THE QUANTUM YIELD OF FLASH-INDUCED PROTON RELEASE BY BACTERIORHODOPSIN-CONTAINING MEMBRANE FRAGMENTS

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ABSTRACT The quantum yield of proton release by bacteriorhodopsin was measured from volume changes after excitation of purple membrane fragments by short flashes. At low ionic strengths, about 0.25 mol of protons is released per einstein absorbed. This agrees well with quantum yields reported recently for the conversion of bacteriorhodopsin into a metastable state (M) that absorbs near 412 nm. However, the quantum yield of proton release increases gradually with increasing ionic strength; it plateaus with a value of 0.43 ± 0.03 at ionic strengths above 200 mM. Changing the ionic strength has no detectable effect on the quantum yield of formation of the M spectral state. It thus appears that as many as two protons can be released and rebound in each photochemical cycle at high ionic strengths. The quantum yield of proton release is essentially independent of pH over the range 6.0–8.75. The quantum yield decreases with increasing flash strength, apparently due to photoreversal of the initial photochemical reaction.

INTRODUCTION

The purple membranes of the halophilic bacterium *Halobacterium halobium* contain the protein bacteriorhodopsin, which uses the energy of light to drive the transport of protons across the membrane. (For a review, see ref. 1.) Retinaldehyde is bound to the protein as a Schiff base in a 1:1 M ratio (2, 3) and is the pigment responsible for the absorption of light. In its resting (but light-adapted) state, bacteriorhodopsin has a strong absorption band at 570 nm. Excitation causes the formation of a product (called K) that has an absorption maximum shifted to the red. After this, a number of transient absorption changes occur, reflecting thermal intermediates that form as bacteriorhopdopsin returns to its original state (4). During the photochemical cycle, protons are released to the solution on one side of the membrane, and taken up on the opposite side (5). The relationship between proton translocation and the spectral transformations of bacteriorhodopsin is still unclear. The complex kinetics of the absorbance changes suggest that the path from K back to the original state may have branches that vary in importance, depending on the pH, temperature, and ionic strength (e.g. 6, 7).

The quantum yield for the conversion of bacteriorhodopsin into its metastable, spectral states has been measured by several groups of investigators. To trap the system in a state [M] characterized by an absorption band at 420 nm, Oesterhelt and Hess (8) illuminated suspensions of purple membranes in solutions saturated with diethyl ether. They reported a quantum yield of 0.79. More recently Becher and Ebrey (9) trapped what appears to be essentially the same intermediate state by illuminating at −40°C, at high salt concentration

and pH 10. They obtained a quantum yield of 0.30 ± 0.03. In a concurrent report, Goldschmidt et al. (10) used flash excitation and rapid kinetic techniques to measure the quantum yield of the M state. Using purple membranes in distilled water (unspecified pH) at room temperature, they obtained a quantum yield of 0.25 ± 0.05, in good agreement with Becher and Ebrey's value. Recent studies of the photostationary states generated by excitation with very strong flashes also support the conclusion that the initial photochemical transformation has a quantum yield below 0.4 (11). No clear explanation is available for the higher value obtained by Oesterhelt and Hess, except that the use of ether might possibly have influenced their results.

A determination of the quantum yield of light-induced proton release is important for an understanding of the mechanism of proton transfer. To address this problem, Lozier et al. (5) have measured flash-induced absorbance changes of the pH-indicator 7-hydroxycoumarin. By calibrating the pH indicator with exogenous acid and by estimating the amount of bacteriorhodopsin cycling from absorbance changes at 420 nm, they concluded that one proton is released per cycle. However, their study was restricted to rather special conditions (distilled water), and did not include an investigation of the effects of pH, salts, or excitation intensity. We felt a need for a more extensive study. As we shall see, the results suggest that under conditions of high ionic strength, more than one proton is released per cycle.

In a previous paper (7), we showed that proton transfer between the purple membrane and the solution can be detected by changes in the volume of the suspension. The volume change that occurs upon excitation of purple membrane suspensions with a flash of light is

$$\Delta V\{T, \mathrm{B}\} = [n_e E_e - n_r \Delta H_r\{\mathrm{B}\}]\alpha\{T\}/\rho C + n_r \Delta V_r\{\mathrm{B}\}. \quad (1)$$

ΔV is a function of the temperature $[T]$ and of the hydrogen ion buffers $[B]$ present in the solution. The term $[n_e E_e - n_r \Delta H_r\{\mathrm{B}\}]\alpha\{T\}/\rho C$ gives the volume change due to thermal expansion or contraction of the solution. n_e is the number of einsteins of light absorbed; E_e, the energy of the photons (calories per einstein); n_r, the number of moles of products generated; and $\Delta H_r\{\mathrm{B}\}$, the enthalpy change associated with the formation of a mole of products (essentially, the amount of energy stored in the system per mole of products). If the flash causes the movement of a proton from the membranes to a buffer in the solution, the enthalpy change will depend on the heat of protonation of the buffer. If the system does no work other than pressure-volume work, the amount of heat released is $(n_e E_e - n_r \Delta H_r\{\mathrm{B}\})$. α is the coefficient of thermal expansion, a strong function of the temperature, T; ρ is the density of the solution; and C is the heat capacity of the solution at constant pressure.

In the second term on the right in Eq. 1, $\Delta V_r\{\mathrm{B}\}$ includes volume changes due to any processes other than heating or cooling. Proton movements can cause substantial volume changes if they involve a change in the number of charged groups in the system. The protonation of $H_2PO_4^-$ by H_3O^+, for example, causes the loss of a negative charge on the buffer and of the positive charge of H_3O^+. This reaction is accompanied by an expansion of 24 ml/mol of phosphate protonated (12). The expansion occurs because water molecules in the vicinity of charged groups are ordered so that the density of the solvent is greater than it is in the absence of the charge, a phenomenon known as electrostriction.

