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KINETICS AND STOICHIOMETRY OF LIGHT-INDUCED PROTON RELEASE AND UPTAKE FROM PURPLE MEMBRANE FRAGMENTS, *HALOBACTERIUM HALOBIUM* CELL ENVELOPES, AND PHOSPHOLIPID VESICLES CONTAINING ORIENTED PURPLE MEMBRANE

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SUMMARY

We have used flash spectroscopy and pH indicator dyes to measure the kinetics and stoichiometry of light-induced proton release and uptake by purple membrane in aqueous suspension, in cell envelope vesicles and in lipid vesicles. The preferential orientation of bacteriorhodopsin in opposite directions in the envelope and lipid vesicles allows us to show that uptake of protons occurs on the cytoplasmic side of the purple membrane and release on the exterior side.

In suspensions of isolated purple membrane, approximately one proton per cycling bacteriorhodopsin molecule appears transiently in the aqueous phase with a half-rise time of 0.8 ms and a half-decay time of 5.4 ms at 21 °C.

In cell envelope preparations which consist of vesicles with a preferential orientation of purple membrane, as in whole cells, and which pump protons out, the acidification of the medium has a half-rise time of less than 1.0 ms, which partially relaxes in approx. 10 ms and fully relaxes after many seconds.

Phospholipid vesicles, which contain bacteriorhodopsin preferentially oriented in the opposite direction and pump protons in, show an alkalinization of the medium with a time constant of approximately 10 ms, preceded by a much smaller and faster acidification. The alkalinization relaxes over many seconds.

The initial fast acidification in the lipid vesicles and the fast relaxation in the envelope vesicles are accounted for by the misoriented fractions of bacteriorhodopsin. The time constants of the main effects, acidification in the envelopes and alkalinization in the lipid vesicles correlate with the time constants for the release and uptake of protons in the isolated purple membrane, and therefore show that these must occur on the outer and inner surface respectively. The slow relaxation processes in the time range of several seconds must be attributed to the passive back diffusion of protons through the vesicle membrane.

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INTRODUCTION

Bacteriorhodopsin in the purple membrane of *Halobacterium halobium* functions as a light-driven proton pump [1, 2]. Using low temperature and flash spectroscopy, we have shown that bacteriorhodopsin undergoes a light-induced reaction cycle, and have identified at least four spectrally distinct intermediates. When pH indicator dyes are added to a suspension of isolated purple membrane fragments, flash spectroscopy also shows that during the reaction cycle, protons are first released and then taken up again [3]. If this release and uptake is an expression of proton pumping, it must occur on opposite sides of the membrane fragments. The availability of model systems consisting of closed vesicles with opposite orientation of the purple membrane and the time difference between release and uptake afford an opportunity to test this point. We report here the stoichiometry of the protonation changes and show that the protons are released from the external surface and taken up on the cytoplasmic surface of the purple membrane.

MATERIALS AND METHODS

Purple membrane was prepared by the method of Oesterhelt and Stoeckenius [4]. Cell envelope vesicles with preferential right side-out orientation were obtained by sonication of cells, followed by differential centrifugation according to MacDonald and Lanyi [5]. Unilamellar vesicles were prepared by a modification of the procedure of Racker and Stoeckenius [2]. Briefly, the purple membrane was suspended in 10 % deoxycholate, then reisolated on a sucrose density gradient and incubated with sonicated lecithin in 2 % cholate. The cholate was removed by dialysis and the excess lipid removed on a sucrose gradient (Hwang and Stoeckenius, in preparation). The pH indicators 7-hydroxycoumarin (umbelliferone) and *p*-nitrophenol were obtained from K&K Laboratories and Eastman Organic Chemicals respectively.

Absorption spectra and light-induced transmission changes were measured with samples contained in 1×1 cm fluorescence type cuvettes. The pH values of the samples, measured in the cuvettes with a glass electrode and pH meter, were adjusted with small volumes of acid or base. The cuvettes were stoppered, except during the measurement and adjustment of pH, to minimize pH drift due to atmospheric carbon dioxide.

