A METHOD FOR MEASURING PICOSECOND PHENOMENA IN PHOTOLABILE SPECIES

THE EMISSION LIFETIME OF BACTERIORHODOPSIN

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ABSTRACT We have measured the emission lifetime of bacteriorhodopsin at physiological temperatures to be 15 ± 3 ps using a technique which employs a mode-locked dye laser, a sum frequency light gate, and a continuous flow system. We observe no concentration dependence of the lifetime over the range of 1.1×10^{-4} M to 1.0×10^{-5} M. We conclude that the emission which we observe comes from bacteriorhodopsin and not one of its photochemically produced intermediates, and that the emission cannot originate from the state into which light is absorbed.

INTRODUCTION

The observation of light emission from bacteriorhodopsin (Lewis et al., 1976) has opened the possibility of investigating the excited state ordering of the retinylidene chromophore, the center of photochemical activity in rhodopsin. Bacteriorhodopsin converts light energy into chemical energy, and the primary processes in this conversion occur in the excited state. Thus a knowledge of the state ordering in the retinylidene chromophore at physiological temperatures is of fundamental importance for the understanding of the initial steps in this energy conversion process.

We present here an experimental determination of the emission lifetime at physiological temperatures of the chromophore of bacteriorhodopsin. The technique we have employed involves the use of a mode-locked continuous wave tunable dye laser, a sum frequency light gate, and a continuous flow system. Our apparatus, which is generally applicable to the study of picosecond phenomena in photolabile species, has allowed us to time resolve the emission lifetime of bacteriorhodopsin without complications due to possible fluorescence from the photochemical intermediates produced by the interaction of a photon with the retinylidene chromophore. This technique, based on the unique characteristics of mode-locked continuous wave tunable dye lasers, can be readily adjusted to study various other picosecond phenomena in molecules, such as the resonance Raman effect of excited states and short-lived transient species.

Bacteriorhodopsin is contained in purple patches in the plasma membrane of the bacterium *Halobacterium halobium.* These purple membrane patches develop when

the bacteria are grown under low oxygen tension and under illumination (Osterhelt and Stoeckenius, 1971; Racker and Stoeckenius, 1974). Biochemical analysis of these purple membrane patches shows that there is only one protein found in these membranes (Osterhelt and Stoeckenius, 1973). This protein has been named bacteriorhodopsin by analogy with the visual pigment rhodopsin, since they both contain a retinal bound to an ϵ-amino group of a lysine residue (Osterhelt and Stoeckenius, 1971) via a protonated Schiff base linkage (Lewis et al., 1974). The absorption maximum of light-adapted bacteriorhodopsin is 570 nm, and its retinylidene chromophore is in the all-trans conformation (Osterhelt et al., 1973).

When illuminated, bacteriorhodopsin undergoes a series of reactions which are stimulated by the absorption of a photon (Lozier et al., 1975; Kung et al., 1975). Light energy absorbed by the retinylidene chromophore is converted into a proton gradient which the bacteria utilize to synthesize ATP (Racker and Stoeckenius, 1974; Danon and Stoeckenius, 1974). Resonance Raman studies on bacteriorhodopsin and its photochemically produced intermediates (Lewis et al., 1974) indicate that a deprotonation of the Schiff base nitrogen occurs on the same time scale as the generation of a proton gradient across the bacterial cell wall. The photochemically induced thermal intermediates generated when light is absorbed by bacteriorhodopsin are strikingly similar to those observed in visual pigments (Busch et al., 1972; Yoshizawa, 1972). Therefore an understanding of the energy conversion processes in this rhodopsin-like protein should advance considerably our knowledge of the energy transduction process in vision.

