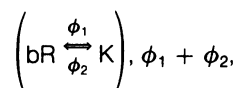


Quantum efficiency of the photochemical cycle of bacteriorhodopsin

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ABSTRACT Values in the literature for the quantum efficiency of the photochemical cycle of bacteriorhodopsin (bR) range from 0.25 to 0.79 and the sum of the quantum yields of the forward and back photoreactions



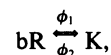
has been proposed to be 1. In the present work, low intensity laser flashes (532 nm) and kinetic spectroscopy were used to determine the quantum efficiency of bR photoconversion, ϕ_{bR} , by measuring transient bleaching of bR at 610 nm in the millisecond time scale. Bovine rhodopsin (R) in 2% ammonyx LO was used as a photon counter. We find that the ratio of the quantum yields of bacteriorhodopsin photoconversion and bleaching of rhodopsin, $\phi_{\text{bR}}/\phi_{\text{R}}$, is 0.96 ± 0.04 . Based on the quantum yield of the photobleaching of rhodopsin, 0.67, the quantum efficiency of bR photoconversion was determined to be 0.64 ± 0.04 . The quantum yield of M formation was found to be 0.65 ± 0.06 . From the transient bleaching of bR at 610 nm with a saturating laser flash (28 mJ/cm^2) the maximum amount of bR cycling was estimated to be $47 \pm 3\%$. From this value and the spectrum of K published in the literature, the ratio of the efficiencies of the forward and back light reactions, ϕ_1/ϕ_2 , was estimated to be 0.67 ± 0.06 and so $\phi_2 \approx 1$ (0.94 ± 0.06). The sum of $\phi_1 + \phi_2 \approx 1.6$. It was found that repeated high-intensity laser flashes ($>20 \text{ mJ/cm}^2$) irreversibly transformed bR into two stable photoproducts. One has its absorption maximum at 605 nm and the other has a well-resolved vibronic spectrum with maxima at 342, 359 (main peak), and 379 nm. The quantum yield of the formation of the photoproducts is $\approx 10^{-4}$.

INTRODUCTION

Bacteriorhodopsin (bR) is the sole protein present in the purple membrane of *Halobacterium halobium*. Its chromophore, retinal, is bound via a protonated Schiff base to the ϵ -amino group of lysine 216. Upon light absorption the light-adapted form of bacteriorhodopsin (bR^{LA}) undergoes a photocycle, during which several spectroscopically distinct species (K, L, M, N, and O) appear and disappear, until finally the parent pigment is restored. During the photocycle transient deprotonation of the Schiff base occurs, and proton translocation takes place from inside the cell to the outside. The light-induced electrochemical gradient of protons is utilized by the cell in the synthesis of ATP, ion transport, etc. (1).

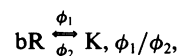
The efficiency of energy storage in purple membrane is directly dependent on the quantum yield of bacteriorhodopsin photoconversion. The values for the quantum yield of bR to M photoconversion that have been reported vary from 0.79 (2) through 0.6 (3) and 0.67 (4) to 0.3 (5)

at room temperature and 0.3 at -40°C (6). From indirect calculations the quantum efficiency of the formation of the primary photoproduct K at room temperature was found to be <0.4 (7), 0.25 (8), 0.6 (9), and 0.33 (10). The ratio of the quantum efficiencies of the forward and back light reactions,



was reported to be 0.4 (7).

In measurements at liquid nitrogen temperatures the ratio of the quantum efficiency of the forward and back light reactions,



was determined to be 0.5 ± 0.1 (11–14) or 0.45 ± 0.03 (15). Estimates of the quantum efficiency of the forward reaction, $\text{bR} \rightarrow \text{K}$, are >0.2 (12), 0.33 (16), and <0.48 (15). The values for the maximum photosteady state concentration of K at low temperature vary from 0.50

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(11–13), 0.53 ± 0.04 (14), and 0.46 ± 0.04 (15) to 0.34 (17) and 0.28 (18). The variability in the data from different laboratories could be due to different methods of measurements and different assumptions made for the calculations. It has also been suggested that bacteriorhodopsin exists in at least two conformations which may possess different quantum yields (15, 19–21). Moreover there is fairly good evidence for the existence of two (22) or more (23) conformers of bR at low temperature.

In the present work the quantum efficiency of the photochemical cycle of bacteriorhodopsin was determined by measuring transient bleaching of the absorption band of bR, using bovine rhodopsin as an actinometer. At room temperature the quantum efficiency of bR photoconversion was found to be 0.64 ± 0.03 .¹

MATERIALS AND METHODS

Halobacterium halobium, strain S9, was grown and purple membrane prepared as described previously (25).

Bovine rod outer segments (ROS) were prepared according to the method of Fung and Stryer (26) except that the retinas were homogenized in 20 mM MOPS buffer (pH 7.2) followed by a single floatation in 38% sucrose. The ROS were then washed twice in 20 mM MOPS. 2% ammonyx LO was used to solubilize the rod outer segments. 20 mM hydroxylamine was added to rhodopsin samples to accelerate the bleaching of rhodopsin during flash photolysis. All procedures with rhodopsin were carried out under dim red light. The quantum efficiency of rhodopsin bleaching was taken to be 0.67 (27).

Light-induced absorbance changes were measured on a single beam kinetic photometer. The sample was placed in a 3-mm square quartz cuvette thermostated at 20°C. The source of actinic illumination was the second harmonic of a Quanta Ray DCR-11 Nd:YAG laser (532 nm; 7 ns pulse; Spectra Physics, Mountain View, CA).

The measurements were made under "magic angle" conditions. Two different schemes for mutual polarization of the actinic and measuring beam were used. In both cases the actinic laser beam was perpendicular to the measuring beam. In the first case (experiments 1 and 2, Table 1) the actinic laser beam was vertically polarized. The polarization of the measuring beam was chosen at the magic angle, 54.7° from the normal to the plane. Under these conditions changes in the orientation of the chromophores due to rotation of rhodopsin and tumbling of purple membrane during the time of measurement will not contribute to the transient absorbance changes (28). In the second case (experiments 3 and 4, Table 1) opal glass was placed before the sample in the path of the actinic beam to produce homogeneous illumination of the sample. Because opal glass depolarizes the actinic irradiation, the polarization of the measuring beam was at 35° from the normal to the plane of measuring and actinic beams, which also corresponds to the magic angle conditions (28). The data obtained under these conditions coincide within the experimental error. Several experiments (5–8, Table 1) were done with opal glass and vertical polarization of the measuring beam which gave close results.