In the transfer of a proton from H_3O^+ to the neutral tris(hydroxymethyl)aminomethane (Tris), the number of ions in solution remains constant and the volume change is quite small, −1 ml/mol (12). Volume changes due to electrostriction are not strongly dependent on the temperature (13). Other processes that can contribute to ΔV, are discussed in our previous paper (7).

In this work, we have determined the quantum yield of proton release from bacteriorhodopsin by employing a sensitive capacitor microphone to measure light-induced volume changes. To focus on volume changes associated with proton release, we compared the volume changes obtained with pairs of buffers that have different heats of protonation or different volume changes associated with protonation.

MATERIALS AND METHODS

Halobacteria halobium strain R_1, a mutant lacking intracytoplasmic vesicles, was grown according to the procedure of Becher and Cassim (14). The cultures were kindly supplied by the laboratories of both W. Stoeckenius and T. Ebrey. Bacteriorhodopsin-containing membrane fragments were isolated from freshly harvested cells as described by Becher and Cassim (14).

Volume change measurements were made with a capacitor microphone mounted on a Pyrex cell, which could be filled through a side arm and then sealed with a Teflon stop cock. A detailed description of the apparatus is given in reference 7. Flash-induced volume changes occurring within the cell distort the polarized (+300 V) inner plate of the capacitor, resulting in a change of the capacitance of the microphone. Voltage changes on the opposite plate are measured with an amplifier with high-input impedence.

The flashes used to excite the bacteriorhodopsin came from either a xenon flash lamp or a dye laser. The xenon flashes had a half-width of 5 μs and were passed through a Schott HA-11 heat filter (Schott, Inc., New York) and a broad-band interference filter (maximum transmittance at 568 nm; width at half-maximum, 40 nm) before being focused on the sample cell. The incident energy, measured with a calibrated Si photodiode, was about 3.4×10^{-4} J/flash. The flash frequency was 0.2 Hz. The dye laser (Phase-R Corp., New Durham, N.H.; model 2100A) gave submicrosecond flashes that were passed through an HA-11 filter to block light from the xenon pumping flash. Rhodamine 6G was used in the dye laser to obtain 588 nm light, and green-9 for 563 nm. The flash frequency was usually 0.05 Hz. The dye laser offers significant advantages as a flash source for volume measurements even when one desires very weak flashes, since the capacitor microphone is exceedingly sensitive to vibration. The collimated beam from the laser allows the microphone apparatus and the laser to be many feet apart so that acoustical noise associated with the discharge is less of a problem than it is with the xenon lamp.

The purple membrane suspensions used for volume measurements had bacteriorhodopsin concentrations of about 20 μM. At the excitation wavelengths, the optical density of the samples in the 1.9 cm light path of the microphone cell was approximately 2.2. To convert as much as possible of the bacteriorhodopsin to the "light-adapted" form, the samples were illuminated with 3–5 strong flashes just before each measurement with attenuated flashes. [Light-adapted bacteriorhodopsin contains all-*trans* retinal (15, 16). The chromophore isomerizes slowly in the dark to a mixture of approximately 1:1 all-*trans*:13-*cis*. The relaxation occurs with a half-time of 21 min at 35°C (15) and causes small changes in bacteriorhodopsin's absorption spectrum. It is slower at the low temperatures at which most of our measurements were made. Both forms of bacteriorhodopsin undergo photochemical cycles (17), but the studies described in the Introduction focused mainly on the light-adapted form.] Although the quantum yield of light-adaptation is not known, the flashes we used for adaptation were strong enough to excite even the bacteriorhodopsin molecules in the back of the cell several times. Approximately half of the sample was in regions around the sides of the cell that were not illuminated directly, but molecules in these regions would make only small contributions to the volume changes, because the samples were unstirred and

each set of measurements required only a few minutes. To determine the extent of light-adaptation, samples were removed from the cell in the dark after exposure to the strong flashes, and their absorption spectra were compared with those obtained after full light- or dark-adaptation. Overall, the samples were approximately 50% light-adapted. The bacteriorhodopsin molecules in the front-central part of the cell, which made the largest contribution to the volume changes, thus must have been predominantly in the light-adapted form. Omitting the light-adaptation procedure seemed to have little or no effect on the volume changes, but we did not investigate this point in detail.

Kinetic analysis of signal-averaged volume change measurements were performed on a PDP-8 computer (Digital Equipment Corp., Maynard, Mass.). The relaxations of the volume changes were fit to a single exponential function, by using an iterative, nonlinear, least-squares program that returned the calculated value of the initial volume change. When necessary, the very slow decay components due to heating (ΔV_{hv}) were resolved by a "curve-peeling" routine during the analysis.

Flash-induced absorbance changes were measured as described previously (18).

