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Proton Transport by Bacteriorhodopsin through an Interface Film

San-Bao Hwang, Juan I. Korenbrot, and Walther Stoeckenius

Department of Physiology and Biochemistry and Cardiovascular Research Institute, University of California Medical School, San Francisco, California 94143

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Summary. Interface films of purple membrane and lipid containing spectroscopically intact and oriented bacteriorhodopsin have been used as a model system to study the function of this protein. Small positive charges in surface potential (< 1 mV) are detected upon illumination of these films at the air-water interface. These photopotentials are not affected by overlaying the interface film with a thin layer (0.3 mm) of decane. However, they are dramatically increased when lipid soluble proton carriers FCCP or DNP are added to the decane. The polarity of the photopotential indicates that, in the light, positive charges are transported through the interface from the aqueous to the organic phase. The action spectrum of the photopotential is identical to the absorption spectrum of bacteriorhodopsin. Since bacteriorhodopsin molecules are oriented with their intracellular surface towards the aqueous subphase, the characteristics of the photopotential indicate that in the light bacteriorhodopsin translocates protons from its intracellular to its extracellular surface. The kinetics of the photopotential reveal that the rate and extent of proton transport are proportional both to the fraction of bacteriorhodopsin molecules excited and to the concentration of proton acceptor. The photopotentials result from changes in the ionic distribution across the decane-water interface and can be cancelled by lipid soluble anions.

Bacteriorhodopsin is a light absorbing protein present in the plasma membrane of *Halobacterium halobium*, where it forms patches of two-dimensional crystal lattice known as purple membrane (Blaurock & Stoeckenius, 1971; Henderson, 1975). *Halobacteria* under anaerobic conditions synthesize ATP in the light but not in the dark (Danon & Stoeckenius, 1974; Oesterhelt, 1975; Bogomolni, Baker, Lozier & Stoeckenius, 1976). Oesterhelt and Stoeckenius (1973) proposed that this transduction of light energy into chemical energy was mediated by

bacteriorhodopsin acting as a light-driven proton pump. The proton electrochemical gradient generated across the membrane by bacteriorhodopsin in the light would be used to synthesize ATP, in accordance with Mitchell's hypothesis of energy coupling (Mitchell, 1961). Confirmation that bacteriorhodopsin may operate as a proton pump was obtained by Racker (1973) and Racker and Stoeckenius (1974) who showed that purple membrane vesicles consisting only of bacteriorhodopsin and lipid, produced light-dependent changes in extravesicular pH which disappeared in the presence of uncouplers known to act as proton carriers. Similar observations have been made by others (Kayushin & Skulachev, 1974). Recently Lozier, Niederberger, Bogomolni, Hwang and Stoeckenius (1976) and Hwang and Stoeckenius (1977) have demonstrated that the change in pH produced by the bacteriorhodopsin model membrane vesicles result from vectorial proton transport across the membrane in the light, and not merely from binding and release of protons. Several groups have shown that this proton transport generates a membrane potential both in model membrane vesicles (Kayushin & Skulachev, 1974; Racker & Hinkle, 1974) and in intact membrane vesicles or bacteria (Renthal & Lanyi, 1976; Bakker, Rottenberg & Caplan, 1976; Bogomolni, 1977).

The reconstitution of proton transport by assembling lipids and purple membrane has opened the possibility of not only confirming that bacteriorhodopsin is a proton translocator, but also of investigating the molecular mechanisms of this transport. The purple membrane vesicles, however, are of limited experimental use mainly because the intravesicular compartment is small and not directly accessible to measurement of ion concentration or electrical potential. Planar lipid films containing purple membrane and separating two large aqueous compartments (Drachev *et al.*, 1974; Drachev *et al.*, 1976; Herrmann & Rayfield, 1976; Shieh & Packer, 1976), or an aqueous and organic compartment (Boguslavsky *et al.*, 1975) overcome some of these limitations. Unfortunately, in the systems available to date, the amount of bacteriorhodopsin in the films and its degree of orientation is not known and only qualitative rather than quantitative results have been obtained. To improve on some of these limitations we have prepared air-water interface films containing known amounts of spectroscopically intact and highly oriented bacteriorhodopsin (Hwang, Korenbrot & Stoeckenius, 1977). We show here that bacteriorhodopsin in these films is also functionally intact and that interface films can be used, under appropriate conditions, to measure ion transport.

Materials and Methods

Materials

Purple membrane fragments, purified lipids and organic solvents were prepared as described in the accompanying paper (Hwang *et al.*, 1977). Decane (over 99% pure) was obtained from Sigma (St. Louis, Mo.) or Aldrich Chemicals and was extensively purified shortly before use either by repeated passes through a 5 × 5 cm column of silica gel G or, by repeated distillation until no photopotentials could be detected at a decane-water interface (*see below*). Purified decane samples were also analyzed by gas liquid chromatography from time to time and no detectable contaminants were found. 2–4 dinitrophenol (DNP) (Calbiochem, San Diego, Calif.), tetraphenyl borate sodium salt (TPB) (Sigma, St. Louis, Mo.) and carbonyl cyanide-*p*-trifluormethoxy phenylhydrazone (FCCP) (Pierce Chemical, St. Louis, Mo.) were used as received without further purification. All inorganic salts were analytical reagent grade. Water used throughout was glass distilled 4 times, including once from alkaline permanganate and once from sulfuric acid.

Film Spreading

Troughs (virgin white Teflon) used in the experiments reported here had outside dimensions of 7.5 × 7.5 × 2 cm and inside dimensions of 5.5 × 5.5 × 1 cm. The troughs were placed within a 12 × 10 × 10 cm electrically shielded plastic box set on a stage designed to minimize mechanical vibration. To obtain reproducible results the troughs were cleaned with great care before every experiment: The trough was thoroughly rinsed with 95% ethanol, washed under running water and then kept for 2 hr in a cleaning solution of concentrated sulfuric acid (12 N) saturated with dichromate. It was then rinsed extensively under running distilled water and kept overnight with constant stirring in 95% ethanol saturated with NaOH. The trough was finally rinsed extensively with twice distilled water, submerged for 10 min in boiling distilled water and dried by suction.

Interface films were formed by spreading onto a clean water surface a suspension of sonicated fragments of purple membrane in a solution of soybean phosphatidyl-choline (soya PC) in hexane prepared as previously described (Hwang *et al.*, 1977). In all experiments the weight ratio of bacteriorhodopsin to soya PC was 7 : 1 and the time of sonication in the hexane phase was approximately 1 min. The Teflon troughs had a fixed surface area of 30 cm^2. The subphase in all experiments reported here was unbuffered distilled water, pH 5.6–6.8. Film-forming material was carefully spread in sufficient amount to bring the surface pressure to 45 dynes/cm, the collapse pressure of the film. This required 50 µl of a purple membrane suspension containing 0.37 mg bacteriorhodopsin/ml. After 10–15 min the hexane had evaporated, and 1 ml of decane was carefully layered drop by drop on top of the purple membrane-soya PC interface film. Thirty min were allowed for the decane to spread into a completely uniform 0.3 mm thick layer which covered all of the interface film. Photoresponses were recorded over the following 4–5 hr.

