THE QUANTUM EFFICIENCY OF PROTON PUMPING BY THE PURPLE MEMBRANE OF *HALOBACTERIUM HALOBIUM*

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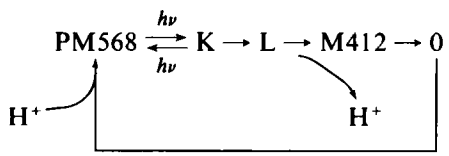
ABSTRACT The quantum yield of H^+ release in purple membrane (PM) sheets, and H^+ uptake in phospholipid (egg phosphatidylcholine, PC) vesicles containing PM, was measured in single turnover light flashes using a pH-sensitive dye, *p*-nitrophenol, with rhodopsin as an actinometer. We have also calculated the ratio of H^+ released per M412 formed (an unprotonated Schiff-base intermediate formed during the photocycle). In PM sheets, the quantum yield of H^+ release depends on the medium. The quantum yield of M412 is independent of salt concentration. The ratio H^+/M412 is ~1.8 in 0.5 M KCl and ~0.64 in 10 mM KCl. Direct measurements of the quantum yield of H^+ give ~0.7 when the PM is suspended in 0.5 M KCl and 0.25 in 10 mM KCl. Using a quantum yield for M412 formation of 0.3 (Becher and Ebrey, 1977. *Biophys. J.* **17**:185.), these measurements also give a H^+/M412 ~2 at high salt. In PM/PC vesicles, the H^+/M412 is ~2 at all salt concentrations. The M412 decay is biphasic and the dye absorption change is monophasic. The dissipation of the proton gradient is very slow, taking on the order of seconds. Additon of nigericin (H^+/K^+ antiporter) drastically reduces the pH changes observed in PM/PC vesicles. This and the observation that the proton relaxation time is much longer than the photochemical cycling time suggest that the protons are pumped across the membrane and there is no contribution as a result of reversible binding and release of protons on just one side of the membrane.

INTRODUCTION

The purple membrane of *Halobacterium halobium* contains a single protein, bacteriorhodopsin, consisting of a retinal moiety attached to its apoprotein via a protonated Schiff base. Bacteriorhodopsin exists in two forms, light- and dark-adapted, with the all-trans isomer of retinal as the chromophore of the light-adaped form. Upon illumination, light-adapted bacteriorhodopsin (PM568) goes through a photocycle consisting of at least four distinct spectroscopic intermediates—K, L, M412, and 0—before returning to its original state. A simpler linear scheme has been proposed to explain the photocycle (Lozier et al., 1975):



Linked with this photocycle is the proton pumping function of the purple membrane. Resonance Raman experiments have shown that the Schiff base has become deprotonated in the M412 intermediate (Lewis et al., 1974; Aton et al., 1977). Because there is both

light-induced H^+ pumping and light-induced deprotonation/reprotonation, there is suspicion among many workers that the proton(s) which is pumped is the Schiff-base proton. If this is true, then only one proton should be pumped per M412 formed. It is this notion we wish to test here, by comparing the quantum yield for proton pumping with the quantum yield for M412 formation.

The quantum yield of the photocycle has been measured in several laboratories. Oesterhelt and Hess (1973) reported a value of 0.79; however, other laboratories obtain yields in range 0.25–0.30 (Goldschmidt et al., 1977; Becher and Ebrey, 1977; Hurley and Ebrey, 1978).

Lozier et al. (1976) have reported the number of protons released per M412 formed during the photocycle to be 1. However, Ort and Parson (1979) have found that the quantum yield of H^+ release in purple membrane sheets ranges from 0.25 to 0.42 depending upon the ionic strength, being higher at high salt concentrations. Taking the quantum yield for the photocycle to be 0.3, the higher value would imply that more than one proton is released per photocycle. Using a pH-sensitive dye and single-turnover light flashes, we have measured the quantum yield of H^+ release in purple membrane sheets, in purple membrane/phospholipid vesicles, and in whole bacterial cells. In the case of the vesicles, the evidence suggests that all the protons we measure are transported across the cell membrane, not just released and rebound on the same side of the membrane. In purple membrane sheets we find one to two or more protons released per M412 formed, depending on the ionic strength. Thus, our measurements support the results of Ort and Parson, suggesting that more than one proton is transported per photocycle under high salt conditions.

MATERIALS AND METHODS

The purple membrane (PM)[1] was prepared from *H. halobium* by the method of Becher and Cassim (1975). All the experiments reported here were done with fresh preparations, <7–10 d old. The PM sheets were suspended in different salt concentrations and used for the spectroscopic measurements, or were incorporated into egg phosphatidylcholine (PC) vesicles. Egg PC was purified according to the method of Singleton et al. (1965). The PM/PC vesicles were prepared by sonicating (Sonifier model W1400; Heat Systems, Ultrasonics, Inc., Plainview, N.Y.) a 1:10 molar ratio of PM and PC in a given concentration of KCl for ~15 min at ~15°C. The preparation was then centrifuged for 1 h at 30,000 rpm. The supernate consisting of single and multilamellar vesicles was used for H^+ measurements.

