J. Membrane Biol. 33, 325–350 (1977)


# Purple Membrane Vesicles: Morphology and Proton Translocation

San-Bao Hwang and Walther Stoeckenius

Cardiovascular Research Institute, University of California, San Francisco, California 94143

Received 28 October 1976; revised 14 January 1977

*Summary.* Purple membrane vesicles prepared by different techniques differ widely in their morphology and ability to establish a proton gradient in the light. The procedures used to prepare active vesicles do not completely dissociate the purple membrane and thus preserve a preferential orientation of the protein, while most of the lipid is exchanged for added lipid. Responses to illumination are largely determined by the size of the vesicles and the degree to which bacteriorhodopsin is preferentially oriented. Any attempt to compare the interaction of different lipids with bacteriorhodopsin by measuring the pH response must take these factors into account.

With an improved technique we have obtained vesicles of rather uniform size and bacteriorhodopsin orientation, which accumulate protons with an initial rate of 160 ng $H^+$ $sec^{-1}$ $mg^{-1}$ protein at light intensities of $10^6$ erg $cm^{-2}$ $sec^{-1}$. The kinetics of the process are complex and at present insufficiently understood.

The purple membrane of *Halobacterium halobium* occurs as well-defined regions in the surface membrane of halobacteria; these regions or patches contain only one protein, bacteriorhodopsin, which accounts for 75% of their dry mass; the remainder is lipid. Bacteriorhodopsin closely resembles the visual pigments of animals. It contains one mole of retinal per mole of protein. The retinal is bound as a Schiff base and the absorption maximum of the chromophore is red shifted to 570 nm by further complexation of the retinal with aromatic amino acid residues of the protein (Oesterhelt & Stoeckenius, 1971; Lewis, Spoonhower, Bogomolni, Lozier & Stoeckenius, 1974). The protein is arranged in the membrane patches in a planar highly ordered hexagonal lattice. The bacteriorhodopsin molecules span the membrane and are all oriented in the same direction (Blaurock & Stoeckenius, 1971; Blaurock, 1975; Henderson, 1975; Henderson & Unwin, 1975; Unwin & Henderson, 1975). The orientation of bacteriorhodopsin in the membrane is visible in freeze-fracture electron-micrographs. The membrane splits along an

interior plane and the outer lamella shows a smooth fracture face, the inner lamella a hexagonal array of particles, presumably representing clusters of bacteriorhodopsin molecules which adhere to the cytoplasmic side of the membrane during fracturing (Blaurock & Stoeckenius, 1971; Stoeckenius, 1976)

In intact cells photon energy absorbed by the purple membrane is converted to other forms of energy that can be used by the cell, e.g. the chemical free energy of ATP (Danon & Stoeckenius, 1974). The chromophores of neighboring bacteriorhodopsin molecules are apparently close enough together for exciton interaction to occur as evidenced by band splitting in the visible CD spectra of isolated purple membrane (Heyn, Bauer & Dencher, 1975; Becher & Ebrey, 1976). In the light energy conversion the first cellular storage form of the energy, which has been unequivocally identified, is an electrochemical gradient generated by the light-driven outward translocation of protons across the cell membrane of *H. halobium* (Oesterhelt & Stoeckenius, 1973). Apparently, bacteriorhodopsin mediates this translocation, i.e. it functions as a light-driven proton pump.

Isolated purple membrane, unlike most other isolated membrane preparations, does not form closed vesicles but exists only in the form of rather stiff membrane sheets (Blaurock & Stoeckenius, 1971). This is probably due to the presence of the planar hexagonal bacteriorhodopsin lattice; the relatively low lipid content of the purple membrane may also contribute to this phenomenon. In the light bacteriorhodopsin undergoes a cyclic photoreaction, which is accompanied by a release and uptake of protons (Lozier, Bogomolni & Stoeckenius, 1975). The kinetics of the photoreaction cycle and the sensitivity and response time of pH measurements with a glass electrode do not allow detection of the proton release and uptake by isolated purple membrane with conventional techniques unless very high light intensities are used or unless the kinetics of the photoreaction cycle are artificially altered so that the deprotonated intermediates accumulate. When this occurs a visible "bleaching" of the preparation is observed. The protons are released on the outer surface and taken up on the cytoplasmic surface of the purple membrane (Lozier, Niederberger, Bogomolni, Hwang & Stoeckenius, 1976). Therefore, when vesicles are formed from the purple membrane sheets and a preferential orientation of the bacteriorhodopsin molecules across the membrane is maintained, protons are translocated across the vesicle wall. The back diffusion is slow enough, so that even at low light intensities an easily detectable change in the pH of the suspension occurs when it is exposed

to light (Racker & Stoeckenius, 1974). In this case the bacteriorhodopsin does not bleach to a significant extent and the pH changes occur more slowly and are much larger than in isolated purple membrane sheets. This response is, therefore, characteristic for preparations where the purple membrane forms or is part of the wall which separates two aqueous phases and where it has retained a preferential orientation across the wall.

## Materials and Methods

### *Materials*

Growth of *Halobacterium halobium* $R_1$ and isolation of purple membrane have been described (Oesterhelt & Stoeckenius, 1974). Asolectin from Associated Concentrates was purified as described by Kagawa and Racker (1971). Soybean phosphatidylcholine was a gift from Dr. H. Genthe (Nattermann & Cie, GmbH).

Cholic acid and deoxycholic acid from Sigma were decolorized with activated charcoal and recrystallized twice from 80% ethanol. Amberlite XAD-2 from BDH Chemical Limited was washed before use as described by Holloway (1973). Triton X-100 and cetyltrimethylammonium bromide (CTAB) were all purchased from Sigma Chemical Co. $^{14}$C-cholate and $^{14}$C-deoxycholate were obtained from ICN Isotope and Nuclear Div.; valinomycin from Calbiochem; carbonyl cyanide *p*-trifluoromethoxyl phenylhydrazone (FCCP) from Pierce Chemical Co.; methyl red from General Chemical Co.; and α-D-glucose-1-phosphate, dipotassium salt and Sephadex G-50-80 from Sigma Chemical Co. All other chemicals were reagent grade commercial preparations.

### *Methods*

#### Analytical Techniques

Bacteriorhodopsin concentrations were determined from the absorbance at 570 nm assuming a molar extinction coefficient of 63,000 $1 \cdot mole^{-1} \cdot cm^{-1}$. Lipid concentrations of vesicle preparations were calculated from phosphorus content determined with the Fiske-Subbarow method (1925). Purple membrane lipid was estimated from the phosphorus content and the known lipid composition of the purple membrane (Kushwaha, Kates & Martin, 1975; Kushwaha, Kates & Stoeckenius, 1976).

The total lipids from the isolated purple membrane, deoxycholate-treated purple membrane, and the reconstituted purple membrane vesicles were extracted by the method of Bligh and Dyer (1959) and chromatographed on Whatman SG-81 with diisobutyl ketone/acetic acid/water (40:25:5, v/v) for 22 hr (Kates, Palameta, Joo, Kushner & Gibbons, 1966).

#### pH Measurements in Vesicle Suspensions

All experiments were carried out on 1-ml samples contained in a thermostatted chamber. The temperature was kept at 30 °C unless otherwise indicated. A 250 W Leitz Prado slide

projector equipped with additional heat filters served as a light source; its intensity was varied by the insertion of neutral density filters. Light intensities were measured with a Kettering Radiant Power Meter probe inserted into the empty sample chamber. The pH was monitored with a Beckman model 39003 glass electrode and Hewlett Packard 680 strip chart recorder. Proton concentration changes were calculated from calibrating additions of standard $10^{-3}$ N KOH.

## Electron-Microscopy

For freeze-fracturing a Balzers BA510 unit was used essentially as described by Moor and Mühlethaler (1963) with 30% glycerol as cryoprotective agent. Shadowed specimens were prepared as described by Stoeckenius and Rowen (1967). For negative staining we used 2% phosphotungstic acid or 1% uranium acetate. Samples prefixed with 2% glutaraldehyde and post-fixed with oxmium tetroxide were used for critical point drying in a Bomar SPC-9-0. The preparations were viewed in a Siemens Elmiskop 1 or 101.

## Preparations of Purple Membrane Vesicles

(a) *Deoxycholate/cholate technique.* 10 mg purple membrane is suspended in 1 ml 10% deoxycholate in distilled water and allowed to stand at room temperature for two days. This suspension is then layered on top of 20 to 60% (w/v) sucrose density gradients and centrifuged at $120{,}000 \times g$ for 20 hr and the purple band, ~1 ml, is collected.[1]

The purple membrane collected from the sucrose density gradient and a sonicated suspension of 8 mg/ml lipid in 2% cholate and 75 mM $K_2SO_4$ are mixed and dialyzed against 1,000 ml 75 mM $K_2SO_4$ for 48 hr with changes of the dialysis solution after 8 hr and 24 hr.One gram hydrated and washed Amberlite XAD-2 is added to the last change of dialysis solution. To check the removal of cholate, 5 μCi $^{14}$C-cholate was added and 10 μl aliquots were withdrawn and counted during the dialysis. A 1.5 ml sample of the dialysate is then layered on an 11-ml 0–45% sucrose density gradient in 75 mM $K_2SO_4$ (or in 75 mM $Na_2SO_4$ or 150 mM KCl) and centrifuged at $120{,}000 \times g$ for 20 hr. The purple-colored band is collected and dialyzed again against 75 mM $K_2SO_4$ to remove the sucrose. In some experiments 75 mM $Na_2SO_4$, 150 mM KCl or 150 mM NaCl was used instead of 75 mM $K_2SO_4$ throughout the procedure.

