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# THE QUENCHING EFFECT OF BLUE LIGHT ON HALORHODOPSIN

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**A mutant of *Halobacterium halobium* which contains halorhodopsin was isolated from strain $S_9$. An absorbance change at 380 nm caused by steady orange light illumination ($\lambda \geq 530$ nm) was observed. This change depended upon the intensity of the actinic light. The bleached envelope vesicles and vesicles derived from nicotine-grown cells showed a small or no absorbance change at 380 nm, suggesting that the change stemmed from the photochemical intermediate of halorhodopsin (referred to as P-380). When blue light was superimposed on orange background illumination, the membrane potential ($\Delta\psi$) of the envelope vesicles decreased. $\Delta\psi$ was determined from the tetraphenylphosphonium cation ($TPP^+$) distribution by means of a $TPP^+$ electrode. When blue light intensity was increased, both $\Delta\psi$ and the amount of P-380 were decreased. An equation was derived which showed that $\Delta\psi$ is proportional to the concentration of P-380 formed by illumination under the assumption that the ionic composition is not significantly changed upon illumination. This equation was checked experimentally from the following three points: The blue light effect, the relationship between $\Delta\psi$ and light intensity, and the effect of gramicidin. The data obtained accorded well with the theoretical relationship.**

## Introduction

It is well known that upon illumination, bacteriorhodopsin in membranes of *Halobacterium halobium* transports $H^+$ from the inside to the outside of cells and that this transport results in acidification of the medium [1,2]. In 1977, Matsuno-Yagi and Mukohata [3] reported that an apparently bacteriorhodopsin-free *H. halobium* strain produced only $H^+$ uptake (alkalinization in the medium), and that the mechanism was different from that of bacteriorhodopsin as suggested by its heat-instability and high resistance to hydroxylamine. MacDonald et al. [4,5] and Greene and Lanyi [6] suggested that the unexpected phenomena observed by Matsuno-Yagi and Mukohata [3] were attributable to a second pigment which acts as a light-driven $Na^+$ pump. Lanyi and Weber [7] succeeded in obtaining the spectrum of this pigment which has an absorbance maximum at 588 nm. This wavelength is consistent with that of the action spectrum of $H^+$ uptake or of an interior negative membrane potential [3,6]. Thus, it has been established that the cytoplasmic membrane of *H. halobium* contains at least two species of light-reactive pigments: bacteriorhodopsin, which has been characterized rather more precisely, and halorhodopsin [8], whose characterization has been started quite recently.

Weber and Bogomolni [9] have shown that halorhodopsin undergoes a photochemical reac-

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Abbreviations: $TPP^+$, tetraphenylphosphonium cation; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

tion which can be detected by flash photolysis. According to the scheme proposed by them, the longest-lived intermediate exhibited an absorbance maximum at 380 nm (referred to as P-380). In this paper, we report a finding that additional blue light ($\lambda \approx 380$ nm) above orange background illumination ($\lambda \geqslant 530$ nm) reduces the membrane potential of envelope vesicles derived from a halorhodopsin-containing mutant isolated from strain $S_9$. The relationship between the photoinduced membrane potential and the amount of P-380 is shown in an equation which can explain the photoinduced membrane potential of envelope vesicles of this mutant.

## Methods and Materials

The strains used were KY-4 and JW-1 (generous gift from J. Weber). In the strain JW-1 (formerly ET-15 [7,9]), it has been shown that residual levels of functional bacteriorhodopsin had to be lower than three molecules per bacterial cell [9]. Strain KY-4 has been isolated by ourselves and used extensively. Colonies of strain $S_9$ were grown on agar plate under white light. A few reddish-brown colonies were found among several hundred colonies of strain $S_9$. Mutant colonies were collected and purified by restreaking twice on agar plate. One clone was picked up and allowed to grow in a peptone medium [10]. Envelope vesicles were prepared by sonication as described by Lanyi and MacDonald [10]. The sidedness of vesicles was checked by NADH-menadion oxidoreductase activity [10] and all preparations showed 85–90% right-side-out vesicles. The envelope vesicles were bleached with hydroxylamine by essentially the same method as that described by Lanyi and Weber [7].