Absorption spectra were measured with a Cary 14 spectrophotometer. The light-induced proton concentration changes were calibrated by adding small aliquots of standardized acid to the indicator-containing cuvette and observing the absorption changes of the indicator with the Cary spectrophotometer.

Transmission changes of bacteriorhodopsin and the pH indicator dyes were measured with a single beam flash kinetic spectrophotometer constructed in our laboratory. The measuring light source consists of a 45 W quartz halogen lamp (G.E. Q6.6A/T4/CL) powered by a regulated d.c. power supply (Lambda 36V 15A) and a 0.25 m f : 3.5 monochromator (Jarrell Ash 82-410) with appropriate optics to focus the image of the lamp filament at the center of the cuvette. After traversing the sample, the measuring beam passes through appropriate colored glass (Corning Glass Works) and interference filters (Baird Atomic) which block light of wavelengths

other than that of the measuring beam. The transmitted light is detected by a 2-inch diameter end-on photomultiplier (EMI 9659QA, S-20 spectral response) and its signal is digitized and stored in a signal averager (Nicolet 1074). Transmission changes are induced by a xenon flashlamp (General Radio type 1531-AB "Strobotac") with 3 μs flash duration or with a photographic flashlamp (R. Bosch "Ultrablitz Cornet 100") with 1 ms duration which is focused with a strong lens onto the sample perpendicular to the measuring beam. The 3 μs flash was used for purple membrane suspensions. The long relaxation time of the pH gradient across the envelope and vesicle membranes limited us to a low flash repetition rate. For this reason, the higher energy longer flash was used for the latter to obtain comparable signal to noise ratios. The actinic light is limited to wavelengths complementary to the measuring wavelength with appropriate colored glass and interference filters so that the amount of stray actinic light reaching the photomultiplier is small. The time base of the signal averager is triggered by a pulse generator (Hewlett Packard 8005A) at an appropriate frequency and a second pulse, after a selected delay time, triggers the actinic source. Differences between experiments performed on samples with and without the pH indicator dye can be obtained by subtraction in the signal averager. The kinetic traces are plotted from the averager on an X-Y recorder (Hewlett Packard 7004B).

Absorbance changes were calculated from transmission changes with equation (1)

$$\Delta A = -\log\left(\frac{\Delta I}{I} + 1\right) \tag{1}$$

where $\Delta I/I$ is the fractional change in the measured transmitted light intensity.

Kinetic analysis of the absorbance changes was done by graphical analysis ("curve peeling"; ref. 6).

Our experiments were designed to insure that differences between the light-induced transmission changes of samples with and without a pH indicator were indeed due to pH changes in the aqueous phase. Criteria for suitable indicators were as follows: (1) the indicator must have a pK near the pH value at which the buffering capacity of the sample is a minimum; (2) the indicator must have a sufficiently large differential extinction coefficient between its protonated and unprotonated forms at a wavelength accessible to our flash kinetic instrument; (3) neither form of the indicator can absorb light within the emission spectrum of the actinic light source; (4) the indicator must not affect the reaction cycle of the bacteriorhodopsin; and (5) the indicator must respond to the pH of the aqueous phase.

RESULTS

Membrane suspensions

Fig. 1 compares light-induced transmission changes of purple membrane suspended in water at pH 7.8 with and without the pH indicator 7-hydroxycoumarin (umbelliferone). Absorption spectra of the indicator alone at various pH values are shown in Fig. 2, and the absolute and difference spectra of the indicator-containing and control samples of Fig. 1 are shown in Fig. 3. The light-induced transmission changes (Fig. 1) were measured at 365 nm, where the non-protonated form of the indicator absorbs maximally, and at 420 nm, where neither the protonated nor the

