Biochimica et Biophysica Acta, 461 (1977) 403–412

BBA 47339

PROBING THE FLUORESCENCE EMISSION KINETICS OF THE PHOTOSYNTHETIC APPARATUS OF *RHODOPSEUDOMONAS SPHAEROIDES*, STRAIN 1760-1, ON A PICOSECOND PULSE FLUOROMETER

V. Z. PASCHENKO, A. A. KONONENKO, S. P. PROTASOV, A. B. RUBIN, L. B. RUBIN[a] and N. Ya. USPENSKAYA

Chair of Biophysics, Biology Department and [a]Chair of Wave Processes, Physics Department, Moscow State University, Moscow 117234 (U.S.S.R.)

(Received November 25th, 1976)

SUMMARY

Using the pulse picosecond fluorometric technique the fluorescence properties of intact cells, isolated chromatophores and photosynthetic reaction centres were studied in bacteria *Rhodopseudomonas sphaeroides*, strain 1760-1.

The fluorescent emission from reduced reaction centres excited by 694.3 nm light has a biphasic character, the lifetimes of the components being $\tau_1 = 15 \pm 8$ ps and $\tau_2 = 250$ ps. The faster component, τ_1, contributes to the integral fluorescence in the long wavelength region. It disappears with oxidation of the reaction centres and is attributed to photoactive bacteriochlorophyll P870. The slow component, τ, is apparently due to both bacteriochlorophyll P800 and bacteriopheophytin. The fluorescence from intact cells exhibits a monophasic pattern and decays with $\tau = 200$ ps.

The fluorescence emitted by chromatophores comprises two components with $\tau_3 = 200$ ps and $\tau_4 = 4200$ ps. The duration of fluorescence τ_3 increases to its maximum of 500–550 ps, as P870 is oxidized chemically or photochemically, while τ_4 remains unchanged. The fluorescence with a lifetime of 200 ps was ascribed to the photosystem and the 4200-ps fluorescence to bacteriochlorophyll which had lost its functional links with the photosystem.

The rise time of the fluorescence emitted by chromatophores varies from 60 or 70 ps to 350 ps depending on the wavelength of the exciting light and the recorded spectral region. On the basis of our findings the rate for energy migration was estimated to be 10^9 s^{-1}.

INTRODUCTION

The advent of the pulsed laser spectroscopic technique, which provides high resolution times, has made available a variety of qualitatively new data on the mechanism of the photosynthetic transformation of light quanta into an electro chemical potential. We have reported earlier [1, 2] the lifetimes of the fluorescent emission from

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Abbreviations: BChl, bacteriochlorophyll; ESR, electron spin resonance.

photosystem I and photosystem II for pea chloroplasts (80 and 300 ps, respectively). Similar estimates were made by another group of investigators for spinach subchloroplast particles enriched in photosystems I and II [3]. Results of picosecond fluorometric study of green microalgae [4], chlorophyll solutions [4, 5] and carotenoids [6] have also become available. In addition, the kinetic parameters of the transient absorption changes induced by laser pulses in pigments have been investigated in isolated chromatophores [7] and photosynthetic reaction centres from purple bacteria *Rhodopseudomonas sphaeroides*, strain R-26 [8–11].

However, thus far very little has been known about the kinetics of the absorbance changes of the photosynthetic pigments in higher plants and about the fluorescence emitted by purple bacteria in the picosecond time domain. In bacteria, the fast component ($\tau_1 = 15$ ps) of the complex decay kinetics of fluorescence from reaction centres was first detected in work [12, 13] on *Rps. sphaeroides*, strain 1760-1. The emission of this component falls within the longest wavelengths. Its intensity depends on the degree of the reduction of photoactive BChl P870. We ascribed this component to the intrinsic fluorescent emission from P870. The value of τ_1 was used for the estimation of the characteristic time for primary charge separation in bacterial photosynthesis [12, 13]. The present work is a further step in this area. The kinetics of the fluorescence from the reaction centre preparations made from *Rps. sphaeroides*, strain 1760-1, were obtained by direct probing under various redox states of the entities and were then compared with the respective kinetics for intact cells and for chromatophores isolated from these bacteria.