The optical arrangement used is given in Fig. 1. The vesicles were illuminated in a $1 \times 1$ cm cuvette by a 1 kW tungsten projector lamp ($L_1$) through a heat filter, a 5 cm water layer containing 1% $CuSO_4$ and a 530 nm cutoff filter (Toshiba Glass Co.). The light passed through these filters will hereafter be referred to as orange light. Blue light (350–420 nm) illumination was provided by a 150 W xenon lamp ($L_2$) through a monochromator. Orange light and blue light were crossed at the center of the cuvette. The illumination was passed through an

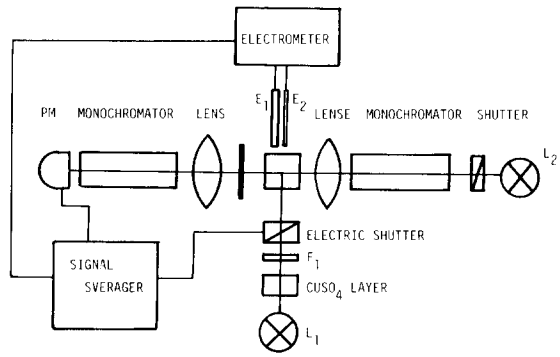


Fig. 1 Optical arrangement for measurements of the steady-state concentration of P-380 and blue light quenching of $TPP^+$ uptake by the envelope vesicles. $L_1$, 1 kW tungsten projector lamp; $F_1$, 530 nm cut off filter; $L_2$, 150 W xenon lamp; $F_2$, blue filter (C-39A, Toshiba Glass); PM, photomultipler; $E_1$, $TPP^+$ electrode; $E_2$, a reference electrode.

electric shutter (DC-495, Copal) controlled by a signal averager (Nihon Koden, Model ATAC 201) or through a shutter operated manually. 10–30 signals were accumulated and averaged. The absorbance change at varying wavelength due to orange actinic light was calculated from the ratio of the change in the output current of photomultipler to the current without actinic light. Light intensity was adjusted with a neutral density filter and measured with a YSI-Kettering radiometer (Model 65A) at the front of the cuvette.

The membrane potential was estimated by the distribution of a liphophilic cation, $TPP^+$, which was monitored by means of a $TPP^+$ electrode as described previously [11,12]. The electromotive force (e.m.f.) arisen between a $TPP^+$ electrode and a reference electrode was measured by an electrometer (TR-8651, Takeda Riken) and stored in the signal averager. We used a small electrode (diameter 7 mm) and reference electrode (diameter 1 mm) which were inserted into a $1 \times 1$ cm cuvette. The $TPP^+$ concentration used was 10 $\mu$M. Illuminating the whole vesicles in the cuvette with actinic orange light, we examined the effect of gramicidin on the photoinduced membrane potential. The apparatus used was essentially the same as described previously [12] except that the light source was replaced by a 1 kW tungsten projector lamp. The temperature was kept constant at 25°C with circulating thermostatically controlled water.

We also used the apparatus shown in Fig. 1, which allowed illumination of only a small part of the cuvette with orange and blue light. In the latter case, the extent of $TPP^+$ uptake was small and we did not calculate the membrane potential from the change in the $TPP^+$ electrode potential, which was plotted in Fig. 6. Changes in pH of the medium were measured by immersing a combination pH electrode (Type 6028-10T, Horiba) connected to a pH meter (F-7AD, Hitach-Horiba). When the action spectrum was measured, the monochromic light was obtained with an appropriate interference filter (half-width 15 nm, Toshiba, KL-series). The internal volume of the vesicle was assumed to be 3 $\mu$l/mg protein [10]. Protein was assayed by the method of Lowry et al. [20] using bovine serum albumin as a standard.

FCCP, gramicidin and all-*trans*-retinal were obtained from Sigma, St Louis, MO. All other chemicals were of analytical grade and obtained from commercial sources.

## Results

The electrical response and $H^+$ movement of KY-4 envelope vesicles upon exposure to orange light were investigated. The interior negative ($TPP^+$ uptake) membrane potential was observed. No acidification in the medium was observed. Addition of 4 $\mu$M FCCP increased both the extent and the rate of alkalinization while a small decrease in $TPP^+$ uptake was observed. These results are consistent with the properties of halorhodopsin reported previously [4–6].