(b) *Cholate technique.* This technique was used as described by Racker and Stoeckenius (1974).

(c) *Sonication technique.* The technique for preparation of purple membrane vesicles is also used essentially as described (Racker, 1973) with the following modifications. The dried lipid is first sonicated alone and lipid vesicle formation is monitored by the light scattering at 500 nm (Miyamoto & Stoeckenius, 1971). Scattering decreases and becomes constant after about 30 min of sonication. The purple membrane is then added and sonication continued for a short time as described in Results.

(d) *Triton technique.* The procedure is based on a technique used by R. Henderson to obtain large membrane sheets (*personal communication*). 10 mg purple membrane in

1 Material remaining in the upper part of the gradient appears slightly orange and spectroscopy reveals bacterioruberin and the Soret band of cytochromes. This indicates a contamination of the isolated purple membrane with ~1% of other cell membrane, the "red membrane" (Oesterhelt & Stoeckenius, 1974).

$H_2O$ is centrifuged at $40,000 \times g$ for 40 min. The pellet is resuspended in 0.4 ml 5% Triton X-100 in 0.1 N acetate buffer, pH 5.0, and allowed to stand at room temperature for two days. The suspension is then centrifuged at $100,000 \times g$ for 30 min, the pellet, if any, discarded and the supernatant dialyzed at 4 °C for three weeks against 0.1 N acetate buffer, pH 5.0, containing Amberlite XAD-2. The resultant suspension is centrifuged through a 0–45% (w/v) sucrose density gradient in 0.1 N acetate buffer, pH 5.0, at $120,000 \times g$ for 40 hr. The purple-colored band is collected and the sucrose removed by dialysis.

### pH Determination with Methyl Red Indicator Dye

Purple membrane vesicles were prepared by technique (c) with asolectin in 75 mM $K_2SO_4$, and methyl red was added to the vesicle suspension to a final concentration of 0.083 mg/ml. To measure pH changes in the interior volume the vesicle suspension with added dye was sonicated and the exterior dye removed through chromatography on a Sephadex G-50-80 (Sigma Chemical Co.) column with 75 mM $K_2SO_4$. 1 mM D-α-glucose-1-phosphate was added as a buffer for the outside solution to reduce the pH change in the medium and therefore the color change of the indicator dye leaking out. The absorption spectra were recorded with a modified Cary 14 scanning spectrophotometer which allowed actinic illumination at right angle to the measuring beam. A 250 W arc lamp (General Electric Co.), filtered through 7 cm of water, two Corning infrared filters (1-75), a Corning yellow cut-off filter (3–69) and two Corning red cut-off filters (2–61 and 2–62) served as an actinic light source. The photomultiplier was protected with two Corning 5–56 blue broadband filters. Spectra were scanned from 580 to 370 nm and stored in a Nicolet 1074 computer. Difference spectra were calculated by the computer and plotted with an X-Y recorder (Hewlett Packard 7004B).

## Results

### *Detergent Treatment of Purple Membrane*

When the purple membrane is exposed to 10% DOC, the sheet-like structure of the membranes apparently remains intact. The material sediments readily at $40,000 \times g$ and electron-microscopy of shadowed preparations from the pellet show sheets indistinguishable from untreated preparations (Blaurock & Stoeckenius, 1971; Oesterhelt & Stoeckenius, 1974). Negatively stained preparations reveal the protein lattice more clearly than it is usually seen in untreated membrane (Fig. 1). For optimal results the electron-microscopy requires the removal of most of the DOC, and reaggregation of the membrane and reformation of the lattice could have occurred. However, X-ray diffraction patterns of purple membrane in 10% DOC after exposure for 48 hr at room temperature also clearly show the presence of the lattice. Some broadening of the reflection and small intensity changes do occur (G.I. King and W. Stoeckenius, *unpublished*). A further test for the integrity of the lattice is the band splitting

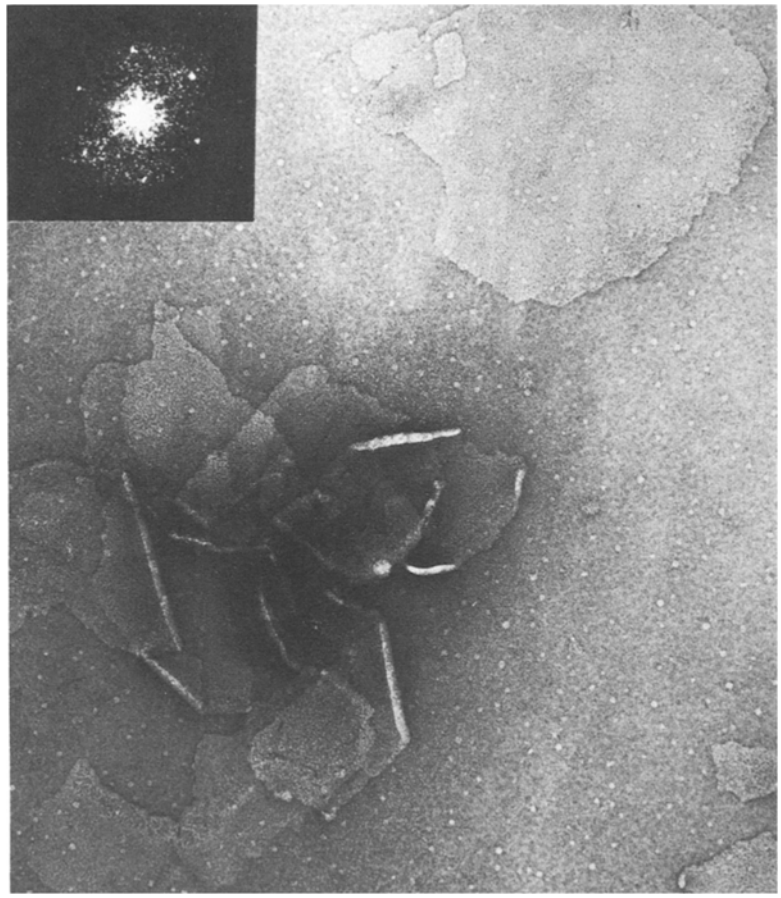

Fig. 1. DOC-treated purple membrane negative stained with 1% uranium acetate. *Inset:* Light diffraction pattern shows presence of the two-dimensional hexagonal lattice in the membrane. Magnification: 60,100×

seen in the visible region of CD spectra (Fig. 2). It is due to exciton interaction between chromophores and disappears when the protein lattice dissociates (Heyn *et al.*, 1975; Becher & Ebrey, 19776). The energy coupling between chromophores is still preserved in 10% DOC-treated purple membrane after 48 hr (Fig. 2). The α-helix content of the protein calculated from the UVCD also remains the same.

While the morphology of the purple membrane sheets is apparently well preserved during the DOC treatment, the lipid composition changes. Differential centrifugation in the presence of detergent or density gradient centrifugation of the membrane suspension in detergent separates most of the native lipid from the membrane sheets. The buoyant density of

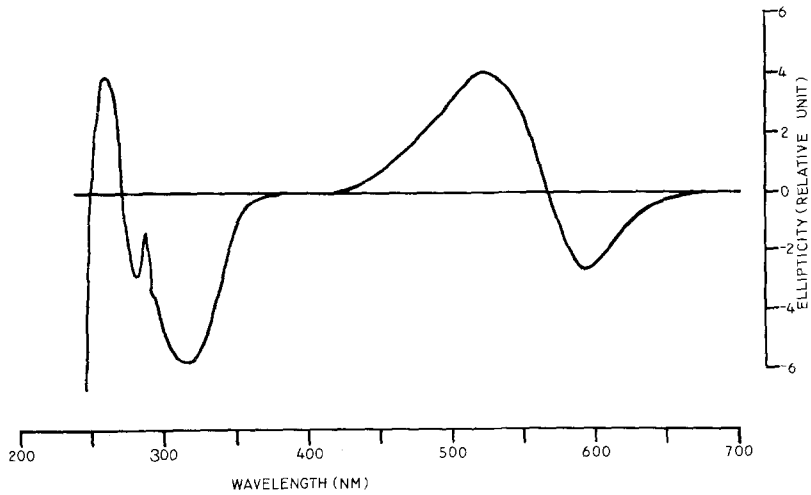


Fig. 2. CD spectrum of the purple membrane (10 mg/ml) treated with 10% DOC for 48 hr recorded at 25 °C with a modified JASCO SS-10. The sample was light-adapted before the measurement. The spectrum is indistinguishable from that of the native membrane. CD scale: $2 \times 10^{-3}$ degrees/cm; cell length: 2 mm

untreated purple membrane containing 24% lipid is 1.18 g/ml. DOC-treated membrane has a buoyant density of 1.21 g/ml and ~80% of the lipid phosphorus remains on top of the gradient. The buoyant density indicates that some of the lipid in the membrane has been replaced by DOC. Paper chromatography of lipids extracted from DOC-treated membrane indicates that 10% DOC extracts all membrane lipids to roughly the same extent (Fig. 3).