Surface pressure was recorded as described previously (Hwang *et al.*, 1977). Surface potential was measured with an ionizing electrode: platinum foil (0.5 × 0.5 cm) was placed 5 mm above the aqueous surface and the gap between the electrode and the surface was ionized with a 5 µC Ra^{226} source (U.S. Radium Corporation). The reference electrode was a calomel half-cell located in a well machined in the wall of the trough and connected to the main compartment below the water surface through a channel 3 mm in diameter. The surface potentials measured were the difference in potential between the two electrodes located across the interface. Potential differences were measured with a unity gain high input impedance differential amplifier (Wilson Electronics Model 1070

S.F.). The signal was further amplified if needed with a variable gain linear amplifier. Surface potentials were expressed as the difference in the potentials measured before and after spreading the purple membrane-soya PC film. Photopotentials were expressed as the light-induced change in surface potential. With the ionizing electrode used, surface potentials and photopotentials were independent of the size of the ionized air gap (up to 10 mm) and of the thickness of the decane layer (up to 0.6 mm).

Photostimulator

For illumination a 250 W quartz iodine lamp (Leitz Prado Slide Projector) was used with a system of lenses and a mirror which uniformly illuminated the trough from above. The surface electrode projected a shadow of about 1.5 × 2 cm onto the film. In addition to the efficient built-in heat filter of the projector, two 1/4 inch thick IR absorbing filters (Edmund Scientific) were used. Calibrated neutral density (Balzers) colored glass (Corning Glassworks and Schott Jena) and narrow band interference filters (Baird Atomic, 15 nm half-band width) controlled the wavelength and intensity of the light as required. A manual shutter controlled the illumination. The light energy flux was measured at the position of the water surface with a Kettering Radiometer (Model 68, Laboratory Data Control Div. Milton Roy Co.). All experiments were carried out in a darkroom dimly illuminated with red safelight (General Electric, BAS 25 W bulbs).

Theoretical Background

In the foregoing paper we described the preparation and properties of air-water interface films which consist of small nonoverlapping fragments of purple membrane separated by a monolayer of lipid. In interface films formed with 7:1 bacteriorhodopsin/soya PC weight ratio the purple membrane fragments are homogeneously distributed, cover 36% of the water surface and ~85% of them are oriented with their intracellular surface towards the aqueous subphase (Hwang *et al.*, 1977). Therefore, if bacteriorhodopsin is intact in these films, protons should be translocated in the light from the aqueous phase into air. This possible charge translocation *through* the monolayer can be measured if the monolayer is covered with a thin layer of suitable organic phase and the surface potential is measured.

The surface potential of an insoluble film at an interface is defined as the difference in the electrical potential measured across the interface before and after forming the film (Davies & Rideal, 1963).

$$\Delta \Psi = \chi_2 - \chi_1 . \quad (1)$$

χ_2 and χ_1 are the potential measured with and without the film.

At an air-water interface, the value of the surface potential is the sum of the potentials due to the dipole moments and to the charges of the film-forming molecules. In addition, reorientation of the water dipoles

about the film-forming molecules also contributes to this potential. If the components normal to the water surface of all dipole moments at the interface are vectorially added, their contribution to the surface potential can be expressed as that of an equivalent dipole moment per molecule μ_D. The surface potential due to n molecules per cm^2 can be expressed as

$$\Delta \Psi = 4\pi n \mu_D. \tag{2}$$

Molecules with net charge contribute to the surface potential an electrostatic potential, *E*, proportional to the magnitude and sign of the charge and to the ionic strength of the subphase (for a complete discussion of this potential *see* Haydon, 1964). The surface potential at an air-water interface therefore can be expressed as

$$\Delta \Psi = 4\pi n \mu_D + E. \tag{3}$$

At an oil-water interface, on the other hand, the surface potential, $\Delta\Phi$, can be expressed as the sum of two potentials (Davies & Rideal, 1955)

$$\Delta \Phi = \Delta \Psi + \Delta V, \tag{4}$$

where $\Delta \Psi$ is the potential due to dipoles and charges in the film-forming molecules as defined above, and ΔV is a distribution potential which results from the unequal solubility of cations and anions between the aqueous and organic phases.

The distribution coefficients of anions and cations between aqueous and nonaqueous phases, *K*, are proportional to the difference in the free energy of the ions in the two phases, and are defined as follows:

$$K_+ = \exp\left[\mu_+^w - \mu_+^o / RT\right] \tag{5}$$

$$K_- = \exp\left[\mu_-^w - \mu_-^o / RT\right], \tag{6}$$

where μ^w and μ^o are the standard chemical potentials in the water and oil phases, respectively. *R* and *T* have their usual meaning. Davies and Rideal (1955) have shown that the value of the distribution potential is simply given by:

$$\Delta V = \frac{RT}{2zF} \ln \frac{K_+}{K_-}. \tag{7}$$

Substituting Eqs. (7) and (3) is Eq. (4), we obtain a general expression for the surface potential at an oil-water interface,

$$\Delta\Phi = 4\pi n \mu_D + E + \frac{RT}{2zF} \ln \frac{K_+}{K_-}. \tag{8}$$

Dean, Gatty and Rideal (1940) and Davies and Rideal (1955) have shown that the value of the surface potential at an oil-water interface depends on the polarity of the oil. The surface potential measured for a film formed at the interface between a thin layer of highly polar oil (e.g., nitrobenzene) in which salts can readily dissolve and water is simply given by the distribution potential of the diffusible ions, ΔV [Eq. (7)], and not by the electrical characteristics of the film-forming molecules. This is because, at equilibrium, the diffusible ions redistribute themselves between the phases on each side of the interface film to restore their electrochemical potentials to their initial values. The contribution of the film-forming molecules to the measured interface potential is thus cancelled.