The difference spectrum between the hydroxylamine-bleached vesicles and the vesicles regenerated with all-*trans*-retinal showed an absorbance maximum around 590 nm (see lower half of fig. 2), which agreed with the value of 588 nm reported by Lanyi and Weber [7]. No shoulder at 570 or 560 nm was found, indicating that the bacteriorhodopsin content was small. According to an analysis using the flash-photolysis technique by J. Weber (personal communication), the present strain contains 2–3-times as much as the JW-1 strain and the contamination of bacteriorhodopsin is less than the limit of the detection of this apparatus (0.2% of the amount which is present in strain R-1). The data in Figs. 2 and 3 indicate that the present

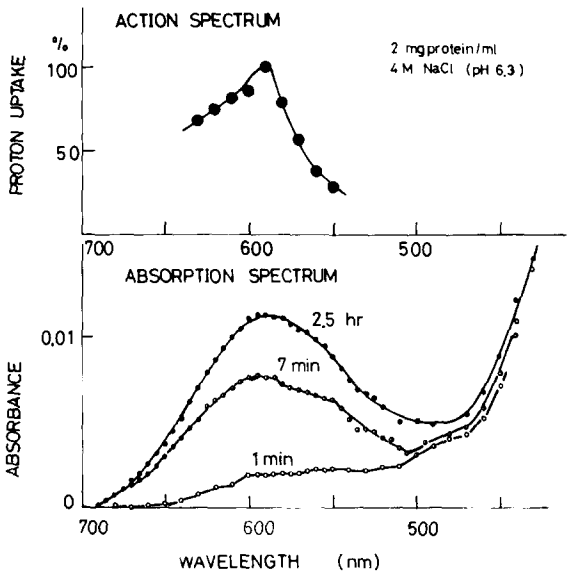


Fig. 2. (Upper) The action spectrum of photoinduced $H^+$ uptake. The ordinate represents the ratio of photoinduced $H^+$ uptake at varying wavelength to that at 590 nm. Vesicles (2 mg protein/ml) were suspended in 4 M NaCl+2 mM Hepes. (Lower) The spectrum of halorhodopsin. The difference spectrum between bleached vesicles and the vesicles (2 mg protein/ml)+all-*trans*-retinal (0.33 ng/ml) was measured with the spectrometer equipped with an end-on photomultiplier (UV-300, Shimazu). The three curves show the spectrum measured 1 min (bottom), 7 min (center) and 2.5 h (top) after addition of retinal.

strain may not contain bacteriorhodopsin. The action spectrum of photoinduced $H^+$ uptake (Fig. 2, upper) peaks at 590 nm, and corresponds to the absorbance maximum of halorhodopsin obtained. The discrepancy at the shorter wavelength is apparent but the reason is not clear.

Weber and Bogomolni [9] have shown that halorhodopsin undergoes photocycling and that an intermediate exhibiting an absorbance maximum at 380 nm is formed. This intermediate is hereafter referred to as P-380, while halorhodopsin in the dark is referred to as P-588. We measured the absorbance change around 380 nm upon steady illumination with orange light. The results are plotted in Fig. 3 (data shown by closed circles). According to the scheme proposed by Weber and Bogomolni [9], P-380 is the longest-lived intermediate and no intermediate other than P-380 exhibiting absorption around 380 nm exists.

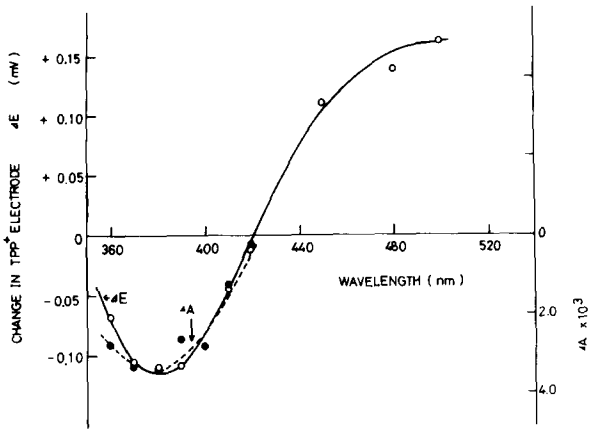


Fig. 3. The absorbance change (●) due to orange light illumination and the change on the potential of the $TPP^+$ electrode (○) by superposition of blue light on the orange background light. The data shown by closed circles are the spectrum of P-380.