The DOC-treated purple membrane has an absorption maximum at 562 nm for the light-adapted and from 560 to 555 nm for the dark-adapted pigment.[2] Such small blue shifts occur in all detergent-treated preparations and are also present in vesicles prepared by sonication of purple membrane with lipids in the absence of detergents. The blue shift is reversible when the detergent is removed and replaced by *H. halobium* lipids. In flash spectroscopy the photoreaction cycle of the DOC-treated membrane shows the same transient absorption changes as with intact purple membrane but with altered kinetics.

Triton X-100 has a much stronger effect on the membrane structure; after two days at room temperature bacteriorhodopsin travels in the included volume of Bio-Gel A 1.5 M column and the negative CD band

2 Dark adaptation is a small reversible blue shift and decrease in the absorption maximum which is observed when the native membrane is kept in the dark for prolonged times (Lozier *et al.*, 1975).

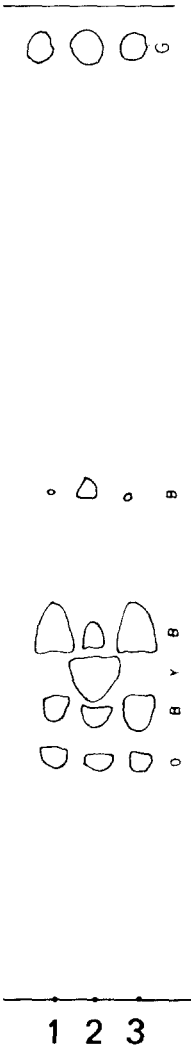


Fig. 3. Paper chromatography of lipids extracted from the intact purple membrane (*1*) DOC-treated purple membrane (*3*), and DOC/cholate vesicles prepared with soybean lecithin (*2*). Staining dye: rhodamine 6G 0.0012%. Abbreviations: *B*, blue; *Y*, yellow; *O*, orange; *G*, grey. The large additional spot in *2* (*Y*) is due to the added soybean PC. The total amount of lipid phosphorus applied was *1*, 1.23 μg; *2*, 4.36 μg; *3*, 1.36 μg

at 590 nm has disappeared indicating solubilization of the membrane. The photoreaction cycle has changed kinetics but is still functioning (Lozier *et al.*, *unpublished*). The stability of the pigment in the Triton-solubilized form is apparently decreased, but light-dark adaptation still occurs.

### *Vesicle Formation and Morphology*

Vesicles form when dispersed lipid is added to purple membrane and the dispersant is subsequently removed. Vesicle formation is indicated by a reversible pH change under illumination in the absence of visible bleaching. If this test is negative, however, it does not distinguish between failure to form vesicles and the formation of vesicles without preferred orientation of bacteriorhodopsin. We have used electron-microscopy to distinguish these cases.

Prolonged dialysis of the Triton preparation leads to reaggregation of bacteriorhodopsin into membranes, which band in sucrose gradients at the same density as intact purple membrane. The small blue shift in the absorption spectrum present in Triton is also reversed and the spectra properties are the same as in intact purple membrane. No bleaching and no pH change in the medium are observed upon illumination. However, in addition to some large crystalline sheets large vesicles are present with diameters of $\sim$ 3–10 μm. They are readily visible in the light microscope. Freeze-fracture electron-microscopy shows smooth and particle-covered areas on the same fracture face (Fig. 4) indicating that the vesicle wall consists of domains which have the bacteriorhodopsin oriented in opposite directions.

Dialysis of preparations containing purple membrane and cholate-dispersed lecithin induces a light-dependent pH response; usually an increase in the pH. During dialysis the pH response increases as the cholate concentration decreases. After 48 hr dialysis more than 90% of the cholate has been removed and the pH response has reached its maximal value (Fig. 5). Electron-microscopy shows that the preparation now consists of two vesicle populations, rather uniform small vesicles with an average diameter of 250 Å and larger vesicles of more variable size. In the following density-gradient centrifugation step the small vesicles are removed; they consist of lipid only. The larger vesicles band at a density of 1.16 g/cm$^3$ and contain all the added bacteriorhodopsin. Their buoyant density indicates that in these vesicles the ratio of lipid to protein is higher than in the native purple membrane and this is borne out by chemical analysis which shows 37.5% lipid content by weight as compared to 24% in the native membrane. If the purple membrane has been pretreated with DOC (DOC/cholate vesicles), 90% of this lipid consists of the phospholipid added with the cholate (Fig. 3).

DOC/cholate vesicles vary in size from 250 to 1400 Å with an average diameter of 850 Å (Fig. 6). The smallest vesicles may represent a contami-

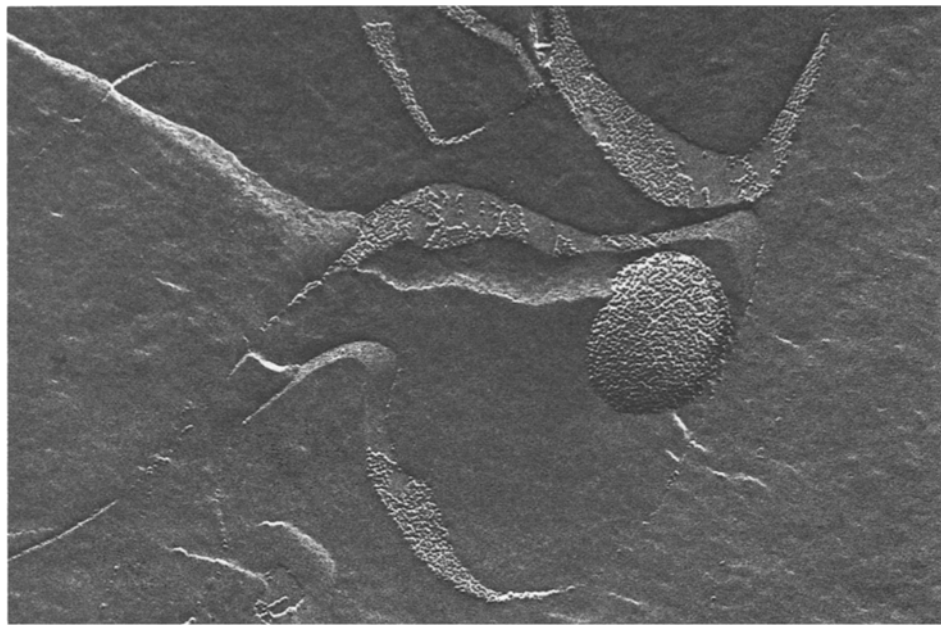

Fig. 4. Freeze-fracture electron-micrograph of vesicles prepared by Triton X-100. The two-dimensional hexagonal array of the proteins is clearly visible; however, smooth and particle-covered areas are seen on the same fracture face, indicating that the vesicle wall consists of domains which have the bacteriorhodopsin oriented in opposite directions. Magnification: 62,900×

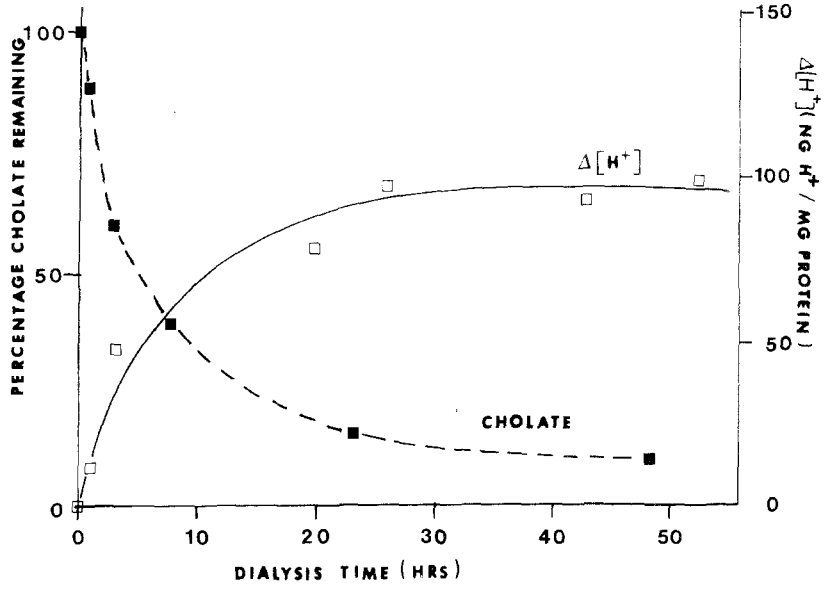


Fig. 5. Changes in the pH response to illumination during cholate removal by dialysis. $\Delta[H^+]_{max}$ is the maximal sustained proton concentration difference in the medium between the dark and the illuminated suspension. In this experiment maximal response was 100 ng ions $H^+$ per mg of bacteriorhodopsin. Light intensity: $10^6$ erg $cm^{-2}$ $sec^{-1}$

