Kinetic Analysis of Light-Induced pH Changes in Bacteriorhodopsin-Containing Particles from *Halobacterium halobium*†

Michael Eisenbach,* Haim Garty, Evert P. Bakker,‡ Gil Klemperer, Hagai Rottenberg,§ and S. Roy Caplan

ABSTRACT: Illumination of a suspension of subbacterial particles from *Halobacterium halobium* or of bacteriorhodopsin-containing proteoliposomes led to acidification or alkalinization, respectively, of the suspending medium. Both processes were reversible upon turning the light off. Each of the "on" and "off" reactions in either of the preparations fitted kinetically a sum of two exponentials, the first phase being faster than the second. The kinetics were the same whether measured electrometrically with a pH electrode or fluorimetrically with the impermeant pH indicator fluorescein isothiocyanate–dextran. The action spectrum of each of the phases overlapped the absorption spectrum of bacteriorhodopsin. The extents of both phases were too large to be accounted for stoichiometrically by the amount of the retinylidene–lysine Schiff base that was deprotonated. The two phases were compared with respect to the effects of altering pH, temperature, and the concentration of a permeant cation. The extent of the slow phase increased considerably either with decreasing the pH or with increasing the temperature or concentration of triphenylmethylphosphonium ion ($TPMP^+$). The extent of the rapid phase, on the other hand, increased only slightly with decreasing pH, decreased with increasing temperature (in the "on" reaction only), and was unaffected by $TPMP^+$. Furthermore, the increased acidification subsequent to the addition of $TPMP^+$ to subbacterial particles during the steady state in the light followed monophasic first-order kinetics, with a rate constant typical of the slow phase. Various interpretations of the observed kinetics are considered. The interpretation which seems to be in accord with the experimental results is that the slow phase represents net proton transport across the membrane, and the rapid phase represents a proton dissociation–association reaction.

It is well established that purple membrane fragments isolated from *Halobacterium halobium* acidify the suspending medium upon illumination (Oesterhelt and Hess, 1973). This led to the suggestion that bacteriorhodopsin, the only protein in the purple membrane (Oesterhelt and Stoeckenius, 1971), serves as a proton pump in the intact bacteria (for reviews, see Oesterhelt, 1976a,b; Henderson, 1977; Lanyi, 1978; Stoeckenius et al., 1978; Bayley and Morton, 1978). Illuminated cells of *H. halobium* extrude protons into the external medium but only after a transient uptake of protons (the net result being acidification) (Oesterhelt and Stoeckenius, 1973; Bogomolni and Stoeckenius, 1974; Danon and Caplan, 1974, 1976; Bakker et al., 1976; Wagner and Hope, 1976). Sonicated bacterial particles extrude protons into the medium like intact bacteria but without the transient initial uptake phase (MacDonald and Lanyi, 1975; Kanner and Racker, 1975), whereas in a suspension of proteoliposomes reconstituted from bacteriorhodopsin and phospholipids (Racker, 1973; Racker and Stoeckenius, 1974; Kayushin and Skulachev, 1974; Racker and Hinkle, 1974; Yoshida et al., 1975; Eisenbach et al., 1976; Bakker et al., 1978) protons are taken up from the medium on illumination. This extrusion or uptake of protons leads to an electrochemical potential difference ($\Delta\tilde{\mu}_{H^+}$) which includes a concentration component ΔpH and an electrical component $\Delta\psi$ (Bakker et al., 1976; Michel and Oesterhelt, 1976; Renthal and Lanyi, 1976; Kayushin and Skulachev, 1974).

Absorption of radiation by bacteriorhodopsin (bR_{570}, the subscript represents the wavelength of maximal absorbance) excites it to the first photointermediate K_{590}, and this relaxes thermally through several further intermediates, probably involving branched pathways (Slifkin and Caplan, 1975; Sherman et al., 1975; Hess and Kuschmitz, 1977; Lozier and Niederberger, 1977; Hurley et al., 1978). Resonance Raman spectroscopy indicates that the Schiff base retinylidene–lysine of bR_{570} is protonated, while that of the M_{412} photointermediate is unprotonated (Lewis et al., 1974; Mendelsohn, 1976; Aton et al., 1977; Marcus and Lewis, 1977). Thus, whenever accumulation of M_{412} occurs under steady-state conditions, it will be accompanied by a stoichiometric release of protons (the stoichiometry being 1:1 according to Lozier et al., 1976). These protons will be termed "stoichiometric protons".

A preliminary analysis of the kinetics of the light-induced acidification and of the alkalinization observed in the dark was performed in our laboratory (Eisenbach et al., 1976; Eisenbach and Caplan, 1976). Both the light and dark reactions appeared to be composed of two simultaneous first-order processes. In the present communication, we try to identify each of these processes under conditions where no accumulation of M_{412} is observed.

Experimental Procedure

Halobacterium halobium M-1 strain was grown as described by Danon and Stoeckenius (1974) using a modified growth medium (Eisenbach et al., 1977a). Purple membrane

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‡ Present address: Lehrstuhl für Biochemie der Pflanzen Abteilung Biochemie von Regulations Vorgängen, Ruhr-Universität Bochum, Postfach 102148, D-4630 Bochum-1, West Germany.

§ Present address: Bell Laboratories, Murray Hill, N.J. 07974.