Therefore, the spectrum shown in the figure is considered to be that of P-380. Hydroxylamine can bleach halorhodopsin and addition of retinal regenerates the pigment [7]. As shown in Fig. 4, hydroxylamine-treated vesicles showed an absorbance change at 380 nm upon orange light illumination. This may be attributed to the incomplete bleaching of halorhodopsin, which is highly resistant to $NH_2OH$ [3,7]. In fact, we did not observe an absorbance change in this wavelength region with the envelope vesicles derived from cells grown in the presence of 3 mM nicotine, which is an inhibitor of retinal synthesis [13]. Addition of all-*trans*-retinal to these preparations increased the change in absorbance at 380 nm upon orange light illumination. Figs. 2 and 4 show that the time courses of these changes are approximately the

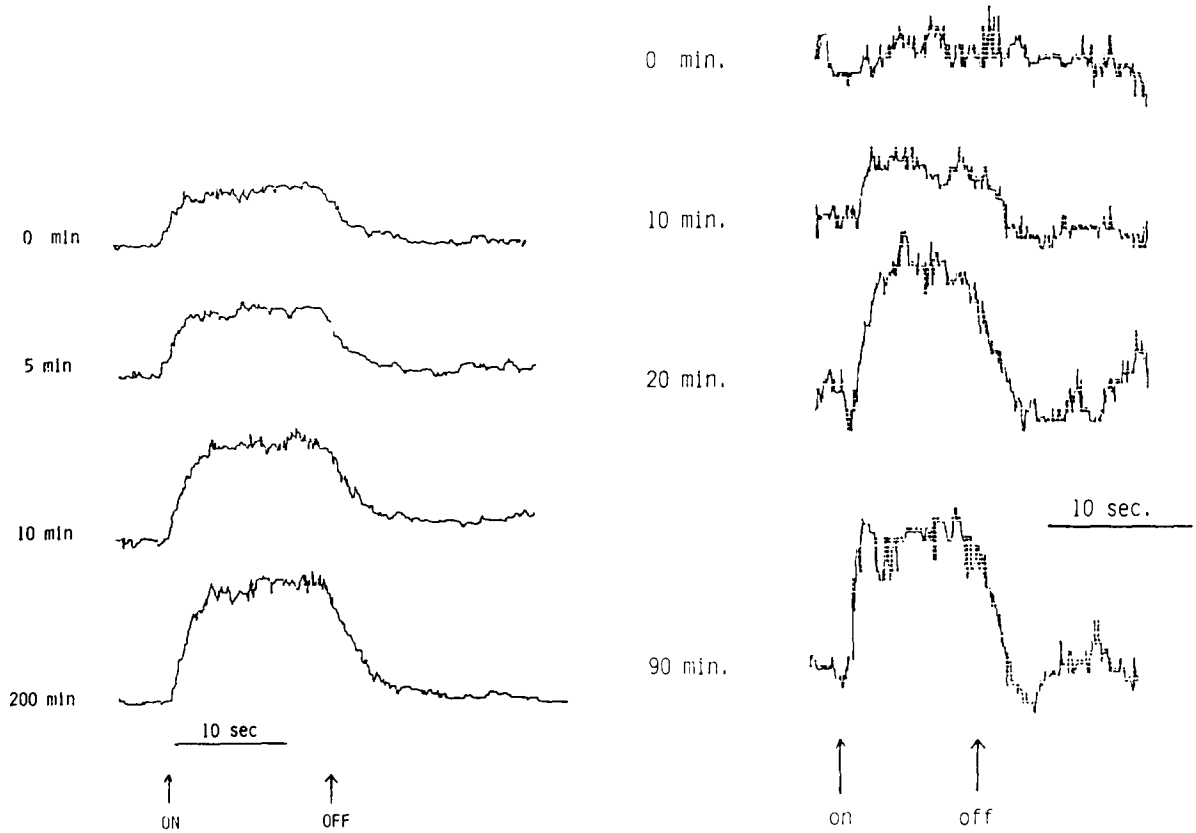


Fig. 4. Recovery of the absorbance change at 380 nm caused by illumination by addition of all-*trans*-retinal (0.33 ng/ml) to $NH_2OH$-treated vesicles (left) and to vesicles derived from nicotine-grown cells (right). The vesicles whose protein concentrations were 2 mg/ml (left) and 4 mg/ml (right), respectively, were suspended in the same solution as that in Fig. 2. At the time indicated by ON, exposure to orange light was commenced by means of an electric shutter controlled by a signal averager, and at the time indicated as OFF, the shutter was closed. The signals were added and averaged. The gain of amplifier for the experiment of nicotine-grown cells (right) was 4-times larger than the other because of low content of halo-opsin of the cells. The time shown to the left of each datum represents the incubation time after addition of retinal.