Measurements with whole cells were made by centrifuging the cells out of the culture medium and resuspending them in basal salts; only freshly harvested cells were used. The samples were light-adapted before each experiment. All experiments were done at room temperature.

Spectroscopic Measurements

The number of protons was calculated by measuring the absorption changes of a pH indicator dye, *p*-nitrophenol ($pK \simeq 6.8$).

Absorption changes were measured with a single-beam kinetic spectrophotometer (Jackson and Crofts, 1971). The signal from the photomultiplier was accumulated by a signal averager (Bowyer et al., 1979). An average of 64 flashes for PM sheets and whole cells and 32 flashes for PM/PC vesicles was taken to improve the signal-to-noise ratio. Light-induced transmission changes were initiated by a xenon flash lamp (~10 μs half-bandwidth) with light of wavelengths above 540 nm was obtained by placing two Corning glass cutoff filters (CS3-66, Corning Glass Works, Science Products Div., Corning, N.Y.) in front of the flash lamp.

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[1] *Abbreviations used in this paper:* PC, phosphatidylcholine; PM, purple membranes.

RESULTS

PM Sheets

PM suspensions, when illuminated, show a monophasic proton release into the medium followed by rebinding (Lozier et al., 1976). By measuring the absorption changes of a pH-sensitive dye, *p*-nitrophenol (pK $\simeq$ 6.8), we have determined the number of protons released per bacteriorhodopsin cycling using single-turnover light flashes. Because the dye absorption change at 400 nm overlaps the M412 absorption change, it was necessary to measure the change in the presence (H^+ and M412 change) and absence (M412 only) of the indicator dye; the difference of the two traces gave the dye absorption change alone. The dye change was calibrated by adding known amounts of HCl. The trace obtained in the absence of the dye was used to calculate the number of moles of the intermediate M412 formed. To measure reliable pH changes, it was necessary to check that the dye did not affect the kinetics of the light-initiated PM absorption changes. This was investigated by adding 10 mM Tris buffer to the cuvette containing the PM suspension plus the dye to eliminate the dye absorption changes and isolate the M412 changes; in another cuvette a PM suspension in 10 mM KCl was used without the dye. The M412 kinetics were identical in the two cases, indicating that the dye had no effect on photocycle kinetics and, that if there was any light-induced dye binding, it did not lead to any absorbance changes at the measurement wavelength.

Fig. 1 *a–d* shows the absorption changes in the presence and absence of the dye at 400 nm and the isolated dye change. All experiments were conducted at pH 6.6. The concentrations of KCl in which the PM is suspended ranged from 10 to 500 mM. The amplitude of the M412 change, and therefore the quantum yield of M412, is not affected by the ionic strength but the dye absorption change increases significantly as the salt concentration is increased. In addition, the decay kinetics of both M412 and the dye become faster at higher salt concentrations (Table I). Because the proton uptake and the M412 decay starts before the H^+ release and M412 formation is complete, it was necessary to plot the absorption changes on a logarithmic scale and extrapolate to time zero in order to get their full amplitude. The amount of M412 formed was calculated using a differential extinction coefficient ($\Delta\epsilon_{400}$) of 30,000.[2] The ratio of protons released per M412 formed (H^+/M412) increases from 0.6 (at 10 mM KCl) to 1.8 or more at 250 mM KCl or higher (Table I).

We were concerned that our results would be influenced if the extinction coefficient of M412 varied with salt concentration. This was checked by measuring the absorption changes at 400 and 580 nm of PM suspensions in several different salt concentrations after exciting with a 530-nm laser flash (model 252 Yag Q-switched laser [Yag Lasers, General Photonics Corp., Santa Clara, Calif.]; frequency doubled to emit at 530 nm). The ΔA400 and the ratio of ΔA580/ΔA400 did not change, implying that the $\Delta\epsilon_{400}$ (and the quantum yield of M412 formation) was not affected by the salt concentration. This latter result is in agreement with Ort and Parson (1979).

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[2]This value of $\Delta\epsilon_{400}$ is slightly less than that determined by Becher and Ebrey (1977) but close to that used by Lozier et al. (1976). We chose to use the lower number so that uncertainty in its value would bias our results towards lower ratios of H^+/M412. A higher value of $\Delta\epsilon_{400}$ would lead to fewer moles of M412 present and even higher values of H^+/M412.