On the other hand, the surface potential measured for a film formed at the interface between a thin layer of parafinic oil (e.g., decane) in which salts do not dissolve and water is given by the potential due to the film-forming molecules, $\Delta\Psi$, [Eq. (3)]. This is because the distribution coefficient of diffusible ions in parafinic oils is essentially zero. Thus:

$$\Delta\Phi = \Delta\Psi = f(\mu, E) \text{ non polar oil;} \tag{9}$$

$$\Delta\Phi = \Delta V = f(K_+, K_-) \text{ polar oil.} \tag{10}$$

In our case, if the purple membrane-PC film is covered with a thin layer of parafinic oil, no change in surface potential would be expected [from Eq. (9)]. However, if upon illumination, protons (positive charges) are transported by bacteriorhodopsin from the subphase into the oil layer, a light-induced distribution potential would be expected [Eq. (7)]. It should be positive, proportional to the amount of charge transported and additive with $\Delta\Psi$ [Eq. (4)]. It is possible, of course, that $\Delta\Psi$ could change with illumination in addition to charge transport across the interface. Light-induced changes in $\Delta\Psi$ should, however, be small, since intramolecular rearrangements in the purple membrane during the photoreaction cycle and the concentration of photosteady state intermediates under the conditions of our experiments should be very small. The energy required to move a proton from the high dielectric constant subphase to the low dielectric constant oil phase may well exceed

the energy available from the photon absorbed. In that case, even with an intact purple membrane at the interface, no light-induced potential would be observed, as is indeed the case (*see below*). It has been shown experimentally (Liberman & Toplay, 1968; McLaughlin, 1972) and explained theoretically (Parsegian, 1969) that uncouplers of oxidative phosphorylation such as FCCP and DNP reduce the energy required to transfer protons from a high into a low dielectric phase. These molecules, therefore, act as proton carriers and acceptors in an oil phase. We have used them to selectively increase the distribution coefficient of protons between water and decane.

Results

The effect of illumination on the surface potential of the purple membrane-soya PC film at an air-water interface is illustrated in Fig. 1. The surface potential changes by $+0.5\,\text{mV} \pm 0.2$ ($\pm$SD) in response to the highest level of illumination used in these experiments. We attribute this small potential change to changes in the charge and/or in the dipole moment of the excited bacteriorhodopsin molecules. Covering the bacteriorhodopsin interface film with a thin layer of decane does not alter the surface potential and does not change the light response. These observations demonstrate that at the decane-water interface the potential change due to dipoles and/or charges in the film-forming molecules, $\Delta\Psi$, has a negligibly small contribution to any photopotential detected.

The failure to detect a photopotential in the presence of decane alone, even with functionally intact bacteriorhodopsin, can be understood by considering Eqs. (5), (6) and (7): The free energy difference which must be overcome to inject protons into decane is probably higher than the energy available in the light transduction process. To specifically reduce the free energy barrier for proton transfer from water to decane, we covered the interface film with a layer of decane containing FCCP, a molecule which, as pointed out above, is a lipid soluble proton acceptor. An interface film covered with decane containing 5×10^{-8} M FCCP does not appreciably change its surface potential in the dark. Light, however, causes a large positive change in surface potential (Fig. 1). For a step of light, the potential quickly reaches a steady value maintained for the duration of the light and returns to its original value when the light is switched off. The polarity of the photopotential indicates a light-

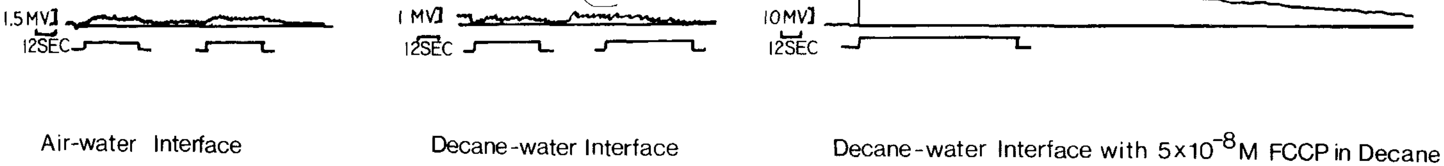

Fig. 1. Photopotentials at the air-water and decane-water interface. The interface was illuminated for 96-sec duration periods with light of wavelength longer than 510 nm of intensity 3×10^5 erg cm^{-2} sec^{-1}. This corresponds approximately to 1 equivalent 570 nm photon for every bacteriorhodopsin molecule at the interface per sec

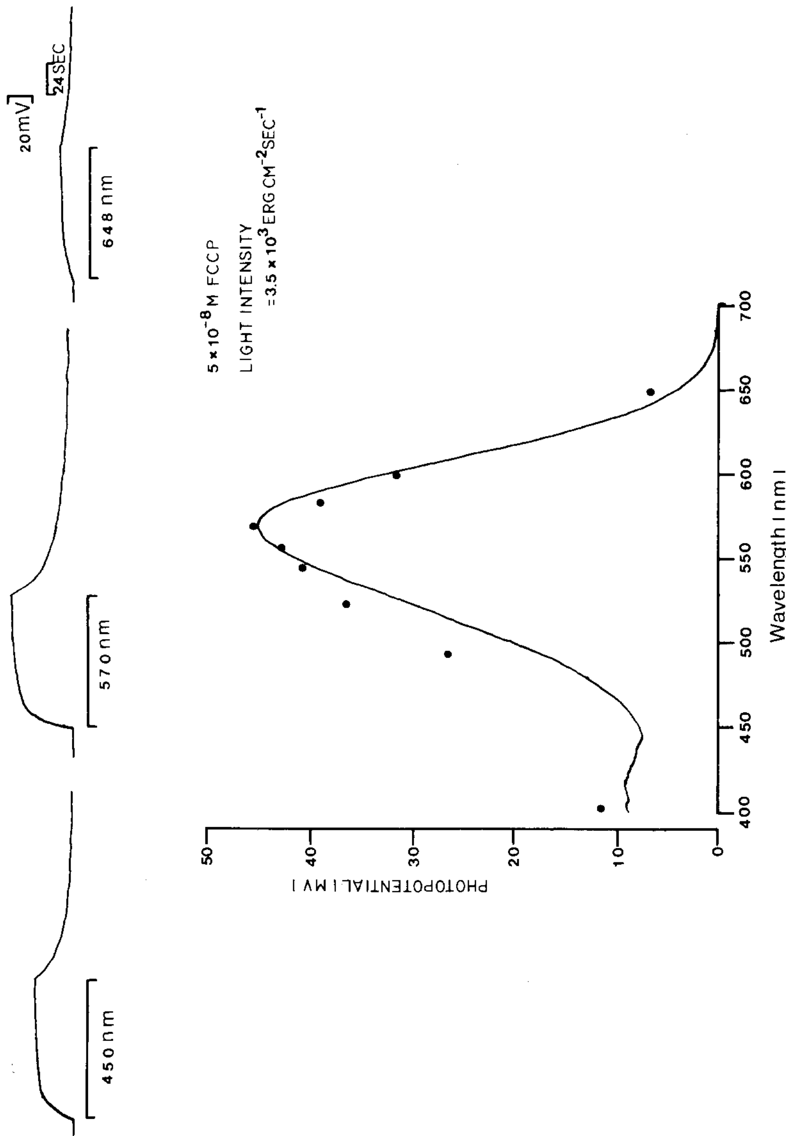

Fig. 2. Action spectrum of photopotentials in the presence of 5×10^{-8} M FCCP in the decane. The interface was illuminated with 96-sec duration light pulses of different wavelength but identical intensity of 3.5×10^{3} ergs cm^{-2} sec^{-1}. The points indicate the photopotentials measured 85 sec after illumination commenced. The continuous line is the absorption spectrum of bacteriorhodopsin scaled to give the best fit to the points

dependent translocation of protons from the aqueous to the organic phase. Since purple membrane is oriented at the interface with its intracellular surface towards the aqueous subphase (Hwang *et al.*, 1977), the protons must be vectorially transported from the intracellular to the extracellular surface of the membrane.

