*FLUORESCENCE AND PHOTOCHEMICAL QUENCHING IN PHOTOSYNTHETIC REACTION CENTERS**

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Photosynthetic bacteria contain bacteriochlorophyll (BChl) that absorbs light and delivers the resulting singlet excitation energy to photochemical reaction centers.[1] The reaction centers, which have been isolated in a form free of light-harvesting BChl,[1-3] contain a photochemically specialized BChl which in *Rhodopseudomonas spheroides* is called P870 after its absorption maximum near 870 nm. Excitation energy reaching the reaction centers causes oxidation (bleaching) of P870 and, presumably, reduction of an electron acceptor. Another specialized BChl, P800, is associated with P870 in the reaction centers and shows a band shift to shorter wavelengths when P870 is bleached. The oxidation of P870 occurs with a quantum efficiency close to 100 per cent whether the light is absorbed by light-harvesting BChl in chromatophores[4, 5] or by either P800 or P870 in reaction center preparations.[6]

Cells or chromatophores of photosynthetic bacteria emit BChl fluorescence when illuminated. This fluorescence comes from the light-harvesting BChl and competes with the utilization of energy at the reaction centers.[1, 7] The yield of fluorescence from P870, measured in the absence of light-harvesting BChl, is small compared with that from light-harvesting BChl measured in chromatophores.[8] The low fluorescence yield of P870 shows that energy reaching this pigment is quenched rapidly, and the high quantum efficiency for P870 oxidation shows that most of this quenching is due to the initiation of photochemistry.

The lifetime of an excited state of a molecule is indicated by the yield of fluorescence corresponding to that state. If the only avenue for de-excitation were the radiative one, the yield would be 100 per cent and the lifetime would assume a maximum value called the intrinsic lifetime, τ_0. If de-excitation can occur by other competing processes such as a photochemical quenching, both the lifetime τ and the fluorescence quantum yield ϕ_f will be reduced in proportion:

$$\tau = \phi_f \tau_0. \tag{1}$$

The intrinsic lifetime can be computed[9] from the expression

$$\frac{1}{\tau_0} = \frac{2.9 \times 10^{-9}}{\langle k^{-3} \rangle_{av}} \int \left(\frac{\epsilon}{k}\right) dk, \tag{2}$$

where k is the reciprocal of the wavelength, cm^{-1}, and ϵ is the molar extinction coefficient, $M^{-1}\ cm^{-1}$, as used in specifying the optical density. The quantity $\langle k^{-3} \rangle_{av}$ is the value of k^{-3} averaged over the fluorescence band:

$$\langle k^{-3} \rangle_{av} = \frac{\int k^{-3} I_f dk}{\int I_f dk}, \tag{3}$$

where I_f is the fluorescence intensity in quanta per unit frequency range. Equa-

tion (2) was shown[9] to be reliable within a few per cent for strongly allowed transitions in several organic dyes.

Using equations (1) and (2), one can deduce the lifetime of excitation in light-harvesting BChl and in P870, provided that the yield of fluorescence from these components can be measured. Applying (2) to absorption and fluorescence spectra of chromatophores and reaction center preparations from *Rps. speroides* gives a value of 18×10^{-9} sec for τ_0, for the lowest singlet excited states of both light-harvesting BChl and P870. The yield of fluorescence from light-harvesting BChl in *Rps. spheroides* is about 5 per cent,[10] which by equation (1) gives a lifetime of 0.9×10^{-9} sec, in good agreement with measured lifetimes for BChl in other photosynthetic bacteria.[11] This is presumably the average time needed for the transfer of singlet excitation energy to the reaction centers. The finding[8] that P870 fluorescence is much weaker than that from light-harvesting BChl then implies that the lifetime of singlet excited P870 is much less than 10^{-9} sec.

This report will show that fluorescence can be measured in reaction centers prepared from *Rps. spheroides*, that some of this fluorescence comes from P870, and that the yield of P870 fluorescence corresponds to a lifetime of 7×10^{-12} sec. This is the time needed to initiate photochemical electron transfer, as shown by the high quantum efficiency of the photochemical oxidation of P870.

Methods.—Reaction centers were prepared from blue-green mutant *Rps. spheroides* as described earlier.[3] The absorption spectrum of a reaction center preparation, measured in strong and in weak light, is shown in Figure 1. Note that strong light causes bleaching of the band at 865 nm (P870) and blue shift of the band near 800 nm, (P800). In all experiments reported here, the reaction center concentration was adjusted to give an optical density of about 0.12 at 810 nm.