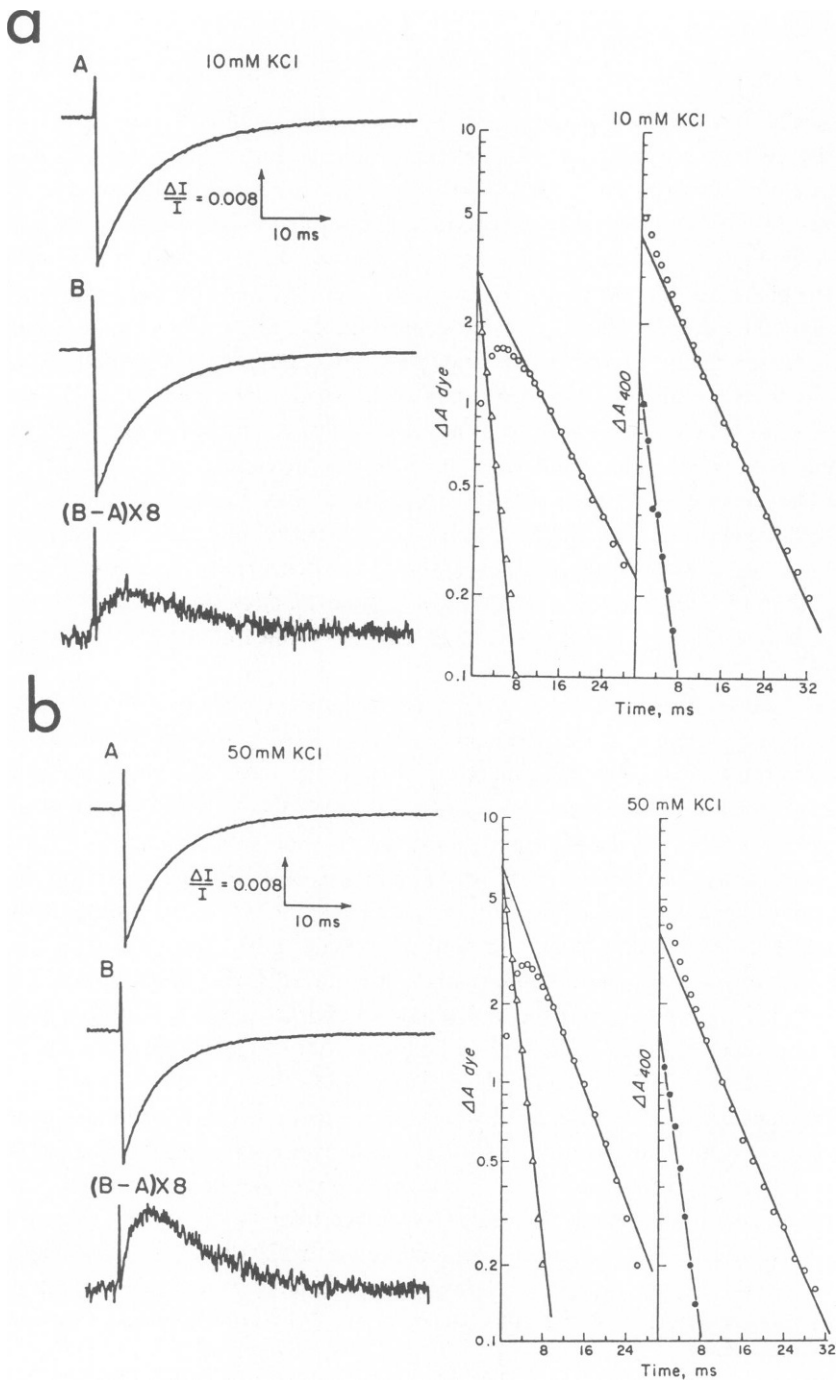
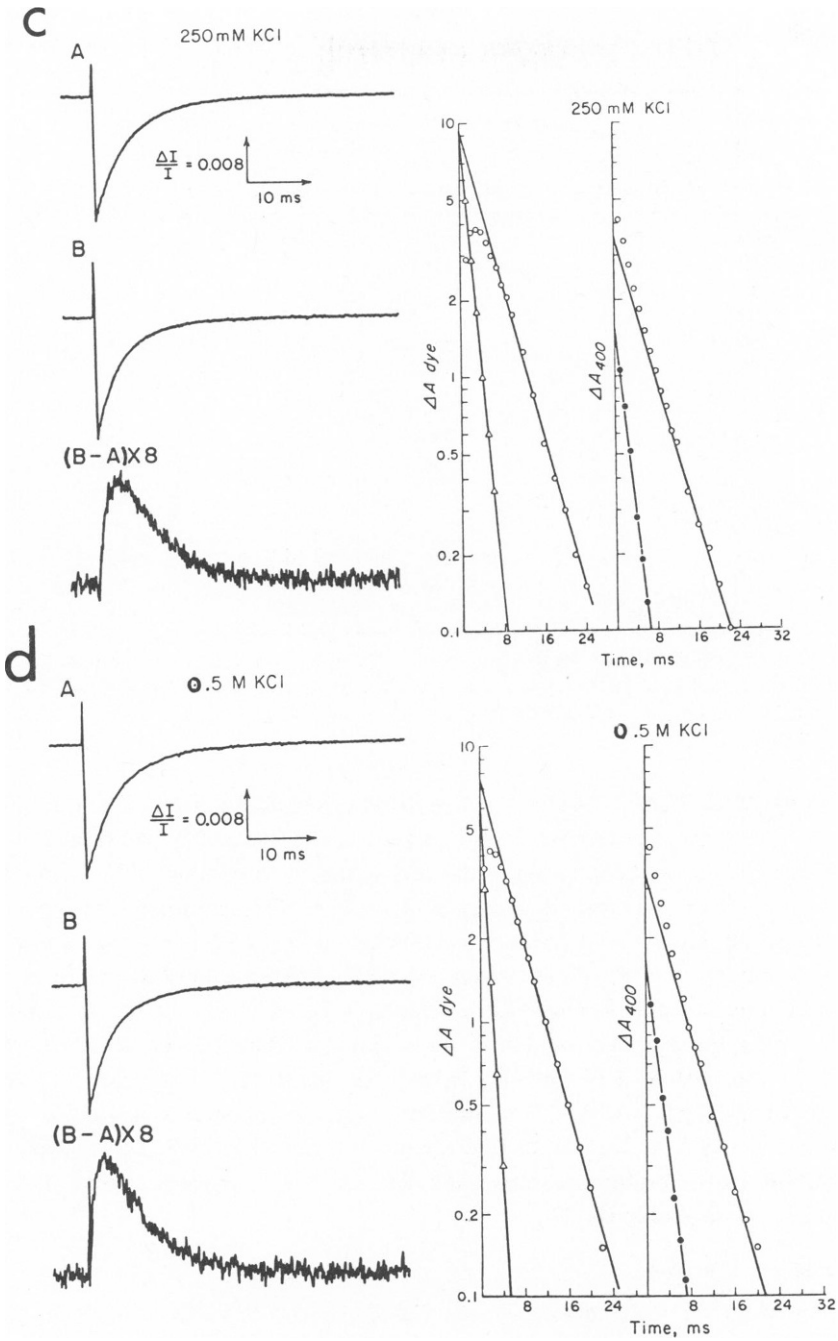