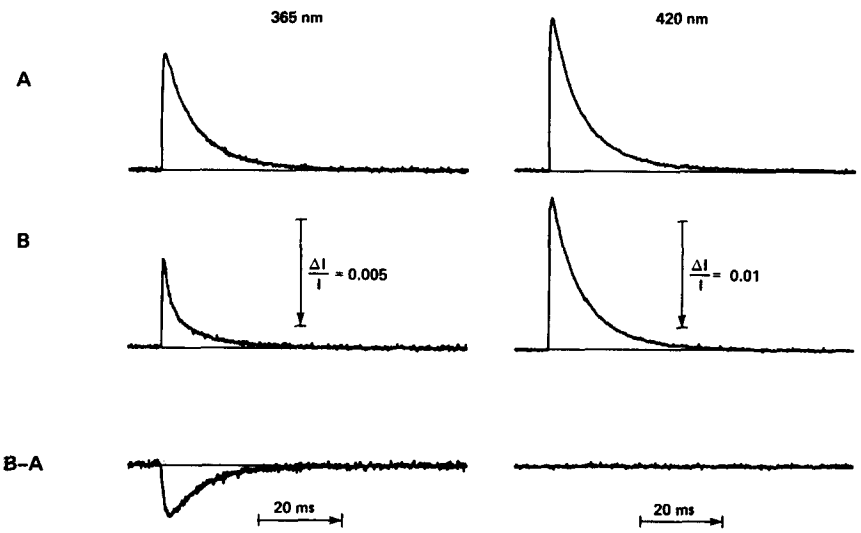

Fig. 1. Light-induced transmission changes at 365 and 420 nm of aqueous suspensions of purple membrane with and without the pH indicator 7-hydroxycoumarin. Each sample contained 36 nmol bacteriorhodopsin in 3.0 ml distilled water at 21 °C (pH 7.85±0.05). The curves are the average of 2048 (365 nm) or 512 (420 nm) actinic flashes from a xenon flashlamp (10 flashes per s of 3 μs duration, wavelength 500–600 nm). A ,without indicator; B, with 300 nmol 7-hydroxycoumarin.

non-protonated form of 7-hydroxycoumarin absorbs appreciably (see Fig. 2). At the control wavelength (420 nm), we see no difference between the two samples, which shows that the samples are well matched (Fig. 1; see also Fig. 3) and that 7-hydroxycoumarin has no measurable effect on the reaction cycle of bacteriorhodopsin*. The kinetics of the transmission changes at both wavelengths in the control sample (sample without indicator) are similar and are due primarily to the rise and decay of

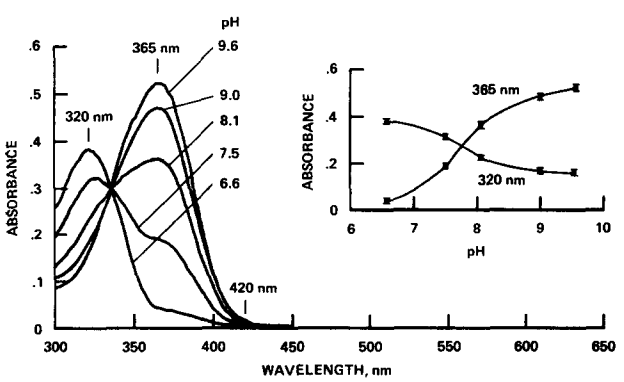

Fig. 2. Absorption spectra of $5 \cdot 10^{-5}$ M 7-hydroxycoumarin in water at various pH values. The inset shows the pH dependence of the absorbance at 365 and 320 nm.

* The reaction cycle of bacteriorhodopsin is sensitive to temperature, pH, and many solutes; thus, a control is required to establish that signals observed are actually attributable to pH changes.