This work gives additional support to the view that BChl P870 is responsible for the fluorescence with $\tau_1 = 15 \pm 8$ ps. The slow fluorescence component, at shorter wavelengths, decaying with $\tau_2 = 700$ ps in the oxidized reaction centre preparations but with $\tau_2 = 250$ ps in the reduced ones, was ascribed to bacteriopheophytin or BChl P800. It is found that the photosystem in intact cells and chromatophores fluoresces with a lifetime of $\tau_3 = 200$ ps. However, whenever P870 becomes chemically or photochemically oxidized, its lifetime increases up to 500–550 ps. The fluorescence with $\tau_4 = 4200$ ps which is apparently due to free BChl was also observed in chromatophores. The time course of the rise of the fluorescence from chromatophores varies from 60 or 70–350 ps, depending on the wavelength of the exciting pulse and recorded spectral region. The findings allowed us to estimate the rate for energy transfer in the photosystem. This was found to be of the order of 10^9 s^{-1}.

MATERIALS AND METHODS

Photosynthesizing non-sulphur purple bacteria *Rps. sphaeroides*, strain 1760-1 (containing carotenoids) were kindly submitted to us by Dr. E. Ohmann from the University of Martin-Luther, Biology Department, Hallevittenberg, G.D.R. The cells were grown as described earlier [12, 13], harvested by centrifugation and resuspended in 0.06 M phosphate buffer, pH 7.0. The absorbance of the sample at 590 nm (A_{590}) was 2.0 cm^{-1}.

After fragmentation of the cells by sonication, chromatophores were isolated by centrifugation and then diluted in 0.05 M Tris · HCl buffer, pH 7.2. The absorbance of the chromatophore samples at 590 nm was 2.5 cm^{-1}.

Photosynthetic reaction centres were prepared by solubilization of isolated chromatophore membranes with the anion detergent sodium dodecyl sulphate. Treatment with detergent was followed by centrifugation in a sucrose gradient. In the experiments use was made of the reaction centres which had been precipitated with ammonium sulphate and then dialized against trickling water or those that had been purified by gel filtration on a G-200 Sephadex column. In the latter case the fragments having the absorption indices $A_{280} : A_{800} = 2.1$ and $A_{765} : A_{800} : A_{870} = 1 : 2 : 1$ were taken for the experiments. They contained at least 75 % of photoactive BChl P870. The total concentration of BChl was determined from the absolute absorbance of the sample at 865 nm by using the extinction coefficient $\varepsilon_{865} = 130\ \mathrm{M}^{-1} \cdot \mathrm{cm}^{-1}$ [14]. It was typically 10–20 μM. The fractionation procedure is described in detail in [12, 13].

For the reduction of chromatophores or reaction centres 1 mM fresh sodium ascorbate was added to the medium and, for their oxidation, 10 mM potassium ferricyanide solution.

All the experiments were carried out in the open air at room temperature.

Measurement technique

The redox state of photoactive BChl was checked before and after the measurements of the fluorescence lifetime by means of ESR detection of the amount of cation radical P870$^+$. For this purpose the samples were placed in standard cuvettes and the amplitude of the ESR signal with $g = 2.0026$ and 10 G bandwidth was measured in the light (700–1100 nm) and after dark adaptation. The intensity of the actinic light (10^5 erg $\cdot$ cm^{-2} $\cdot$ s^{-1}) was saturating for P870 oxidation.

The picosecond fluorescence kinetics were investigated using a pulse fluorometer with a resolution time of 10 ps operated in conjunction with an electron-optic image converter [1, 15].