Special care was taken to ensure a good overlap of the measuring beam and the laser spot. Sample volume was kept to a minimum so that

¹These data were presented at the 34th Annual Meeting of the Biophysical Society of USA, Baltimore, MD, 18–22 February, 1990 (24).

TABLE 1 Relative quantum efficiency of flash-induced transient bleaching of bacteriorhodopsin

Experiment	Polarization*	α_{bR} [‡]	α_R [§]	ϕ_{bR}/ϕ_R	ϕ_{bR}
	<i>act/meas</i>	%	%		
1	0°/55°	3.22	2.12	0.92	0.62
2	0°/55°	4.53	3.08	0.90	0.60
3	OG/35°	3.55	2.40	0.90	0.60
4	OG/35°	3.34	2.07	0.98	0.66
5	OG/0°	4.3	2.9	0.94	0.63
6	OG/0°	4.8	3.0	0.98	0.66
7	OG/0°	5.6	3.6	0.95	0.64
8	OG/0°	2.5	1.6	0.99	0.66

*Polarization of the actinic and measuring beam: 0° corresponds to vertical polarization, OG means random polarization produced by opal glass.

[‡]Suspensions of purple membrane in 10–20 mM MOPS or Hepes, pH 7.0 were used. Optical density of the sample at the excitation wavelength (532 nm) was 0.19. 3 mm square cuvette was used both for flash-induced and absolute absorption measurements.

[§]Bovine rhodopsin solubilized in 2% Ammonyx LO + 20 mM hydroxylamine + 10 mM MOPS, pH 7.2, OD at 532 nm was 0.19.

^{||} ϕ_{bR}/ϕ_R was calculated from Eq. 3.

The quantum efficiency of rhodopsin bleaching was assumed to be 0.67 (27).

the entire volume could be exposed to the actinic laser flash. The optical density of the sample was 0.2 at 532 nm. Absorption spectra were measured on a Cary-Aviv 14 spectrophotometer with an end-on photomultiplier to minimize the effects of scattering. The laser intensity was estimated by using the power per flash, measured with a power meter (Scientech Inc., Boulder, CO), and the area of the flash measured from the burning pattern on a piece of exposed polaroid film. The maximum power was 37 mJ/pulse, the area of the spot, 0.3–0.4 cm². At low light intensities, laser power was calculated from the amount of rhodopsin bleached.

With low energy flashes, when not more than a few percent of the total pigment is excited, the quantum efficiency of bacteriorhodopsin photoconversion, ϕ_{bR} , can be calculated as a ratio of the number of molecules cycling, δn_{bR} , and the number of quanta absorbed ($N[1 - \text{Transmission}]$):

$$\phi_{bR} = \delta n_{bR} / N(1 - T_{bR}). \quad (1)$$

Using rhodopsin as a photon counter, ϕ_{bR} can be obtained from the relative measurements of bacteriorhodopsin and rhodopsin bleaching under the same conditions of excitation with the same dose of photons, N :

$$\phi_R = \frac{\delta n_R}{N(1 - T_R)}.$$

Thus taking the ratio

$$\phi_{bR} = \phi_R \delta n_{bR} \cdot (1 - T_R) / \delta n_R \cdot (1 - T_{bR}). \quad (2)$$

At low optical density (<0.2) the number of molecules transformed (δn) is proportional to the total number of molecules, n , ($\delta n = \alpha n$). The number of molecules n can be determined from the maximum absorbance of the pigments, A_{bR} and A_R , and their extinction coefficients, ϵ_{bR} and ϵ_R , so $\delta n_{bR} = \alpha_{bR} A_{bR} / \epsilon_{bR}$, where α_{bR} is the percent of bR molecules undergoing a photocycle in a single flash.

One can then obtain the following formula for ϕ_{bR} :

$$\phi_{bR} = \phi_R \cdot \alpha_{bR} \cdot \epsilon_R \cdot (1 - T_R) \cdot A_{bR} / [A_R \cdot \alpha_R \cdot \epsilon_{bR} \cdot (1 - T_{bR})]. \quad (3)$$

α_{bR} was calculated as the ratio of the transient bleaching and absorbance of bacteriorhodopsin, $\Delta A/A$, at 610 nm; ΔA was determined by deconvolution of the kinetic traces like those shown in Fig. 1 A; α_R —percent of rhodopsin bleached by a single flash, calculated as a ratio $\Delta A/A$ at 540 or 560 nm; ϵ_{bR} —extinction of bacteriorhodopsin at 570 nm, 63,000 l/mol cm (29); ϵ_R —extinction of rhodopsin at 500 nm, 40,600 l/mol cm (27); $(1 - T_R)$ and $(1 - T_{bR})$ —fractional absorption at 532 nm, of rhodopsin and bacteriorhodopsin, respectively.

As one can see the most critical parameter in the determination of ϕ_{bR} is the percent of molecules undergoing photoconversion, α_{bR} and α_R .

Excitation with saturating laser flashes was used to determine the maximal fraction of bR, α_{bR}^{\max} , undergoing cyclic photoconversion. Under certain conditions, when K completely decays to the L and M intermediates, this value represents the photosteady state fraction of K, x_K , produced during the laser pulse which in turn depends on the ratio of the quantum yields of the forward and back reactions, ϕ_1/ϕ_2 , and relative extinctions of K and bR (14, 23):

$$x_K = (1 + \phi_2 \epsilon_K / \phi_1 \epsilon_{bR})^{-1}. \quad (4)$$

RESULTS

Determination of ϕ_{bR} : transient photolysis of bacteriorhodopsin and rhodopsin with nonsaturating laser flashes