If bacteriorhodopsin is responsible for the proton transport through the interface, the action spectrum of the photopotential should follow the absorption spectrum of the protein. Fig. 2 shows steady state photo-

potentials recorded in response to illumination at various wavelengths, but identical intensities, plotted as a function of wavelength. The action spectrum of the photopotential agrees satisfactorily with the absorption spectrum of bacteriorhodopsin measured in aqueous suspensions of purple membrane (Hwang *et al.*, 1977).

We have reasoned that the positive charges transported into decane are indeed protons, because FCCP has been shown to be a highly selective proton carrier (Liberman & Toplay, 1968) and because the subphase in all these experiments was unbuffered four-times-distilled water in which the concentration of cations other than protons would be extremely low. This reasoning is further supported by the observation that qualitatively similar surface photopotentials are measured when other lipid soluble proton carriers are added to the decane. Typical photopotentials and an action spectrum in the presence of 10^{-5} M DNP are illustrated in Fig. 3.

We have argued above that the proton carriers decrease the energy barrier and allow the light-driven redistribution of protons between aqueous and decane phases. We must also consider, however, that the proton carrier molecules are soluble in both aqueous and organic phases and will distribute themselves between them. At equilibrium, therefore, photopotentials should be proportional to the amount of proton carrier molecules in the system regardless of whether they are initially added to the aqueous or the organic phase. Fig. 4 shows that this is, indeed, the case. Photopotentials are very similar for DNP added to the water or the decane phase. We obtained similar results in experiments using FCCP. Furthermore, we used DNP to directly measure equilibration of these molecules between decane and aqueous phases. DNP is a pH indicator molecule with a characteristic maximum absorbance at 357 nm in an aqueous solution of pH 5.8. It is colorless in decane. Thus, by measuring the absorbance at 357 nm of aliquots collected from subphases covered with decane containing 10^{-9} to 10^{-5} M DNP, it is possible to show (Fig. 5) that the concentration of DNP in the water is linearly proportional to its concentration in decane.

The equilibration of the proton carrier molecules between decane and water implies that these molecules not only facilitate active transport of protons by bacteriorhodopsin, but also passive redistribution of protons between the two phases. That is, the proton carrier molecules render the decane-water interface selectively permeable to protons. In the steady-state, therefore, the net proton transport in light will be the balance of the proton-pump transport from water to decane by bacteriorhodopsin

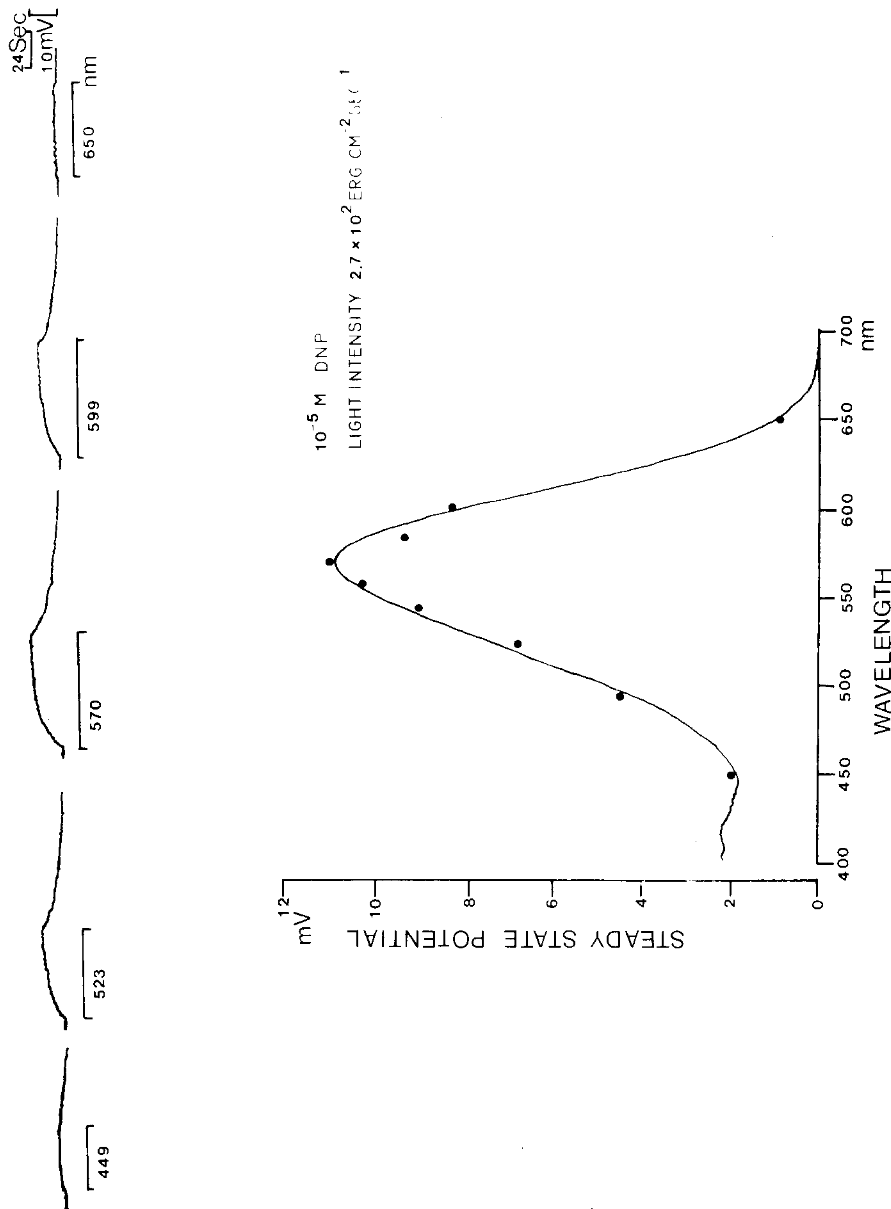

Fig. 3. Action spectrum of photopotentials in the presence of 10^{-5} M DNP in decane. The interface was illuminated with 96-sec duration light pulses of various wavelengths but identical intensity of 2.7×10^2 ergs cm^{-2} sec^{-1}. The points indicate photopotentials measured 85 sec after illumination commences. The continuous line is the absorption spectrum of bacteriorhodopsin scaled to best fit the points

and the proton-leak transport from decane to water by the proton carriers.