Fluorescence was excited with fundamental (694.3 nm) picosecond pulses produced by a ruby laser and second harmonic (530 nm) picosecond pulses from a frequency-doubled Nd-glass mode-locked laser. Measurements of fluorescence were taken over different spectral regions provided by a combination of glass filters. The pulse width at a half-peak measured by the two-photon luminescence technique was 6 ps at 530 nm and approx. 10 ps. at 694.3 nm. A typical laser flash consisted of 20–25 pulses separated from each other by a cavity round-trip time of 10 ns. The average pulse energy density was 10^{-4} J $\cdot$ cm^{-2}. A portion of the actinic light was diverted onto a photocathode for triggering a scanner having a response time of 7–8 ns. In fact, in our experiments we have recorded the fluorescence excited by the third pulse in a pulse train. At the aforementioned concentration of BChl, the absorption of the first three successive pulses of the actinic light caused the oxidation of not more than 2 % of the photoactive pigments in the reaction centre preparation and not more than 5 % of the pigments in chromatophores.

Use of an electron-optic image converter requires strict observation of the procedure for the treatment of densitograms. Particular attention is given to provision of identical conditions under which the photofilms are processed. Moreover, for every new batch of films the dependence of the blackening density on light intensity and contrast factor (γ) has to be determined. If D_1 and D_2 are the densities of blackening at points 1 and 2 on the densitogram, respectively, then the ratio of the intensities of the emissions causing those blackenings will be defined as $\log_{10} I_1/I_2 = (D_1 - D_2)/\gamma$

[16]. It is known that

$$I = I_0 \cdot e^{-t/\tau}$$

where τ is the mean lifetime of the excited state; I_0 is the intensity of the fluorescence at a zero time instant, $t = 0$.

Hence the equation can be derived

$$\tau = \gamma \cdot \log_{10}e[(t_2 - t_1)/(D_1 - D_2)]$$

In analysing, it was assumed that the probability for energy transfer from a short to a long wavelength form of pigment and afterward to the reaction centres was close to unity [14, 21]. Under such conditions the rate for energy transfer from a donor may be given by $K_m{}^D \approx K_D = 1/t_r$ and from an intermediate energy acceptor to the terminal one by $K_m{}^A \approx K_A = 1/t_d$, where K_D, K_A are the sums of the constants for the deactivation of the lowest singlet state S_1 of the energy donor and acceptor ($K_D \gg K_A$) and t_r, t_d are the rise and decay times of the fluorescence emitted by the energy acceptor, respectively.

In binary systems where excitation is applied to an energy donor but the fluorescence emitted by the acceptor is the subject of observation, one has to consider the pattern of the lifetimes of excited states of the components involved in the process. It can be shown theoretically that in binary systems the population $N_1{}^A$ (t) of the S_1 level of the acceptor is described by the equation:

$$N_1^A(t) = \frac{K_{DA} \cdot N_0 \left(e^{-K_A t} - e^{-K_D t}\right)}{K_D - K_A} \qquad (1)$$

where K_{DA} is the constant for energy migration and N_0 is the initial number of donor molecules in the S_1 state induced by a δ-pulse.

As seen from Eqn. 1, $N_1{}^A$ (t) undergoes similar changes both when $K_D \gg K_A$ and when $K_D \ll K_A$ (Fig. 1), a fact not to be discounted when analysing the behaviour of the fluorescence from the energy acceptor. Allowing for a relatively small error, one may accept that when $K_D \gg K_A$, the value of K_A can be determined from the "decay"

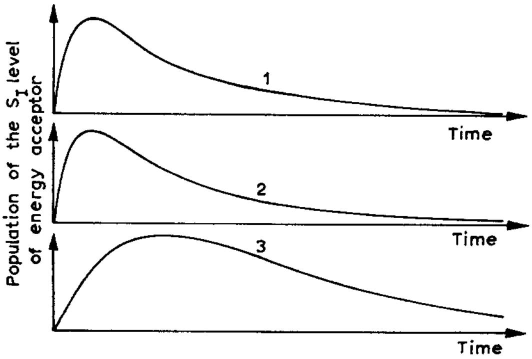

Fig. 1. Dependence of the population of the lowest singlet excited state of an energy acceptor, $N_1{}^A(t)$, on the relationship of K_D and K_A, the constants for deactivation of the energy donor and acceptor, respectively. 1, $K_A \gg K_D$; 2, $K_A \ll K_D$; 3, $K_A \approx K_D$. For the details see the text.