MATERIALS AND METHODS

Optical System and Electronics

The system for detection of the fluorescence and measurement of the lifetime consists of a synchronously pumped, mode-locked dye laser, a "sum frequency" light gate (Mahr and Hirsch, 1975; Duguay and Hansen, 1968), and a computerized signal averaging system as shown in Fig. 1. The output of the rhodamine 6-G dye laser is a continuous train of 10 ps pulses, separated by 10 ns. The dye laser, having a 0.1 nm bandwidth, was tuned to 580 or 590 nm, and the peak power at the sample was approximately 120 W in a 10 ps pulse. The train of identical pulses is split into two beams at the beam splitter (B). One beam is focused by lens (L1) onto the flowing sample while the other goes to a 90° prism (P) mounted on a motor-driven translator. The fluorescence emitted by the sample is collected and collimated by the same lens (L1) used to focus the laser onto the molecular jet, and is then combined at the beam splitter (B) with the laser pulse which is returning from the prism (P).

The fluorescence and the pulse are then focused together into a lithium iodate crystal (C) through a 16 cm focal length lens (L2). Within the crystal the two light waves are mixed to produce a third wave at the sum frequency $\omega_{sum} = \omega_f + \omega_l$ where ω_l is the frequency of the laser pulse and ω_f is a particular frequency within the fluorescence spectrum. This mixing occurs as a result of the high nonlinear dielectric susceptibility of the crystal. The particular range of frequencies within the fluorescence spectrum which is mixed with the laser pulse is determined by the direction of propagation through the crystal by the so-called "phase-matching" conditions (Boyd and Kleinman, 1968). The instantaneous power of the radiation generated at the sum frequency, P_+, is given by $P_+ = k P_f P_L$, where P_f and P_L are the instan-

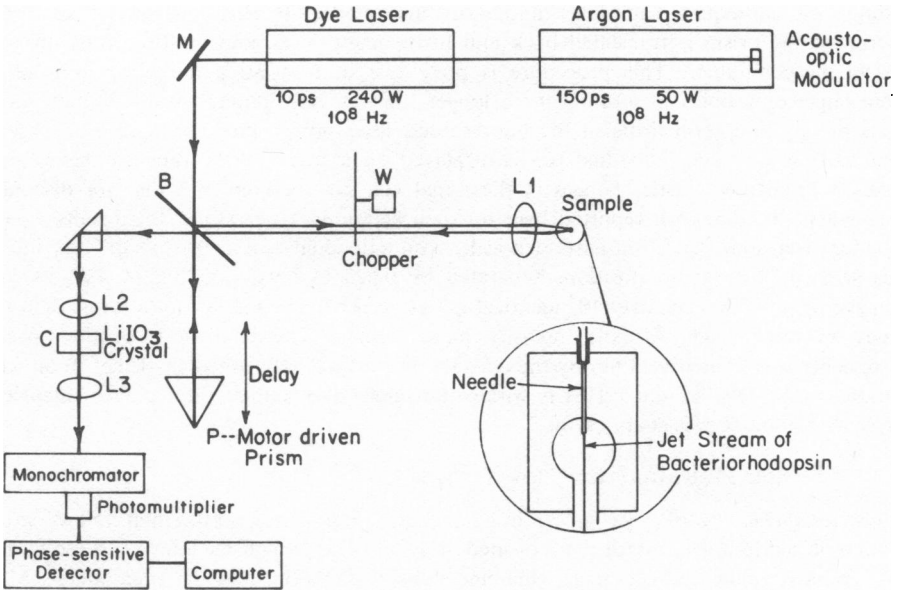

FIGURE 1 Schematic diagram of the experimental arrangement used for emission lifetime measurement.

taneous powers in watts of the fluorescence and the laser, respectively, and k has been measured to be 2.5×10^{-4} W^{-1} in our crystal. Since radiation at the sum frequency can be generated only when the two waves are simultaneously within the crystal, the latter acts as a gate which is open only for the duration of the pulse, namely 10 ps. Thus in this gate the resolution is determined only by the pulse and not by such factors as molecular reorientation which may affect the resolution of a Kerr cell light gate (Duguay and Hansen, 1969; Ippen and Shank, 1975). As prism (P) is translated, the relative arrival time of the laser pulse and the fluorescence at the crystal is varied: If the prism (P) is translated by a distance Δx, the resulting change in relative arrival time is $\Delta\tau = 2\Delta x/c$ where c is the speed of light. (For $\Delta x = 0.1$ in, $\Delta\tau$ is approximately 17 ps.) The time variation of the fluorescence can therefore be mapped out by observing the intensity of the sum frequency radiation as a function of position of the prism.