FIGURE 1 *(a–d)* Light-induced absorbance changes at 400 nm in PM sheets suspended in different concentrations of KCl, (A) without and (B) with the pH indicator dye *p*-nitrophenol; (B-A) shows the absorbance change due to the dye only. An absorption decrease represents acidification of the medium. Traces are an average of 64 flashes, 4 s apart. Bacteriorhodopsin concentration ~2 μM; *p*-nitrophenol, 25 μM; pH ≃ 6.62 ± 0.04. Corresponding semilogarithmic plots of the absorbance changes at 400 nm (data from traces A and B-A) vs. time are shown on the right side of the traces. The rise component of the absorbance change for the dye (△) has been drawn with reversed sign; (○) represent the decay of the dye signal. For △A400 (●) represent the fast component, and the solid line represents the slow component in the biphasic decay of M412. The initial spike seen in all the traces is a flash artifact.



We confirmed the high quantum efficiency of proton release found above by measuring it more directly by (*a*) using rhodopsin as an actinometer to determine the number of photons in the actinic flash and (*b*) by measuring the number of protons released using the same flash. The optical density of the PM suspension and the rhodopsin solution (in 2% ammonyx LO) were adjusted so that they were approximately equal at 560 nm. A 560-nm interference filter

TABLE I

PM SHEETS AND PM INCORPORATED INTO PHOSPHOLIPID VESICLES IN DIFFERENT CONCENTRATIONS OF KCl

	KCl	ΔA dye		ΔA400		H^+/M
		$\tau_{1/2}$ Rise (H^+ release)	$\tau_{1/2}$ Decay (H^+ uptake)	$\tau_{1/2}$ Decay (fast)	$\tau_{1/2}$ Decay (slow)	
	mM	*ms*	*ms*	*ms*	*ms*	
PM sheets	10	1.5	8.0	2.1	7.2	0.64
	50	1.6	5.5	2.1	6.4	1.1
	100	1.3	6.3	2.2	6.5	1.3
	250	1.2	4.0	1.7	4.4	1.7
	500	0.7	4.0	1.8	4.2	1.8
PM/PC vesicles	10		10.8	3.8	23.5	2.18
	50		13.0	3.5	23.0	2.3
	100		14.8	3.5	21.5	2.3
Whole cells	Basal salts	~1.5	~29.0	~1.2	~12	2.2
		~1.8	~28.0	~1.7	~13	1.97
		~1.2	~30.0	~1.6	~15.6	2.18
		~1.8	~30.0	~1.7	~14.6	2.19