segment of the recorded curve $N_1^A(t)$. When $K_D \ll K_A$, the "rise" segment of $N_1^A(t)$ has to be used for making an estimate of K_A. With $K_D \approx K_A$, the fluorescence rise and decay phases do not follow an exponential pattern. Once K_A has been evaluated, one may calculate the theoretical curves $N_1^A(t)$ by varying K_D. Furthermore, by making fit of the theoretical curves $N_1^A(t)$ to the appropriate experiment, one can determine the value of K_D. Analysis of the curves of the fluorescent emission from the acceptor requires that the value either of K_D, or K_A, or their relationship be known.

RESULTS AND DISCUSSION

The fluorescent emission kinetics in reaction centre preparations

Upon excitation of the reaction centres by a 694.3-nm light, when P870 is completely reduced, the decay kinetics of fluorescence exhibit a biphasic pattern over the spectral region from 850 to 1000 nm (Fig. 2/1). The lifetimes of the fast and slow components are $\tau_1 = 15$ ps and $\tau_2 = 250$ ps, respectively. The ratio I_1/I_2 of the intensities for different preparations being 5–6. The rise time of the fluorescence was about 10 ps, i.e. within the resolution time of the instrument. The lifetime of the short-lived fluorescent component was of the same order of magnitude as the resolution time of the instrument, thus commending the method of numerical integration in estimating the value of τ_1. The error introduced in the value of τ_1 which was determined experimentally does not exceed ±8 ps.

At shorter wavelengths, from 730–820 nm, the fast fluorescent component disappeared and there was only the slow one with $\tau_2 = 250$ ps [12, 13]. Bacteriopheophytin having an absorption band centered at 765 nm [14] is part of the reaction centre complex. It absorbs some light quanta at 694.3 nm. This light is also absorbed by the degradation products of BChl (at 692 nm) that are present in the preparations. It is worth noting that the rise time of the fluorescence excited by a picosecond pulse, $\lambda_{exc} = 694.3$ nm, is about 10 ps. This suggests a very rapid transfer of excitation quanta from light-absorbing centres (probably bacteriopheophytin) to fluorescing centres.

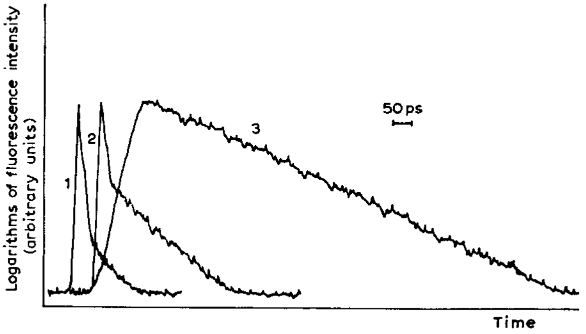

Fig. 2. Decay kinetics of the fluorescence from photosynthetic reaction centre preparations made from *Rps. sphaeroides*, strain 1760-1. $\lambda_{exc} = 694.3$ nm; $\lambda_{meas} = 850$–1000 nm. 1, 100 % reduction of P870; 2, 40–50 % oxidation of P870; 3, 100 % oxidation of P870. The redox state of P870 was poised by adding sodium ascorbate and potassium ferricyanide and monitored by ESR measurements.