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fragments were isolated according to Oesterhelt and Stoeckenius (1971, 1974). Subbacterial particles were prepared and characterized (protein content, intactness, and bacteriorhodopsin content) as described previously (Eisenbach et al., 1977a). The bacteriorhodopsin content of the particles varied from batch to batch within the range 3–8 nmol/mg of protein. Multilayered liposomes were prepared from a mixture of 99% egg phosphatidylcholine and 1% dicetyl phosphate (w/w) or soy bean phospholipids, purified according to Kagawa and Racker (1971). This mixture was preincubated in chloroform at ~3 °C for 24 h or longer. Samples of lipids (25 mg) were freed from organic solvent as described by Racker (1973). To the dry lipids 10–30 nmol amounts of bacteriorhodopsin (in the form of purple membrane fragments) were added, each in 3 mL of aqueous solution. The suspension was shaken gently until all lipid had been dispersed (Bakker et al., 1973). Proteoliposomes were prepared by sonication of this suspension of multilayer liposomes in a 25-mL thick-walled Pyrex glass tube placed inside a Laboratories Supplies thermostated ultrasonic bath-type cleaner (frequency 8×10^4 Hz). Sonication was carried out for 1 h (unless otherwise specified). During sonication the suspension was kept under an argon atmosphere (in the case of phosphatidylcholine only) and the bath temperature was maintained at 22 °C. Some 10 min before ending the sonication it was interrupted to bring the pH of the suspension to the desired value.

The measurements of the external pH were performed either electrometrically with a glass electrode or fluorometrically. In the first case, the pH was measured in a thermostated glass vessel, using a Radiometer (Copenhagen) pH meter (Type 64) connected to a combined pH electrode (Radiometer Type GK 2321C), and recorded on a high-speed recorder (Varian A-25). In the latter case, the fluorescence of the indicator FITC[1]–dextran was followed by a Perkin-Elmer MPF 44A spectrofluorometer, using 480 and 520 nm for the excitation and emission, respectively. The photomultiplier was protected from the illuminating beam by a narrow band interference filter (Baird Atomic B-10). In either case, the pH changes observed were transformed into absolute changes in the number of protons by calibrating the scale with a known amount of standard HCl solution, added to the suspension of particles at the end of each measurement. The samples were illuminated by a slide projector provided with an iodine quartz (24 V, 150 W) lamp through "cuton" filters (Schott-OG 517 or Corning 3-68), unless otherwise specified.

Difference spectra were recorded by a Cary 1605 spectrophotometer equipped with a low light-scattering cuvette housing. Steady-state concentrations of the M_{412} intermediate of bacteriorhodopsin in the light were measured with side illumination of the cuvette, using light from a 24-V, 250-W slide projector concentrated by passing through a condensing lens followed by a Corning 3-69 cuton filter. The photomultiplier was protected from this light by a combination of Corning 7-59 and 4-96 filters. Light intensity was varied by changing the distance of the slide projector from the sample. The light intensity was measured in the cuvette by a YSI-Kettering radiometer (Model 65 A).

The internal pH in the dark was measured as described previously for intact *H. halobium* cells (Bakker et al., 1976). To 450 μL of a particle suspension (~1.5 mg of protein/mL), 40 μL of ^3H_2O (5 mCi/mL) and 5 μL of [^{14}C]DMO (50 μCi/mL) were added. After 20 min of incubation with stirring, aliquots of 200 μL were placed in duplicate in 400-μL microcentrifuge tubes. The tubes were centrifuged at 70 000*g* for 30 min using fitted metal holders to avoid deformation. Sampling of the pellet and supernatant, radioactive counting, and calculations were as described by Bakker et al. (1976).

Taps, Mes, Mops, Pipes, and Hepes were obtained from Sigma Chemical Co., $TPMP^+Br^-$ from K&K Laboratories. [^{14}C]DMO (8.8 mCi/mmol) and ^3H_2O (5 mCi/mL) were from Amersham Radiochemical Center. FITC–dextran (M_r 20 000) was obtained from Pharmacia Fine Chemicals.

Results

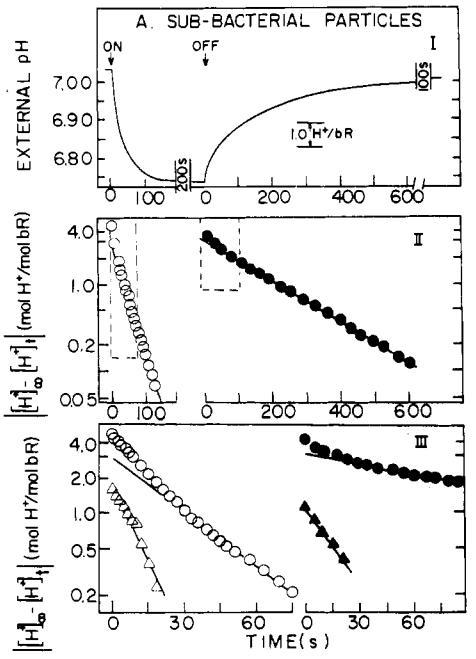
Figure 1 shows light-induced pH changes in suspensions of subbacterial particles (A) and proteoliposomes (B) and their kinetic analyses. Each trace is best described as a sum of two exponentials, either in the "on" or "off" reactions. Note that, in spite of the opposing directions of the pH changes observed in subbacterial particles and proteoliposomes, the kinetics are similar in both preparations.

To verify that the pH electrode used was not rate limiting, we repeated the above experiments using an impermeant pH indicator, FITC–dextran (M_r 20 000), instead of a pH electrode. This indicator measures only the external pH, and its anionic form ($pK = 6.5$) is fluorescent. Illumination caused a biphasic decrease in FITC fluorescence with subbacterial particles and an increase in fluorescence in the case of proteoliposomes, both effects being reversible in the dark (cf. Caplan et al., 1977). Table I compares the kinetic parameters obtained by each method under identical conditions, showing similarity between them within the limits of error. (In all cases where no overlap in the range of standard error was found, the essential identity of the results was established by the Student's *t* test.) The most important result of this comparison is that the value of k_1 (on) (the rate constant of the first phase in the on reaction, which is the faster) as calculated from the fluorometric data, was not greater than that calculated from the electrometric data—indicating that the pH electrode was not rate limiting (the half-time of response of the fluorimeter was 300 ms). We therefore felt free to use the pH electrode in the following experiments, especially since the parameters of prime interest were the relative extents of the two phases.