RESULTS AND DISCUSSION

The quantum yield of proton release by bacteriorhodopsin can be determined from measurements of volume changes, because the heats of protonation and the volume changes associated with protonation are known for several different buffers. In Eq. 1, suppose that n_r represents the number of moles of bacteriorhodopsin converted into metastable states, and that each of these bacteriorhodopsins releases u protons to buffers in the solution. Then

$$\Delta H_r\{\mathrm{B}\} = \Delta H_{\mathrm{BR}} + u\Delta H_p\{\mathrm{B}\}, \tag{2}$$

where $\Delta H_p\{\mathrm{B}\}$ is the heat of protonation of the buffer, and ΔH_{BR} is the enthalpy change associated with the photochemical transformation of the bacteriorhodopsin itself. ΔH_{BR} presumably is independent of the nature of the buffer. Similarly,

$$\Delta V_r\{\mathrm{B}\} = \Delta V_{\mathrm{BR}} + u\Delta V_p\{\mathrm{B}\}, \tag{3}$$

where ΔV_{BR} is a volume change associated with the bacteriorhodopsin, and $\Delta V_p\{\mathrm{B}\}$ is the volume change associated with the protonation of the buffer. From Eq. 1, the difference between the volume changes measured with purple membranes in two different buffers (B_1 and B_2) is therefore

$$\Delta V\{T, \mathrm{B}_1\} - \Delta V\{T, \mathrm{B}_2\} = -un_r[\Delta H_p\{\mathrm{B}_1\} - \Delta H_p\{\mathrm{B}_2\}]\alpha\{T\}/\rho C + un_r[\Delta V_p\{\mathrm{B}_1\} - \Delta V_p\{\mathrm{B}_2\}]. \tag{4}$$

The strong temperature dependence of α allows one to separate the terms on the right side of Eq. 4, and gives two independent ways to measure un_r. For the first method, we worked at the temperature (T_0) at which $\alpha = 0$. In aqueous solutions containing low concentrations of salts, α becomes zero at a temperature between 0° and 4°C. Under these conditions, Eq. 4 reduces to

$$un_r = (\Delta V\{T_0, \mathrm{B}_1\} - \Delta V\{T_0, \mathrm{B}_2\})/(\Delta V_p\{\mathrm{B}_1\} - \Delta V_p\{\mathrm{B}_2\}). \tag{5}$$

To use Eq. 5, the capacitor microphone must be calibrated to give $\Delta V\{T, \mathrm{B}\}$ in milliliters. One can avoid this by measuring the volume change associated with the absorbance of the laser flash with a solution of black ink in distilled H_2O at a temperature (T), where $\alpha \neq 0$, $\Delta V_{hv}\{T\}$. The ink, like the purple membrane suspensions, absorbed essentially

all of the light incident on the solution. Because the ink is not photochemically active, all of the energy of the flash is degraded to heat. According to Eq. 1, the volume change is then

$$\Delta V_{hv}\{T\} = n_e E_e \alpha\{T\}/\rho C. \tag{6}$$

Values of $\alpha\{T\}$ are known for distilled H_2O.

Combining Eqs. 5 and 6 gives the following expression for the quantum yield of proton release.

$$\phi = \frac{un_r}{n_e} = \frac{\Delta V\{T_0, B_1\} - \Delta V\{T_0, B_2\}}{\Delta V_{hv}\{T\}} \left[\frac{E_e \alpha\{T\}/\rho C}{\Delta V_p\{B_1\} - \Delta V_p\{B_2\}}\right]. \tag{7}$$

All of the terms within the brackets at the far right are known constants. Note that the determination of ϕ does not require an independent measurement of the flash strength. The volume changes measured with the purple membranes at the temperature where $\alpha = 0$ are simply compared to the volume change measured with ink at a higher temperature.[1] In experiments where the flash strength was varied, it sometimes was convenient to use strong flashes to measure the volume changes given by ink, and then attenuate the flash with calibrated neutral density filters for measurements with the membrane fragments. ΔV_{hv} thus was calculated from the transmittance of the filter and the volume change measured with the unattenuated flash. The volume change due to the heating of the ink was shown to depend linearly on the flash strength over the range of measurements. In other experiments, ΔV_{hv} and the volume changes with membrane fragments were measured at the same flash strength. The temperature at which $\alpha = 0$ was found empirically, by measuring the volume changes with the purple membrane suspensions at long times after a flash, when the bacteriorhodopsin had returned to its initial state and all of the energy of the flash had been converted to heat (see below and ref. 7), or by using ink in a salt solution comparable to that of the membranes.

Fig. 1 shows the volume changes induced by weak 588-nm laser flashes, for purple membranes suspended in 200 mM KCl and either 5 mM Tris (trace 2) or 5 mM phosphate (trace 3). In each case, the solution undergoes a biphasic expansion (7) and a monophasic relaxation. The expansion is substantially larger in phosphate buffer than it is in Tris. These measurements were made at 2°C, the temperature at which the thermal coefficient of expansion of the salt solution was zero. Consequently, the heating that results when the flash is absorbed by a solution of ink in 200 mM KCl and 5 mM Tris does not cause a detectable volume change (trace 1). The volume change resulting from the absorption of an unattenuated laser flash by a solution of ink in distilled water at 19°C is shown in trace 4; this trace was used to obtain the value of ΔV_{hv}.

The total amplitudes of the flash-induced volume changes were calculated from measurements like those of traces 2 and 3 in Fig. 1, by fitting the relaxation of the volume change to an exponential function, as described in Methods. We have shown previously (7) that proton release is associated only with the faster phase of the expansion, but it was not neces-

[1]When n_e is measured independently, Eq. 6 provides an absolute calibration of the apparatus in $mV \cdot ml^{-1}$. We used a ballistic thermopile to measure n_e to obtain the vertical scales in Figs. 1–3, but the calibration does not enter the calculations of ϕ.