Kinetics of the Photopotential

Photopotentials of a purple membrane interface film in response to steps of light and in the presence of decane containing 5×10^{-8} M FCCP

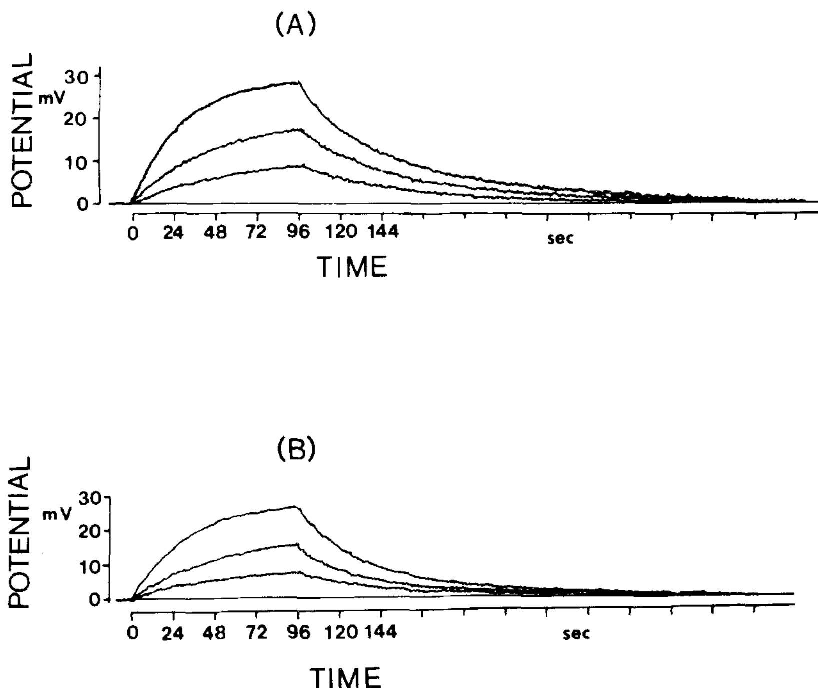

Fig. 4. Photopotentials measured in the presence of 10^{-9} moles DNP. (*A*): the potential recorded when DNP was added to decane. (*B*): The potential recorded when DNP was added to the aqueous subphase. In both cases the illumination was a 96-sec duration step of light filtered through a narrow band interference filter (15 nm half-band width) centered at 570 nm. Light intensity was 4.3; 2.1; 1×10^2 erg cm^{-2} sec^{-1}

are shown in Fig. 6. Three kinetic parameters of the photoresponse were measured: initial slope, measured as the slope of a line tangent to the voltage change recorded immediately after presenting the stimulus; steady-state potential measured 85 sec after presenting a stimulus of 96 sec duration; off-rate, measured as the half-time of the voltage decay after the light was switched off, even though the voltage decay did not completely fit a single exponential, the qualitative nature of the analysis presented makes this necessary simplification acceptable. The dependence of these kinetic parameters on light intensity is presented in Figs. 6 and 7. Over the range tested, the off-rate is essentially independent of light intensity, whereas both the initial slope and the steady-state potential are proportional to it. The initial slope increases linearly with intensity. The steady-state potential, on the other hand, appears to be linearly proportional only at low intensities (Fig. 6) and becomes linearly proportional to the logarithm of the light intensity at higher levels

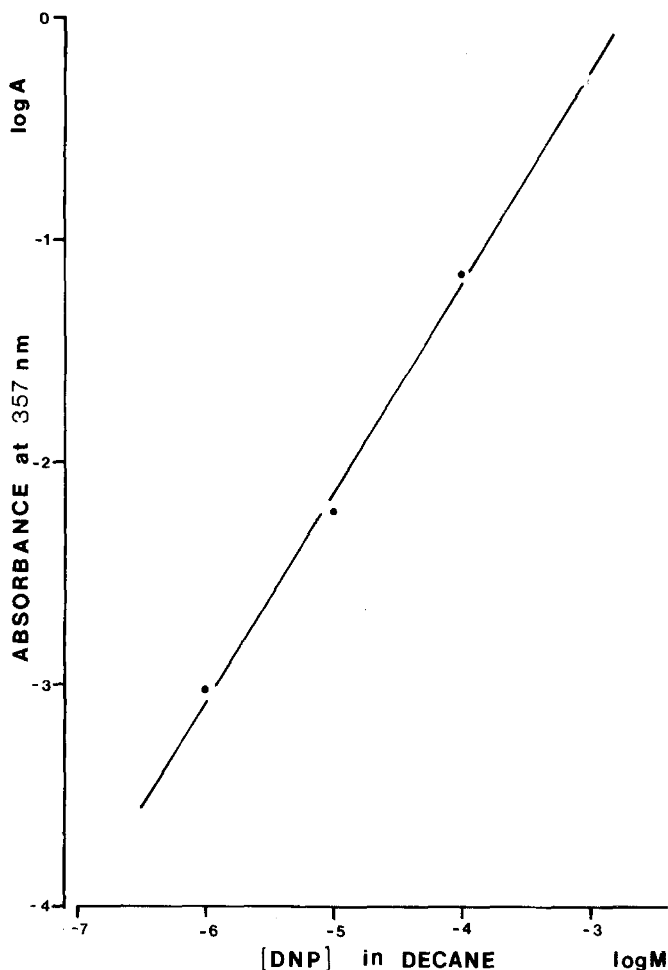

Fig. 5. Absorbance at 357 nm of the aqueous subphase covered with decane containing various concentrations of DNP. The interface film was covered with decane containing DNP and after equilibrium had been attained (30 min), 1 ml aliquots of subphase were collected for spectrophotometric analysis

(Fig. 7). Kinetics of photopotentials recorded with DNP (not shown) are qualitatively identical to those described for FCCP. These data indicate that the number (steady-state potential) and the rate (initial slope) of protons transported from water into decane are proportional to the number of photo-excited bacteriorhodopsin molecules per sec, whereas the back-diffusion of protons from decane to water (off-rate) is independent of light intensity.