Illumination of subbacterial particles or proteoliposomes at wavelengths other than 570 nm did not alter the mode of the kinetics. An action spectrum of the extent of each phase in subbacterial particles is shown in Figure 2. Each process exhibits one peak in the vicinity of 570 nm and thus resembles the absorption spectrum of bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971; Oesterhelt and Hess, 1973; Dencher and Wilms, 1975). Furthermore, the ratio of the extents of both processes seems to be independent of the wavelength (upper line in Figure 2). It therefore seems that absorption of light by a single chromophore triggers both processes. (The rate constants did not vary within the limits of error in the region tested, and consequently the dependence of the initial rate on wavelength was, as expected, similar to that of the extent.)

Table II presents the degree of M_{412} accumulation at various light intensities, both in subbacterial particles and in purple membrane fragments, as measured by a light minus dark difference spectrum. Since each M_{412} molecule is associated with the release of a proton to the medium during the $bR_{570} \rightarrow M_{412}$

[1] Abbreviations used: bR, bacteriorhodopsin; DMO, 5,5-dimethyloxazolidine (2,4-dione); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FITC, fluorescein isothiocyanate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, morpholinopropanesulfonic acid; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid); Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid; $TPMP^+Br^-$, triphenylmethylphosphonium bromide.



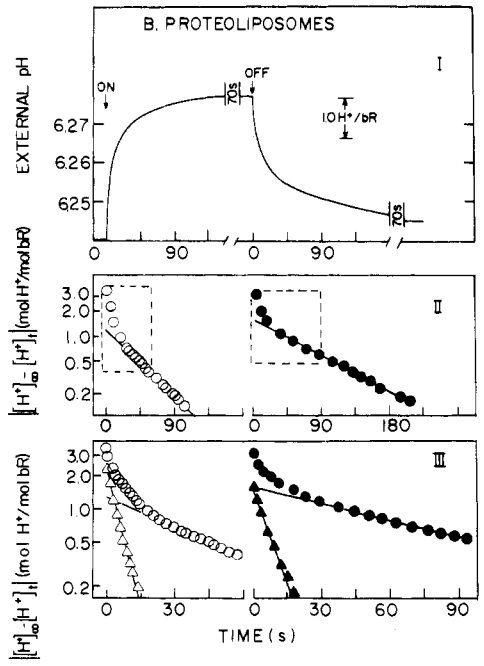


FIGURE 1: Light-induced pH changes and their kinetic analyses in subbacterial particles (A) and proteoliposomes (B). This figure is typical of several hundred experiments, carried out during the last 3 years. The particles (30 nmol of bR, 3.8 mg of total protein), loaded with 4 M KCl, were suspended in 2.5 mL of 4 M KCl at 25 °C, and the proteoliposomes (20 nmol bR), made of soybean phospholipids, were suspended in 1 M KCl. (I) Both samples were preilluminated through a cuton filter OG 517 (not shown), reilluminated after 5–15 min (most of the bacteriorhodopsin is still in the "all-trans" form, cf. Oesterhelt et al., 1973; Ohno et al., 1977) under the same conditions (I = 200 W/m^2), and followed with a pH meter. (II) Semilogarithmic plot of trace I, on the same time scale as the trace. (III) Magnification of the semilogarithmic plots in the frames appearing in diagram II: (○,●) experimental points taken from the trace I; (△,▲) differences between the experimental points and the extrapolated line. The open and closed symbols are for the on and off reactions, respectively. The extent of each phase is calculated from the intercept of the extrapolated line in the semilogarithmic plot on the ordinate at t = 0. (A) Subbacterial particles: k_1 (on) = 0.092 s^{-1}; k_2 (on) = 0.035 s^{-1}; k_1 (off) = 0.096 s^{-1}; k_2 (off) = 0.0063 s^{-1}. (B) Proteoliposomes: k_1 (on) = 0.18 s^{-1}; k_2 (on) = 0.021 s^{-1}; k_1 (off) = 0.13 s^{-1}; k_2 (off) = 0.011 s^{-1}.

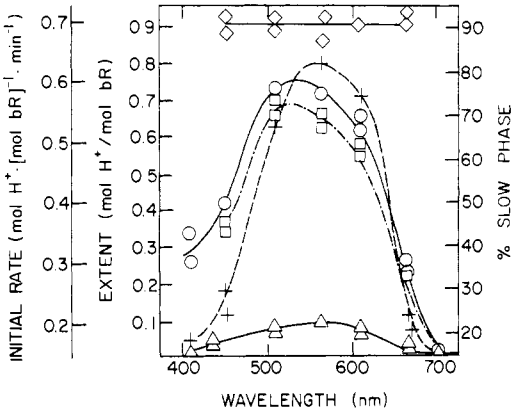


FIGURE 2: Action spectra of the extents and initial rate of light-induced pH changes. KCl-loaded subbacterial particles (79 nmol of bR, 9.5 mg of protein) were suspended in 2.5 mL of 4 M KCl (pH 7.3) at 25 °C. The particles were preilluminated and then illuminated in duplicate at various wavelengths through broad-band interference filters (Balzers K1–K7). The illumination was performed through neutral filters in order to adjust the light intensity at each wavelength to about 4 nmol quanta·cm^{-2}·s^{-1} independent of the energy of the photon. All the data shown in the figure are for the on reaction: (○) total extent; (△) extent of the fast process; (□) extent of the slow process; (◇) extent of the slow process as a fraction of the total extent; (+) initial rate.