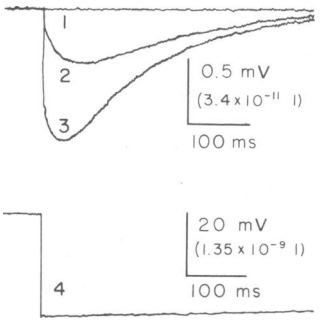

FIGURE 1

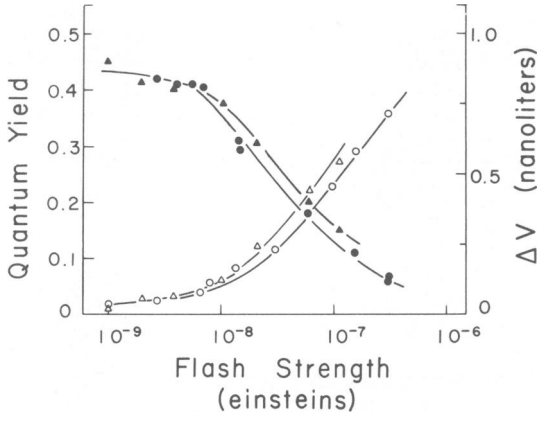

FIGURE 2

FIGURE 1 Flash-induced volume changes in purple membrane suspensions or black ink. A downward deflection is a volume increase. The absorbance of the ink at 588 nm was equivalent to that of the purple membrane suspension. The intensity of the attenuated dye laser flash was 5.7×10^{-9} E. Trace 1 shows the response of a solution of black ink in 200 mM KCl and 5 mM Tris (pH 7.8) at 2°C. At this temperature, $\alpha = 0$. Trace 2 shows light-induced expansion of purple membranes suspended at pH 7.8 in 5 mM Tris and 200 mM KCl at 2°C. Trace 3 is the same, except that 5 mM phosphate buffer replaced the Tris. Trace 4 shows the expansion resulting from the complete absorption of a strong laser flash (3.0×10^{-7} E) by ink in distilled water at 19°C. Each trace was the average of 10 flashes spaced 20 s apart.

FIGURE 2 Quantum yield of proton release by purple membranes, and amplitude of flash-induced volume changes, as a function of flash strength. The volume changes with purple membranes in 200 mM KCl and 5 mM phosphate (pH 7.8) or 5 mM Tris (pH 7.8) at 2°C were used to calculate the quantum yield according to Eq. 7 (filled symbols). The open symbols show the dependence of the amplitude of the volume change on the strength of the flash, for purple membranes suspended in 5 mM phosphate (pH 7.8) and 200 mM KCl at 2°C. An average of 5–36 flashes was used for each measurement. Data were obtained by using both 588 nm flashes (circles) and 563 nm flashes (triangles).

sary to resolve the two phases for the purposes of this work. The amplitude of the slower phase is independent of the nature of the buffer (7).

Table I contains data from a typical experiment, in which the volume changes were measured with purple membranes suspended in 200 mM KCl and phosphate or Tris, with flashes of various intensities. The quantum yields calculated from these data and from other similar experiments are plotted as a function of the flash strength (filled circles, Fig. 2). With relatively weak flashes, the quantum yield is about 0.42 protons released per photon absorbed, and is independent of the flash strength. The quantum yield falls off steeply if the flash strength is increased above about 7.5 nE.

In principle, the drop in the quantum yield that occurs at high flash strengths could be due to saturation of the photochemical apparatus, to a photoreversal reaction in which the initial photochemical product [K] absorbs a photon and the bacteriorhodopsin is driven back to its original form, or to both. Photoreversal of the initial transformation has been well documented (4). Fig. 2 shows that the quantum yield falls off more rapidly when the excitation wavelength is 588 nm (filled circles) than it does when the wavelength is 563 nm (filled triangles). One expects the photoreversal to be more pronounced with the longer wavelength light, because the absorption spectrum of K is shifted to the red relative to that of bacteriorhodopsin in its initial state (4). Photoreversals should be minimal when the

TABLE I

FLASH-INDUCED VOLUME CHANGES AND THE QUANTUM YIELD OF PROTON RELEASE FOR PURPLE MEMBRANE FRAGMENTS EXCITED WITH FLASHES OF DIFFERENT INTENSITIES

Flash strength	$\Delta V_{h\nu}\{19°\}$	$\Delta V\{2°, \text{Pi}\}$	$\Delta V\{2°, \text{Tris}\}$	$\Delta V\{2°, \text{Pi}\} - \Delta V\{2°, \text{Tris}\}$	ϕ
J	mV	mV	mV	mV	
0.0613	34.70	10.54	4.11	6.43	0.07
0.0321	18.17	8.56	3.29	5.27	0.11
0.0030	1.698	2.43	1.03	1.40	0.31
0.00116*	0.657	1.23	0.51	0.72	0.41
0.00083	0.470	0.97	0.45	0.52	0.41
0.00058	0.328	0.77	0.40	0.37	0.42

The purple membrane fragments were suspended in 200 mM KCl and 5 mM buffer, either phosphate or Tris, at pH 7.8 and 2°C. The samples were excited with a dye laser flash (588 nm) and the flash strength was varied with calibrated neutral density filters. Each measurement was the average of between 5 and 25 flashes spaced 20 s apart. The measurements of $\Delta V_{h\nu}$ were made with ink in distilled H_2O at 19°C. The values for the parameters needed for the calculation of the quantum yield according to Eq. 7 are as follows: $\Delta V_p\{\text{Pi}\} = 24.0$ ml/mol, $\Delta V_p\{\text{Tris}\} = -1.0$ ml/mol, $\alpha\{19°\} = 1.9 \times 10^{-4}$ ml $\cdot$ deg^{-1} $\cdot$ ml^{-1}, $\rho = 1.0$ g $\cdot$ cm^{-3}, $C = 1.0$ cal $\cdot$ deg^{-1} $\cdot$ g^{-1}, $E_e = 49$ kcal/E.

*These values are taken from the data shown in Fig. 1.

excitation wavelength is near 560 nm, because the ratio of the extinction coefficient of K to that of the initial state is minimal near this wavelength (11).