The contribution of the fast component to the decay kinetics of the fluorescence emitted by the reaction centres was found to be dependent on the degree of P870 reduction. Thus, at 40–50 % of P870 oxidation the relative intensity of the fast component reduced approximately twice (Fig. 2/2). At 100 % P870 oxidation this component disappears altogether (Fig. 2/3). In the latter case there is a distinctive prolongation of the rise time of the fluorescence ($t_r = 80$ ps) and slowing of the decay of the slow fluorescence, τ_2, to 700 ps.

The fluorescence with a lifetime of 15±8 ps was observed only in the reduced reaction centre preparations in the long wavelength region from 850 to 1000 nm. With oxidation of the reaction centres it faded out until it disappeared altogether. This indicates that photoactive BChl P870 is responsible for this emission.

It is interesting to compare our experimental values of τ_1 for P870 fluorescence with those calculated by other authors through the quantum yield of P870 fluorescence in analoguous reaction centre preparations. The data on these have been given by Zankel et al. [18] and Slooten [19]. Recently these have been analysed in detail by Parson and Cogdell [17]. These investigators measured the quantum yield of fluorescence, φ_f, (0.03–0.04), when P870 was photo-oxidized with a quantum yield close to unity. Assuming that BChl itself fluoresces with an intrinsic lifetime of 18–31 ns (τ_0), they found that the lifetime of the lowest singlet excited state of P870 is 7–9 ps. This value is in a satisfactory agreement with $\tau_1 = 15 \pm 8$ ps measured directly in our experiments. Taking into consideration a high quantum yield of P870 photooxidation ($\varphi_p \approx 1$), as is the case for similar reaction centre preparations [20, 21], one can accept $\varphi_p \gg \varphi_f$. Then, τ_1 measured by us is the time required for an electron to leave the excited P870. This interpretation is confirmed elsewhere [9, 11]. As shown by those workers, the activation of the reaction centres prepared from *Rps. sphaeroides*, R-26, by a laser flash resulted in a transient state of the pigment complex arising for about 10 ps. Whenever the redox conditions are such that a complete turnover of photochemistry takes place, this transient state decays with a halftime of 150 ps [10, 11] to 250 ps [9] until P870 becomes oxidised and the primary acceptor* reduced. Therefore, that $\tau_1 = 15 \pm 8$ ps reflects the most rapid step in the photochemical act, namely the formation of an intermediate P^F (according to the term introduced by Parson and Cogdell [17]).

Let us now consider the nature of the slow component τ_2 of the fluorescence emitted by the reaction centres. It is obvious that the pigments, other than P870, that are present in the reaction centre preparation account for this component. These pigments fluoresce at shorter wavelengths and have a higher quantum yield of fluorescence than P870. An unequivocal identification of the pigment responsible for this component can be made by analysing the fluorescence emission spectra from the reaction centres. Such spectra were recorded by Reed and Ke [22] for the preparations analogous to those studied by us. In particular, they excited fluorescence at the wavelengths which correspond to the absorption band of bacteriopheophytin (530 nm). At 77 K the individual bands of the fluorescence from P870 and P800 (with maxima at 920 nm and 826 nm, respectively) and from bacteriopheophytin and the

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* By our definition, the primary acceptor is the nearest redox (non-porphyrin) agent of the reaction centre pigment complex.

degradation products of BChl (maxima at 775 nm and 707 nm) were distinctly observable in those spectra.

In our case one may expect a similar pattern of the fluorescence emission spectrum and its behaviour. Hence, the fluorescence at short wavelengths of 730–820 nm has to be ascribed to P800 and bacteriopheophytin. The emission at longer wavelengths from 850 to 1000 nm must be ascribed to the BChl P870 (in the reduced reaction centre preparations) and P800.

It is also interesting to discuss our results in the light of the experiments carried out by Reed and Ke [22]. They revealed the components at approx. 800 nm in the circular dichroism (CD) spectra of reduced reaction centres. These indicated strong excitonic interactions [23] between BChl molecules which are responsible for the absorbance in this spectral region. The displacement of an electron from the reaction centre lessened the intermolecular interactions in the pigment complex. The CD spectra for oxidized reaction centre preparations were found to resemble those for the monomeric BChl. A marked increase in the rise and decay times of the reaction centre fluorescence emission upon oxidation of the reaction centre seems to be a reflection of this "loosening" of the interpigment bonds in the reaction centre complex.

