J. Membrane Biol. 36, 115–135 (1977)

Structural and Spectroscopic Characteristics of Bacteriorhodopsin in Air-Water Interface Films

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Received 21 February 1977

Summary. A suspension of purple membrane fragments in a solution of soya phosphatidyl-choline in hexane is spread at an air-water interface. Surface pressure and surface potential measurements indicate that the membrane fragments and lipids organize at the interface as an insoluble film. Electron microscopy of shadow-cast replicas of the film reveal that in the bacteriorhodopsin to soya PC weight ratio range of 2:1 to 10:1, these films consist of nonoverlapping membrane fragments which occupy approximately 35% of the surface area and are separated by a lipid monolayer. Furthermore, the membrane fragments are oriented with their intracellular surface towards the aqueous subphase. Nearly all the bacteriorhodopsin molecules at the interface are spectroscopically intact and exhibit visible spectral characteristics identical to those in aqueous suspensions of purple membrane and in intact bacteria. In addition, bacteriorhodopsin in air-dried interface films show spectral changes upon dark-adaptation and upon flash illumination similar to those observed in aqueous suspensions of purple membrane, but with slower kinetics. The kinetics of the spectral changes in interface films can be made nearly the same as in aqueous suspension by immersing the films in water.

The halophilic bacteria, *Halobacterium halobium*, when grown at low oxygen tension in the presence of light, develop structurally distinct areas in their plasma membrane known as "purple membrane" (Oesterhelt & Stoeckenius, 1971). These specialized membrane patches allow the bacteria, which lack glycolysis, to utilize light as an alternative energy source (Oesterhelt & Stoeckenius, 1973; Oesterhelt, 1975). The transduction of light energy into chemical energy is mediated by the only protein constituent of purple membrane, bacteriorhodopsin. In the light, bacteriorhodopsin acts as a proton pump and thus generates a transmembrane electrochemical gradient of protons (Oesterhelt & Stoeckenius, 1973; Racker & Stoeckenius, 1974; Drachev *et al.*, 1976; Lozier *et al.*, 1976; Renthal & Lanyi, 1976). The energy stored in this gradient may then be used to form ATP according to Mitchell's hypothesis of energy coupling (Mitchell, 1961; Danon & Stoeckenius, 1974; Oesterhelt, 1975). Bac-

teriorhodopsin molecules consist of an apoprotein of about 26,000 mol. wt. and a retinal chromophore covalently bound on a 1:1 mole ratio (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976). Bacteriorhodopsin constitutes 75% of the dry weight of the purple membrane (Oesterhelt & Stoeckenius, 1971; Kushwaha, Kates & Martin, 1975) and exists in a rigid crystalline lattice of space group P3 with 3 protein molecules per unit cell (Blaurock & Stoeckenius, 1971; Blaurock, 1975; Henderson, 1975). Each protein molecule consists of 7 α-helical fragments oriented nearly perpendicular to the plane of the membrane (Henderson & Unwin, 1975). Upon absorption of light, bacteriorhodopsin undergoes a cyclic photoreaction (Dencher & Wilms, 1975; Kung *et al.*, 1975; Lozier, Bogomolni & Stoeckenius, 1975) and in each cycle a single proton is successively released on the exterior surface and taken up on the cytoplasmic surface of the protein (Lozier *et al.*, 1976).

Bacteriorhodopsin, like other membrane-bound proteins, operates at an interface between high dielectric (water) and low dielectric (membrane) phases. Furthermore, the vectorial nature of the proton transport by bacteriorhodopsin demands that the protein be oriented at the interface. To study the mechanism of action of bacteriorhodopsin it would be desirable, therefore, to study the function of the protein in an interface film containing oriented protein molecules. The advantages of the study of membrane-bound proteins at air-water interfaces was originally discussed several years ago (James & Augenstein, 1966), however relatively few studies have been carried out to date. Since protein structure is the result of a balance of forces, including those which arise from the hydrophobic effect, it may be expected that protein structure could drastically change at a dielectric interface. Therefore, any study of interface films containing proteins must substantiate that the structure of the protein at the interface is intact.

Several water insoluble proteins spread from an organic solvent have been shown to maintain at the air-water interface the same secondary structure that they exhibit in the spreading solvent (Malcolm, 1968). On the other hand, water soluble proteins spread from an aqueous solution can take various conformations at an air-water interface depending on (a) the concentration of protein spread (Adams *et al.*, 1971), and (b) the surface pressure at which protein structure is analyzed (Loeb, 1971). In general, the structure of these proteins appears to be "native" only when spread at high concentration of high surface pressures (*see* Miller & Bach, 1973, for review). We have developed a method to form films at an air-water interface or structurally and functionally intact bacteriorhodopsin molecules. The method is based on the concept of

spreading, not the isolated protein, but rather a mixture of purple membrane fragments and lipid. We present here an analysis of the physical and spectroscopic properties of these interface films and, in the following paper (Hwang, Korenbrot & Stoeckenius, 1977), a study of the function of bacteriorhodopsin in the films.

Materials and Methods

Materials

Purple membrane was isolated and purified from the plasma membrane of *Halobacterium halobium* R_1 (Oesterhelt & Stoeckenius, 1974) and stored at 4 °C in basal salt solution (4.3 M NaCl, 0.275 M KCl, 0.080 M $MgSO_4$ and 0.009 M Na citrate, pH 7.0). Soybean phosphatidyl-choline (soya PC) obtained from Nattermann was further purified on silica-gel columns (Hanahan, Dittmer & Warashina, 1957) to remove any contaminating lysophosphatidylcholine. The lipid appeared as a single spot on silica gel 60 TLC plates (E. Merck Labs) developed with either chloroform/acetone/acetic acid/methanol/water (5:2:1:0.5) or chloroform/methanol/acetic acid/water (50:25:8:4) (*see* Fig. 4). Spectrophotometric grade hexane was obtained from Mallinckrodt, Inc. (Palo Alto, Calif.) and redistilled in small volumes before use. All inorganic chemicals were analytical reagent grade. Twice glass-distilled water was used throughout.