same, indicating that the absorbance change at 380 nm stems from halorhodopsin. It is noted that the absorbance change peaks at 380 nm which is clearly different from 412 nm of the M-intermediate of bacteriorhodopsin [1] and that JW-1, the bacteriorhodopsin-free strain [7,9] gives the same spectrum (data not shown). The amplitude of the signal of strain JW-1 was small compared with that of strain KY-4, which is consistent with the halorhodopsin contents of the respective strains. In addition, the absorbance change at 380 nm caused by actinic orange light depends on the intensity of the light employed, and follows a linear relationship when the reciprocal of the absorbance change is plotted against the reciprocal of the light intensity (data not shown, see also Eqn. 1).

Fig. 5 demonstrates the effect of blue light on the membrane potential. Orange light illumination led to $TPP^+$ uptake by vesicles. When blue light was superimposed, a small efflux of $TPP^+$ accumulated previously was observed. Termination of blue light caused the level of $TPP^+$ uptake to revert to the same level as that before blue light illumination. The change in $TPP^+$ uptake caused by blue light illumination can be repeated, indicating that neither halorhodopsin nor membrane integrity was impaired irreversibly by the blue light. We could observe the same effect of blue light irradiation of envelope vesicles derived from strain JW-1. However, the signal of strain JW-1 was small, and then we used strain KY-4 extensively. The reduction in membrane potential may be due to a decrease in the activity of the light-driven ion pump. For only blue light irradiation, a small $TPP^+$ uptake was observed, suggesting that the decrease in the activity of the ion pump cannot be accounted for by the excitation of halorhodopsin in the blue light region. On the contrary, absorption of light by higher excitation states of halorhodopsin can drive the ion pump. Note that the potential of the $TPP^+$ electrode is not influenced by blue light illumination. When the intensity of orange light was kept constant, an increase in blue light intensity (380 nm) led to a decrease in $TPP^+$ uptake by vesicles, i.e., a decrease in the interior negative membrane potential (Fig. 6).