For comparison, the open circles and triangles in Fig. 2 show the amplitudes of the volume changes caused by excitation at 588 nm and 563 nm. The volume changes are indistinguishable at low-flash intensities, but at high intensities they are larger with 563 nm light than they are with 588 nm light. This agrees with the considerations mentioned above, and indicates that photoreversals of the K intermediate are probably an important factor involved in the decline of the quantum yield with flash strength.[2]

When bacteriorhodopsin releases a proton, the enthalpy change associated with protonating the buffer can cause a volume change, and the size of the volume will depend on the temperature, because of the temperature dependence of α. This provides a second method for measuring the quantum yield. Again, we first measure the volume changes with two different buffers at temperature T_0, where $\alpha = 0$. We then repeat the measurements at a higher temperature, T. From Eq. 4,

$$[\Delta V\{T, \text{B}_1\} - \Delta V\{T, \text{B}_2\}] - [\Delta V\{T_0, \text{B}_1\} - \Delta V\{T_0, \text{B}_2\}] = -un_r[\Delta H_p\{\text{B}_1\} - \Delta H_p\{\text{B}_2\}]\alpha\{T\}/C. \quad (8)$$

Calibration of the apparatus can be avoided by measuring the volume change with the purple membranes at the higher temperature [T], at a relatively long time after the flash.

[2]With 588-nm excitation, the drop in the quantum yield begins at about the intensity at which each bacteriorhodopsin molecule in the front region of the cell would be excited once. However, the amplitude of the volume changes is not a simple exponential function of the light intensity (Fig. 2). This probably is due to the high absorbance of the samples at the excitation wavelengths. Because of screening by the front regions of the suspension, the distal regions receive very little light, and they would reach saturation only at extremely high light intensities. In addition, the saturation curves are complicated by the possibility of resonance energy transfer from excited bacteriorhodopsin molecules to neighboring molecules that already have been converted to K (19).

After a sufficiently long time, the bacteriorhodopsin has returned to its initial state, and all of the energy of the flash will have been converted to heat. (The membrane fragments are nonvesicular and have no way of storing energy in the form of electrochemical potential gradients.) Thus, a long time after the flash, the volume change caused by the flash is the same as the $\Delta V_{hv}\{T\}$ given by Eq. 6, except that the value of α is different because the membranes are in a buffered KCl solution, instead of in distilled water. The actual value of α need not be known in this method; α will cancel out of the equations because its value is the same as in Eq. 8. We have shown previously (7) that ΔV_{hv} is independent of the nature of the buffer, and that replacing the membranes by ink gives the same volume change if the temperature and the ionic composition of the solution are kept the same.

Combining Eqs. 6 and 8, we have

$$\phi = \frac{un_r}{n_e} = \frac{[\Delta V\{T, B_1\} - \Delta V\{T, B_2\}] - [\Delta V\{T_0, B_1\} - \Delta V\{T_0, B_2\}]}{\Delta V_{hv}\{T\}} \cdot \left[\frac{E_e}{\Delta H_p\{B_1\} - \Delta H_p\{B_2\}}\right]. \quad (9)$$

Again, the quantities in the brackets at the far right are known constants, and independent measurements of the flash intensity are not necessary.

Fig. 3 shows data from a typical experiment. A weak laser flash at 588 nm was used for

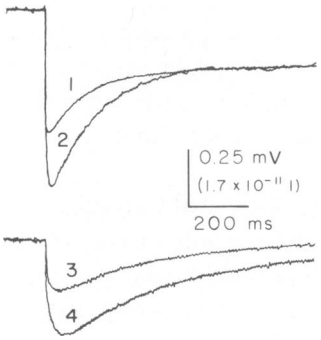

FIGURE 3

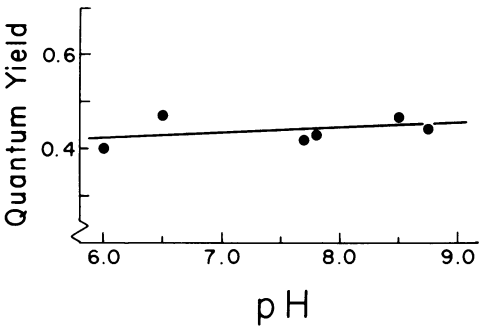

FIGURE 4

FIGURE 3 Flash-induced volume changes of purple membranes in two buffers and at two temperatures. The volume change in 5 mM Tris (pH 8.5) and 200 mM KCl is shown in trace 1 for 10.5°C and trace 3 for 2.0°C. 5 mM pyrophosphate (pH 8.5) was substituted for the Tris in traces 2 (10.5°C) and 4 (2.0°C). The flash strength was 3.15×10^{-9} E. Each trace was the average of 9 flashes, 18 s apart.

FIGURE 4 The pH-dependence of the quantum yield of proton release by purple membrane fragments. Low intensity (< 5 nE) 588-nm laser flashes provided the excitation for most of the measurements; the xenon flash lamp was used for the measurements at pH 8.75. Experimental conditions are detailed in the legends of Tables I and II. The quantum yields at pH 7.8, 7.7, and 6.0 were determined by using the difference in volume change of the protonation of Tris and phosphate (pH 7.8 and 7.7) or phosphate and cacodylate (pH 6.0), according to Eq. 7. In these experiments the purple membranes were suspended in 5 mM buffer and the ionic strength was adjusted to 200 mM with KCl. ΔV_p for cacodylate is 13.2 ml/mol buffer protonated (12). The remaining quantum yield determinations were calculated by using the heat of ionization of two different buffers according to Eq. 9. At pH 8.75, purple membranes were suspended in either 20 mM glycyl glycine or 20 mM pyrophosphate; at pH 8.5 the buffers were 5 mM Tris or 5 mM pyrophosphate; at pH 6.5, they were 5 mM phosphate or 5 mM Aces [*N*-(2-acetamido)-2-aminoethanesulfonic acid]. 200 mM KCl was present in all cases.