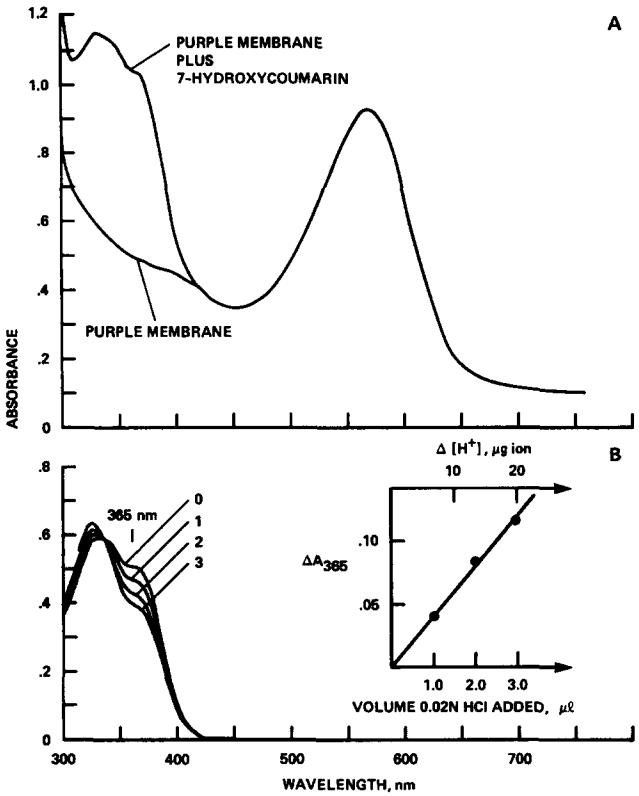

Fig. 3. Absorption spectra (A) and difference absorption spectra (B) of samples used for flash experiment shown in Fig. 1. Part A shows the absolute absorption spectrum of each sample vs. water. The apparent absorption at long wavelengths, and the sloping background at short wavelengths are due to light scattering by the purple membrane. Part B shows the 7-hydroxycoumarin-minus-control sample difference spectrum after addition of 0, 1, 2 and 3 μl of 0.02 M HCl. The inset shows the change in absorbance at 365 nm vs. volume of HCl added to the 7-hydroxycoumarin-containing sample. The slope of the line in the inset is $5.5 \cdot 10^{-3}$ absorbance units per μg ion concentration change of protons.

the M^{LA}_{412} intermediate [3]. The transmission changes at 365 nm in the 7-hydroxycoumarin-containing sample are distinctly different from the control sample, and we attribute this difference to a pH change in the aqueous phase which causes a transient protonation of the indicator. The maximal absorbance change of the indicator is −0.00105 absorbance units. Using the calibration shown in Fig. 3B, this corresponds to a proton concentration change of 0.19 μg ion. The concentration of bacteriorhodopsin cycling can be estimated by the maximal absorbance change at 420 nm (+0.0063) and the differential extinction coefficient between bacteriorhodopsin and the M^{LA}_{412} intermediate, 23000 l/mol per cm [3]. The calculated concentration cycling is 0.27 μM. Thus, the calculated ratio of protons released to bacteriorhodopsin molecules cycling is 0.7. Note that the kinetics of the pH and the bacteriorhodopsin signals are not identical. The kinetic analysis of the data of Fig. 1 is shown in Fig. 4. Here, the calculated absorbance changes are plotted semi-logarithmi-

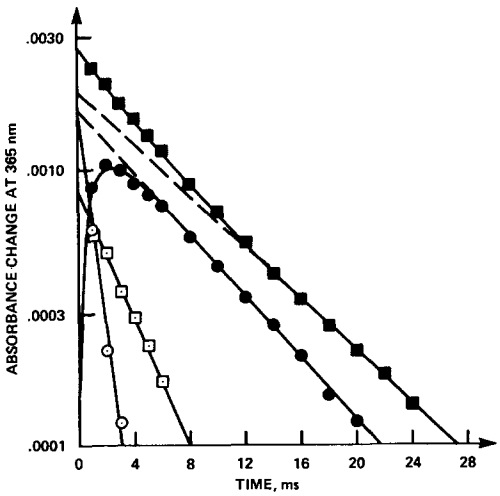

Fig. 4. Semilogarithmic plot of absorbance changes at 365 nm vs. time. Data from Fig. 1 are used for aqueous suspensions of purple membrane (■, curve A in Fig. 1) and for the pH indicator 7-hydroxycoumarin in this suspension (●, curve B-A from Fig. 1). Both cases show a slow component (extrapolated dashed lines) and a fast component (open symbols). Note that the fast component of the indicator is drawn with reversed sign.