The sum frequency light is sent through a Corning 7-54 filter (Corning Glass Works, Corning, N.Y.) and a $\frac{1}{2}$ m monochromator to remove any traces of visible light, and is detected by a UV-sensitive photomultiplier (EMI 6255B; EMI Gencom Inc., Plainview, N.Y.) operated at 1,150 V in a dry-ice cooler. The photomultiplier signal is then detected with a PAR HR-8 phase-sensitive amplifier (Princeton Applied Research, Princeton, N.J.). The mechanical chopper wheel (W) in the sample arm of the system modulates the fluorescence and provides the reference signal for the phase-sensitive detection. The sensitivity of the apparatus could be significantly improved by employing photon-counting techniques.

Translating the prism and recording the signal is done automatically with a Digital Equipment Corp. (Marlboro, Mass.) GT-40 computer system. For each position of the prism, the signal from the phase-sensitive detector is integrated for a desired time interval, digitized, and stored in the computer memory. At the end of the integration interval the prism (P) is stepped to the next position, and the new signal is integrated. At the beginning of each run the laser beam in the prism arm is briefly blocked by a shutter to obtain a background

reading. All subsequent samplings in the run are referred to this zero level. In a given experiment the prism is translated back and forth many times, and multiple runs are averaged in the computer. This procedure is preferable to translating the prism through the whole range only once, integrating for a longer time at each point, since it minimizes the effects of any long-term drifts in the fluorescence, laser power, etc. The curve of observed signal vs time for each individual run is displayed on a cathode ray tube as the run progresses, so that any drastic changes in the signal can be observed and the run discounted if necessary. In the work reported here no such action was necessary; the results of each individual run appeared essentially identical. An individual run consisted of 10-s integrations at each of 28 prism positions separated by 0.025 in corresponding to 4.23 ps steps. Such averaging of the data over 10^9 identical pulses gives better signal-to-noise ratios than techniques involving single variable intensity pulse trains. The bandwidth of the observed fluorescence was limited to 3 nm by the crystal. The crystal was tuned to detect fluorescence over the range 779–782 nm. This is within the range of maximal fluorescence intensity in purple membrane (Lewis et al., 1976).

Sample Preparation and Flow System

Halobacterium halobium R_1 was grown in batches in 2-liter Roux bottles half full of growth medium placed in a water bath thermostated at 39°C. The growth medium consisted of basal salts, essential amino acids, L-malic, thiamine, folic acid, biotin, and the trace metals Mn^{++}, Zn^{++}, Cu^{++}, and Fe^{++} (Lanyi et al., 1976). The medium was passed through soft filter paper and its pH adjusted to 6.8 with NaOH. Purple membrane was isolated from stationary phase cultures by the method of Osterhelt and Stoeckenius (1974). SDS-polyacrylamide gel electrophoresis yielded only one protein band with an apparent molecular weight of 26,000, and the ratio of the 280/560 absorption was 2.05 for the bacteriorhodopsin utilized in our experiments.

The flow system consisted of a small variable speed peristaltic pump (Cole-Parmer no. 7016 Masterflex tubing pump; Cole-Parmer Instrument Co., Chicago, Ill.) inserted into a recirculating system with a 20 ml reservoir. Sample solutions were forced through a 2 in Yale 22 g syringe needle having an internal diameter of 0.41 mm yielding a fine stream through air. We measured the mean flow velocity to be about 360 cm/s by measurement of bulk flow rate which was approximately 0.5 ml/s. We focused the laser beam on the sample solutions less than 1 mm below the tip of the syringe needle. The diameter of the laser beam to the $1/e^2$ points was measured to be 35 ± 3 μm by translation of a razor blade across the beam spot at its focus. With a 35 μm diameter beam spot and the above flow rate, the transit time in the beam for any given molecule was 9.8 μs. There was an average delay of 40 s before a given molecule passed through the beam a second time, thus ensuring that photochemical cycling was complete. The temperature of samples in the laser beam was about 29°C, as measured by a thermocouple attached to the syringe needle.