TABLE II

THE QUANTUM YIELD OF PROTON RELEASE CALCULATED FROM DIFFERENCES IN HEATS OF PROTONATION OF HYDROGEN ION BUFFERS

Buffer	pH	$\Delta H_p\{B\}$	$\Delta V_{hv}\{10.5°\}$	$\Delta V\{10.5°, B\}$	$\Delta V\{T_o, B\}$	ϕ
B		*kcal/mol*	μV	μV	μV	
Phosphate*	6.5	−1.8	515	972	834	0.49
Aces*		−7.2		488	322	
Pyrophosphate‡	8.5	−0.5	366	713	641	0.47
Tris‡		−11.3		450	338	
Pyrophosphate§	8.75	−0.5	133	307	276	0.44
Glycyl glycine§		−10.5		222	179	

The flash-induced volume change of purple membranes was measured at two temperatures, $T_o \sim 2.0°$ and $T = 10.5°$, in each buffer. T_o was always selected to be the temperature at which the thermal coefficient of expansion (α) for the salt solution was zero. The quantum yields were calculated by using Eq. 9.

*5 mM buffer, 200 mM KCl, laser flash of 563 nm (4.4×10^{-9} E), average of 16 flashes. ΔH_p for Aces is from reference 21.

‡5 mM buffer, 200 mM KCl, laser flash of 588 nm (3.15×10^{-9} E), average of 12 flashes. These values come from data shown in Fig. 3.

§20 mM buffer, 200 mM KCl, xenon flash excitation, average of 150 flashes.

excitation. The two buffers used were pyrophosphate (traces 2 and 4) and Tris (traces 1 and 3). For this pair, $\Delta H_p\{PPi\} - \Delta H_p\{Tris\} = 10.8$ kcal/mol. The measurements were made at 10.5° [T] and at 2.0° [T_0]. Table II contains data from experiments using three different pairs of buffers. Both weak laser flashes and xenon lamp flashes were used for excitation. The calculated quantum yields all fall in the narrow range of 0.44–0.49. This agrees fairly well with the results obtained by the first method described above.

The second method for determining quantum yields is not as accurate as the first method. The calculation involves the differences between four measurements of volume changes at two temperatures, instead of the difference between two measurements at the same temperature. In addition, the size of the difference $[\Delta V\{T, B_1\} - \Delta V\{T, B_2\}] - [\Delta V\{T_0, B_1\} - \Delta V\{T_0, B_2\}]$ is relatively small, unless T and T_0 are far apart. (We wanted to keep T and T_0 as close together as practical, to minimize other possible effects that temperature change might have on the membranes; see reference 20.) The major advantage of the second method is that it applies over a wider range of pH, because heats of protonation have been measured for many more different buffers than have volume changes of protonation.

The dependence of the quantum yield of proton release on the pH is shown in Fig. 4. This figure includes data obtained by the first method with two different pairs of buffers and by the second method with three pairs of buffers. The yield is essentially independent of the pH between pH 6.0 and 8.75.

By collecting all of the data obtained by both methods with flash strengths below about 7.5 nE and ionic strengths of 200 mM or greater, the mean value of the quantum yield was found to be 0.43 with a standard deviation of ±0.03. This is the mean of 22 independent determinations. The quantum yield is substantially larger than the quantum yields of 0.25–0.3 reported recently (9–11) for the formation of the M spectral intermediate.

To explore possible reasons for the discrepancy, we investigated the effect of the ionic strength on the quantum yield of proton release (Fig. 5). Purple membranes were suspended

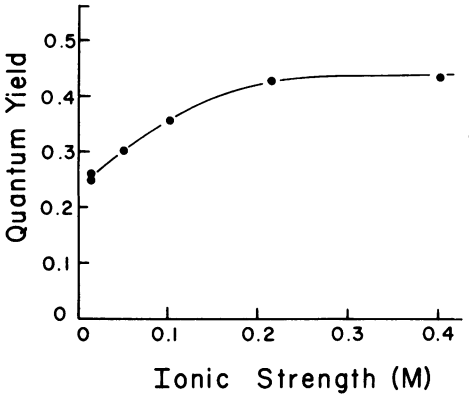

FIGURE 5

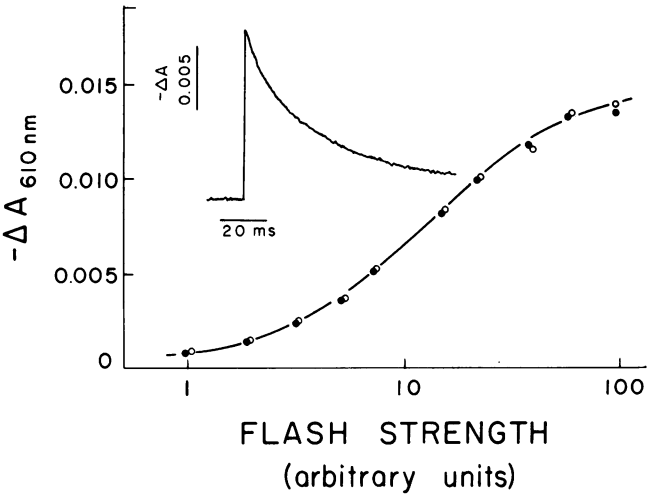

FIGURE 6

FIGURE 5 Quantum yield of proton release by purple membrane fragments as a function of ionic strength. Quantum yields were calculated from the volume change of protonation of the buffer according to Eq. 7. Purple membranes were suspended in 5 mM Tris (pH 7.8) or 5 mM phosphate (pH 7.8) at 2.0°C and KCl was used to vary the ionic strength. The flash strength was 5 nE and the wavelength 588 nm. Volume changes from 16 flashes were averaged.

