (5), we obtain $\bar{\theta} = 5°$. The measured dichroism[11] of chloroplasts is certainly lower than would be expected from this figure, but could be lowered by structural features above the pigment molecular level. On the other hand, most probably the observed fluorescence of Chlorella results from a competitive quenching process which reduces the fluorescence yield from 0.32 to 0.027, so that $\phi/\phi_0 = 0.085$ and we obtain $\bar{n}_r = 141$. This value is more comparable with $\bar{n}$, and taken together indicate only a low degree of orientation. Moreover, this value of $\bar{n}_r$ is similar to those measured in concentrated solutions of chlorophyll *a*, on extrapolation to concentrations known to exist in the chloroplast[12]. Using the data shown in Fig. 3, $\bar{n}_r$ is 8.9 in a $5 \cdot 10^{-3}$ *M* solution of chlorophyll *a*. At this concentration, no concentration quenching took place, and from this value of $\bar{n}_r$ the intermolecular distance at which emission and transfer are equally probable can be calculated at 35 Å. Since $\bar{n}_r$ is linear with concentration in the absence of quenching, by extrapolation $\bar{n}_r$ would be 177 in 10^{-1} *M* solution. The similarity of this value to those for $\bar{n}$ and $\bar{n}_r$ in Chlorella means that concentration quenching must be negligible *in vivo*, despite the existence of chlorophyll concentrations sufficient to produce very strong quenching when observed in solutions of chlorophyll in organic solvents. Formation of non-fluorescent dimers which act as trapping centres undoubtedly produces concentration quenching, and presumably an efficient mechanism exists in the chloroplast which prevents this molecular association.

Both $\bar{n}$ and $\bar{n}_r$ measure the number of different molecules visited during the transfer sequence. Repeated exchanges between the same molecular pair produce no change in observed polarization or quenching provided no changes occur in molecular orientation or the distribution of quenched complexes during the fluorescence lifetime. The transfer process must proceed by a random walk, so that the actual number

of transfers will approach $\bar{n}^2$, or from $2.0\text{-}7.6 \cdot 10^4$. Taking the actual mean lifetime of chlorophyll *a* to be $6.6 \cdot 10^{-9}$ second[13], the time occupied by each transfer lies in the range $3.3\text{–}0.8 \cdot 10^{-13}$ sec. Transfer during time intervals of comparable brevity has been reported between close pairs of molecules in which the donor molecules have undetectable fluorescence yield[14,15].

The efficiency E, with which quanta are trapped by a fraction of trapping centres T, is given by rearranging equation (4),

$$E = \frac{K\bar{n}T}{1 + K\bar{n}T}$$

Assuming that for reasonable photosynthetic efficiency $E \geqslant 0.75$, at this arbitrary value $K\bar{n}T = 3$ or $KT = 3/\bar{n}$. For $\bar{n} = 275$ the required trapping efficiency would be achieved by trapping centres having the same absorption spectrum as chlorophyll *a* ($K = 1$), present as 1.1 of the total pigment. The change in the photochemical yield of the Hill reaction in chloroplasts with progressive fragmentation[16,17] also points to this order of concentration for the trapping centre. An absorption maximum at longer wavelengths, producing an increased overlap with the fluorescence spectrum of chlorophyll *a*, would increase the trapping efficiency in proportion to K. For a trapping centre with an absorption band coincident with the chlorophyll *a* fluorescence band ($\lambda_{max.} = 687$ mμ), $K \backsimeq 3$ and high efficiencies could be achieved with a distribution of one centre in an average of 300 chlorophyll molecules.

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