Blue light of different wavelengths (slit width, 5

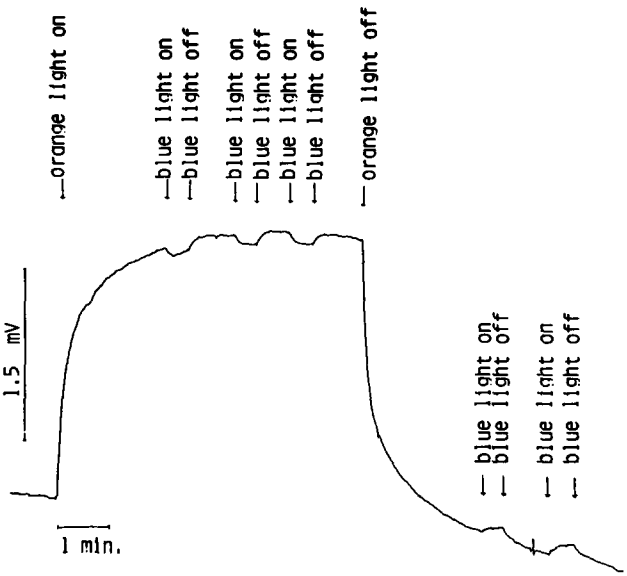


Fig. 5. Blue light quenching of halorhodopsin. $TPP^+$ accumulation due to the orange light illumination ($\lambda \geq 530$ nm, 283 $W/m^2$) and release due to the superposition of blue light ($\lambda = 380 \pm 5$ nm, 28 $W/m^2$). The vertical bar represents the 1.5 mV shift of the read-out in the $TPP^+$ electrode. The medium used contained 4 M NaCl buffered to pH 7.0 with 10 mM Hepes-NaOH.

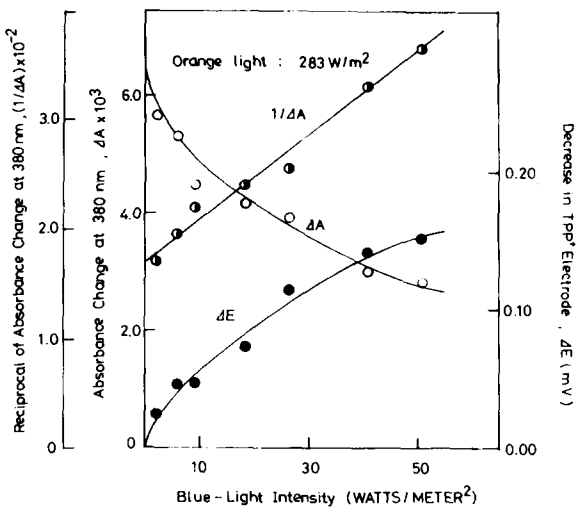


Fig. 6. The decrease in $TPP^+$ uptake and in P-380 caused by blue light irradiation which is superimposed on the background illumination of orange light. Blue light of varying intensities was provided under the condition that orange light intensity was kept constant at 283 $W/m^2$. ●, the decrease in the $TPP^+$ electrode potential; ○, absorbance change at 380 nm , $\Delta A$, which was calculated from $(1/2.3)(\Delta i/i)$ where $i$ and $\Delta i$ stand for the photomultiplier current in the dark and the change in the current upon illumination. ○, $1/\Delta A$ according to Eqn. 1.

nm) was superimposed on the orange light and the extent of change in the $TPP^+$ electrode potential was measured. The signals of 10–30 illuminations were averaged with a signal averager. The results are plotted in Fig. 3 (open circles), showing that the action spectrum agrees well with that of P-380.

## Discussion

The data shown above indicate that absorption of light by P-380 partially inhibits the activity of halorhodopsin. Ormos et al. [14] reported that the membrane potential for bacteriorhodopsin incorporated into a lipid bilayer membrane was smaller with simultaneous blue and green illumination than that with exposure to green light alone. Our results with halorhodopsin are similar to those found with bacteriorhodopsin. The M-intermediate of bacteriorhodopsin (absorption maximum, 412 nm) absorbs blue light and is photoconverted into bacteriorhodopsin through intermediate M′ [14,15]. This branched pathway does not contribute to electrogenic $H^+$ transport. After formation of the M-intermediate, the $H^+$ is released from the protein [16]. Therefore, it is inferred that when halorhodopsin is simultaneously illuminated by orange and blue light, some P-380 formed by orange light illumination goes back to the ground state of halorhodopsin, P-588, via a pathway which reduces the activity of ion pumping. There may be two possibilities as to the interpretation on this reduction. One possibility is that during P-380 formation the ion driven by halorhodopsin (i.e., $Na^+$) resides within the protein moiety and is not released by blue light irradiation. Another possible effect of blue light is that the ion previously pumped by halorhodopsin from the inside to the outside of the vesicles is picked up and sent to the inside by blue light irradiation: The ion is released before the formation of P-380. At present, we cannot identify clearly which possibilities are correct. The time necessary for establishment of the membrane potential and the lifetime of photointermediates will give a clue to this problem [17]. No matter which possibility is correct, absorption of blue light returns P-380 to P-588 via different pathway from the ordinary one.