Fluorescence from a sample in a rectangular cuvette was monitored at right angles to an exciting beam from a tungsten-iodine lamp. The exciting light was filtered with about 2 in. of water, a Corning no. 4-96 (blue) or 2-64 (red) color filter, and an interference filter of about 10 nm in bandwidth. Interference filters provided 810-nm exciting light for reaction centers and 500- or 550-nm light for measurements with Rhodamine B. The exciting light intensity was measured with a Yellow Springs-Kettering Radiometer model 65. The fluorescence was analyzed with a Bausch and Lomb 0.5-m grating monochromator (the grating blazed at 750 nm) with slits set for a bandwidth of 13 nm. Additional filtering was provided by a Corning no. 2-64 or 3-73 filter, the former for measurements with reaction centers and the latter with Rhodamine B. The detector was an Amperex 150CVP photomultiplier.

The fluorimeter could also be used as a single-beam absorption spectrometer by the addition of a monochromatic measuring beam and the use of the same detection system. In this application, which yielded the data of Figure 3, the Bausch and Lomb monochromator was replaced by Wratten 87b and 87c filters plus an interference filter at the wavelength of measurement, 870 nm. Under these conditions the weak fluorescence of reaction centers did not interfere with the measurement of light-induced absorbance changes. In other experiments, a Cary model 14R spectrophotometer was used to measure changes in absorbance as well as absorption spectra. Here the exciting light had the same intensity, and the same geometry in relation to the sample, as in related fluorescence measurements.

The absolute fluorescence yield was measured by comparison with Rhodamine B, which has a yield in ethanol of 97 per cent.[12] Intensities of fluorescence from reaction centers and from Rhodamine B were measured at 900 and 580 nm, respectively, with actinic wavelengths of 810 and 550 nm, respectively. The samples had been adjusted to have equal absorbances at their respective exciting wavelengths and were exposed to measured

exciting light intensities. In addition, the fluorescence spectra were determined by using 810 and 500 nm excitation, respectively. The yields could then be computed from the integrated areas under the fluorescence spectra, normalized to the appropriate responses at 900 and 580 nm which in turn were generated by known rates of quantum absorption. No corrections were applied for effects of polarization, screening, or self-absorption; the samples had optical densities of about 0.12 at the exciting wavelengths and less at the significant emitting wavelengths. The spectral sensitivity of the combined detection system was calibrated with reference to the emission from a tungsten lamp, presumed to be a black body at 2860°K.

Effects of "false light" were found to be negligible in measuring fluorescence of Rhodamine B. Conclusions about reaction center fluorescence will be drawn from light-induced changes in the fluorescence yield, for which the effects of scattered exciting light and emission by components other than P870 are not important. By the use of 810-nm exciting light, it was possible to eliminate most of an annoying background of fluorescence from bacteriopheophytin in the reaction centers.

Excitation at 810 nm implies transfer of energy from P800 to P870; the experiments would have had greater simplicity if the P870 had been excited directly. Direct excitation of P870 was avoided because it entails technical difficulties. However, we have observed that P800 and P870 are about equally effective for driving the photochemical oxidation of P870 and also for sensitizing the fluorescence of this pigment. Therefore, the use of exciting light absorbed directly by P870 would have given essentially the same results as we shall report for excitation by way of P800.