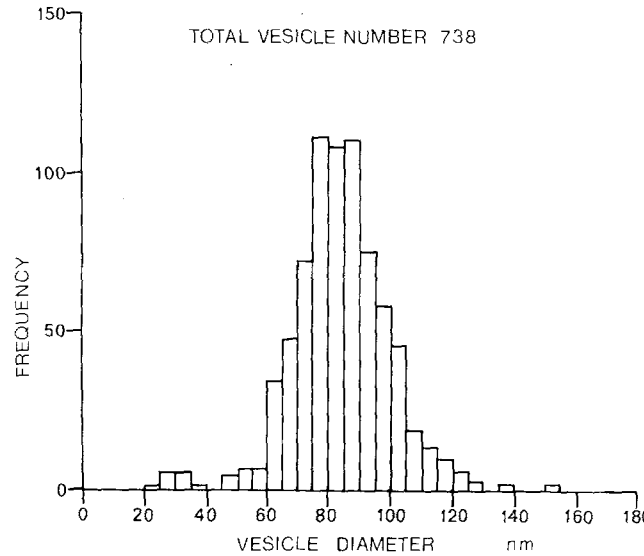


Fig. 6. Size distribution of DOC/cholate vesicles obtained from electron-micrographs of a critical point dried preparation

nation with residual pure lipid vesicles. Freeze-fracture electron-micrographs show a dense particle population on the concave fracture faces and only few scattered particles on the convex faces. This suggests that the bacteriorhodopsin in the vesicles is predominantly oriented inside-out with respect to intact cells, i.e. the cytoplasmic surface faces the medium (Fig. 7). The particles are closely packed on the concave fracture faces, but they do not form a regular lattice comparable to that in isolated purple membrane or whole cells. This decrease in the order of bacteriorhodopsin organization is confirmed by X-ray diffraction diagrams of the preparation which show no sharp reflections and by CD spectra which show no negative component in the 570 nm absorption band region.

DOC/cholate vesicles prepared with soybean PC have an absorption maximum at 550 nm in the dark-adapted preparation; a small reversible red shift to 560 nm and a decrease of the absorption maximum occurs upon illumination. Small changes in the kinetics of the photoreaction cycle compared to intact purple membrane have also been observed (Lozier *et al.*, 1976). The absorption spectrum of DOC/cholate vesicles with soybean PC is pH-dependent, a 480 nm absorbing species is formed reversibly at alkaline pH. A titration curve shows a pK of $\sim$7.5 for this transition (Lozier *et al.*, 1976).

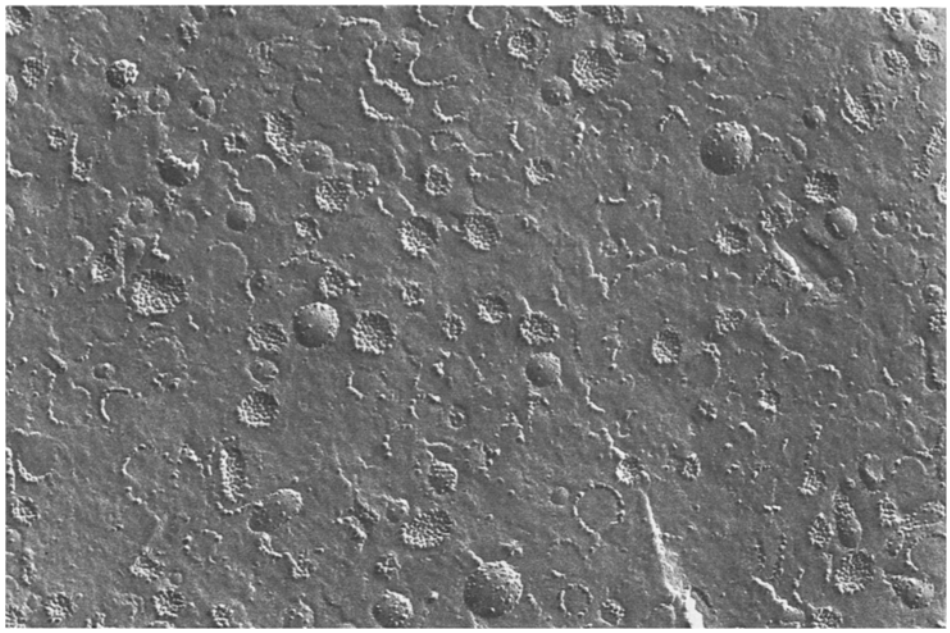

Fig. 7. Freeze-fracture electron-micrograph of purple membrane vesicles prepared with DOC/cholate technique and soybean phosphatidyl choline. A dense particle population is seen on concave fracture faces and only a few scattered particles on the convex faces. Magnification: 83,200×

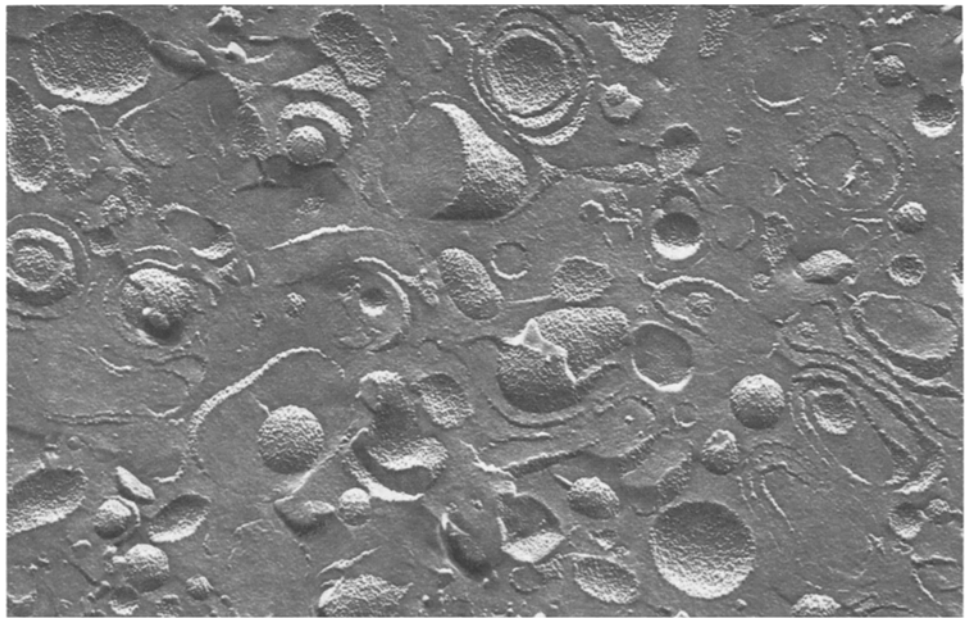

Fig. 8. Freeze-fracture electron-microscopy of purple membrane vesicles prepared in 150 mM KCl and 50 mM $MgCl_2$ with the cholate technique and asolectin. Particles are distributed randomly over both fracture faces. Magnification: 51,100×

Compared to DOC/cholate vesicles electron-micrographs of the cholate vesicles show a larger variation in size, less preferential particle distribution to the concave fracture faces and many vesicles are multilamellar. The degree of preferential bacteriorhodopsin orientation in these preparations varies with the salt concentration used. This aspect has not been explored systematically. However, using 150 mM KCl and 50 mM $MgCl_2$ we have obtained cholate vesicles which gave no pH response and which showed a completely random particle distribution over both fracture faces (Fig. 8).

Purple membrane vesicles can also be formed without the use of detergents by sonication of a mixture of purple membrane and lipids in aqueous suspension (Racker, 1973). We prefer to presonicate the lipid until light scattering reaches a minimum and then add the purple membrane and continue to sonicate for a few minutes. Sonicating purple membrane without added lipids present breaks up the native membrane sheets into smaller fragments. This process can be followed by the decrease in the light scattering of the suspension and by electron-microscopy. The light scattering decreases up to 6 min of sonication and then remains constant up to $>15$ min. At 6 min the average size of the purple membrane fragments has decreased from $\sim 0.5\,\mu m$ to $\sim 0.1\,\mu m$. The absorption spectrum remains unchanged for the first 6 min of sonication; only the light scattering is reduced. At longer times the 570 nm absorbance decreases, indicating a beginning destruction of the chromophore.

Preparations of presonicated lipid sonicated for up to 10 min after adding purple membrane still show a purple band on sucrose gradients, which has the buoyant density of the native purple membrane (1.18 mg/ml) and purple color is also spread out through the gradient in a diffuse band between densities 1.06 to 1.16 mg/ml. The 1.18 mg/ml purple band decreases with increasing sonication time and the amount of purple membrane of buoyant density, 1.16 mg/ml, increases. Apparently the vesicle formation is not very efficient, because purple membrane fragments without incorporated additional lipid are still present, when part of the bacteriorhodopsin has already been damaged by sonication. The maximal pH response in the sonicated suspension is reached after 7 min of sonication and then decreases. We have not established whether this decrease is due to a decrease in vesicle size, denaturation of bacteriorhodopsin or other causes.