cally vs. time. The decay of the absorbance change of bacteriorhodopsin is not a single exponential as can be seen from the non-linearity. The decay can be resolved into two exponential decays, with half-times of 6.4 and 2.6 ms. The rise time of the transmission change is not resolved in Fig. 1, but is less than 0.2 ms. The half time for the proton release of about 0.8 ms is resolved. It is significantly faster than the fast component in the absorbance decay resolved graphically from the semilogarithmic plot. The half time of the proton uptake is about 5.4 ms and may not be significantly different from the slow decay of the bacteriorhodopsin absorbance resolved above.

The delay between bacteriorhodopsin and indicator dye transmission changes requires a correction in the calculation of the number of protons released per bacteriorhodopsin molecule cycling. The value of 0.7 given above is based on the maxima of the absorption changes for indicator dye and bacteriorhodopsin. From the kinetic analysis in Fig. 4, it is evident that the uptake begins before the release is complete. The actual number of protons released given by the y-intercept is 60 % higher than the value obtained from the transient maximum. The same type of correction must be applied to the bacteriorhodopsin changes to determine the amount cycling. This increases the amount cycling by approx. 10 % only, however, because of the larger difference between the rise and decay times. Therefore, the ratio of protons per bacteriorhodopsin molecule cycling is about 1.

Oriented systems

To determine whether the proton release and uptake observed in the purple membrane suspension occur on opposite sides of the membrane (a requirement if the changes are relevant to proton pumping), we have performed experiments on

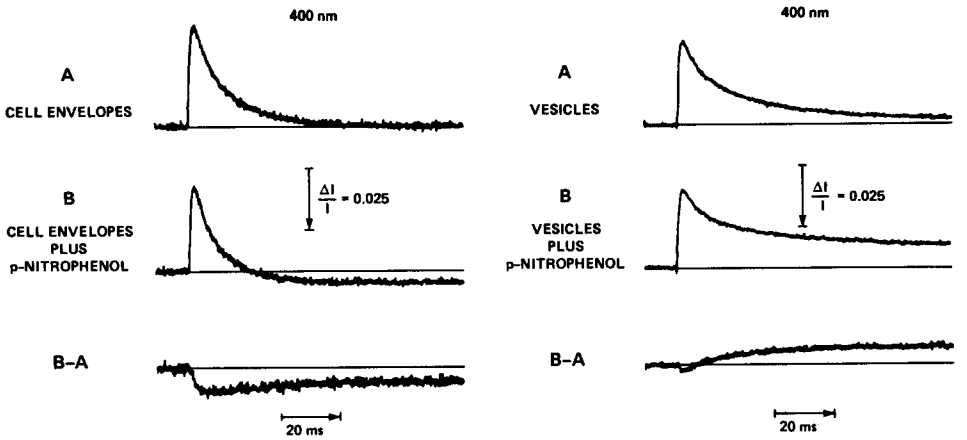

Fig. 5. Light-induced transmission changes at 400 nm of aqueous suspensions of *H. halobium* cell envelopes and purple membrane-containing phospholipid vesicles with and without the pH indicator *p*-nitrophenol. Left side: cell envelopes containing 1.4 mg protein per ml in 3.0 ml 4 M NaCl. Right side: phospholipid vesicles containing approximately 30 nmol of bacteriorhodopsin in 3 ml 75 mM K_2SO_4. Temperature: 22 °C; pH: 6.45±0.15; *p*-nitrophenol concentration: 10^{-4} M. The traces are the average of 8 actinic flashes at 0.1 flash/s. The actinic source consists of an Ultrablitz camera strobe through a Corning yellow glass filter plus a Baird Atomic 5755 A 250 A series interference filter.

