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FLUORESCENCE LIFETIME SPECTRA OF IN VIVO BACTERIOCHLOROPHYLL AT ROOM TEMPERATURE

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Fluorescence lifetime spectra of *Rhodopseudomonas sphaeroides* chromatophores have been measured at room temperature by phase fluorimetry at 82 MHz in order to investigate the heterogeneity of the emission. The total fluorescence was decomposed into two main components. A constant component, F_c, centered at 865 nm, represents about 50% of the total emission from dark-adapted chromatophores (F_o) and has a lifetime of 0.55 ns. A variable component is centered at 890 nm. Upon closing the reaction centers, 5-fold increases take place in both emission yield and lifetime of this component. In the dark-adapted state, its lifetime is about 50 ps and its contribution to the total fluorescence is 70% at 890 nm. In the presence of sodium dithionite, a long-lifetime component ($\tau_D \simeq 4$ ns) is observed. This probably arises from radical pair recombination between P^+ and I^- (P, the primary electron donor, is a dimer of bacteriochlorophyll; I, the primary electron acceptor, is a molecule of bacteriopheophytin). Its spectrum is nearly identical to that of the variable component. This emission seems to be present also under nonreducing conditions, although with a much weaker intensity than when the electron acceptor quinone is prereduced.

Introduction

In green algae and higher plants, a linear relationship between fluorescence lifetime and intensity has been established during the induction transient occurring after switching on continuous illumination [1–5]. These results do not support the existence of a strong heterogeneity of the total emission. However, recent work of Haehnel et al. [6] is not in agreement with this conclusion. These authors suggest that the apparent linear relationship observed in chloroplasts is due to the presence of three distinct emissions whose lifetimes and relative weights produce a proportional increase in the average lifetime (τ) and fluorescence yield (Φ) on closing the reaction centers. The observed enhancement in τ is interpreted as resulting from a dramatic increase (20-fold) in a long-lifetime component (1–2 ns) during the fluorescence induction. This conclusion is in agreement with the attribution by Klimov and Krasnovskii [7] of the variable fluorescence to a charge recombination in the reaction centers.

The behavior of τ and Φ seems different for photosynthetic bacteria. Some measurements by phase fluorimetry have shown opposing variations of lifetime, τ, and fluorescence yield, Φ [8,9]. The authors concluded that a 0.8 ns background fluorescence exists in addition to the variable one. This component could originate from part of the B800-B850 antenna, the contribution of which to the total emission has been shown previously [10]. Such migration times within the antenna are one order of magnitude smaller than in green plants. Other data [11], obtained with *Chloropseudomonas ethylicum*, *Rhodospirillum rubrum* and *Rhodopseu-*

domonas viridis, are at variance with those above. The authors found an enhancement of τ and Φ; in this case, the bacteriochlorophyll (BChl) fluorescence lifetime was of the same order of magnitude as that of chlorophyll in vivo.

On the other hand, it was reported that under reducing conditions, when the primary electron acceptor quinone (X) is kept reduced, the BChl fluorescence lifetime is lengthened drastically to about 5 ns [12]. This delayed fluorescence was attributed to the charge pair recombination bteween P^+ and I^- [7,12–23] (where P is the reaction center BChl dimer and I the bacteriopheophytin acceptor).

In all the above results, fluorescence lifetimes were averaged over the whole spectrum. The present paper reports data obtained by recording simultaneously the spectra of fluorescence intensity and lifetime. The lifetimes were measured from the phase shift and fluorescence demodulation methods. An analysis of both types of spectra under various conditions allows us to determine the spectra and lifetime of two components, a constant one, and a variable one directly connected to the reaction centers.

Material and Methods

Rps. sphaeroides cells (strain Y) were grown anaerobically in the light as described elsewhere [23]. Harvested cells were washed and disrupted in a French pressure cell in 0.1 M sodium phosphate buffer, pH 7.5. Unbroken cells and debris were eliminated by centrifugation at $30\,000 \times g$ for 20 min. Chromatophores were isolated after differential centrifugation (90 min, $200\,000 \times g$) and resuspended in the same buffer. The absorbance of the chromatophores used in the fluorescence experiments was of the order of 0.4 at 850 nm.