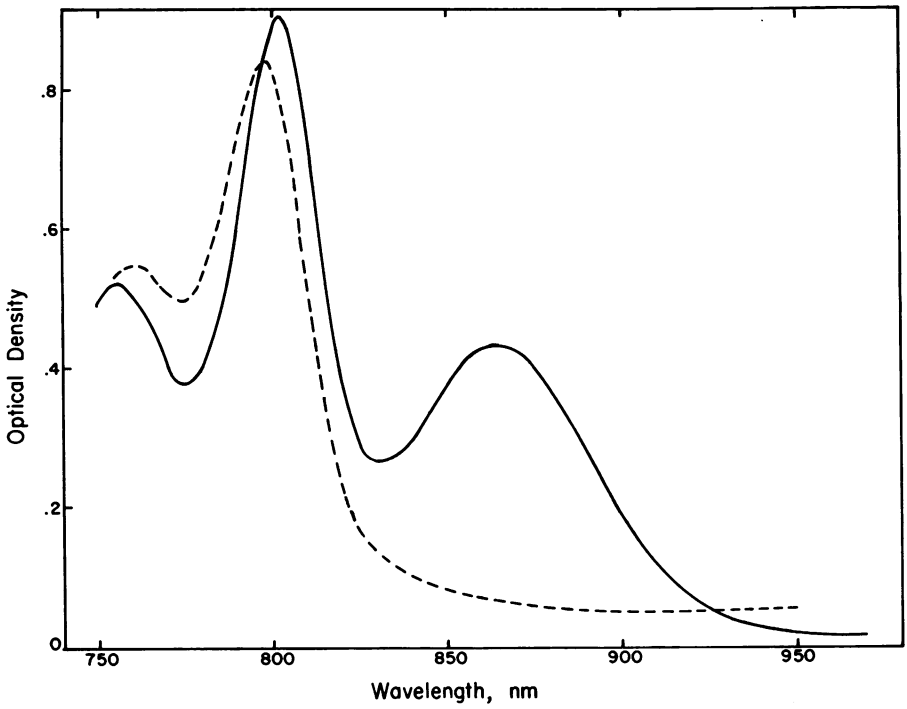

FIG. 1.—Absorption spectra of a reaction center preparation from *Rhodopseudomonas spheroides*, measured with a Cary model 14R spectrophotometer in the IR-1 and IR-2 modes. *Solid line*, IR-1 (sample exposed only to weak monochromatic light during measurement). *Dashed line*, IR-2 (sample exposed to strong white light during measurement). Data in the remaining figures were obtained with this kind of sample, diluted so that the optical density was about 0.12 at 810 nm (1-cm path).

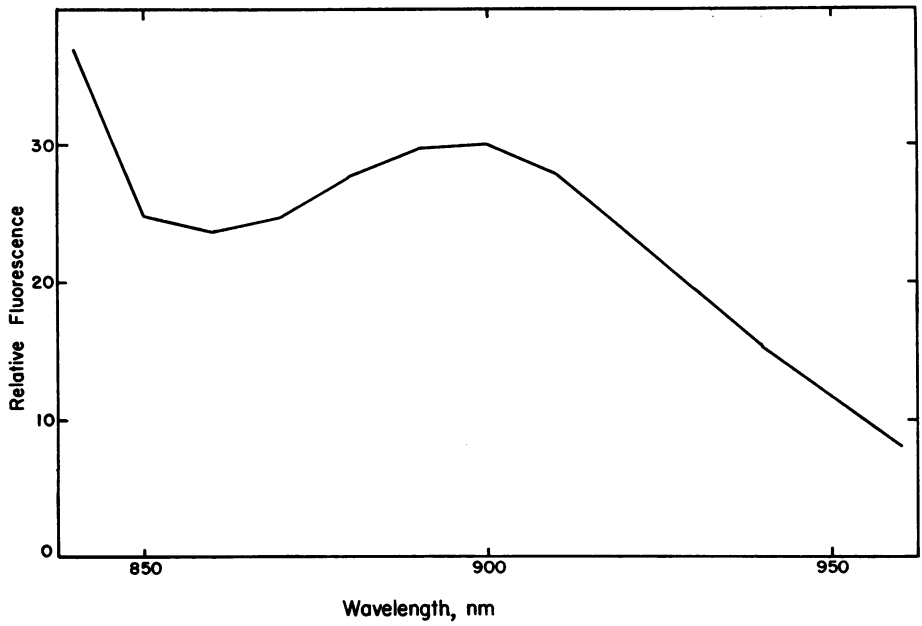

FIG. 2.—Spectrum of fluorescence plus "false light" obtained with reaction centers from *Rps spheroides* exposed to weak (0.02 mw/cm²) 810-nm exciting light. The rise at 840 nm is mainly caused by stray exciting light leaking through the 810-nm interference filter.

Results and Discussion.—Figure 1 shows how the absorption spectrum of a reaction center preparation is changed by strong illumination. The spectrum of fluorescence from reaction centers, excited by weak (0.02 mw/cm²) 810-nm light, is shown in Figure 2. This spectrum shows an emission band at about 900 nm superimposed on a background that increases toward shorter wavelengths. We shall show that most of the 900-nm band can be abstracted and identified as fluorescence coming from P870 (experiments with a phosphoroscope showed that a negligible fraction of this emission could be identified as delayed fluorescence of lifetime greater than 2 msec).