The kinetic parameters of the photoresponse are dependent on the concentrations of proton carrier molecules. Fig. 7 shows steady-state

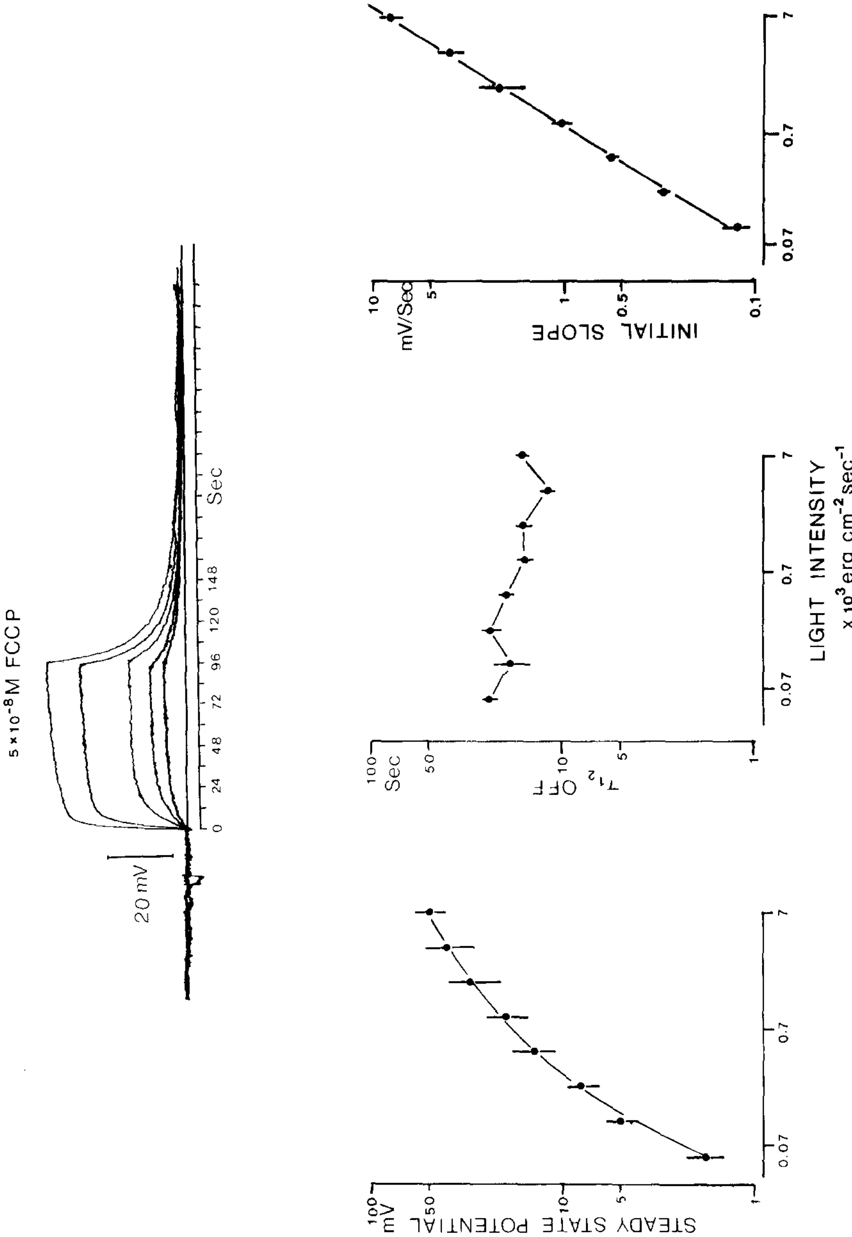

Fig. 6. Kinetics of photopotentials in the presence of 5×10^{-8} M FCCP in decane. Interface was illuminated for 96-sec duration periods with light filtered through an interface filter centered at 570 nm. Light intensity was controlled with neutral density filters. The parameters of the photoresponse were measured as described in the text. Each point is the average of 3 to 5 experiments, the bars are SEM

potentials as a function of light intensity at various concentrations of carriers FCCP and DNP. The analysis of the photoresponse as a function of concentrations of carrier molecules at a constant light intensity should apply to any intensity tested since the lines drawn in Fig. 7 do not cross. Fig. 8 illustrates photoresponses recorded at various

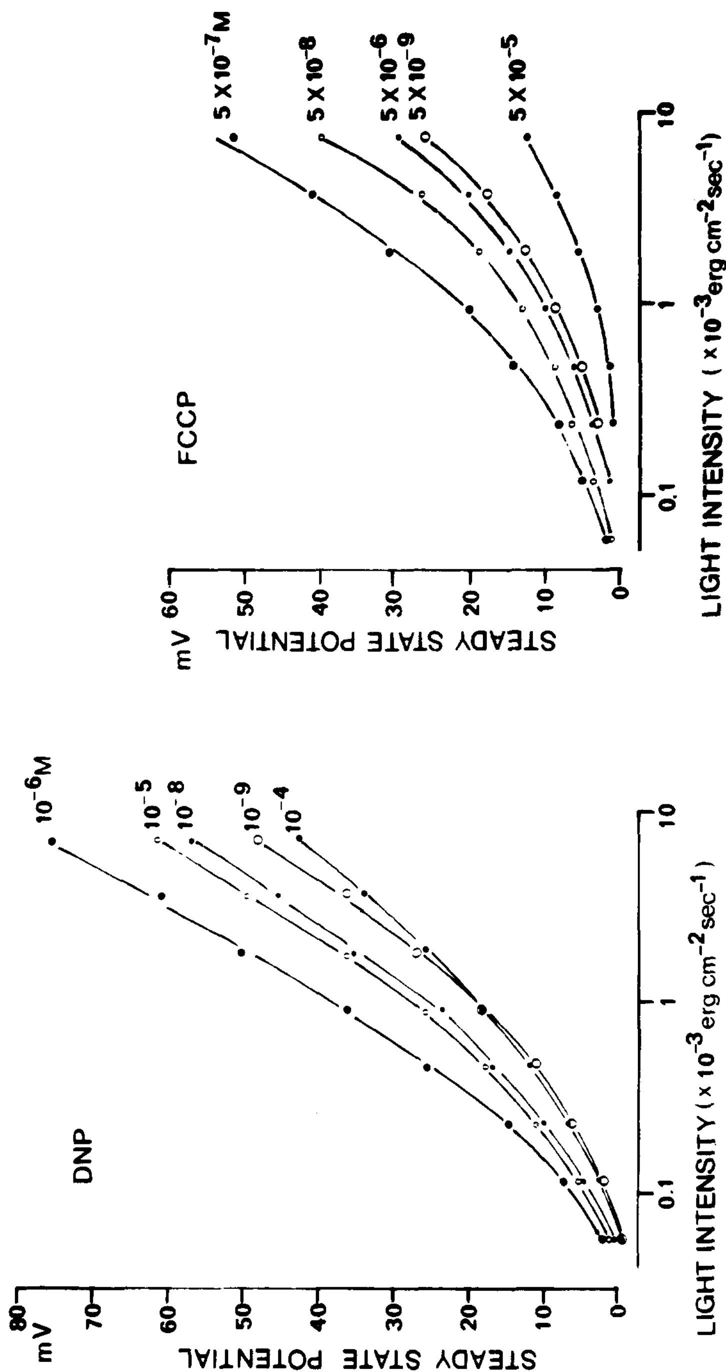

Fig. 7. The dependence of the steady-state potential on light intensity for various concentrations of DNP and FCCP in decane. The interface was illuminated with 96-sec duration steps of 570 nm light at different intensities. In contrast to Fig. 6, this is a semilogarithmic plot to show the dependence of photopotentials on light intensity at high levels of illumination. The points are averages of 3 to 5 experiments. The continuous lines are drawn to best fit the points