Fluorescence lifetimes were obtained by measuring the phase shift (τ_p) and demodulation (τ_m) of the fluorescence in comparison to the exciting light. Then:

$$\tau_p = (1/2\pi\nu)\tan\psi \qquad (1)$$

$$\tau_m = (1/2\pi\nu)(1/M^2 - 1)^{1/2} \qquad (2)$$

where ψ is the phase shift at the light modulation frequency ν. M is the relative modulation coefficient of the fluorescence light. For a homogeneous emission, $\tau_p \equiv \tau_m$. For a mixture of independent emissions [25,26] one can refer to the Appendix. It is clear from Eqns. 1 and 2 that the light modulation frequencies employed must be adapted to the measured lifetimes. The light source is a mode-locked argon laser emitting a continuous train of pulses (full-width at half-maximum approx. 120 ps). The time interval between two consecutive pulses is 12.4 ns. Due to the high harmonic content of the pulse train, measurements can be done at several frequencies (i.e., 82, 164, 246 MHz, etc.). For technical reasons the fundamental (82 MHz) has been used in the present work.

Fluorescence is analyzed at a right angle through an $f/2$ monochromator (band width approx. 15 nm), with optimum transmission at 800 nm. Because the fluorescence wavelength is far from the excitation wavelength, residual scattered light is negligible. A fast photomultiplier (La Radiotechnique 56 CVP) with a rise time of approx. 1.5 ns with an S1 photocathode is operated at 20°C for fluorescence detection. Optimisation of light collection provides a good signal-to-noise ratio and additional cooling is not required.

The d.c. and a.c. components of the anode current are simultaneously measured. The a.c. component is preamplified (wide-band amplifier: 0–500 MHz, gain = 40 dB) and filtered by a narrow-band (4 MHz) passive filter centered at the working frequency. The phase and amplitude of the resulting signal are measured in a vector voltmeter with 1/10 degree phase resolution.

The three parameters (d.c. voltage, a.c. voltage and phase shift) are digitized and averaged in a multichannel averager having three simultaneous inputs. The stepping motor of the monochromator and the channel advance of the averager are controlled by a desk computer HP 85. A 1 nm step size is used for recording the spectra and six scans of about 1 mn duration each are usually necessary to reach the required signal-to-noise ratio.

The phase fluorimeter used in the present work is one of the possible configurations of a general purpose spectrofluorimeter with subnanosecond temporal resolution built by I. Moya at the storage ring of L.U.R.E., Orsay. A complete description of the instrument is in preparation. This instrument

uses both synchrotron radiation and amode-locked argon laser. The white light emitted by the storage ring (modulated at 13.6 MHz, with harmonics up to 82 MHz) has been used to determine the wavelength dependence of the response time of the photomultiplier. Several additional effects can contribute to artifactual phase shift measurements, including dependences of the response time on the light intensity and the cathode area. We have checked this under our conditions between 515 and 850 nm. The overall effect is less than 70 ps. In the spectral range where the spectra have been recorded (830–830 nm) relative wavelength effects are less than 40 ps; in addition, no major phase shift occurs when the amplitude of the signal is divided by 5.

The F_o fluorescent state is obtained by flowing (10 ml/s) the chromatophore suspension through the cuvette, as described in Refs. 5 and 27. The light intensity was adjusted so that the fluorescence level becomes independent of the flow rate. The observed fluorescence corresponds then to the dark-adapted level (open centers). The F_M signal was obtained without flowing the sample with an exciting energy of about 10 mW/cm^2. Fluorescence spectra were not corrected for the wavelength-dependent response of the instrument; however, this does not change our results appreciably.

