

ENERGETICS OF ASSOCIATED MOLECULES OF CHLOROPHYLLS *a* AND *b* AND
BACTERIOCHLOROPHYLL

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It is known that chlorophyll exists in the photosynthetic apparatus of plants either in an associated state or in the form of a monomer, the local concentration of which reaches 0.1 M. It is natural to expect that the mutual influence of pigment molecules may be extremely substantial, especially when the pigment molecule is in an excited state and can enter into a strong dipole-dipole interaction with neighboring molecules.

The main dilemma that arises for investigators of the primary physical steps in photosynthesis is as follows: Can the concept of intramolecular probability transitions in pigment molecules in solution be transferred to highly aggregated biological systems or are the interactions among molecules so great that the concept of intramolecular spectroscopic characteristics loses its meaning? There are extremely few experimental data on the energetics of associates of photosynthetic pigments. It is known that the associates, as a rule, do not fluoresce or fluoresce weakly. And yet, in [1] it was shown that the quantum yields of the triplet states of chlorophylls *a* and *b* in a dry nonpolar solvent (3-methylpentane) are low, and the signal of the observed triplet-triplet absorption is assigned to the residual monomer pigment. Data have appeared in the literature on the triplet states of associates, obtained according to a measurement of phosphorescence [2] and by the method of electron spin resonance [3]. However, the yield of triplet states in the associates has not been estimated.

In this work we present the results of a spectral-kinetic investigation of processes of deactivation of the energy of electronic excitation in associated molecules of the basic photosynthetic pigments: chlorophylls *a* and *b* and bacteriochlorophyll, formed in a dry nonpolar solvent — petroleum ether. In the work we used fluorescent measurements and the methods of pulsed photoexcitation.

Chlorophylls *a* and *b* were produced from Nettles according to the standard procedure [4] and chromatographed twice on powdered sugar. Bacteriochlorophyll was obtained from purple bacteria *Rhodospirillum rubrum* [5]. The measurements of the absorption spectra were performed on a Unicam Sp-800 spectrophotometer. The fluorescence spectra were measured on a Fica-55 spectrofluorimeter. The investigation of triplet states of the pigments was performed according to the T-T absorption spectra on a standard pulsed photoexcitation setup ($E = 100$ J, $\tau_{1/2} = 2 \cdot 10^{-5}$ sec) [6]. All the solutions were degasified on a vacuum setup to a residual air pressure of $2 \cdot 10^{-5}$ mm Hg. Petroleum ether (70–100°C) was thoroughly dried with CaCl_2 and redistilled over a potassium-sodium mirror. When frozen to $T = 77^\circ\text{K}$, petroleum ether formed a glassy matrix, convenient for spectroscopic investigations at low temperature. The working concentrations of the pigments were 10^{-5} – 10^{-4} M and were limited by their solubility in petroleum ether.

Spectral-Luminescent Properties

The absorption spectra of the investigated pigments are evidence of the presence of associated molecules in solution. From Fig. 1 it can be seen that there is a broadening and deformation of the absorption bands (especially the long-wave band) in petroleum ether. Our measurements of the circular dichroism (CD) spectrum of associated chlorophyll *a* showed that

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the CD band in the red region of the spectrum is formed by two components of equal intensity and different signs, with a splitting of about 400 cm^{-1} . This confirms the association nature of the phenomenon and is evidence of a dimer structure of the unit cell [7]. The presence of associates in solution is also indicated by the drop in the relative fluorescence yield of the solution.

Figure 2 presents the fluorescence spectra of chlorophyll α in dry petroleum ether. The fluorescence of monomer chlorophyll α in petroleum ether with an addition of a polar impurity (diethyl ether in a 6:1 ratio) is also cited for comparison. It can be seen that when the fluorescence is excited in the absorption band of the monomer ($\lambda_{\text{exc}} = 430 \text{ nm}$), primarily the emission of the residual monomer chlorophyll α is observed in the fluorescence spectrum (curve 2), although in this case a small broadening of the emission band is observed (half-width $\approx 20 \text{ nm}$) in comparison with a true monomer solution (half-width $\approx 18 \text{ nm}$). When the fluorescence is excited in the band of predominant absorption of the associate ($\lambda_{\text{exc}} = 450 \text{ nm}$), both emission of the monomer ($\lambda_{\text{max}} = 667 \text{ nm}$) and emission of the aggregated form of the pigment ($\lambda_{\text{max}} = 680 \text{ nm}$) are observed. An analogous phenomenon was observed in an investigation of the fluorescence spectra of chlorophyll b and bacteriochlorophyll in dry petroleum ether. It must be noted that decomposition of aggregates of bacteriochlorophyll requires an appreciably larger amount of the polar additive, which is due either to the greater strength of the bond between bacteriochlorophyll molecules in the associate or to the different ability of the pigment molecules for solvation by polar molecules. The fluorescence spectra also depend on the pigment concentration. Thus, when the chlorophyll α concentration is increased to $\sim 10^{-4} \text{ M}$, the emission bands with maxima $\lambda_{\text{max}} = 667$ and 680 nm practically disappear in the fluorescence spectrum, while at the same time new bands with maxima $\lambda_{\text{max}} = 695$ and $735\text{--}736 \text{ nm}$ appear, evidently associated with various forms of enlarged associates, which appear in solution. The data cited above permit an estimation of the absolute quantum yields of the fluorescence of associates. The estimate was performed by a relative method according to the well-known procedure of [8]:

$$B_a = B_m \cdot \frac{(I_{\text{fl}})_a}{(I_{\text{fl}})_m} \cdot \frac{1 - T_m}{1 - T_\Sigma} \cdot \frac{D_\Sigma}{D_a},$$

where B_a and B_m are the quantum yields of the fluorescence of the aggregated and monomer molecules; $(I_{\text{fl}})_a / (I_{\text{fl}})_m$ is the ratio of the areas under the curves of the true fluorescence spectra of the aggregated and monomer solutions; D_Σ and T_Σ represent the summary optical density and transmission of the aggregated solution at the wavelength of excitation; D_a is the optical density due to aggregates at the wavelength of excitation; and T_m is the transmission of a standard solution of the monomer pigment.

The quantum yields of fluorescence of associates measured in this way are cited in Table 1.

Triplet State of Associates

Pulsed illumination of solutions containing associates of the investigated pigments led to the appearance of T-T absorption. It should be noted that in our case in dry petroleum ether, in addition to the aggregated pigment, residual monomer pigment was also present, and their T-T absorption spectra overlap. For a separate recording of the T-T absorption of these forms, measurements were performed at wavelengths where the absorption coefficients of singlet molecules K_S and triplet molecules K_T of one of the forms are equal (isobestic points). Then the transition of part of the molecules of this form to the triplet state does not cause any change in the transmission of the solution. Consequently, all the spectral changes observed at the isobestic point of monomer molecules in a joint solution belong to the aggregated pigment. The isobestic points for the monomer pigment were determined in special experiments, in which a small quantity of a polar impurity, decomposing the associate, was added to petroleum ether. Thus, in petroleum ether the isobestic points of the monomer pigment are situated at 439, 471, and 561 nm for chlorophylls α and b and bacteriochlorophyll, respectively.

In Fig. 3, curve 1 corresponds to clarification of the aggregate, recorded at the isobestic point of monomer chlorophyll b in dry petroleum ether at room temperature. In the observation of the transmission of the solution at the wavelength 474 nm, a complex signal is recorded, consisting of two components (curve 2). One of them, of greater duration but lower intensity, corresponds to clarification of the associate. The more intense and shorter signal is assigned to the T-T absorption of the residual monomer.

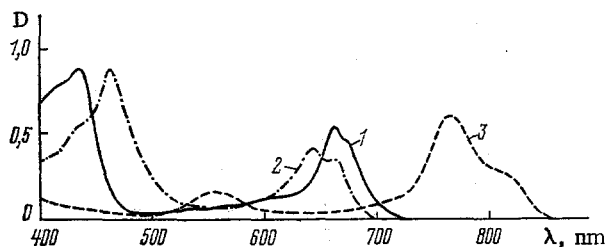


Fig. 1

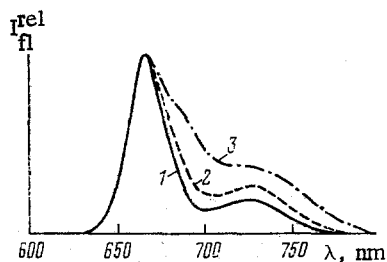


Fig. 2

Fig. 1. Absorption spectra of chlorophylls *a* (1) and *b* (2) and bacteriochlorophyll (3) in dry petroleum ether at $T = 20^\circ\text{C}$.

Fig. 2. Fluorescence spectra of chlorophyll *a* at $T = 20^\circ\text{C}$ in petroleum ether with an addition (6:1) of diethyl ether (1) and in dry petroleum ether (2, 3). $\lambda_{\text{exc}} = 430$ (1, 2) and 450 nm (3).

We also performed the direct excitation of associates of chlorophyll *b* through the corresponding filters in the regions of the spectrum where the absorption of the residual monomer pigment is negligible in comparison with the associated pigment (KS-17). In this case also, the appearance of T-T absorption, belonging directly to the associated form of the pigment, was observed. The differential spectrum of the change in the optical density of aggregated chlorophyll *b* is cited in Fig. 4.