two types of purple membrane-containing vesicle preparations having opposite orientation of the purple membrane. These preparations are the cell envelope vesicles described by MacDonald and Lanyi [5], which have purple membrane preferentially oriented as in intact cells, and the vesicles prepared with purified purple membrane and phospholipids which have the opposite orientation (Hwang and Stoeckenius, in preparation). Light-induced transmission changes in suspensions of these two types of vesicles are shown in Fig. 5. The absorption spectra of these preparations are shown in Figs. 6 and 7. The spectrum of bacteriorhodopsin in the phospholipid vesicles is pH dependent, forming a 490 nm absorbing species at high pH (pK about 7.5)*. Therefore, we chose the indicator *p*-nitrophenol (pK = 7.0) and worked at pH 6.5. The cell envelope vesicles in the absence of pH indicator have transmission changes essentially the same as the purple membrane fragments, whereas the changes in the phospholipid vesicles show somewhat altered kinetics. The pH indicator signal with the cell envelopes shows a rapid decrease in absorption, followed by a slower increase which does not reach the baseline on this time scale. A decrease in dye absorption indicates an acidification of the medium (Fig. 6B). The pH signal of the phospholipid vesicles shows a small transient acidification followed by a net alkalinization. The residual signals decay to the baseline with half times of 3–5 s (data not shown).

* The spectrum of the bacteriorhodopsin in isolated purple membrane is independent of pH in the range of 5 < pH < 10.

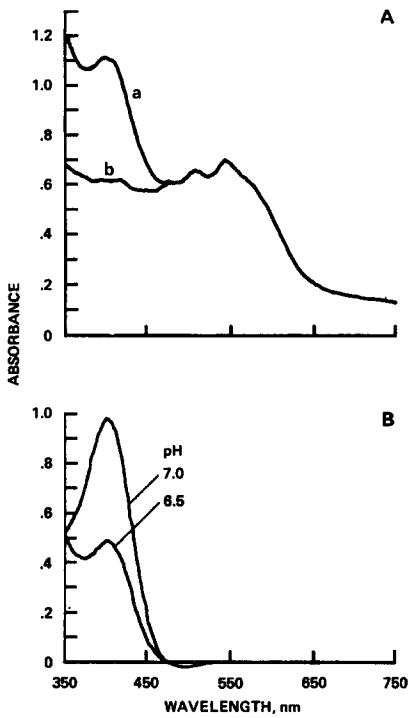

Fig. 6. A. Absorption spectra of cell envelope vesicles at pH 6.5 with (a) and without (b) indicator dye *p*-nitrophenol. The absorption maxima at 475, 506, and 542 nm are due to bacterioruberine present in the plasma membrane. The shoulder at longer wavelengths is due to bacteriorhodopsin. B. Difference spectrum between the samples in part A shown for two different pH values of the indicator-containing sample. Note that the spectrum of the cell envelopes does not depend on pH.

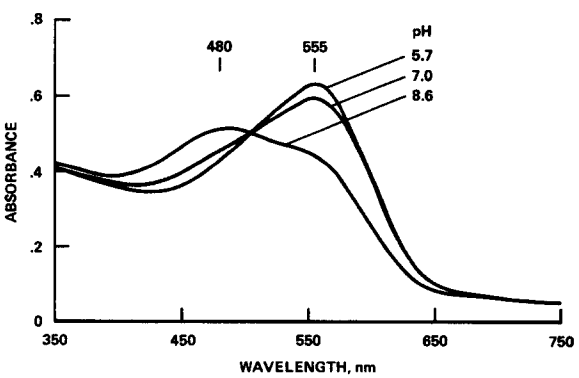

Fig. 7. Absorption spectra of purple membrane-containing phospholipid vesicles at various pH values. The change in the absorption spectrum at higher pH values is discussed in Hwang and Stoeckenius (in preparation).