With all four techniques the properties of the vesicles show no strong dependence on the lecithin used in their preparation, asolectin, purified soybean lecithin, dielaidyl lecithin all yield active preparations. However,

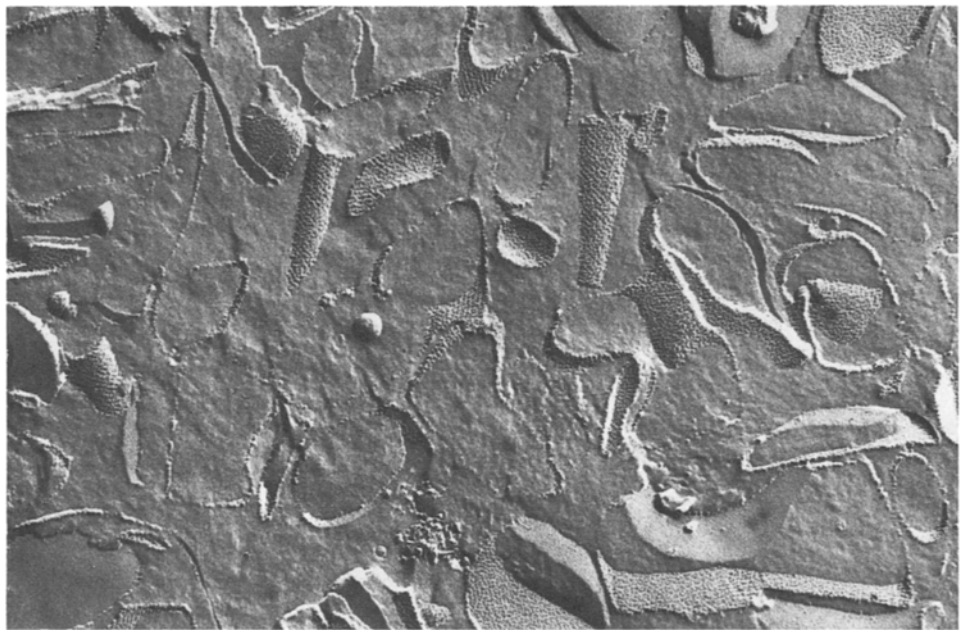

Fig. 9. Freeze-fracture electron-micrograph of purple membrane vesicles prepared by the DOC/cholate technique using the total lipid extract from *Halobacterium halobium*. The vesicles formed are not spherical; many are cylindrical, show flat sides and straight edges. No preferential orientation of particles with respect to the inner and outer fracture faces is apparent, the planar hexagonal lattice is retained. Magnification: 51,100×

as already noted by Racker (1973) when *H. halobium* lipid is used as the added lipid, only relatively small pH responses are obtained. In our hands the responses were even smaller or absent altogether. The reason for this becomes obvious when freeze-fracture electron-micrographs are inspected (Fig. 9). The vesicles formed with *H. halobium* lipid are not spherical; many are cylindrical, or show flat sides and straight edges. No preferential orientation of particles with respect to the inner and outer fracture face is apparent; the in-plane lattice order, however, is very high. The vesicle walls apparently consist of crystalline domains which are oriented inside-out and right-side out with approximately equal frequency.

### *The pH Response*

Comparing the different vesicle preparations we find that the deoxycholate/cholate technique consistently yields the largest pH responses.

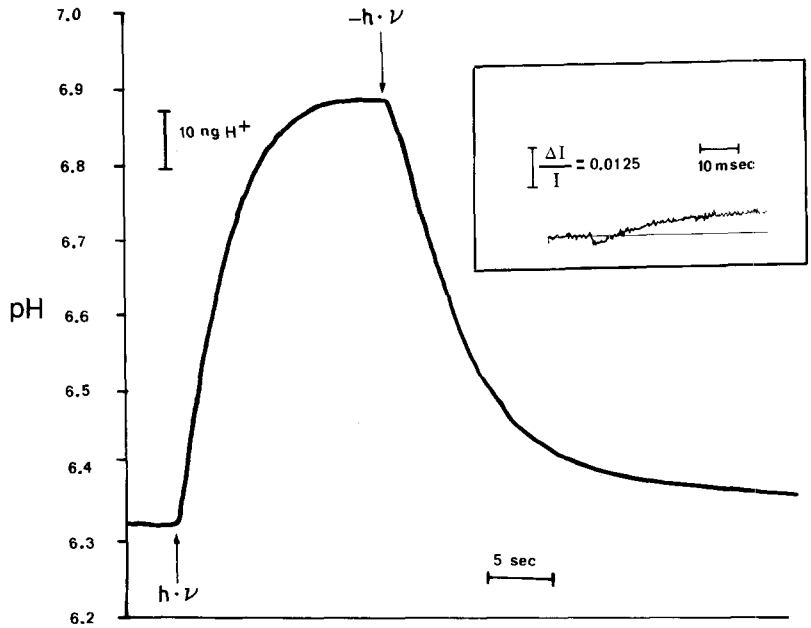


Fig. 10. Typical pH response of reconstituted purple membrane vesicles prepared by DOC/cholate technique with-purified soybean PC in 75 mM $K_2SO_4$; bacteriorhodopsin concentration $\approx 50$ μg $ml^{-1}$. Illumination of the vesicle suspension results in a reversible pH increase of more than 0.5 pH units. The pH is maintained during illumination and decays with half-time $\sim 4.5$ sec when the light is turned off. Light intensity: $10^6$ erg/$cm^2$ sec. The inset shows pH changes after a single microsecond light flash, recorded as absorbance changes of an indicator dye. The initial down deflection of the trace indicates a small rapid acidification, which is followed by a larger sustained alkalinization (from Lozier *et al.*, 1976)

Individual preparation when normalized for bacteriorhodopsin content do not vary by more than $\pm 15\%$ in the extent of the response measured as the difference in proton concentration in the medium between illuminated and dark state, $\Delta[H^+]_{max}$. A typical pH response of vesicles prepared by the DOC/cholate technique is shown in Fig. 10. Illumination of the vesicle suspension with nearly saturating light results in a pH increase of more than 0.5 pH units, which is maintained during illumination and decays when the light is turned off. The size of the response is a function of light intensity (Fig. 11) and bacteriorhodopsin concentration. At the highest light intensities used the proton concentration in the medium decreases by 35 $H^+$ per bacteriorhodopsin molecule. The effect is sensitive to uncouplers, but much higher concentrations are required than in intact cells. Addition of valinomycin has only a small effect but makes the preparation much more sensitive to the action

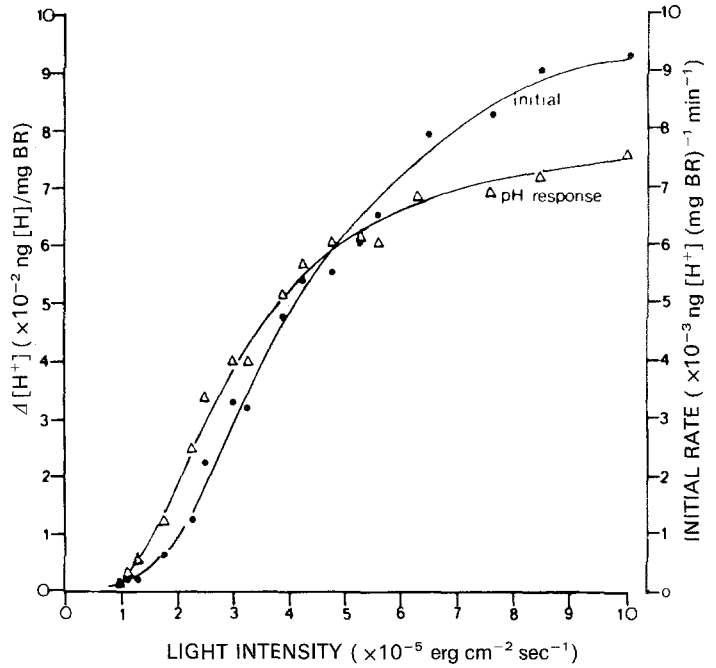


Fig. 11. The initial rate and the size of the response of the reconstituted purple membrane vesicles prepared by the DOC/cholate technique with purified soybean PC in 75 mM $Na_2SO_4$. The initial rate and the size of the pH response are direct functions of light intensity and approach saturation at $\sim 10^6$ erg/cm$^2$ sec

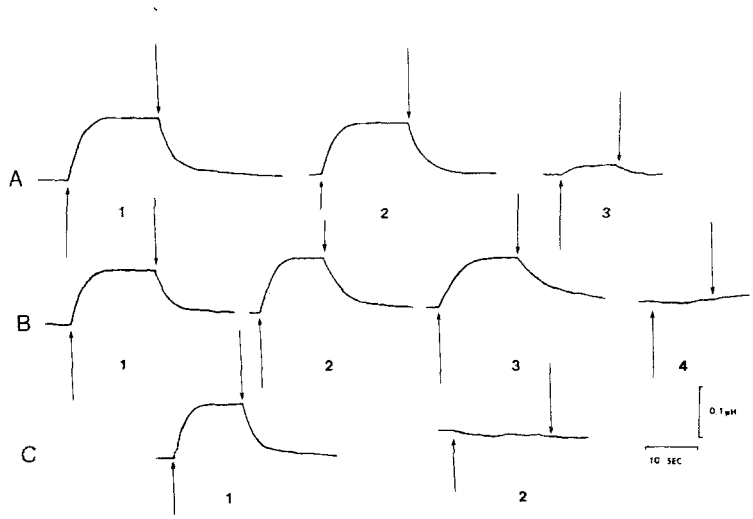


Fig. 12. The effects of FCCP, valinomycin and nigericin on the pH response of the purple membrane vesicles. The vesicles were prepared by the cholate technique with asolectin. *A1*: control; *A2*: after addition of $10^{-6}$ M FCCP; *A3*: after $10^{-5}$ M FCCP; *B1*: control; *B2*: after $10^{-8}$ M valinomycin; *B3*: after $10^{-6}$ M valinomycin; *B4*: after $10^{-6}$ M valinomycin and $10^{-6}$ M FCCP; *C1*: control; *C2*: after $2 \times 10^{-7}$ M nigericin. Arrows indicate the beginning and end of illumination