$\tau_{1/2}$ rise and decay times in milliseconds for the dye and M412 (ΔA400). Concentration of M412 was calculated using a molar extinction coefficient of 30,000 at 400 nm. Number of H^+ was obtained by calibrating the ΔA dye with known amounts of HCl. Dye used, (25 μM) *p*-nitrophenol; approximate concentration of bacteriorhodopsin 2 μM, pH = 6.63 ± 0.05.

and a Corning glass filter CS3-68 were put in front of the actinic flash. Then the ΔA400 for the PM was measured in the presence and absence of the dye and ΔA500 was measured for rhodopsin. For rhodopsin the intensity of the actinic flash was attenuated by a neutral density filter so that <1% of the sample bleached per flash. This eliminated the possibility of photoreversal from any of the intermediates of bleaching. 1 mM NH_2OH was added to insure rapid disappearance of the bleaching products and avoid complications due to absorption from the long-lived intermediates. Using 0.70 as the quantum yield of bleaching rhodopsin (Hurley and Ebrey, 1978), the quantum yield of H^+ release was calculated to be ~0.27 in 10 mM KCl and ~0.67 in 250 mM KCl (Table II). Taking the quantum yield for M412 formation of Becher and Ebrey (1977) of 0.30, the number of protons appearing in solution per M412 formed would be ~2. We also remeasured the quantum yield for M412 formation under the same conditions as the proton measurements and found a value close to that of Becher and Ebrey, ~0.32 ± 0.02 (Table II).

PM/PC Vesicles

To determine if all the proton changes measured above with PM sheets represent the protons actually pumped across the membrane, we measured the light-induced H^+ changes after incorporating the membrane into egg PC vesicles prepared in varying concentration of KCl. Some of these vesicles may be multilamellar; in the inner layers of these vesicles, light-activated bacteriorhodopsin could cycle but probably could not take up protons from the external media. This would tend to lower our quantum yield estimates. Fig. 2 *a–c* and Table I show the M412 and dye absorption changes in vesicles made in 10, 50, and 100 mM KCl.

TABLE II
PM SHEETS SUSPENDED IN 250 mM KCl, OD 560 = 0.115

Exp.	Protons released / rhodopsin bleached	Quantum yield of proton release*	M412 formed / Rhodopsin bleached	Quantum yield M412 formation*
1	0.91	0.64	0.45	0.31
2	1.00	0.70	0.49	0.34
3	0.93	0.65	0.46	0.30
4	1.00	0.70	0.50	0.35

Rhodopsin in 2% ammonyx LO, OD 560 = 0.096. Measuring wavelength is 400 nm (PM) and 500 nm (rhodopsin); actinic wavelength is 560 nm. Concentration of M412 and rhodopsin bleached was calculated using molar extinction coefficients of 30,000 and 40,000, respectively. Number of H^+ released was obtained by calibrating the ΔA of the dye with known amounts of HCl. Dye used, 25 μM *p*-nitrophenol.
*Assuming the quantum yield for bleaching rhodopsin in ammonyx LO is 0.7 (Hurley and Ebrey, 1978).

The orientation of the PM when incorporated into egg PC vesicles is primarily inside-out, resulting in a net uptake of protons (alkalinization of the medium). The small initial overshoot in Fig. 2 *a–c* curve B-A (seen after the spike due to a flash artifact) probably represents a small amount of the right side-out-oriented membrane (cf, Lozier et al., 1976). All our calculations take this fact into account, and the total amplitude of the pH change was obtained by plotting the H^+ uptake, after the initial transients, on a semilogarithmic scale and extrapolating to time zero. Like the PM sheets, the PM/PC vesicles show no significant effect of changing salt concentration on the amplitude and the decay kinetics of M412 [Table I and Fig. 2 *a–c*]. But, unlike the sheets the ΔpH is the same at all salt concentrations. The ratio of H^+/M412 is ~2 at all three salt concentrations, implying that at least two protons are pumped per photocycle. 5 μM nigericin abolished the absorption change due to the uptake of protons and left only a transient acidification attributable to (*a*) the leaking out of protons rapidly released inside the vesicles followed by a slower uptake of protons on the outside and (*b*) some protons released directly outward by right side-out-oriented vesicles (Fig. 3, curve B-A). Moreover, Fig. 2 *a–c* shows that the proton gradient relaxation time (approximately seconds) is much longer than the photochemical cycling time (approximate milliseconds). Rebinding would be expected to occur on this millisecond time scale. Both the long proton relaxation times and the nigericin experiment suggest that the pH change we have observed is due solely to the translocation of H^+ across the membrane and is not due to reversible binding and release of protons on one side only of the membrane.