DNP concentrations in response to identical illumination. The steady-state potential and initial slope both increase with carrier concentration, reach a maximum at 10^{-6} M DNP and then decrease. In contrast, the off-

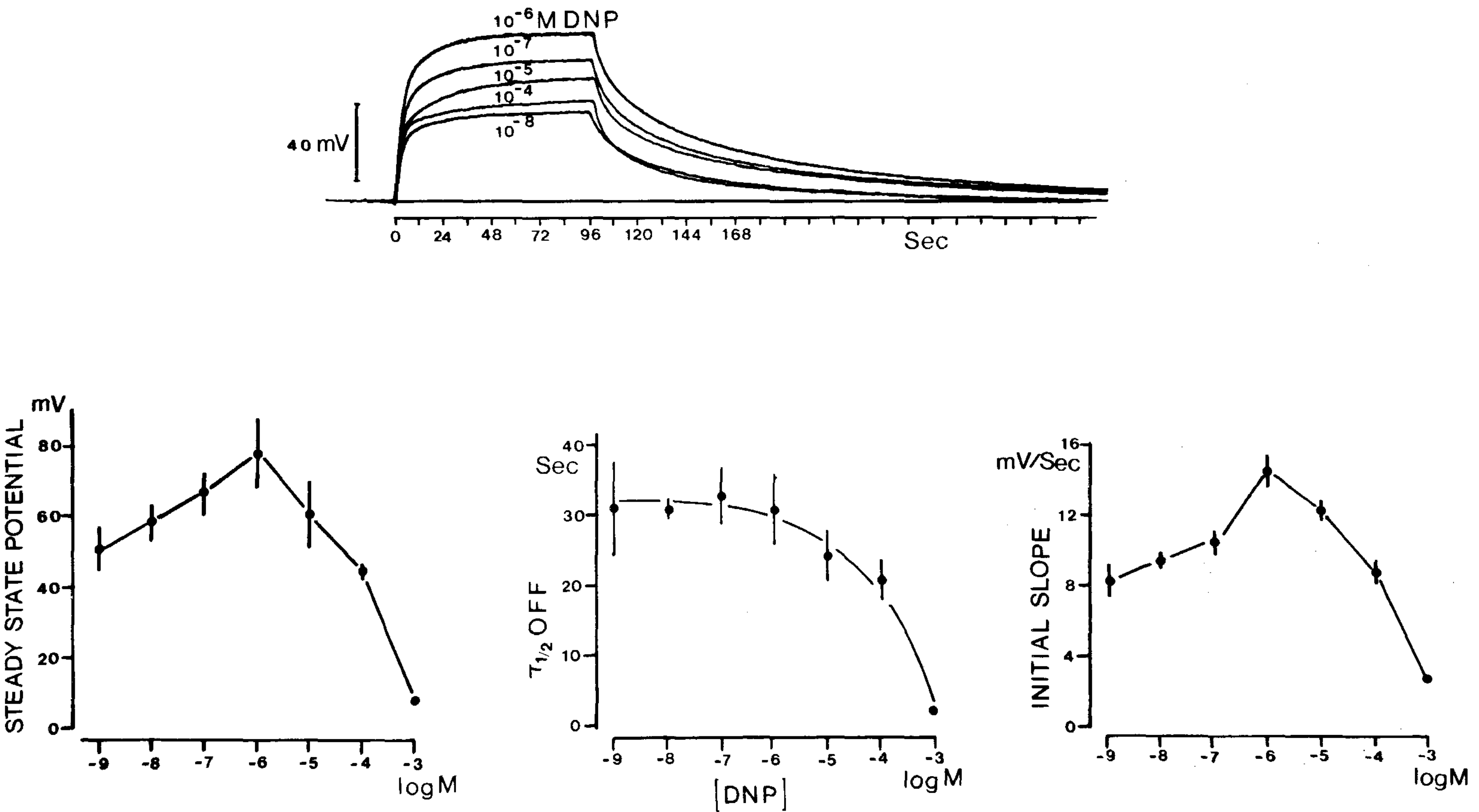

Fig. 8. Kinetics of photopotentials in the presence of different concentrations of DNP in decane. Interface was illuminated with 96-sec duration steps of 570 nm light of intensity 7×10^3 erg cm^{-2} sec^{-1}. Each point is the average of 3 to 5 experiments, the bars are SEM

rates are independent of carrier concentration at low concentrations, and increase with increasing carrier concentrations above 10^{-6} M. It is important to note that the off-rate of the photoresponse accelerates at the same acceptor concentration which produces a decrease in the other kinetic parameters. The kinetics of photopotentials recorded with various FCCP concentrations are qualitatively similar to those described for DNP (Stoeckenius, Hwang & Korenbrot, 1977).

The data presented above confirm the proposition that the rate and number of protons distributed between aqueous and decane phases upon illumination result from the balance between the bacteriorhodopsin pump transport and the carrier molecules leak transport. Thus, at DNP concentrations under 10^{-6} M, the initial slope and steady-state potential increase proportionally with carrier concentration, as the height of the energy barrier for proton distribution between water and decane decreases. The off-rate, determined by the intrinsic permeability of the interface for protons, is independent of carrier concentration. Above 10^{-6} M, DNP increases the proton permeability of the interface to an extent proportional to the concentration of the carrier (McLaughlin, 1972; Liberman & Toplay, 1968). The off-rate hence accelerates with DNP concentration and the net number and rate of proton transported by bacteriorhodopsin decreases.