transition (see Introduction), the degree of M_{412} accumulation under steady-state conditions is a reliable measure of the stoichiometric protons released to the medium. Thus, while the stoichiometric protons ranged from 0.011 to 0.12 H$^+$/bR for subbacterial particles at light intensities between 200 and 2500 W/m^2 (Table II), the observed total extents ranged from 3.8 H$^+$/bR at 200 W/m^2 to 8.8 H$^+$/bR at 1500 W/m^2 (4 M KCl, pH 6.5, 25 °C). Similar results were observed with proteoliposomes when comparing the number of protons taken up from the medium with the degree of M_{412} accumulation. In all the following experiments, we used light intensities of approximately 200 W/m^2 or even lower, where the accumulation degree of M_{412} in subbacterial particles is at most 0.011 (Table II) and the contribution of the stoichiometric protons to the total extent is therefore only 0.3% (100% × 0.011/3.8). In purple membrane fragments this contribution could be somewhat larger, but even under extreme conditions it did not exceed 5% of the total extent (compare Table II with the extents in Garty et al., 1977). It should be stressed therefore that stoichiometric protons (as defined in the Introduction) play no significant role in the kinetics observed in this report. The reader should guard against identifying the light-induced pH changes described here, measured under steady-state conditions, with the flash-induced proton release and uptake measured by Lozier et al. (1976) under fast transient conditions.

Figure 3 summarizes the typical effect of external pH on the extent of each phase in subbacterial particles suspended and loaded either in 4 M KCl (A and B) or in 4 M NaCl (C and D) and in proteoliposomes (E and F). The total extent increased with decreasing pH, as was observed by Kanner and Racker (1975), and reached a value of 59 H$^+$/bR at pH 4.3 in KCl subbacterial particles (Figure 3A). Unfortunately, in these particles the kinetic analysis of the on reaction at pH 5.4 and below was difficult to perform, since the lines on the semilogarithmic plot were not straight (their slopes increased with time for reasons at present unknown). However, in the off reaction the phases were separable, and the extent of the slow phase increased to 31.5 H$^+$/bR (90% of the total extent) while that of the rapid phase only increased to 3.5 H$^+$/bR (Figure 3B).

TABLE I: A Comparison between the Kinetic Parameters of the Light-Induced pH Changes as Measured by FITC-Dextran Fluorometrically and by a pH meter.[a]

light	parameter	subbacterial particles		proteoliposomes	
		fluorometer	pH meter	fluorometer	pH meter
on	k_1 (s^{-1})	0.129 ± 0.020 (3)	0.198 ± 0.003 (5)	0.179 ± 0.029 (4)	0.181 ± 0.032 (7)
	k_2 (s^{-1})	0.026 ± 0.006 (3)	0.048 ± 0.016 (5)	0.038 ± 0.012 (4)	0.021 ± 0.006 (7)
	total extent (H^+/bR)	4.7 ± 0.9 (4)	2.8 ± 0.4 (5)	1.14 ± 0.11 (4)	2.0 ± 0.2 (8)
	rapid-phase fract (%)	51 ± 13 (3)	53 ± 13 (5)	45 ± 7 (4)	49 ± 6 (7)
off	k_1 (s^{-1})	0.090 ± 0.016 (3)	0.109 ± 0.027 (5)	0.176 ± 0.026 (7)	0.136 ± 0.023 (10)
	k_2 (s^{-1})	0.0094 ± 0.0016 (3)	0.011 ± 0.003 (5)	0.021 ± 0.005 (7)	0.017 ± 0.004 (10)
	total extent (H^+/bR)	3.8 ± 0.9 (5)	2.5 ± 0.5 (5)	1.18 ± 0.11 (8)	1.8 ± 0.2 (8)
	rapid-phase fract (%)	41 ± 4 (3)	51 ± 8 (5)	43 ± 9 (7)	50 ± 4 (10)

[a] To a 3-mL suspension of subbacterial particles (51 nmol of bR) in 4 M KCl + 1 mM Pipes (pH 6.5) or of proteoliposomes (made of soybean phospholipids with 15-39 nmol of bR) in 1 M KCl (pH 6.5), FITC-dextran (0.5 mg/mL final concentration) was added. Each of these suspensions was measured first in the pH meter and then in the fluorometer (or vice versa). Note that the measurements in the pH meter also included FITC-dextran, because its presence in the medium increased the rate constant of the pH changes (but had no effect on the extent within the limits of error). Each portion was preilluminated (I = 225 ± 25 W/m^2) and then reilluminated and measured in duplicate as described in the legend to Figure 1 and under Experimental Procedure. The kinetic parameters were calculated from semilogarithmic plots as in Figure 1. The values given are averages of three to ten similar experiments (as indicated in the brackets), and the plus or minus (±) signs refer to standard errors. All the data for subbacterial particles were obtained from a single batch, and those for proteoliposomes were obtained from various preparations of proteoliposomes which had been made from a single batch of purple membrane fragments: pH 6.5; temperature 25 °C.

TABLE II: Degree of M_{412} Accumulation as a Function of Light Intensity.[a]

preparation	light intensity (W/m^2)		
	200	400	2500
PM in 20 mM KCl	0.009 ± 0.001 (3)	0.017 ± 0.001 (2)	0.037 ± 0.004 (2)
PM in 1 M KCl	0.003 ± 0.003 (3)	0.015 ± 0.002 (3)	0.048 ± 0.003 (3)
SBP in 4 M KCl	0.011 ± 0.004 (4)	0.047 ± 0.005 (7)	0.119 ± 0.007 (3)

[a] The degree of accumulation $\{[M_{412}]/([M_{412}] + [bR_{570}])\}$ was calculated from light minus dark difference spectra (yielding peaks at 412 nm), using ϵ 33 000 $M^{-1}\cdot cm^{-1}$ to calculate the concentration of M_{412} and ϵ 63 000 $M^{-1}\cdot cm^{-1}$ for that of bR_{570} (Oesterhelt and Hess, 1973). KCl-loaded subbacterial particles (SBP) (27.3 nmol bR) were suspended in 2.5 mL of 4 M KCl at pH 6.5, 25 °C. Purple membrane fragments (PM) from a single batch (38-55 nmol of bR) were suspended in KCl solution (at the indicated concentration) under the same conditions. Each preparation was illuminated at the indicated light intensity, while its spectrum (against water) was recorded as described under Experimental Procedure. The dark spectrum was taken immediately after turning the light off to ensure that the bacteriorhodopsin was still in its light-adapted form. The difference spectra used for the calculation were manually drawn by subtracting the dark spectra from the appropriate light spectra. The values given are averages of two to seven similar experiments (as indicated in the parentheses), and the plus or minus (±) signs refer to standard errors.