Whole Cells

Experiments similar to those described in sections "PM sheets" and "PM/PC vesicles" are difficult to perform using whole bacterial cells due to light-scattering and settling of the cells during the measurements. Moreover, other processes involving H^+ changes, e.g. H^+reentry into the cells due to ATPase and other ion pump activities could affect the measurements. However, an advantage in using whole cells is that the PM is in its native environment so that problems of misorientation, etc., are eliminated. Restricting the pH measurements to a short time after the flash, e.g. <25 ms, probably minimizes the contribution of processes other than the light-activated H^+ pumping. The signal-to-noise ratio can be improved considerably by

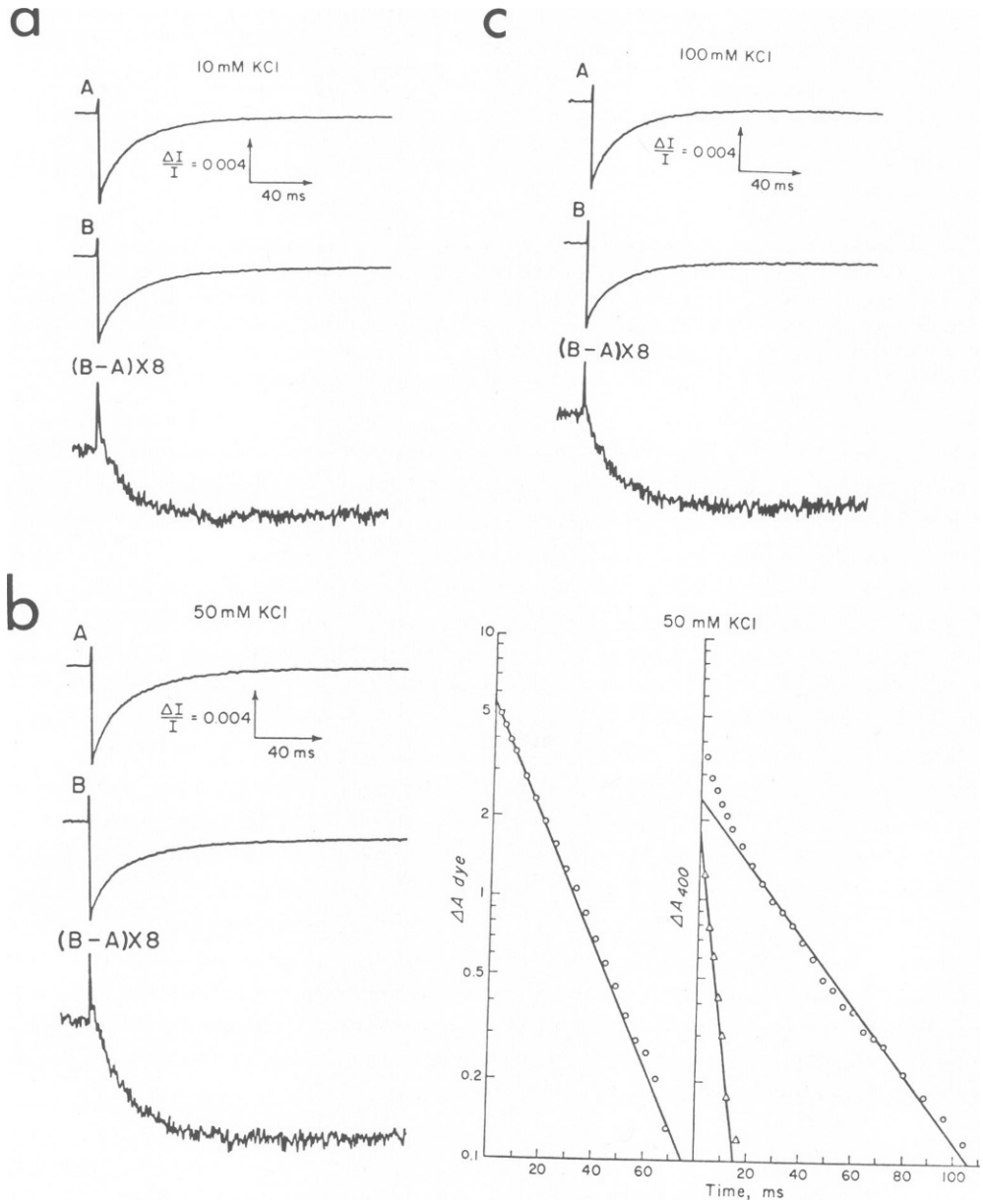


FIGURE 2 *(a–c)* Light-induced absorbance changes at 400 nm in PM/phospholipid vesicles prepared in different concentration of KCl, (B) with and (A) without the pH indicator dye *p*-nitrophenol; (B-A) shows the absorbance change due to the dye only. Traces are an average of 32 flashes, 8 s apart. OD ≃ 0.12 at 560 nm; *p*-nitrophenol 25 μM; pH ≃ 6.63 ± 0.05. A semilogarithmic plot of the absorbance changes at 400 nm (data from traces A and B-A) vs. time for vesicles in 50 mM KCl is shown on the right. For ΔA_{400} (○, △) represent the fast component, and the solid line represents the slow component in the biphasic decay of M412.