A direct confirmation of the population of the triplet levels of aggregates of bacteriochlorophyll was obtained in experiments on triplet-triplet transfer of energy of excitation. We used pheophytin *a*, which is not subject to aggregation in dry nonpolar solvents, as the donor of triplet energy. The effective transfer of energy to the triplet level of the associate was directly confirmed, on the one hand, by direct clarification of the absorption band of the associate ($\lambda_{\text{max}} = 810$ nm) with excitation in the absorption band of pheophytin *a* ($\lambda_{\text{exc}} = 405$ nm) and, on the other hand, by the substantial shortening of the lifetime of the triplet state of the donor. As we indicated earlier, residual bacteriochlorophyll, which might serve as an acceptor of triplet energy, is also present in dry petroleum ether. A measurement of the fluorescence intensity of residual monomer molecules of bacteriochlorophyll in dry petroleum ether permitted a calculation of its amount in solution. Thus, in the initial solution of bacteriochlorophyll with concentration $1.45 \cdot 10^{-5}$ M, after aggregation, $0.23 \cdot 10^{-6}$ M monomer pigment remains.

Special experiments on T-T transfer of energy from pheophytin to monomer bacteriochlorophyll permitted a calculation of the rate constant of the transfer of triplet energy $K_t = 2.3 \cdot 10^9$ liters \cdot mole $^{-1}$ \cdot sec $^{-1}$. Considering the rate constant of transfer of triplet energy to associates also equal to K_t , and using the Stern-Volmer equation

$$\frac{\tau_0}{\tau} = 1 + K_t \cdot C_{\text{sum}} \cdot \tau_0,$$

where τ_0 is the lifetime of the donor without quencher; τ is the lifetime of the donor with quencher; K_t is the rate constant of T-T energy transfer; and C_{sum} is the summary concentration of the quencher, we can calculate the summary concentration of monomers and associates in solution. Thus, for the pair pheophytin *a* - bacteriochlorophyll in dry petroleum ether, the summary concentration of quenchers is equal to

$$C_{\text{sum}} = \frac{\frac{\tau_0}{\tau} - 1}{K_t \cdot \tau_0} \approx 1.2 \cdot 10^{-6} \text{ M.}$$

Consequently, the concentration of associates in solution is $C_{\text{as}} = C_{\text{sum}} - C_{\text{mon}} = 1.2 \cdot 10^{-6} - 0.23 \cdot 10^{-6} \approx 1 \cdot 10^{-6}$ M. Knowing the initial concentration of the pigment in solution, we can also estimate the quantitative composition of the associate *n*. According to our calculations, $n = [(C_{\text{init}} - C_{\text{mon}}) / C_{\text{as}}] \approx 10-15$ molecules.

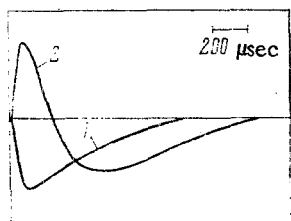


Fig. 3

Fig. 3. Oscillograms of spectral changes of chlorophyll b in dry petroleum ether (excitation KS-10, T = 20°C). $\lambda_{\text{obs}} = 471$ (1) and 474 nm (2).

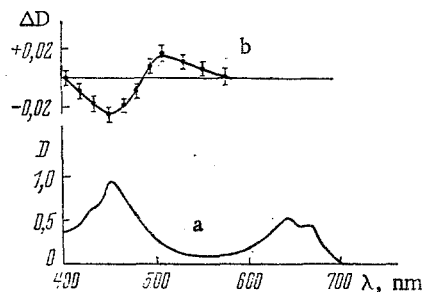


Fig. 4

Fig. 4. Absorption spectrum of chlorophyll b (a) and differential spectrum of the change in the optical density of chlorophyll b in dry petroleum ether with pulsed photoexcitation (KS-17, T = 20°C) (b).

TABLE 1. Quantum Yields and Probabilities of Intramolecular Translations

Pigment	B_S	$B_{\text{in.c}}$	γ	$f \cdot 10^{-7}, \text{sec}^{-1}$	$r \cdot 10^{-7}, \text{sec}^{-1}$	$d \cdot 10^{-8}, \text{sec}^{-1}$	$q \cdot 10^{-3}, \text{sec}^{-1}$	n
Chl. a-M	0,32	—	0,64	4,7	9,4	—	0,6	—
Chl. a-AG	0,015	0,86	0,13	4,7	41,6	31,2	0,6	—
Chl. b-M	0,12	—	0,88	2,7	19,8	—	0,3	—
Chl. b-AG	0,004	0,71	0,29	2,7	195,7	48,5	0,4	—
Bchl.-M	0,35	—	0,65	6,2	11,3	—	3,85	—
Bchl.-AG	0,01	0,93	0,06	6,2	37,2	57,3	—	10-15