A similar dependence on pH for the on reaction was observed with NaCl-loaded particles (Figure 3C,D).[2] As shown in Figures 3E,F, essentially the same phenomenon was observed with proteoliposomes in spite of the smallness of the alkalinization:[2] the relative extent of the slow process increased from the vicinity of zero (in the on reaction) or 23% (in the off reaction) at pH 7.7 to 58% (on) or 73% (off) at pH 4.5.

In an attempt to understand the steep increase in the extents seen in subbacterial particles at low pH values, we followed the internal pH of the KCl-loaded particles (from the same batch as in Figure 3), kept in the dark, after overnight incubation at various external pH values at 4 °C. The internal pH and ΔpH values (measured as described under Experimental Procedure) are plotted in Figure 4 vs. the external pH. It may be seen that decreasing the external pH from 8.3 to 6 was accompanied by a parallel decrease in the internal pH, so that the measured ΔpH in the dark was nearly zero. However, a further decrease in the external pH was not accompanied by a similar decrease in the internal pH (probably because the system had not reached equilibrium), indicating a high internal buffer capacity below pH 6. This may be the reason for the steep increase of the extent of the light-induced pH change below pH 6 (Figure 3).

The effect of increasing the temperature on the light-induced pH changes is shown in Figure 5. It is seen that the extent of each phase depended differently on the temperature (as on the pH). The extent of the slow process (both in the on and off reactions) increased with increasing temperature up to 65 °C, where it reached a value of 7.3 H^+/bR at pH 6.3. Above 65 °C, bacteriorhodopsin denatures (Mendelsohn, 1976) and as a result the extent was dramatically decreased. On the other hand, the extent of the rapid process in the on reaction (but surprisingly not in the off reaction) decreased with increasing temperature, until above 35 °C it could not be distinguished and the reaction seemed to follow monophasic kinetics at the rate of the slow process. The net effect was an increase in the total extent with increasing temperature. We observed a similar tendency in the ratio of the extents of the phases in 4 M NaCl-loaded subbacterial particles (pH 7.2) and in proteoliposomes (in 2 M NaCl at pH 7.6). An increase in the *total* extent with the temperature was observed also by Racker and Hinkle (1974) in proteoliposomes made of halobacteria phospholipids.

$TPMP^+$ is a permeant cation (Grinius et al., 1970) which

[2] The extents of the light-induced pH changes were smaller in NaCl-loaded subbacterial particles than in those loaded with KCl, because of the low light intensity used with the former. This is because at light intensities higher than 45 W/m^2, alkalization follows the light-induced acidification in NaCl-loaded particles as a consequence of the operation of a Na^+/H^+ antiport (Eisenbach et al., 1977a; Caplan et al., 1977). The small extents observed with proteoliposomes are the result of their 1000-fold smaller volume (Hwang and Stoeckenius, 1977) in comparison with subbacterial particles and their lower internal buffer capacity (Bakker, E. P., unpublished).

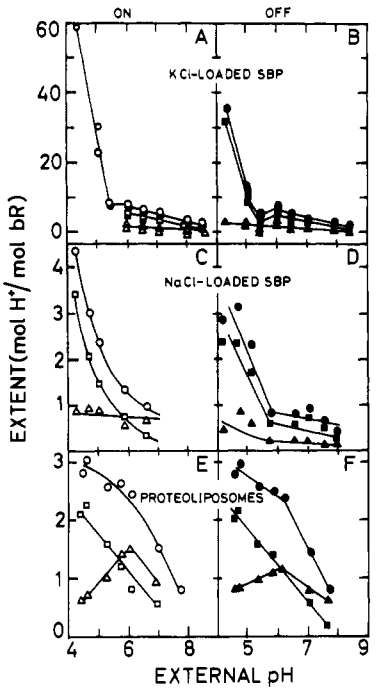


FIGURE 3: Effect of initial pH on the extents of each phase of the light-induced pH changes. (A and B) Portions of KCl-loaded subbacterial particles (SBP) (36 nmol of bR, 4 mg of protein) were suspended in 2.5 mL of 4 M KCl and either 2 mM Taps, 3 mM Mops, or 3 mM Mes, 7.5 mM acetate, or 12 mM citrate, according to the pK of the buffer. (These buffers were required to keep the external pH as close as possible to the original value during the illumination. This was especially important at pH values below 6, where otherwise the external pH could drop by 0.5 pH unit. In a parallel experiment, a homogeneous solution of all these buffers at smaller concentrations was added to all the samples, and the same results were obtained.) The pH was adjusted by the addition of small amounts of NaOH or HCl, and the suspensions were incubated overnight at 4 °C. After incubation, the light-induced pH changes were followed at 25 °C as described in the legend to Figure 1. (C and D) NaCl-loaded subbacterial particles (18 nmol of bR, 2 mg of protein) were suspended in 2 mL of 4 M NaCl (no buffer), the pH was adjusted to the desired value, and the sample was preilluminated and measured as described above. It was then titrated to a new pH value. (E and F) Different preparations of egg phosphatidylcholine proteoliposomes (3.0 nmol of bR/mL) were prepared in media of 150 mM NaCl (no buffer) and final pH as indicated in the figure. Sonication time was 30 min. Note the difference in scale on the extent axis between each type of preparation, I = 200 W/m^2 (except for NaCl-loaded subbacterial particles, where I = 25 W/m^2): (○,●) total extent; (□,■) slow process; (△,▲) fast process. The open and closed symbols are for the on and off reactions, respectively.