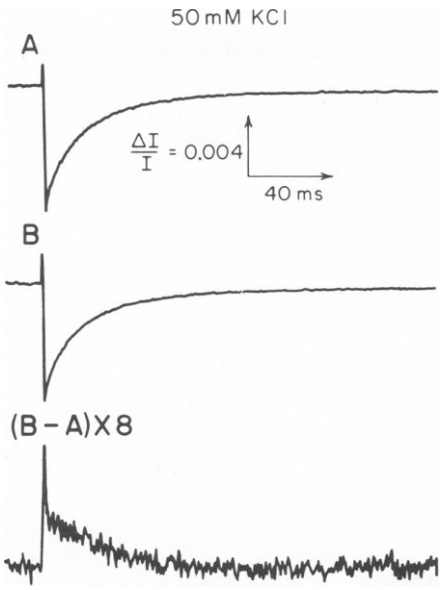


FIGURE 3 Same as Fig. 2 plus 5 μM nigericin. Note that the pH change as seen in trace B-A is greatly reduced and is over in a relatively short time.
p-nitrophenol 25 μM; pH $\simeq$ 6.78 $\pm$ 0.05.

taking an average of a large number of flashes. However, this has to be compromised somewhat because the problems due to settling of the cells over a long period of time become more pronounced. Taking an average of 64 flashes 4 s apart gave acceptable results.

Preliminary experiments using whole cells give a ratio of $H^+/M412 = 2$ (Table 1 and Fig. 4). In calculating the ratio of $H^+/M412$ we have used the initial slope of the dye decay curve, (Fig. 4, curve B-A), for two reasons: (*a*) it gave the lowest number of protons and hence the lowest ratio of $H^+/M412$; and (*b*) it probably has the least contribution from H^+ changes associated with various other ion pump activities of the cells.

DISCUSSION

To understand the mechanism of light-induced proton translocation by the PM, it is necessary to know the number of protons pumped during each photocycle. We have measured the number of protons released by light from PM sheets, the number taken up by PM containing vesicles, and the number expelled by whole cells. There are conflicting results in the literature on the number of protons released by light from PM sheets ranging from 1 H^+/photocycle (Lozier et al., 1976; U. Fisher in Hartman et al., 1977) to 2 H^+/photocycle (Ort and Parson, 1979). Different experimental conditions used by different investigators could possibly account for the different results. We find for fresh preparations of PM sheets that the $H^+/M412$ ratio is $\sim$1 at low salt concentrations and is $\sim$2 at high salt concentrations (Table I). Moreover, we find the quantum yield of proton release of $\simeq$0.27 at low salt and 0.67 at high salt concentration. These values are similar to those of Ort and Parson (1979), 0.25 at low salt concentrations, and 0.43 at high salt concentrations although we find slightly higher quantum yield values at high salt concentration: this difference may be due to a difference in the age of