Results

Fig. 1 shows the emission spectra of fluorescence intensity and lifetime in *Rps. sphaeroides* chromatophores in the F_o and F_M states. As can be seen, the ratio F_M/F_o is greater than τ_M/τ_o at any wavelength (τ_M and τ_o are the fluorescence lifetimes associated with the F_M and F_o levels). The increase in both F and τ during the induction is minimum in the short-wavelength range. The heterogeneity of fluorescence is thus obvious. To explain our data, we postulate the existence of two independent emission with different spectra. The first one is a 'live' emission, the yield and lifetime of which (F_v, τ_v) are modulated by the state of the reaction centers. Thus, for each wavelength $F_{vM}/F_{vo} = \tau_{vM}/\tau_{vo} = K$. Where F_{vM}, F_{vo}, τ_{vM} and τ_{vo} are the intensity and lifetime values of this component with closed or opened centers, respectively. This variable part of the total fluorescence should mainly come from the B870 antenna, which is well connected to the reaction centers [10]. This hypothesis concerns only the initial and the final states of the fluorescence induction, and is independent of the model of energy migration in the antenna.

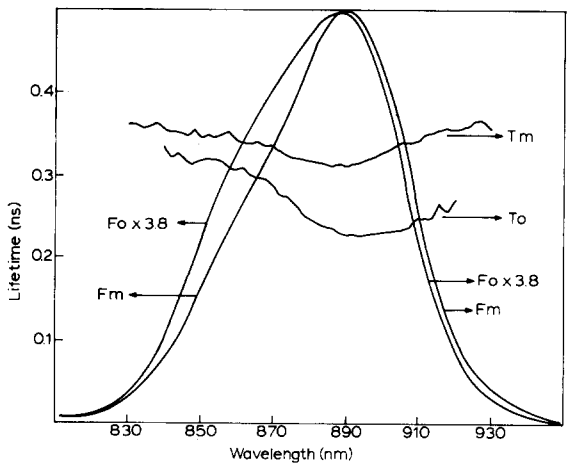

Fig. 1. Fluorescence (F_o, F_M) and lifetime spectra (τ_o, τ_M) of *Rps. sphaeroides* chromatophores suspended in 0.1 M sodium phosphate, pH 7.5. Subscript o refers to the dark-adapted state, subscript M to the high fluorescence level (closed centers).

On the other hand, the B850 antenna is known to be less linked to the reaction centers. Thus, a constant part of its fluorescence, not sensitive to the state of the reaction centers, must be present in the fluorescence of chromatophores. Calling this constant fluorescence component F_c, and its lifetime τ_c, our hypothesis can then be written:

$F = F_v + F_c$ for each wavelength of the spectrum. In the Appendix, the solutions of this decomposition are given. It is shown that τ_c, τ_{vo}, and τ_{vM} can be obtained in a unique way for a given value of K. However, one can see in Fig. 1 that the lifetime spectra are enhanced above 890 nm. A third component, with a long lifetime, is necessary to fit well our data in this region of the spectrum.

The K value is reached by assuming two criteria. Firstly, F_c, the spectrum of which must be similar to that of the B850 antenna is centered at 865 nm [28], and is negligible over 910 nm. The second point is that the fluorescence lifetime of this homogeneous chromophore must be constant over the whole range of its spectrum. In other words, τ_c

must be a nonwavelength-dependent parameter.

The above criteria restrict the K value to narrow limits: $K = 5 \pm 0.2$. Thus, F_v is found centered at 890 nm, which confirms that the variable fluorescence arises mainly from the B870 antenna. Its nonwavelength-dependent lifetime varies from 50 to 250 ps depending on the states of the reaction centers. The average lifetime of the constant emission is then found to be 0.55 ns.

Variations of K within the indicated limits do not change the results of this analysis by more than 10%. The obtained solution is therefore stable. However, as indicated above, the two-component model is not able to explain the experimental data above 890 nm. This can be seen in the behavior of the τ_c spectrum which rises strongly above 890 nm (Fig. 2).

Addition of a long-lifetime component (approx. 4 ns) with nearly the same spectrum as F_v is then necessary to fit correctly our experimental curves (Fig. 3). This emission does not represent more than 5% of F_o or F_M. Its possible significance will be examined later.

Low redox potential results

In the presence of excess sodium dithionite, a significant increase in lifetime is observed in the dark-adapted level. It is then possible to measure the demodulation lifetime spectrum. It is obvious from this spectrum, sensitive to long-lifetime components, that a nanosecond emission is occurring in the long-wavelength range of the spectrum in agreement with previous results [7,12–23].