Then we measured the relative quantum yield of the formation of triplet states of the pigment aggregates. The measurements were performed as follows. The values of the clarification $\Delta D_T/D$ (where ΔD_T is the change in the optical density in the long-wave absorption maximum during pulsed photoexcitation, D is the optical density of this maximum) in the monomer and aggregated solutions were compared. The fraction of molecules passing into the triplet state did not exceed 10% in all cases. Triplet-triplet excitation in this region of the spectrum was neglected on account of the smallness of K_T . Excitation was performed through interference filters. To consider excitation we introduced a correction factor K , considering the fraction of light absorbed by each sample. The coefficient K was determined as follows:

$$K = \frac{\int T_f \cdot (v) \cdot dv - \int T_{f,s} \cdot (v) \cdot dv}{\int T_f \cdot (v) \cdot dv},$$

where T_f is the transmission of the filter; $T_{f,s}$ is the transmission of the filter with solution. The values of $\Delta D_T/D$ are directly proportional to the concentration of triplet molecules, and, consequently, to the quantum yields of intercombination conversion γ . The quantum yields of intercombination conversion in associates of the investigated pigments, found in this way, are cited in Table 1.

Table 1 presents values of γ for monomer pigments, measured by a relative method. The standard was chlorophyll a in diethyl ether ($\gamma = 0.64$) [1]. The value of the quantum yield γ for chlorophyll b was in good agreement with the early measurements ($\gamma = 0.88$) [1]. We have found no values of the quantum yields of fluorescence and intercombination conversion for bacteriochlorophyll in the literature. The values that we determined, $B_S = 0.35$ and $\gamma = 0.65$, confirm the rule that for porphyrins and a number of aromatic molecules, the sum of the yields B_S and γ is equal to one. The values of the quantum yield of fluorescence B_S for monomer solutions of the pigments chlorophyll a and chlorophyll b were taken from [9]. The quantum yield of fluorescence of monomer bacteriochlorophyll was also measured relative to chlorophyll a ($B_S = 0.35$). The errors in the determination of B_S were 15%, and in the determination of γ , 25%.

From Table 1 it can be seen that the quantum yield of fluorescence of associates of the investigated pigments is close to zero, while the quantum yield of intercombination conversion γ is $\approx 0.1-0.3$. Then the quantum yield of internal conversion is equal to $B_{in.c} = 1 - (B_S + \gamma) \approx 0.7-0.9$. In a discussion of the quantity $B_{in.c}$, attention must be paid to the following experimental observations. When the concentration of chlorophyll α in petroleum ether is increased, long-wave bands (695, 735 nm) appear in the fluorescence spectrum, and the band 680 nm is weakened, which is an indication of the formation of pigment associates, which might serve as traps for electronic excitation of the oligomer matrix. An analogous phenomenon is observed when dry solutions of chlorophyll α in petroleum ether are frozen. These results are qualitative evidence that the quenching of the fluorescence of associates is due to rapid migration of the energy of electronic association to traps, the role of which may be played by aggregates with lower singlet excited levels. Thus, the deactivation of the energy of electronic excitation in these associates occurs chiefly emissionlessly as a result of internal and intercombination conversion. The values cited for the quantum yields B_S , $B_{in.c}$, and γ , as well as a knowledge of the lifetimes of fluorescence τ_S of the monomer pigment and the lower triplet state τ_T of monomers and associates, permit a calculation of the rate constants of intramolecular transitions in the investigated associates [10]. An analysis of the absorption spectra and calculation of the Kravets integrals show that the probability of emissionless transition from the singlet state is practically unchanged by aggregation. Then

$$\frac{B_{mon}}{B_{as}} \cdot \frac{\gamma_{as}}{\gamma_{mon}} = \frac{r'}{r}$$

The rate constants of intercombination conversion r' in associates calculated in this way are cited in Table 1. It can be seen that in all cases, association of molecules leads to an increase in the probability of intercombination transition to the triplet state. The rate constants of internal conversion d , calculated within the framework of the three-level scheme of Yablonskii, are also cited in Table 1.

An investigation of the kinetics of deactivation of the triplet state of associates of chlorophyll α and b at low temperatures showed that aggregation does not lead to any increase in the emissionless monomolecular constant of deactivation $q(T_1 \rightsquigarrow S_0)$.

Hence, on the basis of the experimental results, the quantum yields of intramolecular transitions in the investigated associates of photosynthetic pigments were determined, and their probabilities were calculated. It was shown that the association of molecules leads to an increase in the probability of intercombination transition (3-10-fold) with unchanged probability f . The basic channel of degradation of energy of singlet excitation is emissionless transitions by internal and intercombination conversion. Association of the molecules does not lead to any appreciable change in the emissionless monomolecular rate constant $q(T_1 \rightsquigarrow S_0)$.

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