Fig. 1 A shows the light-induced absorbance changes, ΔA , of light-adapted bacteriorhodopsin (pH 7) at 610, 570, and 410 nm produced by a nonsaturating laser pulse (0.15 mJ/cm²) which converts ~4.5% of the total bacteriorhodopsin. The difference spectrum (ΔA vs. wavelength, inverted and normalized at 610 nm) is plotted together with the absorption spectrum of bR in Fig. 1 B. One can see that the difference spectrum overlaps the absorption spectrum of bR at wavelengths longer than 590 nm but differs significantly at shorter wavelengths. The difference between the ΔA and A spectra is clearly seen in Fig. 2 A, where the ratio $\Delta A/A$ is plotted. The ratio is not constant and drops significantly at shorter wavelengths (between 570 and 500 nm). Because M does not absorb beyond 510 nm (6, 30), this difference must be due to the presence of some other photocycle intermediates, most likely L (21) (and perhaps N). This intermediate would comprise ~9–10% of the pigment cycling in the sample at the time of measurement. The estimation of the amount of intermediates, other than M, was done as follows. Based on the room and low temperature measurements of the spectra of L (11, 14) and N (31, 32), we take the absorbance of these intermediates (L or N) at 545 nm to be ~0.68 of that of bR at 568 nm. Thus the amount of the intermediate was calculated by dividing the difference between spectra 1 and 2 at 545 nm in Fig. 1 B by 0.68 \pm 0.02 and

the absorbance of bR at the maximum (spectrum 1). For the case shown in Fig. 1 B this results in 10% (of the amount of bR cycling) in L or N. To minimize the contribution of this species in the quantum efficiency determination, measurements of transient bR bleaching were made at 610 nm where the signal is still large and the absorption of L (or N) is relatively small (~ one-third of the absorption of bR at 610 nm [11, 14, 31, 32]). Thus the observed transient bleaching at 610 nm should be ~0.97 of the fraction cycling.

In the case of rhodopsin, measurements were made at 540–560 nm. Low intensity laser flashes converting only a small fraction of both rhodopsin and bacteriorhodopsin (1.6–5.6%) were used. Under these conditions no isorhodopsin is formed and back photoreactions from bathorhodopsin and the K-photoproduct are insignificant. An average of 64 flashes was used in the case of bacteriorhodopsin (2 s between flashes) and 4–16 traces were averaged for rhodopsin using a fresh sample for each trace. The typical signals are shown in Fig. 1 A (for bR) and Fig. 1 C (for R). The average value for the ratio of the quantum efficiency of bacteriorhodopsin photoconversion and that for the photobleaching of rhodopsin (ϕ_{bR}/ϕ_R) is 0.96 ± 0.04 (Table 1). Taking 0.67 for the quantum efficiency of rhodopsin, the quantum yield of bR appears to be $\phi_{bR} = 0.64 \pm 0.03$. The data obtained under magic angle conditions (experiments 1–4) give an average value for the quantum yield of 0.62. Taking into account that the measured percent of cycling is ~0.97 that of the actual, the value for the quantum yield obtained under magic angle conditions would be 0.64 ± 0.04 .

We also made an independent set of measurements using a power meter for determination of light energy of the actinic laser flash. The quantum yield of bR photoconversion was found to be 0.7 ± 0.1 which is in agreement with the data obtained with rhodopsin as a photon counter.

From the light-induced absorbance changes at 410 nm we can also determine the percent of bR transformed into M, α_M , and quantum yield of M formation, ϕ_M . The difference in the extinction coefficients of M and bR at 410 nm was taken to be 0.53 ± 0.03 of bR extinction at 570 nm (this value was obtained from the extinction of M at the maximum being 0.71 ± 0.02 that of bR [14, 33] and extinction of bR at 410 being 0.18 ± 0.01 [34]; the same value is obtained by linear extrapolation of the data measured at -180°C and -100° to 20°C). The percent of bR transformed into M under nonsaturating conditions was found to be $4.1 \pm 0.2\%$ whereas the percent of transient bleaching of bR at 610 nm was $4.03 \pm 0.06\%$ under the same conditions of excitation. Consequently the quantum yield of M formation calculated by Eq. 3 in which α_M was introduced instead of α_{bR} was determined to be 0.65 ± 0.06 .