Starting from the above analysis under normal conditions, we explain our data by adding a nanosecond emission (F_D, τ_D), probably a delayed

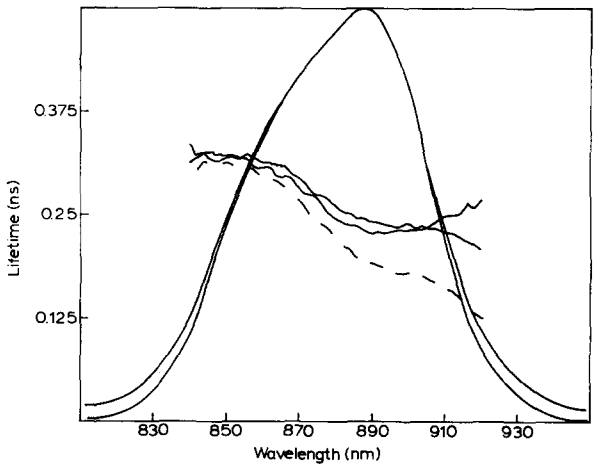

Fig. 3. Fitting of the experimental curves F_o and τ_o by the calculated spectra assuming for τ_c (see the text) a constant value of 0.55 ns and a 5% component with a 4 ns lifetime with the same spectrum as F_v. Dashed line: calculated τ_o spectrum without adding this nanosecond component.

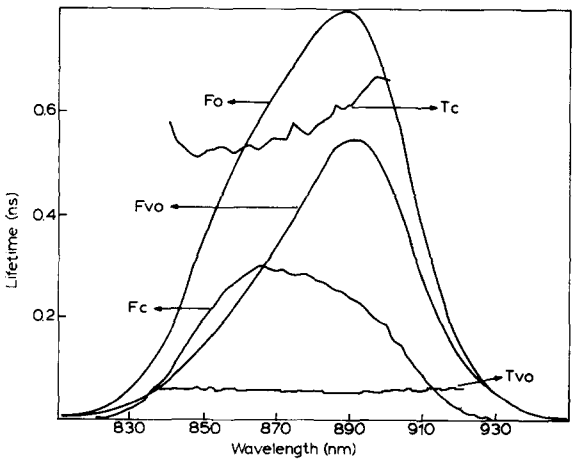

Fig. 2. Decomposition of the F_o spectrum of *Rps. sphaeroides* chromatophores into two main components: F_{vo} (variable) and F_c (constant), τ_{vo} and τ_c are the lifetime spectra associated with these components.

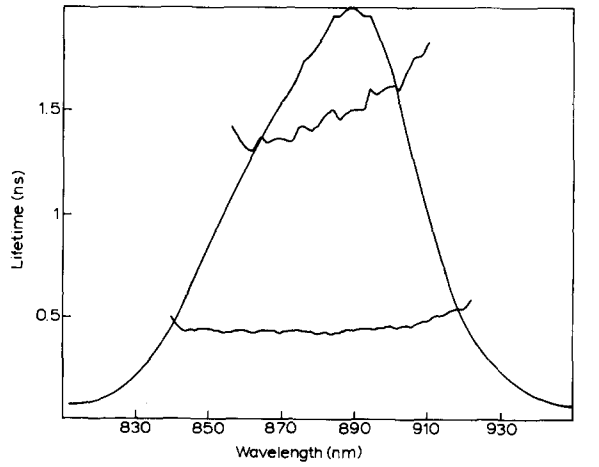

Fig. 4. Fluorescence and lifetime spectra of *Rps. sphaeroides* chromatophores in the presence of sodium dithionite. The lower spectrum is measured by the phase shift method at 82 MHz. The upper spectrum is measured from the relative modulation of the fluorescence signal at the same frequency.