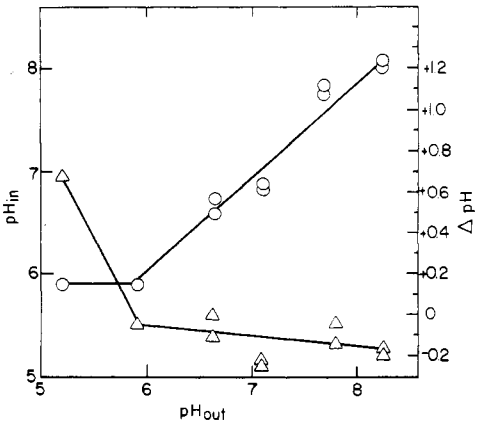


FIGURE 4: Effect of external pH on internal pH and ΔpH. Six portions of the KCl-loaded subbacterial particles of the same batch that was used in Figure 3 (13.5 nmol of bR/mL, 1.5 mg/mL) were incubated overnight at 4 °C with different buffers (50 mM): acetate (pH 5.2), Mes (pH 5.9 and 6.5), Mops (pH 7.1), and Taps (pH 7.7 and 8.2). After this period, the internal pH and ΔpH were measured at 25 °C in the dark as described under Experimental Procedure: (○) pH_{in}; (△) ΔpH (= $pH_{in} - pH_{out}$).

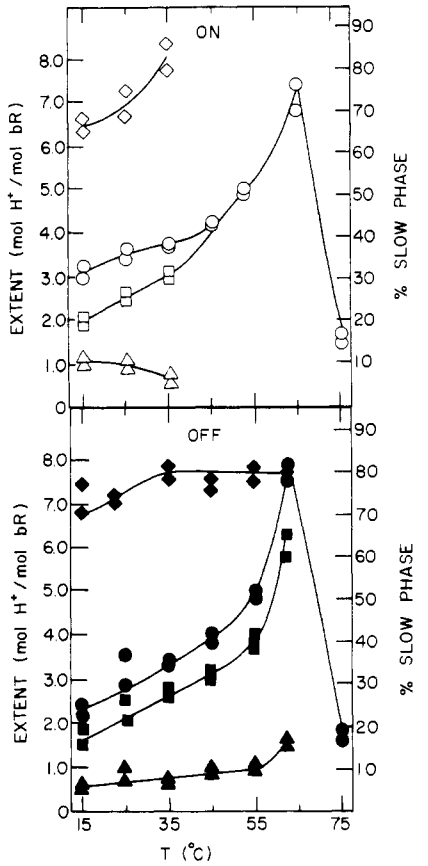


FIGURE 5: Effect of temperature on the extents of light-induced pH changes. KCl-loaded subbacterial particles (32 nmol of bR, 3.9 mg of protein) were suspended in 2.5 mL of 4 M KCl (pH 6.3) and treated as described in Figure 1. The experiment was initiated after a 30-min incubation at each temperature, I = 200 W/m^2: (○,●) total extent; (△,▲) extent of the fast process; (□,■) extent of the slow process; (◇,◆) extent of the slow process as a fraction of the total extent. The open and closed symbols are for the on and off reactions, respectively.

decreases or even abolishes (depending on its concentration) the membrane potential difference $\Delta\psi$ in subbacterial particles of *H. halobium* (Renthal and Lanyi, 1976) and thus causes an increase in ΔpH in the light (Bakker et al., 1976; Garty, Cooper, and Eisenbach, unpublished results). It was, therefore, of interest to examine the effect of this ion on the observed pH changes. As shown in Figure 6, when $TPMP^+$ was added in the dark which preceded the illumination, the extent of the rapid process was unaffected by $TPMP^+$ but that of the slow process increased with increasing concentration of $TPMP^+$. In agreement with this result, when $TPMP^+$ was added during the steady state under illumination (Figure 7A) further acidification was observed, the increase having a rate constant typical of the slow process (compare the slope in part II of Figure 7B with the slope of the slow phase in part I). In these two experiments, we used NaCl-loaded subbacterial particles, since $\Delta\psi$ is much higher in them (Renthal and Lanyi, 1976), and the effect of $TPMP^+$ was, therefore, more pronounced than in KCl-loaded particles. However, qualitatively similar results were obtained with KCl-loaded particles.

Discussion

We have shown that stoichiometric protons do not contribute significantly to the observed light-induced pH changes (Table II). Likewise, only one photosystem is involved in triggering the pH change (Figure 2). Nevertheless, two phases are ob-

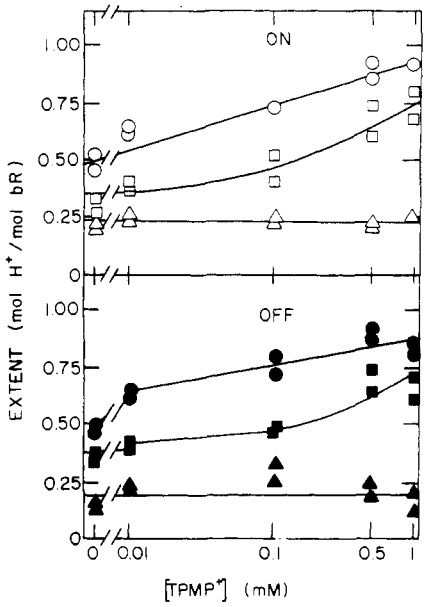


FIGURE 6: Effect of $TPMP^+$ on the extents of light-induced pH changes. NaCl-loaded subbacterial particles (69 nmol of bR, 6.6 mg of protein) were suspended in 2.5 mL of 4 M NaCl, pH 6.5 (25 °C), and then $TPMP^+$ was added and the particles were preilluminated through a 580 ± 10 nm interference filter and treated as described in Figure 1, I = 25 W/m²: (△,▲) fast process; (□,■) slow process; (○,●) total extent. The open and closed symbols are for the on and off reactions, respectively.

served (Figure 1), and, since each of them responds differently to a change in the external pH or temperature or to the presence of $TPMP^+$ (Figures 3, 5, and 6), they most probably represent distinct processes. These processes occur *simultaneously*, as can be seen in Figure 1 (part III); the kinetic analysis (sum of two exponentials) does not fit, for example, a single reaction with a rate decreasing with time. We shall devote this discussion to a consideration of possible explanations for the kinetics.