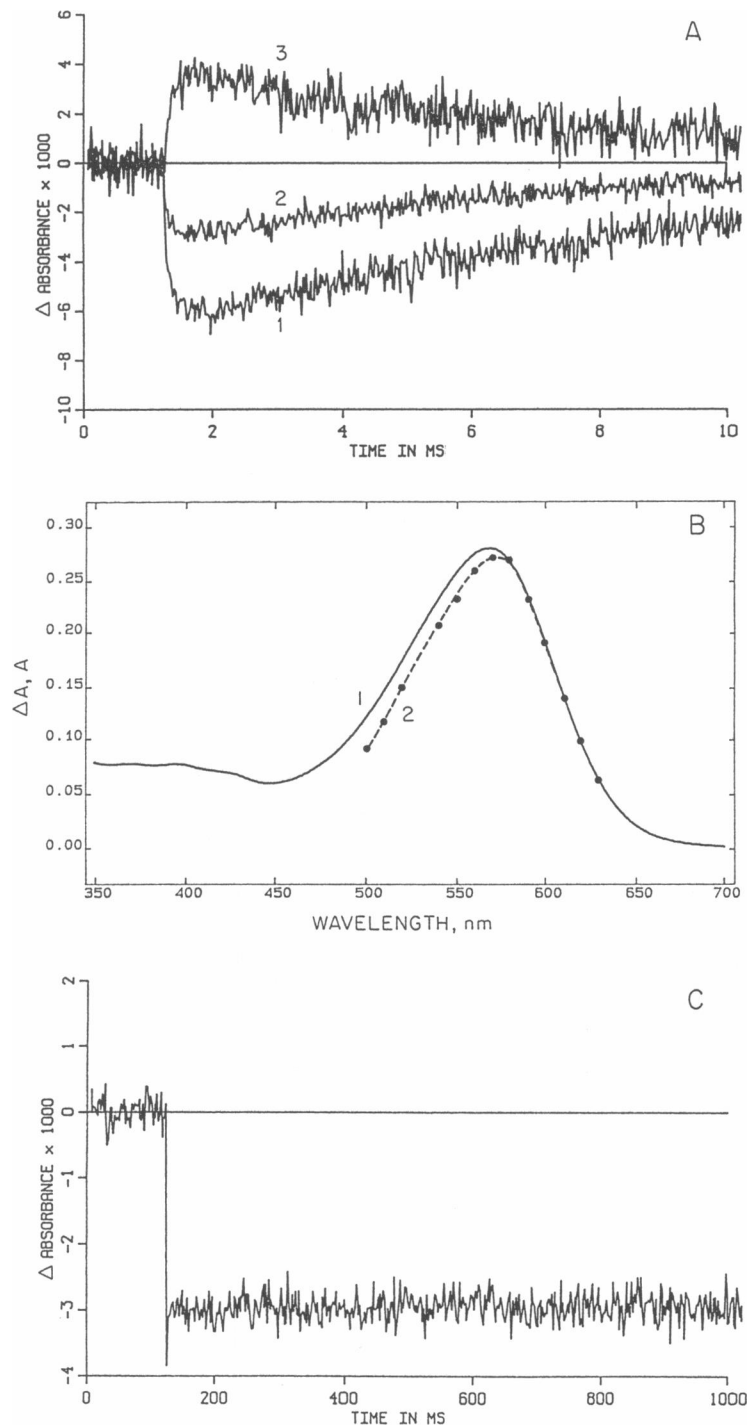


FIGURE 1 (A) Absorbance changes of light-adapted purple membrane (10 mM HEPES, pH 7) at 570 nm (1), 610 nm (2), and 410 nm (3) produced by a nonsaturating 532 nm laser flash (0.15 mJ/cm^2). Each curve is an average of 64 flashes. Optical density of the sample at 570 nm was 0.28. (B) Absorption spectrum of light-adapted purple membrane, pH 7 (1), and difference absorption spectrum produced by nonsaturating (0.15 mJ/cm^2) 532 nm laser flashes (2) (spectrum 2 is shown here with the opposite sign and normalized at 610 nm with spectrum 1). (C) Absorbance changes produced by 532 nm low intensity (0.15 mJ/cm^2) laser flashes in bovine rhodopsin in 2% Ammonyx LO and 20 mM hydroxylamine, pH 7.2, average of eight flashes.

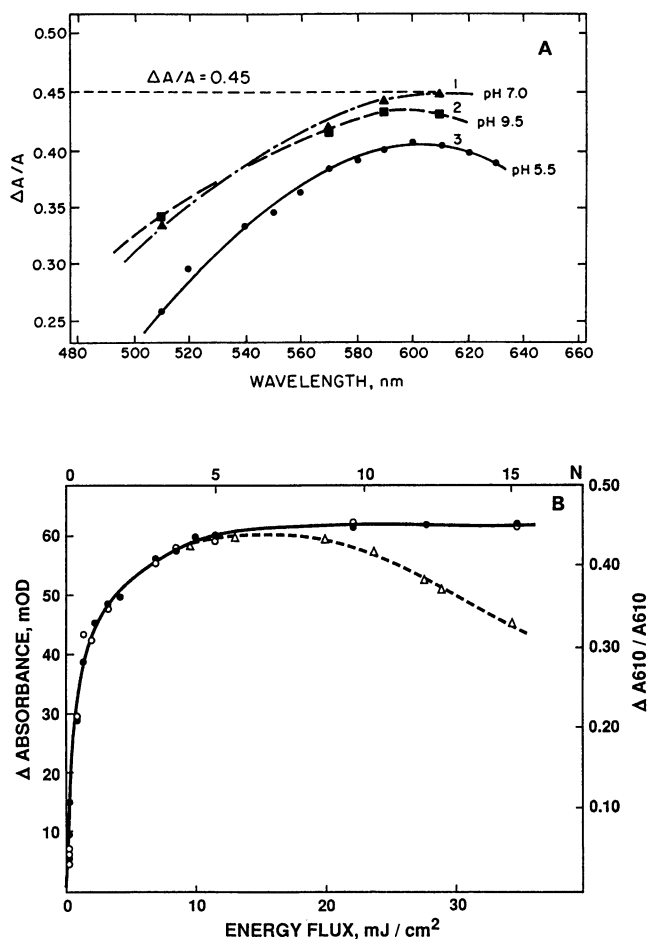


FIGURE 2 (A) Fractional absorbance changes, $\Delta A/A$ (in %), produced by a saturating laser flash (28 mJ/cm^2) in suspensions of light-adapted purple membrane at pH 7 (1), 9.5 (2), and 5.5 (3). (B) Light energy dependence of absorbance changes at 610 nm (open circles; data shown with the opposite sign) and 412 nm (closed circles) in a suspension of light-adapted purple membrane, pH 6.96 (10 mM Hepes), absorbance at 568 nm is 266.8 mOD, 20°C . Measurements were done with actinic beam being perpendicular to the measuring beam, and both beams were vertically polarized. At light energies 0–1 mJ/cm^2 64 flashes were averaged for each point, at energies 1–10 mJ/cm^2 eight flashes were averaged from each sample, and at energies more than 20 mJ/cm^2 four flashes were averaged for each point, using a fresh sample for each flash. To illustrate the destruction of the pigment produced by high energy laser flashes the amplitude of the signal at 610 nm is shown in the sample pretreated with 100 flashes (triangles). On the right side of the figure the relative transient bleaching, α_{br} , calculated as the ratio of $\Delta A/A$ at 610 nm, is shown on the Y axis on the right. The average number of quanta absorbed per bR molecule during the flash, N , is shown on the top of the figure. Optical density at 610, 137.9 mOD.

The value obtained for ϕ_{M} is close to the quantum efficiency of ϕ_{br} determined from the transient bleaching at 600–610 nm. This suggests that all or almost all of the L intermediate relaxes through M at room temperature.

To study the pH dependence of the quantum efficiency of bacteriorhodopsin, we made comparative measurements of bR cycling at pH 5.5, 7.0, and 9.7 under identical conditions of excitation. The values obtained for ϕ_{br} were: 0.63 ± 0.03 (pH 5.5), 0.64 ± 0.03 (pH 7), and 0.61 ± 0.03 (pH 9.7). They indicate that ϕ_{br} remains almost unchanged at these pHs, which is in agreement with the data published earlier (35). The small drop in the quantum efficiency at pH 9.7 most likely could be explained by the formation of a small amount of intermediates having maxima at 500 and 370 nm and long lifetimes at alkaline pHs (S. Balashov, unpublished data).