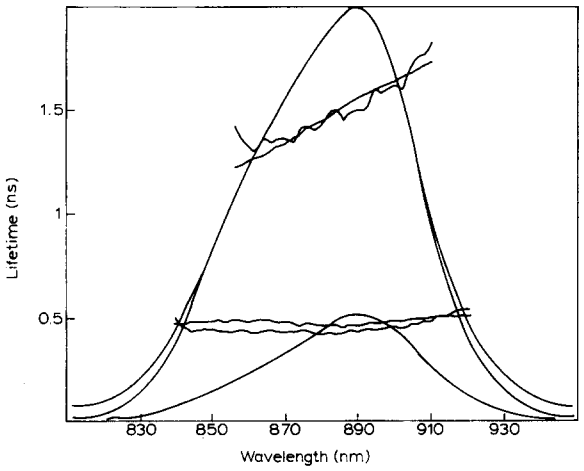

Fig. 5. Experimental (same as in Fig. 4) and calculated fluorescence and lifetime spectra of *Rps. sphaeroides* chromatophores in the presence of sodium dithionite (see the text). The lower spectrum represents the relative contribution of the delayed fluorescence.

fluorescence arising from radiative deactivation of the radical pair state P^F when X is prereduced. This component, simply added to F_v and F_c, can be characterised in a unique way thanks to the measurement of the demodulation lifetime spectrum.

Assuming a constant value of τ_c, F_D and τ_D can be determined by successive iterations. A good adjustment of experimental curves is obtained for $\tau_D = 4 \pm 0.3$ ns and F_D proportional to the F_v spectrum within 2 nm (Fig. 5). The unity of the solution excludes the possibility of F_D being different from F_v. Both intensity (approx. 30% of the total emission in the F_o state) and lifetime values are in good agreement with data found previously by the single-photon-counting method on *R. rubrum* chromatophores [12].

Discussion

Two types of antenna complexes (B800, B850 and B870) drive energy to reaction centers in *Rps. sphaeroides* but the greater part of fluorescence emission arises from the B870 antenna [28]. The shoulder at 865 nm has been assigned to the B850 antenna emission. Using a new method of time-resolved spectroscopy, we have identified the 'background' fluorescence with the last emission. Depending on the relative weight and lifetime of this 'constant' component, one can obtain different patterns of τ vs. F relationships. If the F_c contribution remains minor, τ varies in the same way as F (this work and ref. 10). For high F_c contribution, opposing variations of these two parameters can take place, as observed by Borisov and Godik [8] and also by us in some chromatophore preparations (unpublished results).

The quantum yield of the constant emission is $\Phi_c = \tau_c/\tau_i$ (τ_i, intrinsic BChl lifetime) and the quantum yield of F_{vo} is $\Phi_{vo} = \tau_{vo}/\tau_i$. Thus, we have $\Phi_c \simeq 10\Phi_{vo}$.

Since the amount of B800-B850 bacteriochlorophyll is similar to the amount of B870 bacteriochlorophyll one would expect to obtain a fluorescence intensity about 10-times greater for F_c than for F_{vo}. From the decomposition of Fig. 2, we have concluded that $F_{vo} \simeq 2F_c$. This strong discrepancy can be taken into account if we suppose that F_c arises from a small amount of B800–B850 BChl, the majority of them being nonfluorescent because of the efficient transfer of their energy to B870 in agreement with the results of Monger and Parson [29]. The knowledge of the F_c spectrum allowed us to calculate the fluorescence lifetime and quantum yield rises of the live fluorescence F_v arising from B870 antenna.

The K value, 5 ± 0.2, suggests that closed centers remain partially quenchers, as postulated previously [30] and as has been shown for green plants [3,5].

The 50 ps fluorescence lifetime found in the PIX state is of the same order of magnitude as observed previously [8,31]. It can be compared to the emission lifetimes in the Photosystem I antenna [32–34]. However, it is much shorter than emission lifetimes in Photosystem II antenna.

Under reducing conditions, a 4 ns delayed fluorescence emission was found. This was previously interpreted as a radiative charge recombination of the transient state P^F. We find its spectrum to be identical to that of the variable fluorescence. This delayed emission seems then to occur from BChl molecules in the same surroundings as are the B870 antenna molecules.

To explain the presence of a similar nanosecond component under nonreducing conditions, we suggest that a small fraction of centers (approx. 5%)

may remain in the reduced form even in the dark-adapted state. Alternatively, this long-lifetime component may arise in part from BChl molecules which have lost their links with the photosystem. This hypothesis was proposed by Paschenko et al. [35] in discussing their finding of a 4.2 ns component in *Rps. sphaeroides* chromatophores under normal conditions.