DISCUSSION

The kinetics of spectral changes in the photoreaction cycle are slightly but significantly different in the isolated purple membrane and the vesicle preparations. We therefore take the kinetics of the spectral changes as our frame of reference when we compare the pH response in the different preparations. The proton release from purple membrane suspensions which consist of membrane sheets [7] is most closely correlated in time with the rise of the M^{LA}_{412} intermediate [3]. The rise time of the pH signal is, however, significantly slower than the rise time of M^{LA}_{412} and faster than the fastest component in the decay at 400 nm. This appears to link the proton release to the formation of M^{LA}_{412}. There are at least three possible explanations for the short delay in the appearance of the pH signal. First, the time constant of the pH response could be diffusion limited. This is unlikely; we estimate that diffusion is complete in less than 10^{-4} s. Second, the bacteriorhodopsin reaction cycle may not be properly described by a linear unidirectional model, but could have back reactions or branch points, in which case the measured half times for the absorbance change would not correspond to single steps in the model. Third, the release of the proton itself does not cause an absorbance change, but is linked indirectly to the reaction giving rise to the spectral change. Further experiments will be required to distinguish these possibilities. However, the results are unlikely to influence the conclusions drawn in this paper.

At lower temperatures (−28 and −40 °C) Chance et al. [8] have reported a correlation between absorption changes of bacteriorhodopsin and a fluorescence intensity change of 7-hydroxycoumarin, and attributed the latter to a pH change. At an intermediate temperature (1.8 °C), we find that the rise time of the pH change measured with brilliant yellow is still significantly different from the kinetics of bacteriorhodopsin. An alternative method for distinguishing pH changes from spectral changes of bacteriorhodopsin might be to include pH indicator in both samples, but to buffer one of the samples heavily. This method was used by Dencher and Wilms [9] and led to results which conflict with ours. In our hands, this method proved to be unsatisfactory since buffer alone (Tris or phosphate) altered the kinetics of bacteriorhodopsin. Also, we find the kinetics of bacteriorhodopsin to be extremely dependent on pH and temperature at the wavelength and pH used by Dencher and Wilms [9], thus making sample matching difficult.

In continuous light, cell envelope vesicles acidify the medium whereas lipid vesicles show an alkalinization [5, 2]. Apparently the cytoplasmic surface of the purple membrane is exposed to the medium in the lipid vesicles and to the vesicle interior in the envelopes. This is consistent with the preferential orientation of the purple membrane in these preparations as observed with freeze-fracturing (see Fig. 8). With flash illumination, the direction of the main and sustained pH response in both preparations is the same as that evoked by continuous light. The time constant for the acidification in envelope vesicles corresponds to the proton release seen in isolated purple membrane suspensions and the alkalinization in the lipid vesicles to the proton uptake in isolated purple membrane. We conclude, therefore, that protons are released on the exterior side of the purple membrane and are taken up on the cytoplasmic side.

The slow decay of the response over several seconds must be attributed to the back diffusion of protons across the vesicle membrane. Its time constant is comparable

Fig. 8. See opposite page for legend.

to that obtained for the decay of the proton gradient after continuous illumination.

The initial small acidification seen in the lipid vesicles can be attributed to the small unoriented fraction of bacteriorhodopsin which is directly observed in freeze-fracture preparations (see Fig. 8). The fast decaying component in the acidification response of flash illuminated envelopes is similarly explained by a fraction of mis-oriented purple membrane present in these preparations. This fraction can be estimated by the menadione reductase assay [5] and amounts to approx. 20 % in our preparation.