The rate of M formation is much faster at pH 9.7 as compared with that at pH 7.0. At pH 9.7 60% of M is formed with a rise time $\leq 5.5 \mu\text{s}$ and 40% with a rise time of $100 \mu\text{s}$, whereas at pH 7 only 8–10% of M is formed with $\tau \leq 6 \mu\text{s}$, the major part (90%) has a rise time $\approx 85 \mu\text{s}$. However, the percent of M formed under low intensity laser flashes at pH 7 and pH 9.7 is the same within the experimental error (4.1 ± 0.2 at pH 7 and 4.2 ± 0.2 at pH 9.7) indicating that the quantum yield, ϕ_{M} , is approximately the same for the fast and slow M components.

Maximum transient bleaching of bR

It was interesting to estimate the maximum fraction of bR cycling, $\alpha_{\text{br}}^{\text{max}}$. Previous measurements gave the value of 37% with 530 nm, 40 ns laser pulse excitation (7). At the same time, higher values for the photosteady state concentration of K ($\sim 50\%$) were obtained at low temperatures (11–15) and also proposed for room temperature (36).

To find the maximum fraction cycling we measured the light intensity dependence of the amplitude of transient bleaching of bR at 610 nm and M formation at 412 nm. The data are presented in Fig. 2 B. The magnitude of the relative transient bleaching, α_{br} , calculated as $\Delta A/A$ at 610 nm, is shown on the Y axis on the right. The average number of quanta absorbed per bR molecule is shown on the top of the figure. The measurements were made with parallel (vertical) polarization of measuring and actinic beams. One can see that saturation is reached at energies higher than 10 mJ/pulse (energy flux 23 mJ/cm^2 , or 6.2×10^{16} quanta/ cm^2). At this light intensity molecules with transition dipole moment oriented parallel to the polarization of the actinic beam absorb more than 10 quanta during the pulse, which is enough for saturation, taking into account high quantum yields of the forward and back light reactions. As was pointed out by Nagle et al. (37), the saturating light intensity depends on the mutual polarization and geometry of actinic and measuring beams being minimal for the parallel polarization of both beams. Comparison of our data with the correspond-

ing theoretical curve (V) presented in reference 37 indicates that under our conditions at a laser power of 10 mJ, 99.7% of saturation should be achieved. Thus, based on the shape of the light curve, the number of quanta absorbed, and from comparison with the theoretical curve (37), we conclude that saturation was achieved in our experiments. At the magic angle polarization of the measuring beam (54.7°) the value of ΔA was 88% of saturation which is in good agreement with the value 91% predicted in reference 37.

The maximal transient bleaching observed under saturating conditions, α^{\max} , is $45.6 \pm 1\%$. Two corrections should be made to obtain the actual percent of bR cycling. The first correction is necessary because with high intensity laser pulses part of the pigment is irreversibly transformed into stable photoproducts (see next section). To avoid artifacts due to these transformations during signal averaging, we used a fresh sample for each flash at high laser energies (>15 mJ/cm²). Because only 0.3% of the pigment was destroyed by a single 15 mJ flash (see below) the correction for the amount bleached would be negligible. Introducing this correction we get 45.7% for the maximal transient bleaching. The second correction must be made to compensate for the presence of 10% of the L (and possibly N) intermediate at the time of maximum M formation. Because the extinction of these intermediates is $\sim 1/3$ that of bR at 610 nm the percent cycling would be $45.7 \pm 1 / (0.97 \pm 0.01) = 47 \pm 3\%$.

The fraction cycling can also be calculated from the absorption changes at 412 nm. Assuming that the difference in the extinction coefficient of M and bR at 412 nm is 0.53 ± 0.03 of the extinction of bR at 568 nm, we estimate that $43.8 \pm 3\%$ M is formed under saturating conditions. Because only $\sim 90\%$ of the pigment is in M, the actual percent of cycling would be $43.8/0.9 = 48.7 \pm 3\%$ which is close to that estimated at 610 nm.

The relative transient bleaching, $\Delta A/A$, was found to be both wavelength and pH dependent (Fig. 2 A). The maximum value of (45.6%) was observed at 590–620 nm at pH 7. The decline of $\Delta A/A$ at wavelengths shorter than 590 nm is apparently due to the presence of the intermediates L or N. The decline of the $\Delta A/A$ in 610–640 nm region at pH 5.5 is most likely due to the formation of the long wavelength photointermediate O. The maximum transient bleaching at pH 9.5 is almost the same as at pH 7; at pH 5.5 it is slightly smaller (0.41).

Irreversible transformation of bR with high laser power excitation

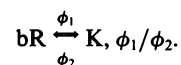
Illumination of the purple membrane with 532 nm laser pulses of high intensity (more than 10 mJ/cm²) causes an irreversible change in the pigment which results in the

loss of the ability for the cyclic photoconversions and changes in the absorption spectrum. As shown in Fig. 2 B, 100 flashes of light intensity 34 mJ/cm² produce a 27% decrease in transient absorption changes at 610 and 410 nm. From this one can calculate that $\sim 0.3\%$ of bR molecules are destroyed in each flash, and the quantum yield of bR degradation at this energy is 2×10^{-4} (0.003/15 quanta absorbed). Fig. 3 A shows that illumination of the sample with 100 flashes of higher energy (96 mJ/cm²) causes a 60% decrease in the transient light-induced absorbance changes. The sample turns blue and the absorption maximum shifts to longer wavelengths (Fig. 3 B). Also a triple peaked structure is observed at 342, 359, and 379 nm and the absorption decreases in the tryptophan absorption band (280 nm). Excitation with 380 nm light produces a strong fluorescence emission with maxima at 441, 461 (main), and 482 nm. The excitation spectrum (of emission at 490 nm) has the same maxima as seen in the absorption spectrum (342, 359, and 379 nm). These data indicate that absorption bands in the UV and at 605 nm belong to two different photoproducts. The absorption spectrum of the mixture of these two photoproducts is shown in Fig. 3 B, curve 3. The absorption maximum of the blue species is at 605 nm. It was assumed in the calculation that the fraction of native bR left is proportional to the light-induced transient absorbance change (Fig. 3 A). It was found that light-induced difference absorption spectra of purple membrane containing 38% of the photoproducts were different only in the amplitude but similar in shape which suggests that species absorbing at 605 nm does not undergo light-induced transient absorption changes (0.2–1 ms time scale) and does not form an M-like photoproduct.

The addition of cations (0.2 M KCl) did not restore the purple color or prevent the photodegradation of bR. This reaction was more prominent at alkaline pH (9.7) than at neutral or acid pH. To prevent any problems arising from the irreversible bleaching of bR, a fresh sample was used before each flash in the data shown in Fig. 2 B and only eight flashes were averaged per point in Fig. 2 A.