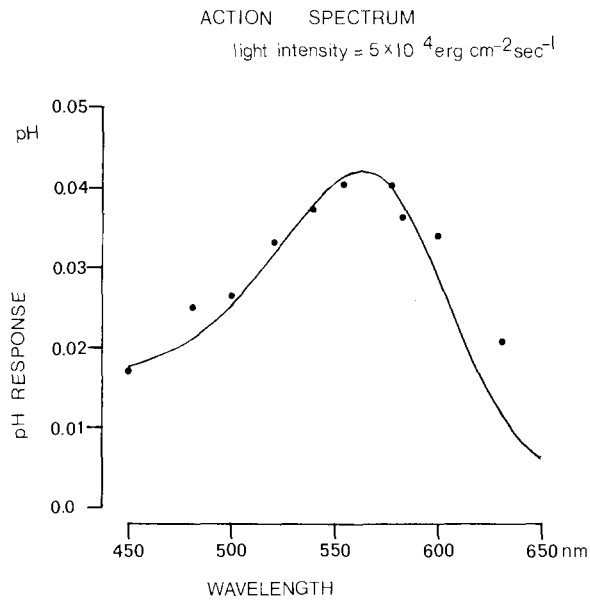


Fig. 13. Action spectrum of the pH response in purple membrane vesicles prepared by the DOC/cholate technique with dipalmitoyl PC in 75 mM $K_2SO_4$ at 50 °C. Light of different wavelengths was selected by the use of 15 nm bandwidth interference filters

of uncouplers. Nigericin is as effective in abolishing the response as valinomycin and FCCP combined (Fig. 12).

The extent of the pH changes strongly suggests that they are due to a light-driven translocation of protons from the medium to the vesicle interior, which as the action spectrum shows is mediated by bacteriorhodopsin (Fig. 13). The direction of translocation is consistent with the pigment orientation seen in the freeze-fracture electron-micrographs. The action of uncouplers further strengthens this argument, because at the concentrations used here they have no effect on the photoreaction cycle of bacteriorhodopsin (Oesterhelt & Stoeckenius, 1973; R.H. Lozier and W. Stoeckenius, *unpublished*). Finally we can directly demonstrate the acidification of the vesicle interior with an indicator dye for which the vesicles have a low permeability. When methyl red is present in the medium it indicates an alkalinization upon illumination (Fig. 14); when it is present in the interior of the vesicles it indicates acidification (Fig. 15).

The kinetics of the pH response are complex; for the DOC/cholate vesicles we find at least two simultaneous first-order components when we plot the pH rise on a semilogarithmic scale. The fast component has the opposite sign of the main component and appears as an efflux

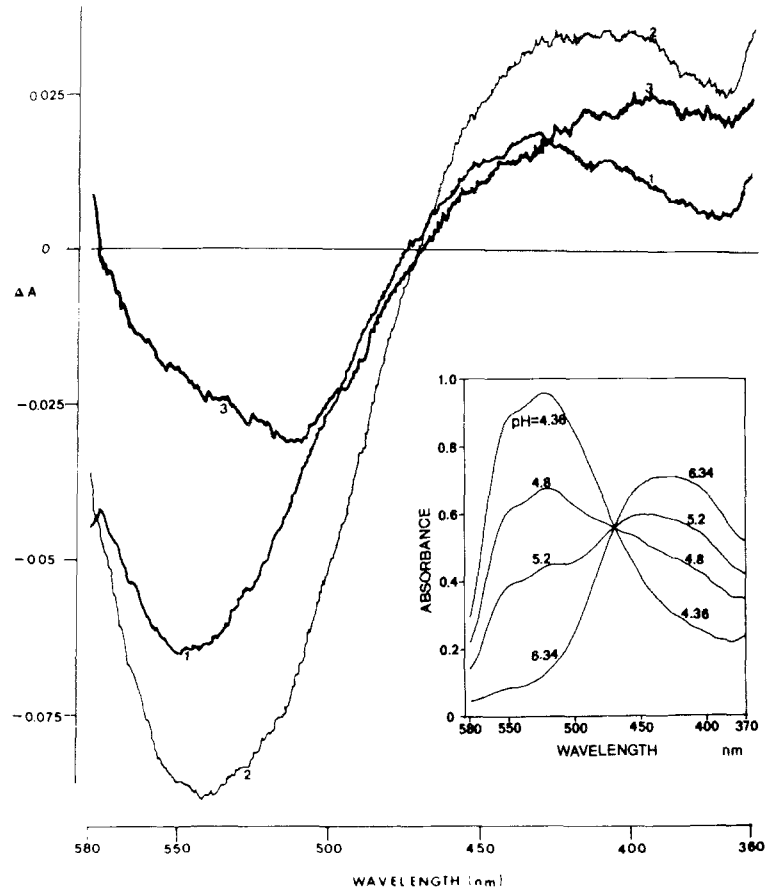


Fig. 14. Color change of methyl red in the exterior medium of the vesicle suspension. Vesicles were prepared by the sonication technique with 24 mg asolectin and 2.2 mg purple membrane in 2.0 ml 75 mM $K_2SO_4$. Curve (*1*) shows the difference spectrum of the vesicle with and without illumination. The color change is due to the photosteady-state accumulation of 412 nm intermediate. Curve (*2*) shows the difference spectrum of the vesicle with methyl red with and without illumination; the color change is due to the accumulation of 412 nm intermediate and the color change of the dye caused by the pH change in the exterior medium. Curve (*3*) is the difference spectrum of curve (*2*) minus curve (*1*), showing the color change of the dye only, caused by the pH change of the exterior medium. The decrease in absorbance around 520 nm indicates an alkalinization of the exterior medium occurs during illumination. pH: 6.23; light intensity: $4 \times 10^5$ erg/cm$^2$ sec; path length: 1 cm; concentration of bacteriorhodopsin: 0.37 mg/ml; concentration of methyl red: 0.083 mg/ml. The inset shows the absorption spectra of the indicator dye at different pH values

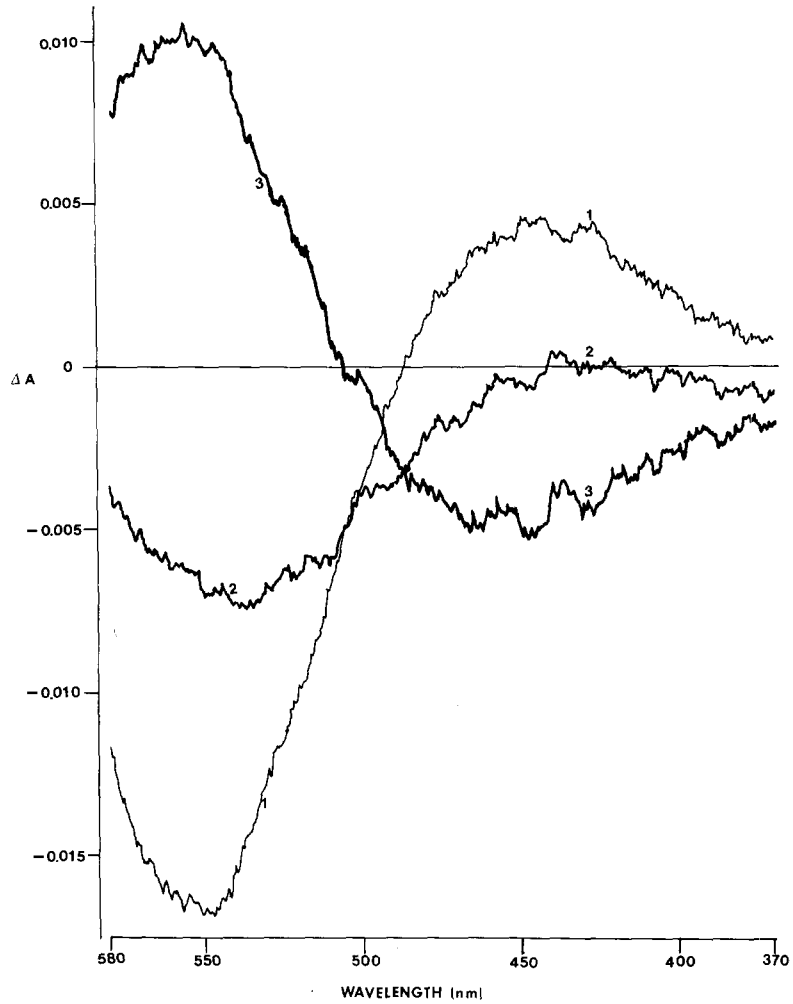


Fig. 15. Color change of methyl red in the interior of the vesicle. Preparation as in Fig. 14; after addition of methyl red the suspension was sonicated and the dye was removed from the exterior medium by passing the vesicle suspension through Sephadex G-50-80. Curve (*1*) is the difference spectrum of the vesicles (without dye) in the light and in the dark. This shows the color change due to the photosteady-state accumulation of 412 nm intermediate. Curve (*2*) is the difference spectrum of vesicles in the light and in the dark with dye in the vesicle interior. This shows the sum of the color changes due to accumulation of 412 nm intermediate and the dye color change in the interior medium. Curve (*3*), the difference spectrum of curve (*2*) minus curve (*1*), therefore shows the dye color change due to the pH change of the vesicle interior. The change is opposite to that in Fig. 14, indicating acidification of the vesicle interior. pH: 6.2; light intensity: $4 \times 10^5$ erg/cm$^2$ sec. Path length: 0.1 cm; concentration of bacteriorhodopsin: 0.37 mg/ml; concentration of methyl red in the vesicle interior: 1.0 mg/ml