Before attempting to obtain a signal from the purple membrane, a weak emitter, we aligned and calibrated our instrument with an easily observable signal from the dye 3,3'-diethylthiodicarbocyanine iodide (DTDC), a strong emitter. We pumped this dye through the flow system and maximized our signal with the monochromator set at 334.4 nm, the sum frequency of the fluorescence at 772 nm and the dye laser frequency at 590 nm. The dye concentration was 5×10^{-4} M. After measurement of the DTDC fluorescence we flushed the pump system with ethanol, and then with doubly distilled water. After flushing the pump system thoroughly, 20 ml of sonicated purple membrane was introduced into a new reservoir. The only part of the flow system that was common to both samples was the syringe needle. The position of the sample stream was not altered during the course of these manipulations, and only in this manner were we able to observe a signal from bacteriorhodopsin which has a quantum

efficiency less than 10^{-3} (Lewis et al., 1976). Various concentrations of bacteriorhodopsin ranging from 1.1×10^{-4} M to 1.0×10^{-5} M were utilized in these experiments. To verify that the signal was actually from the purple membrane, distilled water was flowed through the system and background measurements were taken. This background signal was the same level as our dark current noise level.

THEORY OF THE MEASUREMENT

As discussed above, the lithium iodate crystal acts as a light gate. The observed signal at the sum frequency as a function of the delay time τ, $S(\tau)$, is given by

$$S(\tau) = \int_{-\infty}^{\infty} F(t)P(t - \tau)\mathrm{d}t, \tag{1}$$

where $P(t)$ is the laser pulse shape and $F(t)$ is the sample fluorescence intensity. The delay time τ is determined by the position of the motor driven prism. Assuming a single exponential decay and an instantaneous rise time for the sample fluorescence, $F(t)$ is given by

$$F(t) = \int_{-\infty}^{t} P(t')\exp[-(t - t')/\tau_F]\mathrm{d}t', \tag{2}$$

where τ_F is the lifetime of the observed fluorescence.

The response of the light gate and the character of the laser pulse is obtained by measuring the autocorrelation function $A(\tau)$ of the laser pulse given by

$$A(\tau) = \int_{-\infty}^{\infty} P(t)P(t - \tau)\mathrm{d}t. \tag{3}$$

This is measured by replacing the sample and lens L1 in Fig. 1 with a second right-angle prism.

At small delay times, where the laser pulse is still apparent, the observed signal is complicated by its presence. However, if the delay time, τ, is large enough the observed signal will be an exponentially decreasing function of τ with the correct fluorescence decay time τ_F.

RESULTS

Emission lifetime measurements were done on suspensions of purple membrane fragments at physiological temperature over the concentration range 1.0×10^{-5} M to 1.1×10^{-4} M. A semi-log plot of the observed fluorescence signal vs. delay time for a typical set of runs is shown in Fig. 2. Also shown in Fig. 2 is the laser pulse autocorrelation function. It is evident that the fluorescence rises with the laser pulse indicating that there is no resolvable time delay for the onset of fluorescence. Also the decay time of the bacteriorhodopsin emission is clearly resolved. Lifetimes are obtained by a least squares fit to data points detected after the exciting pulse intensity has decayed by a factor of 10.