Any fluorescence coming from P870 should be missing under conditions (such as strong light) that bleach this pigment. The fluorescence should be seen to disappear as the P870 becomes bleached and, if certain conditions are met, the kinetics of decline in the fluorescence should parallel the kinetics of bleaching. Sufficient conditions are that the individual reaction centers (molecules of P870 with their associated P800 and other components) act independently of each other and that each unbleached reaction center has the same fluorescence yield. Figure 3 shows that the intensity of fluorescence does change in a way that mirrors the bleaching of P870. In Figure 3*a*, the bleaching of P870 was nearly complete. The fluorescence did not go to zero, but the time-varying part, when suitably normalized, showed the same kinetics as the absorption change. The time-independent residue, about one third of the total initial value, could be attributed to scattered exciting light and to fluorescence from substances other than photochemically active P870. With addition of phenazine methosulfate

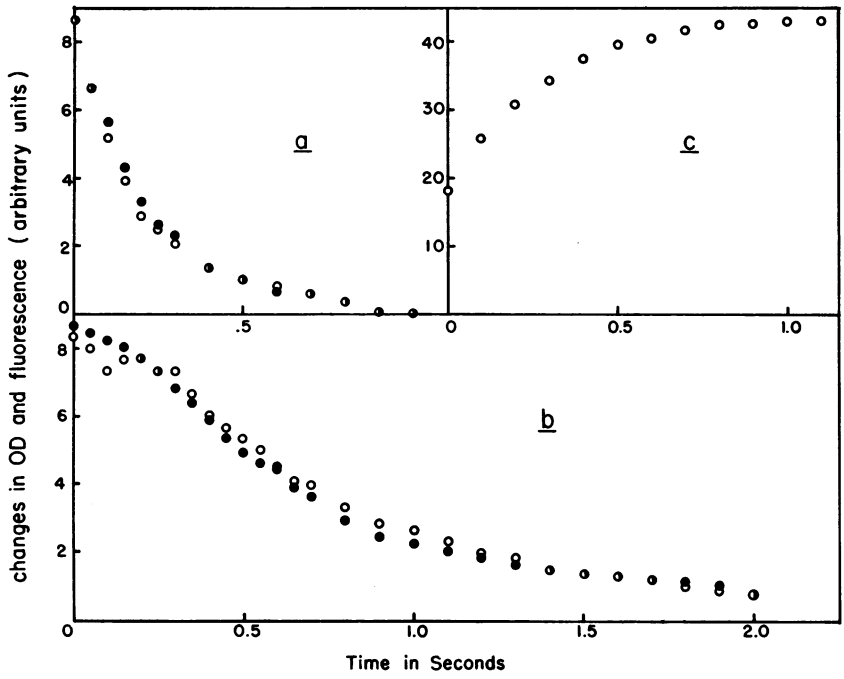

FIG. 3.—Changes in optical density and fluorescence of reaction centers from *Rps. spheroides*, with time after illumination. The exciting light intensity was 0.1 mw/cm². In (*a*), the total changes in absorbance and fluorescence from 0 to 20 sec were normalized to be equal. The same normalization factor was used in (*a*) and (*b*). The numerals on the ordinates were chosen so as to be commensurate in the three plots. (*a*) Untreated reaction centers; (*b*) reaction centers with 50 μM phenazine methosulfate; (*c*) reaction centers with 50 μM phenazine methosulfate and 1 mM dithiothreitol. *Open circles*, fluorescence. *Filled circles*, absorbance.

(PMS), the kinetics of the bleaching of P870 were altered markedly. Again the kinetics of the time-varying fluorescence mirrored those of the absorbancy change (Fig. 3*b*). Note especially that the same normalization factor was used in both cases (Fig. 3*a* and *b*). From these results it appears certain that the time-varying fluorescence comes from excited P870. This conclusion is verified by the spectrum of the time-varying fluorescence, which is appropriate for emission from P870 (see below, Fig. 4*a*).

If fluorescence from P870 competes with photochemical quenching, any inhibition of the latter should enhance the former. One way to inhibit the photochemistry is to reduce the primary electron acceptor so that it can no longer function in its photochemical role. This can apparently be done by adding sufficient amounts of either dithionite or dithiothreitol (DTT) under anaerobic conditions. Either of these treatments eliminates observable light-induced bleaching of P870 and at the same time causes a marked increase (about 5×) in the fluorescence band centered at 900 nm. Addition of DTT with PMS to reaction centers in an open cuvette has another effect: a reversible light-induced increase in the fluorescence, unaccompanied by any apparent change in the state of P870. Probably in the light steady state some of the acceptor molecules are reduced