Effects of Lipid Soluble Anions

The photopotentials of the purple membrane interface films described above result from the unequal distribution of positive and negative charges between decane and water phases. Hence, from Eq. (7), it can be predicted that if negative charges were allowed to cross the interface in parallel with the protons, steady-state photopotentials should become smaller. We tested this prediction by measuring photopotentials in the presence of the lipid soluble anion tetraphenyl borate (TPB). Purple membrane films were spread on subphases containing NaTPB in the concentration range 10^{-8} M to 10^{-4} M or no NaTPB, while the concentration of DNP added to the decane was kept constant at 10^{-6} M. The time course and magnitude of the photopotential are a function of light intensity and TPB concentration. Fig. 9 illustrates the photoresponse recorded at various light intensities in the presence of 10^{-6} M TPB. At low light intensities, the steady-state potential is about 1/10 the value of that recorded in the absence of TPB. At high intensities, the photore-

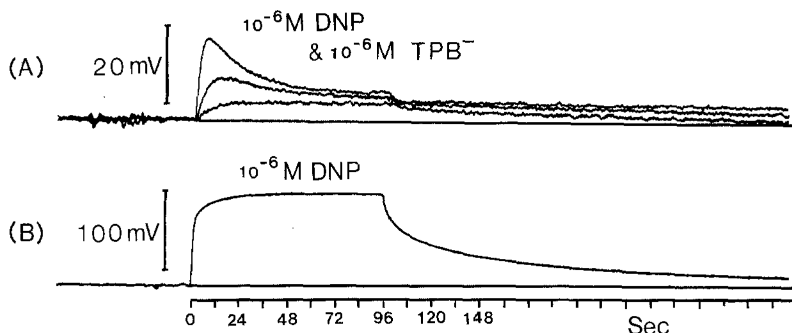

Fig. 9. Photopotentials in the presence of 10^{-6} M DNP (*B*) or in the presence of 10^{-6} M DNP and 10^{-6} M TPB (*A*). Interface was illuminated with 96-sec duration steps of light longer than 510 nm of different intensities: 7.57×10^3; 3×10^4 and 1.2×10^5 erg cm^{-2} sec^{-1}. The gain in the two sets of records is different. The steady state photopotential in the presence of TPB is about 1/10 that in its absence. The intensity of illumination in the absence of TPB was 1.2×10^5 erg cm^{-2} sec^{-1}

sponse transiently rises to a peak and then declines to the steady-state value. The decrease in the photoresponse in the presence of TPB can arise in part from a dependence of the rate of proton transport by bacteriorhodopsin on the electrochemical gradient for protons. Thus, in the presence of TPB, the proton gradient between aqueous and decane phases may be smaller than in its absence and the proton transport may be reduced. No independent direct evidence exists now on whether the rate of proton transport by bacteriorhodopsin indeed responds to an electrochemical gradient. In addition, however, it must also be considered that Ketterer, Neumcke and Lauger (1971) have shown that in the steady-state the flux of TPB across an interface is limited by its rate of aqueous diffusion (*see also* Haydon & Hladky, 1972). Therefore, the rate of transfer of TPB from the aqueous into the decane phase is limited by its flux rate through the thick unstirred layers underneath the interface film. This consideration can account for the appearance of an overshot in the photoresponse. Thus, at low light intensities the rate of proton transport by bacteriorhodopsin is slow (*see* Fig. 6) and does not outrun the TPB flux induced by the electrical potential. In contrast, at high light intensities the proton transport by bacteriorhodopsin is fast compared with the rate of TPB flux through thick unstirred layers. The TPB flux may lag behind the proton flux, resulting in the observed transients.

The hypothesis presented above to explain the overshoot can be experimentally tested. Since Ketterer *et al.* (1971) have shown that the aqueous flow of TPB is linearly proportional to its concentration, by

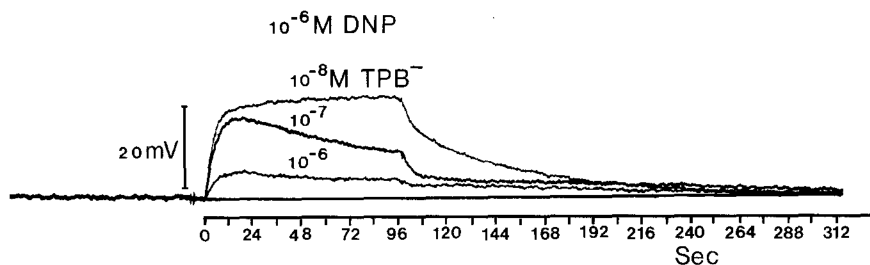

Fig. 10. Photopotentials in the presence of 10^{-6} M DNP and various concentrations of TPB. The interface was illuminated with 96-sec duration light of 570 nm and 7×10^3 erg cm^{-2} sec^{-1}

increasing TPB concentration, its flow through unstirred layers could be made fast enough compared to the rate of proton transfer, to eliminate transients in the photoresponse. This is indeed the case. Fig. 10 illustrates photopotentials in response to identical illumination recorded at various concentrations of TPB. The transient in the photopotential observed at 10^{-7} M is nearly abolished at 10^{-6} M TPB. In summary, the positive photopotential which results from the transport of protons by bacteriorhodopsin from the aqueous into the decane phase can be reduced by the simultaneous transfer of lipid soluble anions.

Discussion

The results described here and in the accompanying paper show that it is possible to form interface films from isolated purple membrane and lipids in which known amounts of bacteriorhodopsin are highly oriented and spectroscopically intact. Furthermore, it is possible to measure light-dependent charge transfer through these films from the aqueous subphase into an overlaying thin oil phase. The requirement of specific lipid-soluble proton acceptors in the oil phase and the action spectrum of the photopotentials demonstrate that bacteriorhodopsin functions as a light-driven pump, translocating protons from its intracellular to its extracellular surface in the light without the need of a preexisting electrochemical gradient.

Interface films have been formed independently by Bogulavsky *et al.* (1975) by adsorbing purple membrane at a water-octane interface from a suspension in the aqueous phase. Although these films cannot be compared directly to the ones described here because the amount, orientation

and organization of purple membrane at the interface have not been described, Bogulavsky *et al.* have described photopotentials in the films. The amplitude of these photopotentials was reported to increase to a saturating value as a function of FCCP concentration and not to decline at higher concentrations. This differs from our observations that the amplitude (steady-state potential) reaches a maximum and declines at higher concentrations. However, in recent experiments, they have obtained results similar to ours in the presence of lipid and their earlier results remain unexplained (V. Skulachev, *personal communication*).

Mixed lipid-protein interface films have been elegantly used as model systems to study the mechanisms and regulation of some enzymatic activities (Romeo, Hinckley & Rothfield, 1970). We have shown here that under appropriate conditions, interface films may also be used as model systems to study charge transport. Thus, interface films can be useful tools in the analysis of membrane-bound protein function, in particular, since interface films of structurally and functionally intact membrane proteins may be formed by spreading membrane fragments. For example, an intestinal aminopeptidase has been found to be enzymatically active in interface films formed with brush-border membrane (Verger & Pattus, 1976). Vertebrate rhodopsin has been found to be spectroscopically intact and chemically regenerable in interface films formed with rhodopsin reconstituted membrane fragments (Korenbrot, 1977). Once structurally intact interface films are formed, they can be used to study parameters of biological function not easily measurable in other preparations.

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