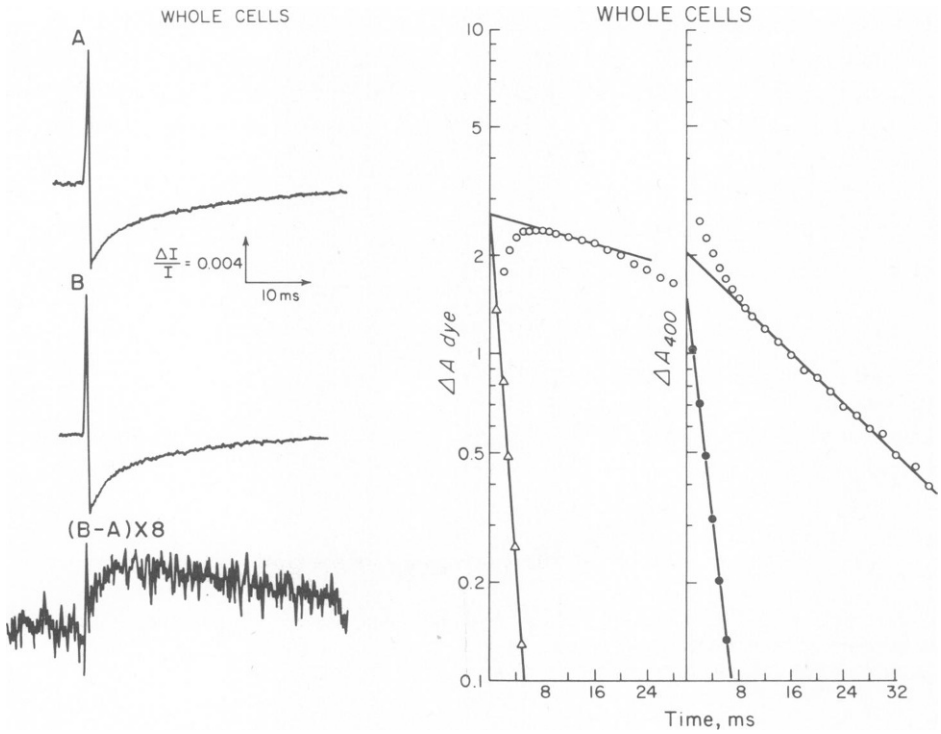


FIGURE 4 Light-induced absorbance changes at 400 nm in *Halobacterium halobium* cells suspended in basal salts, (B) with and (A) without the pH indicator dye *p*-nitrophenol, (B-A) shows the absorbance change due to the dye alone. An absorption decrease represents acidification of the medium. Corresponding semilogarithmic plots of the ΔA dye and ΔA400 (from traces of B-A and A, respectively) are shown on the right. The rise component of the dye change (Δ) has been drawn with reversed sign; (○) represent the decay of the dye signal. For ΔA400 (●) represent the fast component, and the solid line represents the slow component in the biphasic decay of M412. Traces are an average of 64 flashes, 4 apart. OD 560 ≃ 0.14; *p*-nitrophenol 25 μM; pH ≃ 6.78 ± 0.05.

the PM preparations. We find that the freshly prepared sheets give higher quantum yields than older samples. Assuming a salt concentration-independent quantum yield for M412 formation of ~0.30 (Becher and Ebrey, 1977; Ort and Parson, 1979; Results), the direct quantum yield measurements also give a H^+/M412 ratio from ~1 to >2, depending on salt concentration.

In vesicles and whole cells, we find a H^+/M412 ratio of 2 or greater (Table I). In the PM/PC vesicle experiments, the effect of nigericin (Fig. 3) and the long relaxation time of the pH signal (Fig. 2 *b*) both strongly suggest that all of the protons are pumped across the membrane (see below). Interestingly, our H^+/M412 measurements in phospholipid vesicles show no salt concentration dependence. Bogomolni (1977) and Hartman et al. (1977) have reported quantum yields for proton expulsion from whole cells in the range 0.4–0.6. If we take a quantum yield for M412 formation of ~0.3, then these workers' results require a H^+/M412 ratio of >1 (Becher et al., 1978). Thus these previous results are entirely consistent with the H^+/M412 ratio determined here for vesicles and whole cells.

Caplan et al. (1977) and Eisenbach et al. (1976; 1978) have proposed that not all the

light-induced proton changes measured with PM sheets are the result of the pumping process; rather, they suggest that only part of the protons seen are actually pumped and some are simply released and rebound on the same surface of the membrane. However, in sheets one might expect that the kinetics of the pH changes due to pumped protons and to those that are reversibly released and rebound may be different. But the kinetics of proton release and uptake in sheets are monophasic (Ort and Parson, 1979: Lozier et al., 1976; and our measurements). In vesicles there certainly would be a difference; the pumped protons would decay back quite slowly, requiring several seconds to diffuse back across the cell membrane while the reversible release and rebinding would be over in several milliseconds, the photocycling time. The relaxation time of the pH gradient in PM/PC vesicles is very slow; in addition, the abolition of the pH changes in PM/PC vesicles by nigericin also suggests that the pH change observed is due to the translocation of H^+ across the membrane.

Calculations of proton release relative to M412 however assume that all bacteriorhodopsin goes through this intermediate, which may not be true under all conditions. However, our results do require that the ratio of moles of protons pumped to moles of the unprotonated Schiff-base intermediate (M412) formed be >1.

A H^+/M412 ratio of one is easy to rationalize in terms of the deprotonation of the retinylidene Schiff base; although we have no evidence for this appealing mechanism, our measurements do allow such a model in the case when PM is suspended in water or in low salt concentrations; where more than one proton is pumped per unprotonated Schiff base (M412) formed, it is necessary that at least one of the pumped protons not be the Schiff-base proton. And if one of the pumped protons is not from the Schiff base, then the great simplicity of the model for how the Schiff base directly pumps protons is removed. Clearly, more elaborate models (Honig et al., 1979) will have to be proposed.

We thank John Bowyer for the use of his computer programs, Gerry Tierney for electronics help, Burr Nelson for preparing the purple membrane and the phopholipid vesicles, and Don Ort for many useful discussions.

This work is supported by National Science Foundation grant PCM 76-82764 and the University of Illinois Research Board.

Received for publication 11 September 1979 and in revised form 15 January 1980.

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