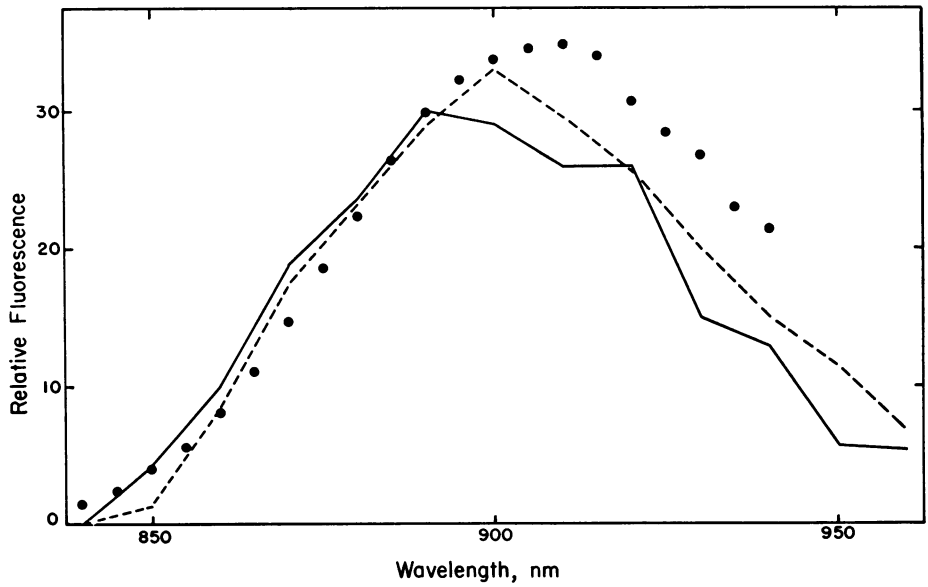

FIG. 4.—Spectra of components of the fluorescence from reaction centers. *Dashed line*, change in fluorescence yield (energy per unit wavelength interval) obtained by varying the exciting light intensity from 0.02 to 2 mw/cm^2. *Solid line*, total change in fluorescence during 30 sec after illumination at 2 mw/cm^2 (810 nm), with 50 μM phenazine methosulfate present. *Circles*, spectrum predicted theoretically from the shape of the absorption band of P870 (see text).

and those reaction centers give more P870 fluorescence, but in the dark the reaction centers all return to their functional form. This phenomenon is shown in Figure 3*c*.

Because light bleaches one of the fluorescing components (P870), the over-all quantum yield of fluorescence decreases with increasing actinic light intensity. The difference between the yield in dim light and that in bright light should then show a spectrum corresponding to fluorescence from P870. Figure 4 shows that it does. This figure also shows the time-varying fluorescence discussed earlier. These spectra can be compared with the shape of a fluorescence spectrum predicted theoretically from the shape of the absorption band of P870. The theoretical shape is obtained by multiplying the absorption at each wavelength by the Planck radiation formula for a black body at room temperature.[13] This procedure rests on the assumption that thermal relaxation among substrates of the excited state is complete, and this assumption may well be invalid because of the short lifetime of excited P870. In any case the spectrum computed in this way for P870 fluorescence is in reasonable agreement with the other spectra plotted in Figure 4. Furthermore, any deviations due to incomplete thermal relaxation would change the theoretical spectrum so as to correspond more closely to the observed spectra (smaller interval between absorption and fluorescence maxima).

Having identified part of the fluorescence as emission from P870, we determined the absolute quantum efficiency of this part as outlined in *Methods*. The

P870 fluorescence was abstracted both as the time-varying component in the presence of PMS and as the component responsible for the variations of the steady-state quantum yield with exciting light intensity. When probable errors were taken into account, the yield was between 0.25×10^{-3} and 0.55×10^{-3}. With an intrinsic lifetime of 18×10^{-9} sec, the average of these values for the yield gives an actual lifetime of 7×10^{-12} sec for the lowest singlet excited state of P870 in the reaction center preparation. This is probably the time needed for an excited electron to move from P870 to the primary acceptor and to become sufficiently stabilized so that the reverse process (which would restore singlet excited P870) is unlikely. There is no reason to believe that the photochemical time in the living cell is different from that inferred from the measurements with reaction centers.

Summary.—Reaction centers prepared from *Rhodopseudomonas spheroides* emit fluorescence, part of which can be identified, from its spectrum and its time variations, as coming from P870. The fluorescence from P870 has an absolute quantum yield of about 0.4×10^{-3}, corresponding to a lifetime of 7×10^{-12} sec for the lowest excited singlet state of P870. Since most of the singlet excitation energy accepted by P870 is used for its photochemical oxidation, 7×10^{-12} sec is apparently the time needed for transfer of an electron from excited P870 to an acceptor molecule.

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