Fig. 7 illustrates the highly simplified photocycle of halorhodopsin adapted from the scheme proposed by Weber and Bogomolni [9]. The rate of formation of P-380 is given by $k_1 I$[P-588] and the decay is given by $k_2$[P-380] + $k_b I_b$[P-380] because P-380 returns to P-588 via a different pathway from the ordinary one upon blue light illumination. Here, $I$ and $I_b$ represent the light intensities of orange and blue light, respectively. Under the condition of a steady state, we obtain the following equation (hR, halorhodopsin):

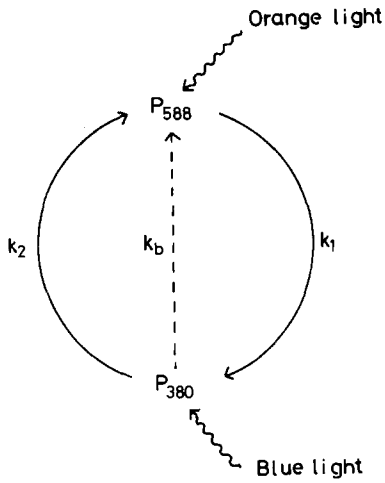


Fig. 7. Highly simplified photocycle of halorhodopsin adapted from the scheme of Weber and Bogomolni [9].

$$[\text{P-380}] = \frac{k_1 I}{k_1 I + k_2 + k_b I_b}[\text{hR}] \quad (1)$$

where [hR] = [P-580] + [P-380]. According to this equation, when $I_b = 0$, a linear plot of $1/$[P-380] vs. $1/I$ should be obtained, which is experimentally justified (data not shown). In addition, this equation indicates that a linear relation is obtained when $1/$[P-380] is plotted vs. $I_b$, as shown in Fig. 6.

We will consider the dependence of the membrane potential on P-380 concentration. The basic postulate is that the passive current of an ion species is the product of the conductance of the ion and motive force of the ion [18]. We assume that light-driven ion transport by halorhodopsin is only an electrogenic process and that other ion movements are passive when the vesicles are illuminated. The flux of the ion pumped by

halorhodopsin (which is considered to be $Na^+$ [4,6]) is denoted by $J_x$. The electric current caused by passive movement of ions such as $Na^+$, $Cl^-$ and $H^+$ (or $OH^-$) is given by $g_j(\Delta\psi - E_j)$, where $g_j$ is the conductance of the $j$-th ion, $E_j$ the equilibrium potential of the $j$-th ion, and $\Delta\psi$ the membrane potential. Then, the total ionic current across the vesicle membrane, $i$, is given by the following equation:

$$i = FJ_x + \Sigma g_j(\Delta\psi - E_j)$$

Under the steady-state condition, the total current, $i$, must be zero. Therefore, we obtain

$$\Delta\psi = \frac{\Sigma g_j E_j}{\Sigma g_j} - \frac{F}{\Sigma g_j} J_x$$

The ion species present in the system are $Na^+$, $Cl^-$ and $H^+$ (or $OH^-$), and the ionic compositions inside and outside the vesicles are the same. The concentrations of these ions are assumed to be kept constant because of a high concentration (4 M NaCl) even though halorhodopsin expels ions upon illumination. Actually, the rate of $Na^+$ extrusion by halorhodopsin is small (unpublished observation and see Ref. 19). The change in pH upon illumination was negligibly small provided that sufficiently high concentration of buffer (50 mM Hepes, pH 7) was used for both sides of the vesicles. Under these assumptions, we obtain the equation $E_j = 0$. Then, we have

$$\Delta\psi = -\frac{F}{\Sigma g_j} J_x \tag{2}$$

This equation indicates that the photoinduced membrane potential is determined by $J_x$ (the flux of the ion pumped by halorhodopsin upon illumination) and $g_j$ (conductance of ions).