It seems evident that the slow process represents net proton transport (light-induced proton extrusion in subbacterial particles and proton uptake in proteoliposomes), it is affected by $TPMP^+$ (Figures 6 and 7) and ionophores (Bakker et al., 1978; Garty et al., 1978), and its extent may increase until the ratio H^+/bR becomes so high (Figures 3 and 5) that any chemical process other than transport would be unlikely. However, since the proton pump which drives transport is associated with the photocycle (Kozlov and Skulachev, 1977; Stoeckenius, 1978), an explanation for the 1000-fold slower rate of proton transport (as compared to the turnover number of the photocycle) is required. Considering a subbacterial particle as a sphere with a radius of 2.5×10^{-7} m (Eisenbach et al., 1977a) and using a capacity value of 1 μF/cm², which is typical of the membranes of most living cells (Cole, 1968), one arrives at a capacity *per vesicle*, C_{ves}, of 8×10^{-15} F. The number of protons, N_{H^+}, which are *electrogenically* transferred by a single vesicle to establish the measured $\Delta\psi$ is given by $N_{H^+} = C_{ves}\Delta\psi/e$, where e is the protonic charge (1.6×10^{-19} C). Substituting the highest measured value of $\Delta\psi$, i.e., 0.12 V (Renthal and Lanyi, 1976), one obtains $N_{H^+} = 6 \times 10^3$ protons. The extent seen in Figure 1A, i.e., 5 H^+/bR (equivalent to 45 nmol of H^+/mg of protein), may be used for calculating the *actual* number of protons transferred by a single particle at a light intensity of 200 W/m². Using the value of 2.7 μL/mg of protein as the internal specific volume of the vesicles (Eisenbach et al., 1977a), one finds that, under these conditions, 6.7×10^5 protons are actually extruded from one

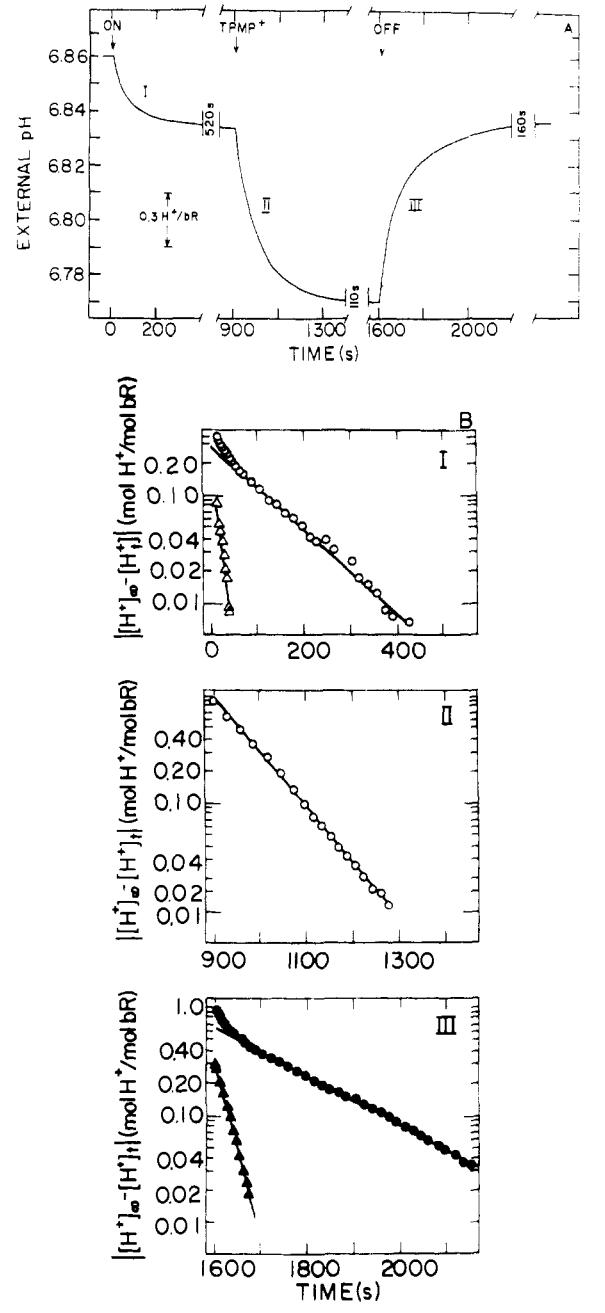


FIGURE 7: Effect of $TPMP^+$ when added during illumination. NaCl-loaded subbacterial particles (120 nmol of bR) were suspended in 2.5 mL of 4 M NaCl plus 0.5 mM Pipes + 0.5 mM Mes (pH 6.9) at 26 °C, and treated as described in Figure 1. I = 14 W/m² (580 ± 10 nm interference filter): (A) trace of the pH changes; (B) semilogarithmic plots of the different sections of trace A, analyzing the kinetics of (I) the light-induced pH changes prior to the addition of $TPMP^+$, (II) the pH changes induced by the addition of $TPMP^+$ (1 mM) in the light, and (III) the pH changes of the off reaction. (I) $k_1 = 0.088\ s^{-1}$; $k_2 = 0.0092\ s^{-1}$. (II) $k = 0.011\ s^{-1}$. (III) $k_1 = 0.039\ s^{-1}$; $k_2 = 0.0052\ s^{-1}$. (○,●) experimental points taken from the trace; (△,▲) the rapid phase obtained by subtraction as described in Figure 1. The open and closed symbols refer to the on and off reactions, respectively.