FIGURE 6 Flash-induced absorbance decrease in purple membrane suspensions at two ionic strengths. Purple membranes (approximately 1.6 μM bacteriorhodopsin) were suspended in 5 mM Tris pH 7.8, with KCl to give ionic strengths of 14.6 mM (open circles) or 400 mM (filled circles), and were excited with laser flashes at 588 nm. The temperature was 1.5°C. Each measurement is an average of 5–10 flashes, 10 s apart. The inset shows a typical measurement. Three or four flashes with a strong flash were given before each measurement with a weak flash, to insure light adaptation of the bacteriorhodopsin. The absorbance changes measured at the higher ionic strength have been multiplied by 1.04 to correct for a small difference between the apparent extinction coefficients of bacteriorhodopsin at the two ionic strengths. The abscissa also was corrected for the small difference in the amounts of light absorbed. Note that the bacteriorhodopsin concentration was much lower for these measurements than in the measurements of volume changes, so that screening was less important. About 20% of the excitation light was absorbed in the 1-cm optical path.

at pH 7.8 in either 5 mM Tris or 5 mM phosphate and KCl was used to vary the ionic strength (the lower ionic strength of Tris was compensated for with KCl). As the ionic strength was lowered, the quantum yield fell off gradually, from about 0.43 at an ionic strength of 400 mM to 0.25 at 14.6 mM.[3] The amplitudes of both components of the flash-induced expansion decrease by approximately an equivalent proportion with decreasing ionic strength (20).

The quantum yield of about 0.25 for proton release at low ionic strength (Fig. 5) agrees very well with the recent values for the quantum yield of M. This corroborates the report of Lozier et al. (5) that purple membranes suspended in distilled water release 1 mol of protons/mol of M intermediate formed. The higher quantum yield of proton release at higher ionic

[3]From the expressions given by Redlick and Rosenfield (reference 13) one can calculate that ΔV_p{PPi} – ΔV_p[Tris] would decrease by about 4% as the ionic strength is decreased from 400 mM to 0. We have neglected this small effect in our calculations and used values of ΔV_p measured (12) at ionic strengths near 100 mM. Including a correction for this effect would slightly increase the dependence of the quantum yield on the ionic strength, but the correction would be only marginally significant.

strength (Fig. 5) could reflect either a change in the quantum yield of the photochemical transformations of bacteriorhodopsin or a change in u, the number of protons released per photochemical cycle. To investigate this point, we measured the flash-induced bleaching of the chromophore at several different ionic strengths. Fig. 6 shows the absorbance decreases at 610 nm caused by flash excitation of purple membranes at ionic strengths of 14.6 and 400 mM. The measurements were made approximately 2 ms after the flash, when the concentration of the M intermediate is maximal. The abscissa gives the flash intensity on a logarithmic scale; an increase in the quantum yield of the M intermediate would shift the experimental curve to the left. Varying the ionic strength causes no detectable change in the quantum yield. This agrees with Becher and Ebrey (9) and Goldschmidt et al. (10), who measured essentially the same quantum yields of the M intermediate at widely different ionic strenths. The number of protons released per cycle thus appears to increase from one to approximately two with increasing ionic strength.

There is one area of uncertainty regarding the conclusion that the number of protons released per cycle increases at high ionic strength. The measurements of the quantum yield of photochemistry (references 5, 9–11, and Fig. 6) rely on the assumption that all bacteriorhodopsin cycling passes through the M intermediate. But a comparison of the kinetics of proton release and M intermediate formation and decay has demonstrated that these are not synchronous processes (7). Possibly, some of the bacteriorhodopsins release protons but return to their initial state by a shortcut that bypasses the formation of M; these molecules would not be detected in the experiments of Fig. 6. To explain the data this way, however, would require additional ad hoc assumptions about how the quantum yield of the putative shortcut depends on the ionic strength. The photoreversal from K back to the initial state probably cannot be significant in this regard, because the quantum yield of proton release does decline when photoreversals occur (Fig. 3).

After this work had been submitted for publication, we learned of two other relevant studies. First, Hartmann et al. (22) have found that intact cells of *H. halobium* extrude 0.4–0.6 protons per photon absorbed. This measurement, made in a medium with a high ionic strength, agrees well with our value of 0.43 for the quantum yield of proton release by purple membrane fragments. Second, Hess and Kuschmitz (23) have found that purple membrane fragments release approximately two protons per cycle (i.e., per M intermediate formed) at high ionic strengths, and about one proton per cycle at ionic strengths near 10 mM. These conclusions are identical to ours. Hess and Kuschmitz (23), however, have further found that the yield of protons becomes even less than unity as the ionic strength approaches zero. The apparent discrepancy between this finding and the results obtained by Lozier et al. (5) remains to be resolved. Hess and Kuschmitz (23) attribute the dependence of the proton release on ionic strength to a shift in the pK_a of a proton-binding group, caused by the surface potential of the negatively charged membrane. If this explanation is correct, the experiments of Figs. 4 and 5 indicate that the pK_a is below 6 at ionic strengths near 200 mM, and above 7.8 at ionic strengths near 10 mM. Because *H. halobium* thrives only at high salt concentrations, the yield approaching two protons per photochemical cycle would seem to be of greater physiological significance than the lower yields obtained at low ionic strengths.