The fluorescence emission kinetics in intact cells and chromatophores of bacteria

Table I represents the parameters of the fluorescence emitted by intact cells and isolated chromatophores, strain 1760-I, in the media where the redox potential was sufficient to retain the photoactive BChl P870 in the reduced state.

The fluorescence from the cells has a monophasic pattern with a lifetime of 200 ps. The fluorescence from chromatophores consists of two components with lifetimes of 200 ps (τ_3) and 4200 ps (τ_4).

It seems likely that the photosystem of the studied bacteria fluoresces for 200 ps, while the 4200-ps emission found only in chromatophore preparations is due to free molecules of BChl whose functional links have been lost in the course of the isolation

TABLE 1

The fluorescence emission kinetics for intact cells and chromatophores from bacteria *Rps. sphaeroides*, strain 1760-1. The redox potential of the medium was sufficient to retain BChl P870 in the reduced state. For the details of the experiment see Materials and Methods.

Experimental conditions		Fluorescence kinetics			
$\lambda_{excitation}$ (nm)	$\lambda_{recorded}$ (nm)	Cells		Chromatophores	
		t_{rise} (ps)	t_{dec} (ps)	t_{rise} (ps)	t_{dec} (ps)
694.3	730–1000	60–70	200	60– 70	200
694.3	850–1000	–	–	180–200	200
530	650–1000	60–70	200	60–70	200* 4200**
530	730–1000	–	–	220–250	200
530	850–1000	–	–	350	200

* 1st component
** 2nd component

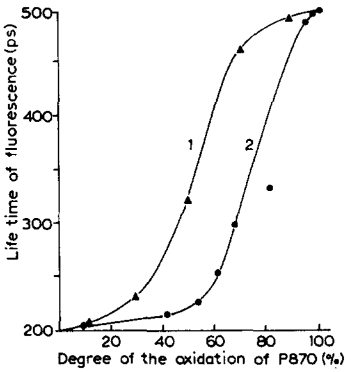

Fig. 3. Lifetime of the fluorescence emitted by chromatophores from *Rps. sphaeroides*, 1760-1, as a function of a redox state of BChl P870 of photosynthetic reaction centres. λ_{exc} = 694.3 nm; λ_{meas} = 730–1000 nm. The redox state of P870 was monitored by ESR measurements. P870 was oxidized either chemically by adding potassium ferricyanide (1) or photochemically with background illumination from a He-Ne laser (2).

of photosynthetic membranes.

In order to lend experimental support to this view the duration of the components τ_3 and τ_4 of the chromatophore fluorescence was studied as a function of the state of the photochemically active BChl. In one series of experiments P870 was oxidized by addition of potassium ferricyanide. As the reaction centres were oxidized, τ_3 increased from 200 to 500–550 ps (Fig. 3/1), while τ_4 = 4200 ps remained unchanged. In another series of experiments P870 was oxidized with a background illumination from a He-Ne laser. The dependence of BChl oxidation on the intensity of the background illumination was monitored in parallel by means of ESR measurements. On increasing the intensity, τ_3 increased up to 500–550 ps (Fig.3/2), while τ_4 did not vary.

Hence, in bacteria under investigation, the photosystem fluoresces with a lifetime (τ_3) of 200 ps.

It is generally accepted that BChl in vitro fluoresces longer than BChl in vivo because of a great effectiveness of energy migration from long-wave light-harvesting pigments to active reaction centres in which an ultrafast energy conversion ($\tau_I = 15 \pm 8$ ps, as measured by us) in an electrochemical potential occurs due to charge separation. With this in view, one can estimate the rate for energy transfer from long wave light-harvesting BChl to reaction centres. Basing on the BChl fluorescence lifetime of 200 ps, we estimate it to be $5 \cdot 10^9$ s^{-1}.