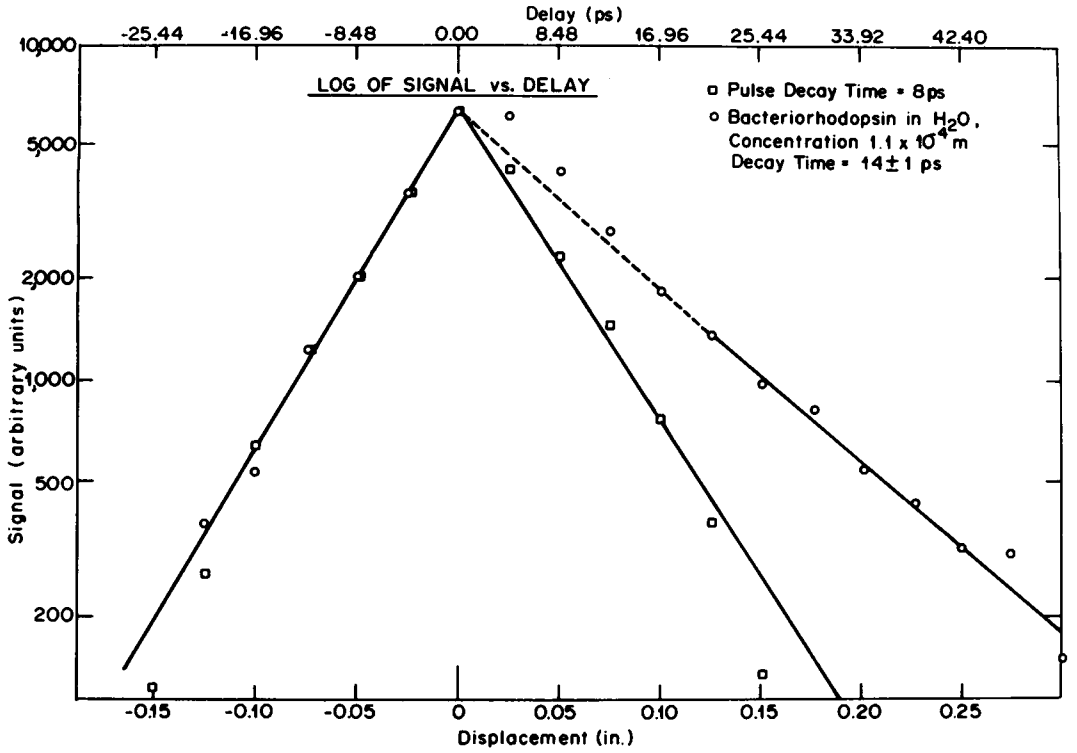

FIGURE 2 Plot of log of measured signal vs. delay time τ for bacteriorhodopsin (○) and the laser pulse (□). The solid line on the bacteriorhodopsin trace is after pulse has decayed by a factor of 10. The dotted line is extrapolated to zero time. The vertical dimension of each data point shown represents the uncertainty in the measured intensity.

Results of data for various concentrations are summarized in Table I. No significant variation in the observed emission lifetime is found to occur over the range of concentrations and wavelengths investigated.

DISCUSSION

In determining the emission lifetime it is important to minimize the concentrations of the photochemically produced intermediates of bacteriorhodopsin which may themselves fluoresce. Upon absorbing a photon bacteriorhodopsin (bR 570) is converted to a red-shifted intermediate (batho-bacteriorhodopsin) with a formation time of less than 6 ps (Kaufmann et al., 1976). This intermediate which has an absorption maximum at ~635 nm (Lozier et al., 1975; Kung et al., 1975) decays in 2 μsec to a blue shifted species absorbing maximally at 540 nm. The next intermediate in the sequence has an absorption maximum at 412 nm and is formed on the order of 50 μs (Lozier et al., 1975; Kung et al., 1975; Marcus and Lewis, 1976) and has been shown by resonance Raman studies (Lewis et al., 1974) to have a chromophore with an unprotonated Schiff base. We have flowed the sample with a speed fast enough to remove the photo-

TABLE I

LIFETIMES OF EMISSION FROM BACTERIORHODOPSIN FOR VARIOUS CONCENTRATIONS

Values of σ are given. Calculations are done by a least squares fit to data.

Concentration	Lifetime
mol	*ps*
1.1×10^{-4}	15.0 ± 1.5
0.9×10^{-5}	14.9 ± 2.1
4.5×10^{-5}	12.1 ± 6.0
1.0×10^{-5}	14.8 ± 4.9

lyzed bacteriorhodopsin before the 412 nm intermediate is formed. Thus the emission lifetime we detect can arise from either bacteriorhodopsin, batho-bacteriorhodopsin (635 nm), or the 540 nm species which precedes the formation of the 412 nm intermediate. However, Lewis et al. (1976) have argued that these intermediates do not contribute to the observed fluorescence when excited with 580–590 nm excitation. Our data tend to support this conclusion especially as far as the 540 nm intermediate is concerned, since the rise time of the emission is coincident with the laser pulse as indicated in Fig. 2. Thus we conclude that the emission lifetime of 15 ± 3 ps which we observe at 29°C is for bacteriorhodopsin in its light-adapted 570 nm form.