First, we will make further discussion on the assumption that the first possibility mentioned above is correct. If photocycling of halorhodopsin couples tightly with the ion transport, $J_x$ should be proportional to the rate of the photocycle (P-588 $\overset{k_1}{\rightarrow}$ P-380 $\overset{k_2}{\rightarrow}$ P-588), and this is given by $k_2$[P-380] under steady illumination. Therefore, we obtain the equation:

$$FJ_x = \alpha k_2[\text{P-380}]$$

where $\alpha$ is the proportionality constant. This equation and Eqn. 2 indicate that $\Delta\psi$ is proportional to [P-380]. Then, we have

$$\Delta\psi = -\alpha\left(\frac{[\text{hR}]}{\Sigma g_j}\right)\frac{k_1 k_2 I}{k_1 I + k_2 + k_b I_b} \tag{3}$$

If the second possibility is correct, $J_x$ should be composed of two terms: the ion pumping by the ordinary pathway ($\alpha k_2$[P-380]) and the reverse pumping by blue light ($\alpha k_b I_b$[P-380]). Then, we have the following equations:

$$FJ_x = \alpha(k_2 - k_b I_b)[P\text{-}380]$$

and

$$\Delta\psi = -\alpha\left(\frac{[\text{hR}]}{\Sigma g_j}\right)\frac{k_1(k_2 - k_b I_b)}{k_1 I + k_2 + k_b I_b} \tag{3'}$$

Since the change in $TPP^+$ electrode, $\Delta E$ in Fig. 6, corresponds to the change in $\Delta\psi$, the data shown in Fig. 6 are consistent with either Eqn. 3 or 3'. Next, we will consider the case when $I_b = 0$. These equations show that the membrane potential under varying light intensities obeys a linear relationship when $1/\Delta\psi$ is plotted against $1/I$. In addition, when $g_j$ is increased by addition of appropriate ionophores, only the maximum value of $\Delta\psi$ is decreased while the light intensity giving the half-maximum value of $\Delta\psi$ stays constant, provided that $k_i$ ($i = 1$ and 2) is not altered. The steady-state concentration of P-380 was not changed by addition of gramicidin (data not shown), suggesting that the assumption of a constant $k_i$ is valid. In Fig. 8, $1/\Delta\psi$ is plotted against $1/I$ at varying concentrations of gramicidin, indicating that Eqn. 3 holds.

In the discussion made above, it is assumed that the membrane contains only halorhodopsin as a light-reactive pigment which is associated with the ion pumping. Now that halorhodopsin is not isolated as functional single protein, we have used the envelope vesicles from the mutant containing halorhodopsin. We cannot rule out the possibility that the membrane contains a retinal protein other than bacteriorhodopsin and halorhodopsin whose photointermediate shows a maximum absorbance at 380 nm. If this is the case, the interpretation becomes complicated. Since until now such a pig-

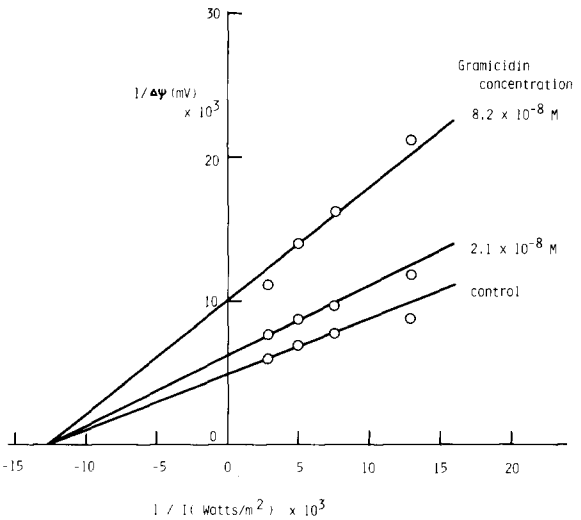


Fig. 8. Double-reciprocal plot of the photoinduced membrane potential and the light intensity in the presence of varying concentrations of gramicidin.

ment has not been reported, we analysed our data on the basis of the assumption that the membrane contains only halorhodopsin.

In conclusion, the photoinduced membrane potential of the vesicle containing halorhodopsin follows Eqn. 3 or 3′. If we can analyse the dependence of $\Delta\psi$ on $I_b$ quantitatively, we can identify which possibility is correct by comparing the data with Eqns. 3 and 3′. Further study with high accuracy may be necessary and the correlation between photocycling and ion pumping of halorhodopsin should be investigated.

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