It is possible that some of the protons released when membrane fragments are excited with a short flash are rebound at the same site rather than at a site on the opposite side of the

membrane. Protons released and rebound on the same side of the membrane would not contribute to net translocation of protons across the membrane. Our measurements do not address the sites of proton release and rebinding directly. We have shown previously, however, that both the release of protons from the membrane fragments into solution and subsequent uptake of protons occur with monophasic kinetics (7). The proton uptake precedes the return of bacteriorhodopsin's absorption band at 570 nm, and appears to be fast enough to be consistent with the proton translocation occurring in intact cells. Our results suggest, therefore, that each cycle of bacteriorhodopsin at high ionic strength may be coupled to the translocation of two protons across the membrane. This suggestion is strengthened, though certainly not proved, by the agreement between the quantum yield we measured and that obtained by Hartmann et al. (22) with intact cells. However, current thinking about the mechanism of proton transport (1), which emphasizes the deprotonation of the retinylidine Schiff base, does not readily account for the transport of more than one proton per cycle.

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REFERENCES

1. HENDERSON, R. 1977. The purple membrane from *Halobacterium halobium*. *Annu. Rev. Biophys. Bioeng.* **6**:87–109.
2. OESTERHELT, D., and W. STOECKENIUS. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nat. New Biol.* **233**:149–154.
3. PAPADOPOULOS, G., T. HSIAO, and J. CASSIM. 1978. Determination of the retinal/protein molar ratios for purple membranes of *Halobacterium halobium* and *Halobacterium cutirubrum*. *Biochem. Biophys. Rev. Commun.* **81**:127–132.
4. LOZIER, R., R. BOGOMOLNI, and W. STOECKENIUS. 1975. Bacteriorhodopsin: a light-driven pump in *Halobacterium halobium*. *Biophys. J.* **15**:955–962.
5. LOZIER, R., W. NIEDERBERGER, R. BOGOMOLNI, S. HWANG, and W. STOECKENIUS. 1976. Kinetics and stoichiometry of the light-induced proton release and uptake from purple membrane fragments, *Halobacterium halobium* cell envelopes, and phospholipid vesicles containing oriented purple membranes. *Biochim. Biophys. Acta.* **440**:545–556.
6. LOZIER, R., and W. NIEDERBERGER. 1977. The photochemical cycle of bacteriorhodopsin. *Fed. Proc.* **36**: 1805–1809.
7. ORT, D., and W. PARSON. 1978. Flash-induced volume changes of bacteriorhodopsin-containing membrane fragments and their relationship to proton movements and absorbance transients. *J. Biol. Chem.* **253**:6158–6164.
8. OESTERHELT, D., and B. HESS. Reversible photolysis of the purple complex in the purple membrane of *Halobacterium halobium*. *Eur. J. Biochem.* **37**:316–326.
9. BECHER, B. and T. EBREY. 1976. The quantum efficiency for the photochemical conversion of the purple membrane protein. *Biophys. J.* **17**:185–191.
10. GOLDSCHMIDT, C., O. KALISKY, T. ROSENFELD, and M. OTTOLENGTHI. 1977. The quantum efficiency of the bacteriorhodopsin photocycle. *Biophys. J.* **17**:179–183.
11. GOLDSCHMIDT, C., M. OTTOLENGHI, and R. KORENSTEIN. 1976. On the primary quantum yields in the bacteriorhodopsin photocycle. *Biophys. J.* **16**:839–843.
12. NEUMAN, R., W. KAUZMANN, and A. ZIPP. 1973. Pressure dependence of weak acid ionization in aqueous buffers. *J. Phys. Chem.* **77**:2687–2691.
13. MILLERO, F. 1971. The molal volumes of electrolytes. *Chem. Rev.* **71**:147–176.
14. BECHER, B., and J. CASSIM. 1975. Improved isolation procedures for the purple membrane of *Halobacterium halobium*. *Prep. Biochem.* **5**:161–178.
15. OESTERHELT, D., M. MEENTZEN, and L. SCHUHMANN. 1973. Reversible dissociation of the purple complex in

bacteriorhodopsin and identification of 13-*cis* and all-*trans*-retinal as its chromophores. *Eur. J. Biochem.* **40**:453–463.

16. PETTEI, M. J., A. P. YUDD, K. NAKANISHI, R. HENSELMAN, and W. STOECKENIUS. 1977. Identification of retinal isomers isolated from bacteriorhodopsin. *Biochemistry.* **16**:1955–1959.
17. DENCHER, N., and M. WILMS. 1975. Flash photometric experiments on the photochemical cycle of bacteriorhodopsin. *Biophys. Struct. Mech.* **1**:259–271.
18. PARSON, W., R. CLAYTON, and R. COGDELL. 1975. Excited states of photosynthetic reaction centers at low redox potentials. *Biochem. Biophys. Acta.* **387**:265–278.
19. EBREY, T., B. BECHER, B. MAO, P. KILBRIDE, and B. HONIG. 1977. Exciton interactions and chromophore orientation in the purple membrane. *J. Mol. Biol.* **112**:377–397.
20. ORT, D., and W. PARSON. 1979. Enthalpy changes during the photochemical cycle of bacteriorhodopsin. *Biophys. J.* **25**:355–364.
21. MCCLOTHIN, C., and J. JORDAN. 1976. Thermo-dynamic parameters of some biochemically significant buffers. *Anal. Lett.* **9**:245–255.
22. HARTMANN, R., H.-D. SICKINGER, and D. OESTERHELT. 1977. Quantitative aspects of energy conversion in Halobacteria. *FEBS(Fed. Eur. Biochem. Soc. Lett.* **82**:1–6.
23. HESS, B., and D. KUSCHMITZ. 1978. Establishments of electrochemical gradients: general views and experiments on purple membrane. *In* Frontiers of Biological Energetics. P. L. Dutton, J. Leigh, and A. Scarpa, editors. Academic Press, Inc., New York. In press.