vesicle. Comparing this value with the theoretical value of 6×10^3 protons extruded electrogenically, we see that 0.9% of the protons pumped under these conditions is sufficient to bring about the measured value of $\Delta\psi$. The significance of this calculation is that practically all the protons transported are accompanied by other ions to maintain electroneutrality. Since in experiments with subbacterial particles the only ions present in the suspension in addition to protons or hydroxyl ions are

Na^+, K^+, and Cl^-, and since the membrane permeability for the latter three is lower than for protons (Lanyi and Hilliker, 1976; Eisenbach et al., 1977a), the rate of proton transport is undoubtedly limited by the flux of co-ions or counterions.[3] Consequently, the observed proton transport is relatively slow in spite of the rapid turnover of the photocycle. In the off reaction, the slow phase reflects proton back-diffusion under the influence of $\Delta\tilde{\mu}_{H^+}$ and it is therefore limited by the flux of the accompanying ions as well as by the proton permeability. Thus, no quantitative correlation between the rates of the on and off slow processes is to be expected. The same argument holds for proteoliposomes.

Possible explanations for the observed kinetics are the following. (1) The systems examined contain two types of purple membrane or represent a heterogeneous population of particles. This possibility does not seem to us likely, since the two phases were quite differently (and even oppositely) affected by pH, temperature, and $TPMP^+$, as shown in Figures 3, 5, 6, and 7. (2) The rapid phase represents a net charge transfer in the absence of a fully developed $\Delta\psi$ (electrogenic protons), while the slow phase is limited by $\Delta\psi$; i.e., it depends on the rate of co-ion and counterion transport. In this case, one would expect the rate constant to decrease gradually with time, resulting in a totally different kinetic picture (cf. Eisenbach and Gutman, 1975).[4] Furthermore, according to the calculation performed above, the fast phase should be at least tenfold smaller than actually observed. This argument holds equally well for any explanation based on a pump–leak model. (3) The rapid phase, in contrast to the slow phase, does not represent H^+ transport but rather protons dissociated from the membrane of subbacterial particles (or associated to that of proteoliposomes), a phenomenon similar to the "membrane Bohr effect" suggested for mitochondria and chromatophores (Chance et al., 1970; Chance, 1972).[5] Azzi (1969; for a review, see Azzi and Montecucco, 1976) has suggested that in the case of mitochondria the Bohr protons are observed as a result of conformational changes in the membrane upon energization. In discussing this model, we shall refer to purple membrane fragments. Although no transport can be obtained in these preparations (both sides of the membrane are exposed to the same medium), illuminating them under physiological conditions causes a pH change that cannot be explained by the 412-nm intermediate accumulation (Table II). Since the magnitude and direction of this pH change are affected by both pH and temperature, it was concluded that it is the net result of protonation and deprotonation processes occurring on opposite sides of the membrane upon illumination. Thus, Garty et al. (1977) have shown that in purple membrane sheets, increasing the temperature decreases the initial rate of light-induced acidification to zero, after which the rate increases but in the opposite direction (alkalinization). This could occur if the observed kinetics reflected the difference between two opposite reactions, since the rate of a single reaction invariably increases with increasing temperature (except in the case of denaturation). It seems to be evident that in the processes of pumping the number of protons taken up on one side of the membrane is not balanced completely by the number of protons released on the other, giving rise to a net dissociation and/or association of protons to the protein–lipid complex. These processes should take place in closed vesicles too, and we suggest that they are responsible for the first phase we observed (association in proteoliposomes, dissociation in subbacterial particles). We also propose that these processes are due to a change in the number of protonatable groups on the membrane exposed to the medium (either the external or the cytoplasmic) as a result of light-induced conformational changes. Since the fast phase does not in this view represent translocation of protons from one side of the membrane to the other, it should not be sensitive to the presence of $TPMP^+$ or the internal buffer capacity, as indeed was observed. Moreover, it has been shown that FCCP decreases the rate constant of the slow process by 90%, while it practically does not affect that of the rapid process (Garty et al., 1978). Several alternative interpretations of the rapid process (according to this picture) can be suggested: (a) It may be due to the same acidic or basic groups as are involved in the transport process. (b) It may be a side effect of electric-field-induced conformational changes. (c) It may be due to vertical displacement of the bacteriorhodopsin molecules in the membrane (Borochov and Shinitzky, 1976) as a consequence of the local electric-field and viscosity changes. In order to establish any one of these hypotheses, more evidence is required. However, the conformational changes we invoke in all the above alternatives are unlikely to be seen in flash experiments, since they necessarily occur on a time scale much slower than that of the photocycle. Under steady illumination, each rotation of the photocycle (after switching on the light) produces an incremental change, which increases progressively until the final stationary state is achieved.

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[3] Counterions and co-ions here denote anions and cations, respectively, coupled to proton transport. Anions (e.g., Cl^-) are transported in the same direction, whereas protons and cations (e.g., K^+) are transported in the opposite direction.

[4] It should be emphasized that kinetic data which are expressable as a sum of exponentials fit both parallel and sequential reactions. However, this type of kinetics does not fit a decrease in the rate constant of a single process. Indeed, any explanation which does not take into account the fact that both the rapid and slow processes occur simultaneously cannot hold.

[5] Oesterhelt (personal communication) has suggested naming these protons "Bohr protons". This phenomenon should be distinguished from "vectorial Bohr mechanism" proposed by Papa (1976) for proton transport across the mitochondrial membrane (via a series of p*K* shifts). The similarity between the original Bohr effect (Bohr et al., 1904; Kilmartin, 1977) and this phenomenon is, however, still in doubt.

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