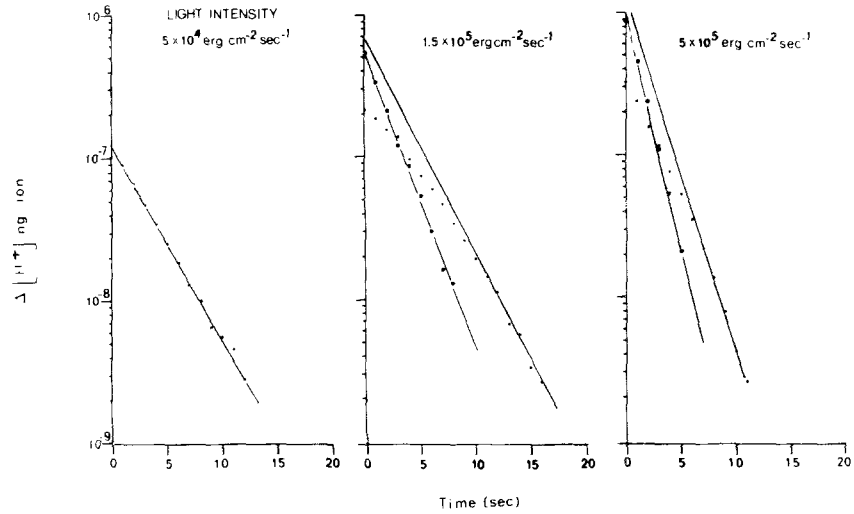


Fig. 16. Semilogarithmic plot of the pH rise at different light intensity. The purple membrane vesicles were prepared with soybean PC by the DOC/cholate technique in 75 mM $K_2SO_4$ at 4 °C and the pH response was recorded at 30 °C. The kinetic analysis was done by the curve peeling method (Shipley & Clark, 1972). The fast component was obtained by subtracting the real data value from the extrapolated value. Bacteriorhodopsin concentration: ~50 μg/ml

of protons (Fig. 16). The decay of the pH gradient after the light has been turned off is even more complex and can be resolved into at least three first-order processes (Fig. 17). The initial transient fast component may not be completely time resolved; it indicates a brief fast uptake of protons after the light has been turned off. Because after absorption of a photon bacteriorhodopsin first releases a proton on the outer surface of the membrane and only later takes up a proton on the cytoplasmic side, and because these are dark reactions (Lozier *et al.*, 1976), we may tentatively attribute the initial fast proton uptake after the light has been shut off to the main fraction of bacteriorhodopsin molecules which are oriented inside-out and which are completing their photoreaction cycle in the dark. Similarly, the inflection seen at the beginning of the pH rise may be attributed to the first fast release of protons from the fraction of bacteriorhodopsin molecules which is oriented in the direction opposite to the majority of molecules which dominate the response at later times. In experiments with better time resolution, this initial release by the misoriented bacteriorhodopsin has been observed as a fast initial acidification (Fig. 10 inset). The remaining kinetic components cannot be conclusively interpreted at this time and will require further study. The other vesicle preparations show similarly complex kinetics.

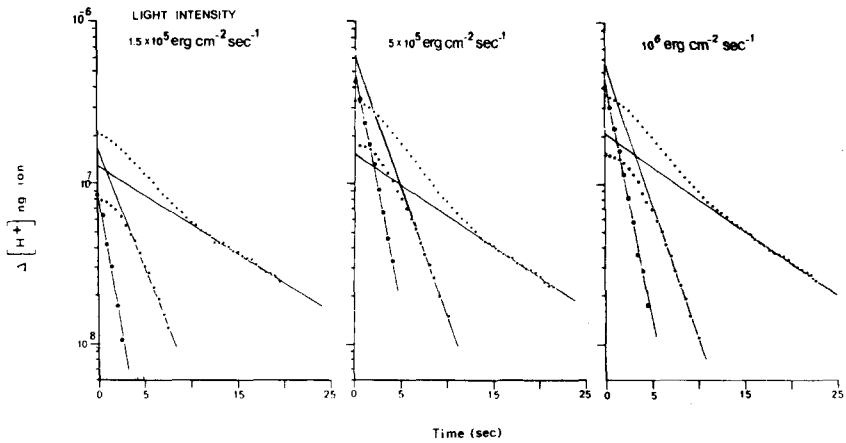


Fig. 17. Semilogarithmic plot of the decay of the pH response. The preparation and assay conditions were the same as in Fig. 16. The fastest component is plotted with reversed sign; it indicates an acidification

Comparing the steady-state values for the pH response of cholate vesicles to that of DOC/cholate vesicles, we find a much smaller response in cholate vesicles amounting only to 3–4 protons/molecule of bacteriorhodopsin, less than one-tenth the value found in DOC/cholate vesicles. This is only partly due to a greater permeability of the cholate vesicles to protons; the half-times for the decay of the proton gradient are 4.3 sec for DOC/cholate and 2.4 sec for cholate vesicles, whereas the initial rates of proton uptake determined from the slope of the pH rise extrapolated to the zero time (and ignoring the initial inflection) are 160 ng $H^+$ $sec^{-1}$ $mg^{-1}$ protein for deoxycholate/cholate vesicles *vs.* ~50 ng $H^+$ $sec^{-1}$ $mg^{-1}$ protein for cholate vesicles and ~80 ng $H^+$ $sec^{-1}$ $mg^{-1}$ for the sonicates vesicle. This result agrees with the more uniform orientation of bacteriorhodopsin seen in freeze-fracture electron-micrographs of DOC/cholate vesicles. However, other factors which may contribute such as the size of the vesicles and differences in the kinetics of the photoreaction cycle have not been analyzed in detail.

## Discussion

The results are summarized in Table 1. The amount of protons disappearing from the medium when the more active preparations are illuminated makes it unlikely that the pH changes are caused by protonation changes of the protein and/or lipid of the vesicle wall. In addition we

Table 1.

| | Action of dispersing agent | Effect of added lipid | Vesicle morphology | pH response |
|---|---|---|---|---|
| DOC/cholate +DPC | removes lipids, membrane fragments intact | exchanges for membrane lipid | ~800 Å diameter ~80% inside-out, no lattice | large, e.g. 160 ng ion $H^+$/ sec/mg protein |
| DOC/cholate +polar lipids from *H. halobium* | removes lipids, membrane fragments intact | exchanges for membrane lipid | larger irregular shape, crystal lattice present, no preferential orientation | small or absent |
| Sonication +DPC | produces smaller membrane fragments, destruction of bacteriorhodopsin after 6 min | exchanges for membrane lipid | large size variation, no lattice, preferentially inside-out, often multilayered | large, e.g. 80 ng ion $H^+$/ sec/mg protein |
| Triton X-100 (no added lipid) | solubilizes membrane, destabilizes pigment | | 3–10 μ diameter, crystal lattice present, no preferential orientation | negligible |
| Cholate +DPC | | exchanges for membrane lipid | variable size and orientation depending on ionic strength, no lattice | medium or small |

show here that alkalinization of the medium is accompanied by acidification of the vesicle interior. The action of uncouplers alone or in the presence of other ionophores further emphasizes this point because they are without effect on the light-induced protonation changes in isolated purple membrane, at least in the concentrations used here (Oesterhelt & Stoeckenius, 1973). The action of these ionophores also strongly suggests that the light-driven proton translocation is an electrogenic process; a conclusion for which more direct evidence has already been published on similar model systems (Drachev, Jasaitis, Kaulen, Kondrashin, Liberman, Nemecek, Ostroumov, Semenov & Skulachev, 1974; Drachev, Kaulen, Ostroumov & Skulachev, 1974; Kayushin & Skulachev, 1974; Racker & Hinkle, 1974; Hwang, Korenbrot & Stoeckenius, 1977; Stoeckenius,

Hwang & Korenbrot, 1977; Yaguzhinsky, Boguslavsky, Volkov & Rakhmaninova, 1976).

The organization of bacteriorhodopsin in the vesicle wall is obviously a dominating factor determining the direction and extent of the light response. Objections could be raised against use of the freeze-fracture technique to establish bacteriorhodopsin orientation. The amount of lipid on both sides of the native membrane is approximately the same and the protein spans the membrane. Therefore, after exchange of the lipid the fracture behavior could conceivably change. The consistency of the observed pH response and the freeze-fracture topology itself is an argument against such a possibility. Furthermore, the misorientation of a small part of the bacteriorhodopsin in the DOC vesicles deduced from the electron-micrographs is confirmed by the small and rapid initial acidification seen in such preparations by flash spectroscopy in the presence of a pH indicator dye.