More precision in our data is needed to interpret this component quantitatively. Further experiments using phase fluorimetry and single-photon-counting decay techniques will help to solve this problem.

Acknowledgements

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Appendix

Let us consider a fluorescence emission (τ, lifetime) originating from two elementary components (1 and 2), the lifetimes of which are τ_1 and τ_2. In the phase fluorimetry method, samples are excited by a harmonically modulated light beam.

Therefore, fluorescence lifetimes are measured by the phase lag and by the relative modulation coefficient, as compared to the exciting light. The above scheme shows that:

$$\tilde{F}^2 = \left(\tilde{F}_1 \cos\psi_1 + \tilde{F}_2 \cos\psi_2\right)^2 + \left(\tilde{F}_1 \sin\psi_1 + \tilde{F}_2 \sin\psi_2\right)^2 \quad \text{(A-1)}$$

and

$$\tan\psi = \frac{\tilde{F}_1 \sin\psi_1 + \tilde{F}_2 \sin\psi_2}{\tilde{F}_1 \cos\psi_1 + \tilde{F}_2 \cos\psi_2} \quad \text{(A-2)}$$

Knowing that, at frequency ν, the phase angle ψ_i of the ith component is such that:

$$\tan\psi_i = 2\pi\nu\tau_i$$

where τ_i is the fluorescence lifetime of the ith component, Eqn. A-2 can be written as:

$$\tau_p = \frac{\tilde{F}_1 \cos\psi_1\tau_1 + \tilde{F}_2 \cos\psi_2\tau_2}{\tilde{F}_1 \cos\psi_1 + \tilde{F}_2 \cos\psi_2} \quad \text{(A-3)}$$

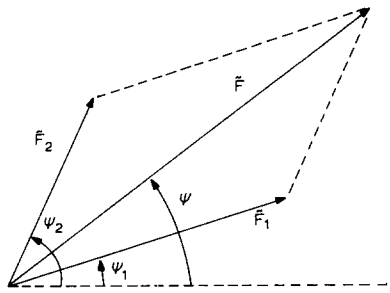

Fig. 6. Vector diagram of the total alternating emission ($\tilde{F}$) and of its two components $\tilde{F}_1$ and $\tilde{F}_2$.

where τ_p is the average lifetime obtained by the phase lag measurement. For small ψ_i angles, $\cos\psi_i$ is taken as approx. 1. In our experiments with the modulation frequency of the exciting light (82 MHz), this assumption was made and justified a posteriori.

Then Eqns. A-1 and A-3 become:

$$\tau_p = (F_1\tau_1 + F_2\tau_2)\cdot(F_1 + F_2)^{-1} \quad \text{(A-4)}$$

$$F^2 = (F_1 + F_2)^2 \quad \text{(A-5)}$$

Where F, F_1 and F_2 are the d.c. parts of the total emission, and of its two elementary components.

From our model (see the text), it follows that:

$$F_o = F_{vo} + F_c$$

$$F_M = F_{vM} + F_c$$

$$\frac{\tau_{vM}}{\tau_{vo}} = \frac{F_{vM}}{F_{vo}} = K$$

These imply:

$$F_{vo} = \frac{1}{K-1}(F_M - F_o)$$

$$F_{vM} = \frac{K}{K-1}(F_M - F_o)$$

$$F_c = \frac{1}{K-1}(KF_o - F_M)$$

Lifetimes are thus deduced:

$$\tau_{vo} = \frac{1}{(K^2-1)}\left(\frac{\tau_M F_M - \tau_o F_o}{F_{vo}}\right)$$