Other workers (Alfano et al., 1976) have measured the emission lifetime of bacteriorhodopsin. However they could not resolve the room temperature emission using a mode-locked Nd:glass laser at 530 nm and an intensity of approximately 6×10^{14} photons/cm^2. The resolution of the equipment of Alfano et al. was 8 ps; thus they concluded that the lifetime for the observed emission is less than this value. The present experiments utilized a laser pulse intensity of 3.2×10^{14} photons/cm^2.

Let us now examine reasons for the difference between these results, including the possibility of annihilation phenomena occurring in the samples. Mauzerall (1976) and Campillo et al. (1976) have demonstrated that in photosynthetic systems with well-ordered interacting pigment molecules the emission lifetime decreases with higher peak excitation intensities. Since bacteriorhodopsin also has a well-ordered pigment structure and exhibits excitonic interactions (Heyn et al., 1975; Becher and Ebrey, 1976; Bauer et al., 1976) similar effects may be observable in its emission lifetime.

It is a well known fact that molecules in an excited singlet state which are near one another can interact, leading to singlet-singlet annihilation. Such interactions have been investigated previously by Frölich and Mahr (1966) for the case of energy transfer in crystals and by Mauzerall (1976) and Campillo et al. (1976) in photosynthetic systems. This type of singlet-singlet annihilation process which can take place over distances as large as 100 Å might lead to a shortening of the observed emission lifetime and a corresponding decrease in the quantum efficiency, since the fluorescence intensity is dependent on the excited state population. Such processes may account for

the differences in emission lifetime observed by different investigators. Förster (1949) and Frölich and Mahr (1966), for example, have shown that if a dipole-dipole coupling exists between excited molecules, the fluorescence decay rate immediately following excitation increases as the initial concentration of excited states increases.

We now examine the likelihood of such singlet-singlet excited state interactions occurring in our experiments. From the known values of pulse duration, average power and focused beam diameter, the photon flux at the sample was calculated to be 3.2×10^{14} photons/cm^2 per pulse. The number of photons absorbed per molecule per pulse is given by $I_o \sigma_A \tau_p / \pi r_o^2$, where I_o equals the number of photons per second, σ_A is the absorption cross section at 5,800 Å, r_o is the radius of the laser beam at the sample, and τ_p is the width of the laser pulse. Under the conditions utilized in our experiments this number is 0.067 photons absorbed per molecule per pulse. It has been shown by Unwin and Henderson (1975) that the purple membrane is arranged in a two-dimensional hexagonal array with a 62 Å by 62 Å cell with a trimer of molecules about each lattice point. Thus there are 21 bacteriorhodopsin molecules within a 62 Å radius. The cross section of absorption at 5,800 Å in the purple membrane is 2.1 Å^2. Becher and Ebrey (1976) and Bauer et al. (1976) have demonstrated excitation coupling in a trimer. Utilizing this fact, the effective absorption cross section of the purple membrane becomes about 6.3 Å^2, that of a trimer. At our photon density we present one photon every 31.3 Å^2. This implies that about one out of every five trimers is excited by each pulse. At this density of excited trimers singlet-singlet annihilation phenomena may occur to a limited extent. These annihilations will reduce the excited state population even further so that the rate of decay will rapidly approach that of an isolated excited molecule. We therefore believe that the decay time we observe is close to the natural lifetime of bacteriorhodopsin emission in the absence of singlet-singlet annihilation phenomena.

It is tempting to suppose that singlet-singlet annihilation phenomena could explain the seemingly shorter emission lifetime observed by Alfano et al. However, the intensities in the two experiments were so similar that a convincing argument cannot be made at this time. A possible alternate explanation could be the fact that the uncertainties in the measured intensities in our experiment (see Fig. 2) were significantly less than those of Alfano et al., because of the greater number of events observable with a continuous train of identical laser pulses.