It was found, too, that the risetime of fluorescence depends, to a large extent, on both the wavelength of an exciting pulse and recorder spectral region. Thus, when excited at 694.3 nm, t_r of the fluorescence from chromatophores varies from 60–70 ps at wavelengths 730–1000 nm to 180–200 ps at wavelengths 850–1000 nm. At 530 nm excitation it varies from 60–70 ps at λ = 650–1000 nm to 350 ps at λ = 800–1000 nm (Table I).

In fact, these data indicate that the 694.3 nm and 530 nm laser irradiation excites one collection of pigments, while fluorescence is emitted by another. Between the two an effective migration of energy occurs. If the probability for energy transfer between them is about unity, then the rise times of fluorescence from 60 to 350 ps reflect in proportion the rates of energy transfer of $16 \cdot 10^9$ to $3 \cdot 10^9\ s^{-1}$, a fact which supports the Förster mechanism of energy migration [24].

It is relevant that our estimates of the times for energy transfer are of the same order of magnitude as those measured earlier in chloroplasts [1]. However, as shown by some authors, in green plants they are somewhat shorter [4, 5]. Nevertheless, relatively long risetimes of the fluorescence in chromatophores and cells of bacteria do not seem to be an artifact. Firstly, at equipment time response of about 10 ps and under the same experimental conditions, reduced reaction centre preparations showed much shorter times (Fig. 2). Secondly, the dependence of the fluorescence risetimes on the recorded spectral region was observed at a constant wavelength of exciting light (Table I). It seems probable that the use of pulse trains of exciting light may be one of the reasons for a longer fluorescence rise. However, Harris et al. [25] did not observe any marked difference in the time course of the fluorescence from the chlorella cells after excitation with a pulse train and with a single pulse. Clearly, further elucidation is needed to give insight into descrepancy in the data.

It should be emphasized that for both green plants [1] and for the preparations used in our experiments the duration of the fluorescence from a light-harvesting BChl, even at the 100 % oxidation of reaction centres, is 10 times shorter than from chlorophyll in solutions.

If Duysens's hypothesis that the fluorescence from the photosystem is quenched by reaction centres is true, then it can be concluded that the reaction centre, both in the reduced and oxidized form, is capable of quenching the excitation of the BChl antenna. The quantum yields of these processes, as determined by us, are 0.96 and 0.90 for reduced and oxidized reaction centre, respectively, indicating that the primary act of excitation quenching is of a nature more complex than originally proposed.

NOTE ADDED IN PROOF (Received June 14th, 1977)

We obtained (in collaboration with Dr. E. P. Lukashev) the fluorescence spectra (at $\lambda_{exc} = 360$ to 580 nm and $I_{exc} \simeq 10^5$ erg $cm^{-1}s^{-1}$) of SDS - preparations of the reaction centres from *Rps. sphaeroides* 1760–1. The typical spectrum shows distinctive bands which can be atributed to BChl P870 (maxima near 890 nm and 920 nm at 295 K and 80 K respectively), bacteriopheophytin (772 nm, 776 nm) and the degradation products of BChl (704 nm, 706 nm). In contrast to the observation of Reed and Ke [22] fluorescence from BChl P800 was not detected at all. This allows us to suggest that the slow component of fluorescence with $\tau_2 = 250$ ps from laser-activated reaction centres belongs only to bacteriopheophytin. If so, the good agreement of τ_2 value in our experiments with the characteristic time of discharge of P^F state in reaction centres [9–11], is obviously not a coincidence. Thus our data strongly support the current view on the obligatory participation of bacteriopheophytin in the formation of an intermediate of the photochemical reaction in bacterial photosynthesis. Experiments on the redox titration of picosecond fluorescence in reaction centres poised at high and low potentials are in progress now.

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