Another explanation for the fast transient components in the pH signal is the diffusion of indicator dye into the vesicles. We believe that this possibility is less likely, since experiments using 7-hydroxycoumarin gave the same results as those shown here using *p*-nitrophenol. The permeability of biologic membranes for 7-hydroxy-coumarin is very low [10] and the slow back diffusion of protons shows that the vesicle walls are not leaking. Also, the small interior volume and the relatively high buffering capacity of the vesicle interior due to the high surface to volume ratio should render any contribution from enclosed dye very difficult to detect. Moreover, the misorientation of part of bacteriorhodopsin, which we believe to be responsible for the fast components, can directly be demonstrated by electron microscopy and in envelope vesicles also with the menadione reductase assay.

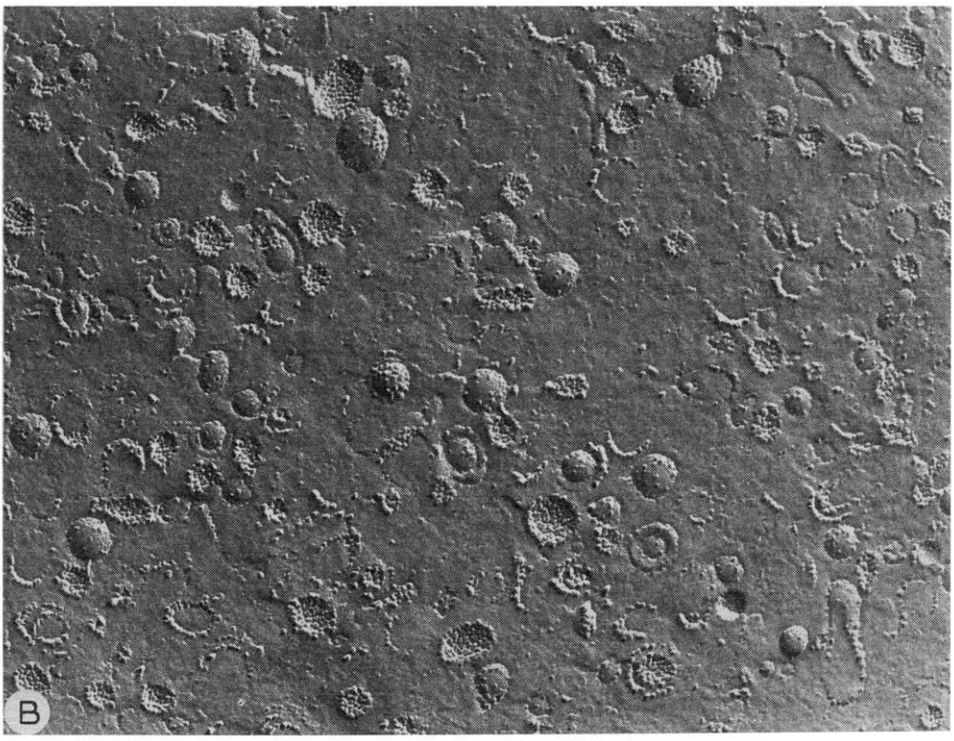

Fig. 8. Freeze-fracture electron micrographs of cell envelope vesicles (A) and purple membrane lipid vesicles (B). Freeze-fracturing splits membranes along an interior plane and reveals intramembranous proteins as particles on the fracture face. In intact cells, a dense population of particles is seen on the always convex "half membrane" adjacent to the cytoplasm (the A face) and few particles on the always concave outer "half membrane" (the B face). The A face of the purple membrane patches shows a regular hexagonal array of particles; the B face is smooth [7]. The same topography is present in the cell envelopes shown here, indicating that they have the same orientation as intact cells. An A face of a purple membrane patch is marked with a single arrow, a B face with a double arrow. In the purple membrane lipid vesicles (B), the particles appear closely packed only on the concave surface, which therefore corresponds to the A face in intact cells, but in these vesicles this "half membrane" faces the medium. However, a small but significant number of particles is also present on the convex surfaces, indicating that part of the bacteriorhodopsin is oriented in the opposite direction. Magnification: A, 49 300×; B, 77 080×.

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