DISCUSSION

In this paper we have addressed two problems: (a) the quantum efficiency of bR photoconversion, and (b) the maximum percent of bR cycling and related to it the ratio of the quantum efficiencies of the forward and back photoreactions of bR,



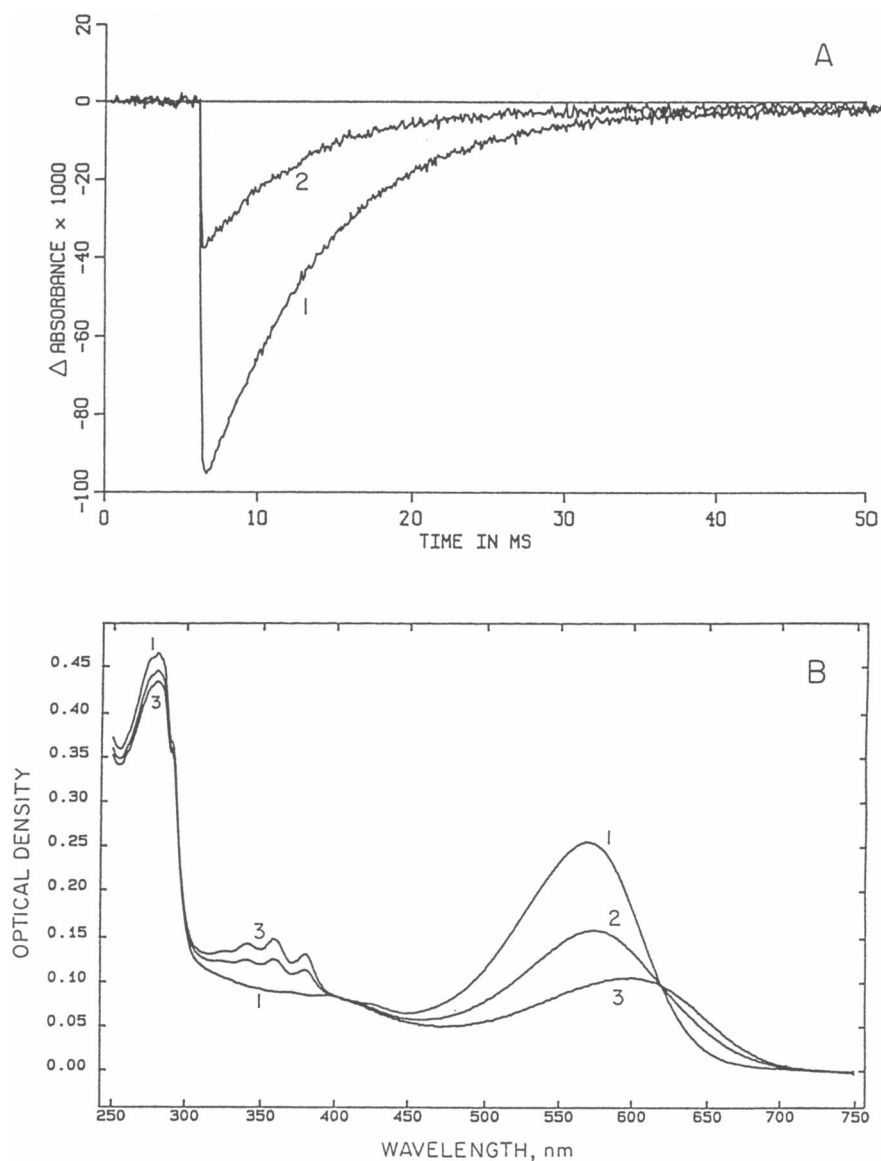


FIGURE 3 Transformation of purple membrane into a nonphotoactive form under high intensity 532 nm laser flashes. (A) Decrease in light-induced transient bleaching of bR at 610 nm after 100 flashes: (1) average of first eight flashes in a series of 100 flashes; (2) average of last eight flashes in a series of 100 flashes. Light intensity 94 mJ/cm². (B) Absorption spectrum of light-adapted purple membrane (pH 7) before (1) and after (2) illumination with a series of 100 flashes. (Curve 3) Spectrum of the photoproduct(s) produced by illumination of purple membrane with high intensity laser flashes. Spectrum 3 was obtained by subtracting the spectrum of remaining amount of bR from spectrum 2, assuming that the concentration of initial bR in the mixture is proportional to the amplitude of the transient light-induced absorbance changes (Fig. 3 A).

In addition we have found an irreversible phototransformation of bR under high intensity laser pulses.

Quantum efficiency of bR photoconversion

Most of the determinations of the quantum efficiency of bR photoconversion in the past were based on the amount

of M intermediate formed. However, in some conditions part of the L intermediate may decay to bR bypassing M. This happens at low temperature (12, 17) and probably in *Halobacterium* cells with a high membrane potential (23). Recently it has been suggested that some portion of bR may cycle without M formation in suspensions of purple membrane at room temperature (20, 21). To test these hypotheses we determined the quantum efficiency of

bacteriorhodopsin by measuring the amount of transient bleaching of the main bR absorption band.

The major problem in such determinations is to find a proper wavelength for measurements. The choice of the right wavelength requires that there be no major overlap of bR with any of the photointermediates. At wavelengths shorter than 600 nm absorbance due to L and N is high. In the 600–650 nm range K and O intermediates are the major absorbers. However, the life-time of K is very short, and the half-time of O formation is relatively slow. We therefore made measurements of the photobleaching of bR around 610 nm in the time scale from 4 μ s to 2 ms, which allowed us to minimize the contributions from the photointermediates.

As seen in Fig. 1 *B* the difference spectrum (when inverted and normalized at 610 nm) overlaps the absorption spectrum rather well on the long wavelength side, which lends support to the idea that contribution from the intermediates is negligible around 610 nm (at the time of maximum M formation). Our estimation shows that at pH 7 the maximum transient bleaching at 610 nm is 0.97 of the actual fraction cycling. To minimize the effects due to the photoreversal of the primary light reactions of rhodopsin and bacteriorhodopsin, we made the measurements with very low laser energy.