At present we can only speculate on the causes for vesicle formation and the preferential orientation of bacteriorhodopsin in the vesicles. When the native lipid is replaced by other phospholipids, effective formation of vesicles with preferential bacteriorhodopsin orientation only appears to take place if the protein lattice is loosened by uptake of additional lipid, but a complete dissociation of the membrane is avoided. A difference in the charge density on both sides of the membrane may be responsible for preferential closure in one direction when the membrane sheets form vesicles. It seems that in the preparations with an intact lattice only a limited curvature of the membrane is possible and that either very large vesicles are formed as in the Triton preparations or more box-like structures with relatively flat sides, as in the vesicles obtained with added *H. halobium* lipids. In both cases crystalline domains with opposite orientations occur with about equal frequency.

The observation of at least two major kinetic components not only in the rise but also the decay of the pH response suggests that they are caused by the passive permeability of the vesicles and not by the proton pump. Mitchell (1969) has shown how biphasic kinetics will result from the presence of several ionic species with different permeabilities. The data presented here do not appear promising for a more detailed analysis, partly because of additional complexity introduced by the rather large size variation in the vesicle population, partly because of the misorientation of part of the bacteriorhodopsin and because the interior volume unfortunately is rather small in the vesicles of the most uniform

size and bacteriorhodopsin orientation. Planar films of purple membrane offer better conditions for the measurement of light-induced membrane potentials (Hwang *et al.*, 1976).

Model systems of the kind described here are widely used to investigate the interaction of lipids and proteins in natural membranes. Our results demonstrate that changes in vesicle structure rather than more direct lipid protein interactions play a dominant role when transport processes are investigated and must be taken into account. The purple membrane as a model system has the advantage that the turnover of the pump can be monitored by following the spectroscopic changes during the photoreaction cycle; however, to take full advantage of this possibility the ambiguities in the interpretation of the spectral changes which presently still exist must be resolved (Lozier *et al.*, 1976), and the kinetics of these changes should provide a better indicator of lipid protein interactions, if care is taken to eliminate any possible contribution from electrochemical gradients, which should not present a serious problem.

The work reported here is part of San-Bao Hwang's Ph.D. thesis. We thank Drs. Justine Tseng, G. King and R. Lozier for supplying CD spectra, X-ray diffraction and flash spectroscopy data for some of the preparations and Dr. R.A. Bogomolni for discussion and help with some of the experiments. The work was supported by USPHS Program Project Grant HL-06285 (NHLBI) and NASA Grant NSG-7151.

## References

Becher, B., Ebrey, T. 1976. Evidence for chromophore-chromophore (exciton) interaction in the purple membrane of *Halobacterium halobium*. *Biophys. J.* **16**:100a (Abstract TH-AM-G7)

Blaurock, A.E. 1975. Bacteriorhodopsin: A trans-membrane pump containing α-helix. *J. Mol. Biol.* **93**:139

Blaurock, A.E., Stoeckenius, W. 1971. Structure of the purple membrane. *Nature New Biol.* **233**:152

Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem.* **37**:911

Danon, A., Stoeckenius, W. 1974. Photophosphorylation in *Halobacterium halobium*. *Proc. Nat. Acad. Sci. USA* **71**:1234

Drachev, L.A., Jasaitis, A.A., Kaulen, A.D., Kondrashin, A.A., Liberman, E.A., Nemecek, I.B., Ostroumov, S.A., Semenov, A.Yu., Skulachev, V.P. 1974. Direct measurement of electric current generation by cytochrome oxidase, $H^+$-ATPase and bacteriorhodopsin. *Nature (London)* **249**:321

Drachev, L.A., Kaulen, A.D., Ostroumov, S.A., Skulachev, V.P. 1974. Electrogenesis by bacteriorhodopsin incorporated in a planar phospholipid membrane. *FEBS Lett.* **39**:43

Fiske, C.H., Subbarow, Y. 1925. The colormetric determination of phosphorus. *J. Biol. Chem.* **66**:375

Henderson, R. 1975. The structure of the purple membrane from *Halobacterium halobium*: Analysis of the X-ray diffraction pattern. *J. Mol. Biol.* **93**:123

Henderson, R., Unwin, P.N.T. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature (London)* **257**:28

Heyn, M.P., Bauer, P.-J., Dencher, N.A. 1975. A natural CD label to probe the structure of the purple membrane from *Halobacterium halobium* by means of exciton coupling effects. *Biochem. Biophys. Res. Commun.* **67**:897

Holloway, P.W. 1973. A simple procedure for removal of Triton X-100 from protein samples. *Analyt. Biochem.* **53**:304

Hwang, S.-B., Korenbrot, J.I., Stoeckenius, W. 1977. Light-dependent proton transport by bacteriorhodopsin incorporated in an interface film. *Prog. Clin. Biol. Res.* (*in press*)

Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. XXV. Reconstitution of vesicles catalyzing $^{32}P_i$-adenosine triphosphate exchange. *J. Biol. Chem.* **246**:5477

Kates, M., Palameta, B., Joo, C.N., Kushner, D.J., Gibbons, N.E. 1966. Aliphatic diether analog of glyceride-derived lipids. IV. The occurrence of di-O-dihydrophytylglycerol ether containing lipids in extremely halophilic bacteria. *Biochemistry* **5**:4092

Kayushin, L.P., Skulachev, V.P. 1974. Bacteriorhodopsin as an electrogenic proton pump: Reconstitution of bacteriorhodopsin proteoliposomes generating $\Delta\psi$ and $\Delta$pH. *FEBS Lett.* **39**:39

Kushwaha, S.C., Kates, M., Martin, W.G. 1975. Characterization and composition of the purple membrane and red membrane from *Halobacterium cutirubrum*. *Can. J. Biochem.* **53**:284

Kushwaha, S.C., Kates, M., Stoeckenius, W. 1976. Comparison of purple membrane from *Halobacterium cutirubrum* and *Halobacterium halobium*. *Biochim. Biophys. Acta* **426**:703

Lewis, A., Spoonhower, J., Bogomolni, R.A., Lozier, R.H., Stoeckenius, W. 1974. Tunable laser resonance Raman spectroscopy of bacteriorhodopsin. *Proc. Nat. Acad. Sci. USA* **71**:4462

Lozier, R.H., Bogomolni, R.A., Stoeckenius, W. 1975. Bacteriorhodopsin: A light-driven proton pump in *Halobacterium halobium*. *Biophys. J.* **15**:955

Lozier, R.H., Niederberger, W., Bogomolni, R.A., Hwang, S.-B., Stoeckenius, W. 1976. 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. *Biochim. Biophys. Acta* **440**:545

Mitchell, P. 1969. Oriented chemical reactions and ion movements in membranes. *In*: The Molecular Basis of Membrane Functions. D.C. Tosteson, editor. p. 483. Prentice Hall Inc., Englewood Cliffs, N.J.

Miyamoto, V.K., Stoeckenius, W. 1971. Preparation and characteristics of lipid vesicles. *J. Membrane Biol.* **4**:252

Moor, H., Mühlethaler, K. 1963. Fine structure in frozen-etched cells. *J. Cell Biol.* **17**:609

Oesterhelt, D., Stoeckenius, W. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nature New Biol.* **233**:149

Oesterhelt, D., Stoeckenius, W. 1973. Function of a new photoreceptor membrane. *Proc. Nat. Acad. Sci. USA* **70**:2853

Oesterhelt, D., Stoeckenius, W. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *In*: Methods in Enzymology, Vol. 31, Biomembranes, Part A. S. Fleischer and R. Estabrook, editors. Academic Press, New York

Racker, E. 1973. A new procedure for the reconstitution of biological active phospholipid vesicles. *Biochem. Biophys. Res. Commun.* **55**:224

Racker, E., Hinkle, P.C. 1974. Effect of temperature on the function of a proton pump. *J. Membrane Biol.* **17**:181

Racker, E., Stoeckenius, W. 1974. Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. *J. Biol. Chem.* **249**:662

Shipley, R.A., Clark, R.E. 1972. Tracer Methods for In vivo Kinetics. Academic Press, New York & London

Stoeckenius, W. 1976. Structure of biological membranes: Bacteriorhodopsin and the purple membrane. Proceedings of a Conference held in Rome, April 14–19, 1975; Pontifical Academy of Sciences "Biological and Artificial Membranes and Desalination of Water"

Stoeckenius, W., Hwang, S.-B., Korenbrot, J. 1977. Proton translocation by bacteriorhodopsin in model systems. Nobel Symposium 34: The Structure of Biological Membranes, S. Abrahamson and I. Pescher, editors. pp. 479–496. Plenum Press, New York

Stoeckenius, W., Rowen, R. 1967. A morphological study of *Halobacterium halobium* and its lysis in media of low salt concentration. *J. Cell Biol.* **34**:365

Unwin, P.N.T., Henderson, R. 1975. Molecular structure determination by electron microscopy of unstained crystalline specimens. *J. Mol. Biol.* **94**:425

Yaguzhinsky, L.S., Boguslavsky, L.I., Volkov, A.G., Rakhmaninova, A.B. 1976. Synthesis of ATP coupled with action of membrane protonic pumps at the octane-water interfaces. *Nature (London)* **259**:494