Since the sample was in the beam for many pulses in our experiments, the possibility of excited state interactions which arise from the fact that we were exciting with multiple pulses should be considered. Each molecule was in the beam for about 10^3 pulses, time enough for about 67 photons to be absorbed per molecule. Now suppose that the quantum efficiency of intersystem crossing into the triplet manifold is great enough to build up a concentration of molecules in the triplet state. This requires the quantum efficiency for intersystem crossing, ϕ_{ISC}, to be at least 0.015. Molecules in this triplet state can interact with excited singlets via the following mechanism:

$$S_1 + T_0 \rightarrow T_1 + S_0.$$

It is possible that this type of singlet-triplet state fusion process may deplete the S_1 population and thereby shorten the observed lifetime for emission. However, there is no evidence at present that the quantum yield of intersystem crossing is great enough to populate the triplet state. Fisher and Weiss (1974) have found $\phi_{ISC} < 0.001$ in a protonated Schiff base of retinal. Also Alfano et al. (1976) did not observe changes in the emission from pulse to pulse. Thus we do not believe that we are observing multiple pulse phenomena in our emission lifetime measurements.

As is evident from Table I, no concentration dependence of the emission lifetime was observed. This lack of a concentration effect over the range investigated can be readily explained. At the highest concentration utilized in these experiments the average center to center distance between molecules is calculated to be about 250 Å. This is much greater than the intermolecular distance of molecules on the same patch. Thus the maximal intermolecular interactions occur between molecules on the same purple membrane patch irrespective of the gross macromolecular concentration.

We prepared a sample of bacteriorhodopsin without the intrapatch interactions in order to verify that the shortness of the lifetime is due to interactions among neighboring molecules. To do so we solubilized 100 mg of bacteriorhodopsin in 1% Triton X-100 at a concentration of 1.0×10^{-4} M. This was performed overnight at room temperature in the dark. Unfortunately we were unable to obtain a good estimate of the emission lifetime from this sample due to its photolability. The laser intensities necessary for our measurements were high enough to bleach the flowing solubilized membranes in too short a time for reliable data to be taken.

We have shown that an excited state of bacteriorhodopsin and not one of its photochemically produced intermediates is responsible for the emission lifetimes which we observe. Lewis et al. (1976) have measured the quantum efficiency of emission as being between 1.2×10^{-4} and 2.5×10^{-4}. The radiative lifetime for emission (τ_o) is given by τ_f/ϕ where τ_f is the observed emission lifetime and ϕ is the quantum efficiency. From our measured value of τ_f and the value of ϕ given above we obtain a radiative lifetime of 60–125 ns. However, integration of the bacteriorhodopsin absorption spectrum yields a radiative lifetime for the allowed transition from the ^1B_u state of only about 7 ns. Similar discrepancies between calculated and experimental radiative lifetimes have been discussed for model polyenes by Hudson and Kohler (1973). There is also a large separation between absorption and emission spectra of model polyenes Hudson and Kohler, 1973) as is the case in bacteriorhodopsin (Lewis et al., 1976). Since the radiative lifetime of the emitting state we observe differs so radically from the lifetime for the allowed transition as calculated from the absorption spectrum, we conclude as did Hudson and Kohler for model systems that the emission from bacteriorhodopsin cannot originate from the ^1B_u state.

The lifetime of 15 ± 3 ps for the emitting state is equal to the lifetime of a transient reported by Kaufmann et al. (1976) in their experiment to determine the formation time of batho-bacteriorhodopsin (K_{635}). It is therefore possible that this transient absorption originates from the low-lying state from which we observe the emission. Finally the absorption of the transient is red shifted from that of bR_{570}. Thus it is

possible that we are observing emission from an electronic state which is a precursor to the batho intermediate.

In this article we have been able to resolve and to measure at physiological temperatures the emission lifetime for bacteriorhodopsin using a continuous flow method employing a mode-locked tunable dye laser and a fast optical gate. This lifetime has been determined to be 15 ± 3 ps. We have discussed the possibility that annihilation processes may shorten the apparent lifetime for sufficiently high excitation intensities. We believe, however, that such effects were minimal in our measurements. Finally, it has been shown that the emission cannot originate from the ^1B_u state, thus adding support to the conclusions of Lewis et al. (1976).

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