Under these conditions we find the quantum yield of the bR photoconversion to be 0.64 ± 0.04 . Measurements of the quantum yield of M formation gave similar value, 0.65 ± 0.06 . These data indicate that all or at least 90% of bR cycles through M. The value for the quantum efficiency of bacteriorhodopsin obtained by us is considerably higher than the earlier value of 0.3 (5, 8, 16) but somewhat less than 0.79 (2) and closer to the values reported in references 3 and 4.

Our previous measurements where we reported that $\phi_M = 0.33$ (5) were based on the measurement of M using rhodopsin as a photon counter. There are several reasons to believe that those measurements may have been an error. A large source of error may lie in the experimental procedures. We found that it is extremely important to keep the sample volume as small as possible so as not to exceed the size of the laser spot. This minimizes the problems arising from the diffusion of unphotolyzed sample into the light path, which if present reduces the amplitude of the absorbance changes significantly. The actinic flash used in our previous experiments was a xenon flash lamp together with a 560-nm interference filter. The broader band pass from the interference filter (≈ 10 nm) could have caused an error in the estimation of the quanta absorbed by rhodopsin, and the longer duration of the flashlamp could induce some branching from L. The measurements of the quantum yield of M were done also at -40°C (6), which gave a value of 0.3. This result is not

in contradiction with the quantum yield of M being equal to 0.65 at room temperature because the yield of M was found to be temperature dependent, dropping sharply below -20°C (38) due to spontaneous branching from L to bR (12).

Dancshazy et al. (20) have recently suggested that the variations in the quantum yield may be due to the fact that light-adapted BR exists in at least two distinct pH-dependent forms. The pK of the two forms is 10 in low salt (10 mM KCl) and 8.5 in 1 M KCl (19, 20, our unpublished observations). However, the quantum efficiency of bR photoconversion at pH 9.6 is quite similar to that observed at pH 7 and pH 5. Thus, whereas it may be true that there are two or more photochemically active conformers of bR, it appears that their photocycles have similar quantum yields. Moreover, at least 90% of the bR cycles through M at high pH, which does not support the view that a significant amount of bR, at room temperature, cycles without M formation (20, 21).

The finding that photochemical quantum yield is equal to 0.64 ± 0.04 , taken together with the value of the maximum quantum yield for proton transfer, 0.6–0.7 (5, 39–41) suggests that only one proton is translocated in the photocycle of bR. This conclusion is in agreement with the value for protons transferred per M formed, H^+/M , being close to 1 (41–44).

Estimation of the ratio of the quantum efficiencies of the forward and back photoreactions of bacteriorhodopsin

If one assumes that at room temperature K completely decays to L and M, the transient bleaching $\Delta A/A$ or percent of bR cycling would be equal to the photosteady state concentration of K and the quantum yield of K formation ϕ_1 would be equal to ϕ_{bR} . Knowing the concentrations of K and bR (C_K and C_{bR} , respectively) in the photosteady state and their extinction coefficients at the wavelength of excitation, the ratio of the quantum efficiencies ϕ_1/ϕ_2 (or ϕ_{bR}/ϕ_K) can be calculated (7, 11–15):

$$\phi_1/\phi_2 = C_K \epsilon_K / C_{bR} \epsilon_{bR}. \quad (5)$$

At liquid nitrogen temperatures K is stable and $\text{bR} \rightleftharpoons \text{K}$ equilibrium can be studied directly. Such studies have been done by several groups (11–18). The quantum yield ratio was found to be ≈ 0.5 (11–16). The photosteady state concentration of K varies from 50% (11–14) to 28% (18). The last value was obtained assuming that K converts completely into M upon warming the sample from -196° to -50°C . However, it has been shown that at low temperature L decays not only to M but also back to bR (12, 17). As a result of this branching only part of K

transforms into M. Because the quantum yield of K is proportional to the maximal photosteady state concentration of K, which is ~53% (14) rather than 28% (18) the corrected value for the quantum yield of bR to K photoreaction at low temperature can be estimated to be ~0.60 (instead of 0.30 [17, 18]).

At room temperature the quantum yield ratio was determined by Goldschmidt et al. (7). Taking ϵ_K/ϵ_{bR} at 532 nm equal to 0.70, and the fraction of bR cycling to be 0.37, ϕ_{bR}/ϕ_K was calculated to be 0.40 ± 0.05 (7). This value would require that the upper limit of the quantum yield of bacteriorhodopsin, ϕ_{bR} , must be equal to or less than 0.45. According to our data the maximal fraction cycling is $47 \pm 3\%$. The source of disagreement is apparently in the method of determination of the α^{max} . In reference 7 it was measured as a ratio of maximum transient bleaching at 570 nm. However, at this wavelength α is smaller than at 610 nm. We recalculated the data of Goldschmidt et al. (7) using $\alpha^{max} = 0.47$ instead of 0.37. The ratio of the extinction coefficients of K to bR at 532 nm was found to be 0.76 and the quantum yield ratio ϕ_K/ϕ_{bR} equal to 0.67. Introducing the yield $\phi_{bR} = 0.64$, the quantum yield of the back reaction ϕ_K was calculated to be 0.96.

Shichida et al. (45) also determined the fraction cycling by measuring the transient bleaching at 570 nm. The maximal fraction of bR cycling under saturating conditions was 33–37%. We recalculated the ϵ_K/ϵ_{bR} for the case of $\alpha^{max} = 0.47$ (see Table 2).

The ϕ_{bR}/ϕ_K and ϕ_K were also calculated for other values of ϵ_K/ϵ_{bR} published in the literature (9, 11, 33, 46, 47) (see Table 2) using maximal percent of cycling $\alpha^{max} = 0.47$. The quantum efficiency of photocycling of bR, ϕ_{bR} , was taken to be 0.64.

One can see in Table 2 that in all the cases (except one) the ratio of the quantum yields fits within a narrow interval 0.65 ± 0.03 and the quantum yield of the back reaction is equal to 0.96 ± 0.04 . Taking into account the error in determination of $\alpha^{max} = 47 \pm 3\%$ and $\phi_{bR} = 0.64 \pm 0.03$, the values for ϕ_{bR}/ϕ_K and ϕ_K would be 0.67 ± 0.06 and >0.9 . The value for ϕ_K obtained with the data of Shichida et al., measured at 100 ps after the pulse, is >1 indicating that the value for ϵ_K/ϵ_{bR} obtained in reference 45 is incompatible with our results. Pollard et al. (9) also calculated the spectrum of K for the same time of measurement (100 ps), assuming the quantum yield of bR to be 0.6 and got a larger value for ϵ_K/ϵ_{bR} (0.77 at 532 nm).