$$\tau_{vM} = K\tau_{vo}$$

$$\tau_c = \frac{\tau_o F_o - F_{vo}\tau_{vo}}{F_c}$$

References

1 Tumerman, L.A. and Sorokin, E. (1967) Mol. Biol. 1, 628–638
2 Sorokin, E.M. and Tumerman, L.A. (1971) Mol. Biol. 5, 753–765
3 Briantais, J.-M., Merkelo, H. and Govindjee (1972) Photosynthetica 6, 133–141
4 Mar, T., Govindjee, Singhal, G.S. and Merkelo, H. (1972) Biophys. J. 12, 797–808
5 Moya, I. (1974) Biochim. Biophys. Acta 368, 214–227
6 Haehnel, W., Nairn, J.A., Reisberg, P. and Sauer, K. (1982) Biochim. Biophys. Acta 680, 161–173
7 Klimov, V.V. and Krasnovskii, A.A. (1981) Photosynthetica 15, 592–609
8 Borisov, A.Yu. and Godik, V.I. (1970) Biochim. Biophys. Acta 223, 441–443
9 Borisov, A.Yu. and Godik, V.I. (1972) Bioenergetics 3, 211–220
10 Zankel, K.L. (1978) in the Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 341–348, Plenum Press, New York
11 Govindjee, Hammond, J.H. and Merkelo, H. (1972) Biophys. J. 12, 809–814
12 Godik, U.I. and Borisov, A.Yu. (1979) Biochim. Biophys. Acta 548, 365–369
13 Shuvalov, V.A. and Klimov, V.V. (1976) Biochim. Biophys. Acta 440, 587–599
14 Godik, V.I., Kotova, E.A. and Borisov, A.Yu. (1979) Dokl. Akad. Nauk S.S.S.R., 249, 993–996
15 Parson, W.W., Clayton, R.K. and Cogdell, R.J. (1975) Biochim. Biophys. Acta 387, 265–278
16 Shuvalov, V.A. and Parson, W.W. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 957–961
17 Cogdell, R.J., Monger, T.G. and Parson, W.W. (1975) Biochim. Biophys. Acta 408, 89–199
18 Rockley, M.G., Windsor, M.W., Cogdell, P.J. and Parson, W.W. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2251–2255
19 Kaufmann, K.J., Dutton, P.L., Netzel, T.L., Leight, J.S. and Rentzepis, P.M. (1975) Science 188, 1301–1304
20 Holten, D., Windsor, M.W., Parson, W.W. and Thornber, J.P. (1978) Biochim. Biophys. Acta 501, 112–126
21 Van Grondelle, R., Holmes, N.G., Rademaker, H. and Duysens, L.N.M. (1978) Biochim. Biophys. Acta 503, 10–25
22 Van Bochove, A.C., Van Grondelle, R. and Duysens, L.N.M. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Vol. 3, pp. 989–996, Balaban International Science Services, Philadelphia
23 Schenk, C.C., Blankenship, R.E. and Parson, W.W. (1982) Biochim. Biophys. Acta 680, 44–59
24 Reiss-Husson, F., De Klerk, H., Jolchine, G., Jauneau, E. and Kamen, M.D. (1971) Biochim. Biophys. Acta 234, 73–82
25 Spencer, D. (1976) Ph.D. Thesis, University of Illinois at Urbana, IL
26 Spencer, D. and Weber, G. (1969) Ann. N.Y. Acad. Sci. 158, 361–372
27 Lavorel, J. (1959) Plant Physiol. 34, 204–211
28 Zankel, K.L. and Clayton, R.K. (1969) Photochem. Photobiol. 9, 7–15
29 Monger, T.G. and Parson, W.W. (1977) Biochim. Biophys. Acta 460, 393–407
30 Godik, V.I. and Borisov, A.Yu. (1977) FEBS Lett. 82, (2) 355–358
31 Campillo, A.J., Hyer, R.C., Monger, T.G., Parson, W.W. and Shapiro, S.L. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1997–2001
32 Moya, I. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.) pp. 163–172, Balaban International Science Services, Philadelphia
33 Searle, G.F.W., Barber, J., Harris, L., Porter, G. and Tredwell, C. (1977) Biochim. Biophys. Acta 459, 390–401
34 Borisov, Y. and Il'Ina, M.D. (1973) Biochim. Biophys. Acta 305, 364–371
35 Paschenko, V., Z., Protasov, S.P., Rubin, A.B., Timofeev, K.N., Zamazova, L.M. and Rubin, L.B. (1975) Biochim. Biophys. Acta 408, 143–153