Shichida et al. (45) have found that on a time scale of 0.1–150 ns the K intermediate undergoes some changes which were interpreted as transition into a new state named KL. Stern and Mathies (48) using Resonance Raman spectroscopy also found evidence for some relax-

TABLE 2 Ratio of the quantum yield of the forward and back light reactions of bR, ϕ_{bR}/ϕ_K , and the quantum efficiency of the back reaction, ϕ_K , calculated from the maximum fraction of bR cycling (0.47) and the relative extinctions of bR and K at 532 nm at room temperature taken from the literature

Reference from which value for ϵ_K/ϵ_{bR} was taken	t_K^*	$\epsilon_K/\epsilon_{bR}^\ddagger$	ϕ_{bR}/ϕ_K^\S	$\phi_K^!$
	ns			
Lozier et al., 1975 (11)	~200	0.70	0.62	1.0
Goldschmidt et al., 1976 (7)	300	0.76 [†]	0.67	0.96
Shichida et al., 1983 (45)	0.1	0.62 [†]	0.55	1.16
Shichida et al., 1983 (45)	150	0.72 [†]	0.64	1.00
Poland et al., 1986 (9)	0.1	0.77	0.68	0.94
Maurer et al., 1987 (46)	>1,000	0.75	0.67	0.96
Hofrichter et al., 1989 (47)	>10	0.75	0.67	0.96
Zimanyi et al., 1989 (33)	60	0.76	0.67	0.96

* t_K is time after the flash at which the difference spectrum K minus bR was measured.

[†]Calculated from the spectra presented in the papers cited.

[‡] ϕ_{bR}/ϕ_K was calculated using Eq. 5, assuming $C_K/C_{bR} = 0.47/0.53$.

[§] ϕ_K was calculated from the ratio ϕ_{bR}/ϕ_K taking $\phi_{bR} = 0.64$.

[!]The data shown is a recalculation of the original data of the authors for the fraction of bR cycling equal to 0.47 (instead of 0.37 measured by the authors). The original data of Goldschmidt et al. (9) was: $\epsilon_K/\epsilon_{bR} = 0.70$, photosteady state concentration of K, 0.37, which resulted in the value for $\phi_{bR}/\phi_K = 0.4$ (9). The original data of Shichida et al. are: $\epsilon_K/\epsilon_{bR} = 0.52$ and $\epsilon_{KL}/\epsilon_{bR} = 0.64$ at 532 nm (40).

ation process in the bathproduct on this time scale. Milder and Kliger (49) showed that the process is accompanied by a small absorption change and is accomplished within 10 ns after the flash. On a time scale from 10 ns to 3 μ s it uniformly decays to the next intermediate (L). Because the duration of the laser pulse in our experiment was 7 ns we suggest that during the flash at high light intensity both states were present and bR was in equilibrium with both K and KL. If one of the states predominates, for example KL, then the maximal fraction of cycling would be determined mainly by $bR \rightleftharpoons KL$ photoreactions. It seems most likely that photochemical properties of KL are close to K. This form is supposed to be present in the 10 ns–10 μ s time scale and is usually called K (11, 46, 47).

Earlier, the sum of the quantum efficiencies of the forward and back photoreactions, $bR \rightleftharpoons K$, was proposed to be close to 1 (8, 16, 50) which was used to suggest that bR and K share a common excited state (16). However, the present results show that the sum of ϕ_{bR} and ϕ_K is >1 (≈ 1.6) and consequently bR and K (KL) would not have a common excited state. This however does not exclude the possibility that precursor of the K intermediate J may have a common excited state with bR.

On the nature of the irreversible photochemical transformation of bR under high intensity laser pulses

The absorption spectrum of the longwave photoproduct produced by high intensity laser pulses (Fig. 3 B) is characterized by a broad maximum at 605 nm similar to the acid blue (51) and deionized forms of bacteriorhodopsin (blue membrane) (52, 53). The purple to blue transition is controlled by some amino acid residue which is protonated in the blue form and is negatively charged (deprotonated) in the purple (51). The state of this group is controlled by cations (directly or indirectly through surface pH) (52, 53) and apparently by neighboring groups. It may be that excitation of bR with high energy pulses alters the state of this group.

Bands in the near UV region at 342, 359, and 379 nm represent a second photoproduct. Oesterhelt et al. (54, 55) have described a photoproduct with a similar spectrum produced by the photoreduction of the Schiff base in bR in the presence of borohydride. Retinal is linked to the lysine residue by a single C—N bond in this photoproduct and shows retinylprotein fluorescence (55). The similarity of the two photoproducts indicates that under high intensity laser flashes photoreduction of the chromophore occurs in the absence of exogenous donors of electrons.

The photochemical reactions produced by high intensity laser flashes are accompanied by a decrease in the intensity of the absorption band of the tryptophan residues. This indicates that they are probably directly involved in the formation of 605 nm or UV absorbing photoproducts. Because two tryptophans are located in the vicinity of the chromophore (56) one may also suggest that the decrease in absorption at 280 nm is caused by the change in the state of the chromophore.

The quantum yields of the 605 and 359 nm photoproducts are in the order of 10^{-4} (at laser power 30–90 mJ/cm²). The photosteady state concentration of the excited molecules (during the 30 mJ/cm² laser pulse) is $\sim 10^{-3}$ of the total molecules in the sample (assuming cross-section of bR at 532 nm is equal to 1.79×10^{16} cm² and the lifetime of the excited state is 0.5 ps [9, 57, 58]). These molecules can absorb a second quantum with transition into a higher excited state (9, 57) which may lead to the destruction of bR molecules. A second mechanism may involve formation of iso bR (23, 59) and pseudo bR (23, 59, 60). These photoproducts have longer excited state lifetimes (≥ 7 ps and ≈ 70 ps, respectively [23]) which may facilitate further degradation of the pigment.

We thank A. H. Xie for useful discussions.

This work was supported by DMB 8815824 from the National Science Foundation, 88ER13948 from the Department of Energy and the

exchange program between Midwest Universities Consortium for International Activities and Moscow State University (Dr. Balashov).

Received for publication 3 January 1990 and in final form 14 May 1990.

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