Film Spreading

Bacteriorhodopsin interface films were formed by spreading onto a clean air-water interface a suspension of purple membrane fragments in a solution of soya PC in hexane. This suspension was prepared by a procedure based on protocols of Das and Crane (1964) and Gitler and Montal (1972): Purple membrane fragments in the concentration range of 0.16 to 0.8 mg/ml of bacteriorhodopsin (0.33 to 1.67 O.D. at 570 nm assuming a 54,000 extinction coefficient) were suspended in 2 ml of basal salt and sonicated for 30 sec in a bath sonicator (Model G-80-80, Laboratory Supply Co., Hicksville, N.Y.). 0.5 ml of a solution of 0.16 mg/ml of soya PC was then layered on top of this suspension. After vigorous mixing with a Vari-Whirl Mixer (VWR Scientific) for 5 sec, three immiscible phases were separated by centrifugation at $1500 \times g$ for 10 min: A lower, clear aqueous phase; a middle, narrow turbid phase in which the purple membrane fragments were concentrated, and an upper, clear hexane phase. The aqueous phase was carefully removed with a disposable pipette. To the remaining two phases, 2.2 ml of redistilled hexane were added, and the preparation was sonicated for 60 sec in the bath sonicator immediately before spreading. The length of this sonication was critical because denaturation of the protein was detectable after about 3 min of sonication. A new suspension was prepared for every interface film formed. To form the film, 100 to 200 λ of the suspension were applied with a Hamilton microsyringe onto the clean water surface. The weight ratio of bacteriorhodopsin to PC in the hexane phase determined the physical properties of the interface film. All films described here were prepared with weight ratios of bacteriorhodopsin/soya PC between 2:1 and 10:1.

Surface Balance

The recording surface balance used in these experiments has been described in detail elsewhere (Goerke, Harper & Borowitz, 1970). The balance was contained within an electri-

cally shielded plastic enclosure and consisted of a Teflon trough (10 cm × 40 cm × 0.5 cm) with a tight-fitting Teflon barrier which could be mechanically driven at variable rates. An electrical signal indicating the relative position of the Teflon barrier and, therefore, the film area was fed into an *X-Y* plotter, and surface pressure and surface potential *vs.* area isotherms were recorded directly. Surface pressure was measured by the Wilhelmy method (Gaines, 1966). A strip of sand-blasted platinum foil (4 × 0.5 cm), cleaned by flaming before each experiment was suspended from a pressure transducer (Statham Instruments Gold Cell U-5 with a microscale accessory). The output of the transducer was recorded with a stabilized bridge-balance amplifier. Surface potential was measured by the ionizing electrode method (Gaines, 1966). Platinum was used as the electrode, and the air gap between electrode and water surface was ionized with a 5 μC radium 226 source (U.S. Radium Corporation). The reference electrode in the trough was a calomel half cell. The signal from the surface electrodes was recorded through a high-input impedance differential amplifier (Winston Electronics Model 1090, San Francisco, Calif.). All experiments were carried out at room temperature (20–21 °C) and with unbuffered distilled water (pH 5.6–5.8) as the subphase.

Deposition of Interface Films on Solid Support

The interface films were transferred to a hydrophilic solid support, either mica or glass slide, by inserting and withdrawing the slide across the interface at a rate of 2 cm/min (Blodget, 1935). During deposition, surface pressure was maintained at the collapse pressure of the film with the use of a servo mechanism which automatically advanced a movable barrier as material transferred from the interface to the slide. When mica was used as a support, it was cleaved immediately before use. When glass was used, pre-cut glass coverslips (Scientific Products, #2 0.18–0.22 mm thick) were thoroughly cleaned in warm dichromate-sulfuric acid and extensively rinsed in distilled water immediately before use. The slides emerged wet and were allowed to dry completely between successive depositions.

Spectroscopy of Multilayers

Absorption spectra in the visible range of 40–120 layers on glass were recorded with a Cary 118C or a Cary 14 scanning spectrophotometer equipped with scattering transmission accessories (Varian Instruments, Palo Alto, Calif.). For flash spectroscopy measurements, the multilayers were illuminated with 1 μsec actinic flashes at 575 nm, while their transmittance at 410 nm was continuously monitored. Changes in transmittance were average with a Nicolet 1074 computer (Lozier *et al.*, 1976).

Electron Microscopy

Single films transferred to glass or mica were shadowed with platinum-carbon at 20° followed by carbon evaporation at 90° in a Varian vacuum evaporator (VE-61, Varian Instruments, Palo Alto, Calif.). The replicas on mica were floated off on water; the replicas on glass were floated off on 50% hydrofluoric acid. After washing with methanol/water (1:1) and then water, the replicas were transferred to 400 mesh carbon-coated grids.

Freeze-Fracture

The purple membrane-PC film was transferred to glass pretreated with low molecular weight polylysine to bind the film more firmly (Fisher, 1975). The glass slide with the

interface film was then dipped through a PC monolayer spread from hexane and maintained at its collapse pressure. The PC monolayer transfers to the solid support, presumably creating a bilayer in the area between the purple membrane fragments. A clean copper disc was then placed against the glass-supported bilayer film. This sandwich assembly was frozen in Freon 22, maintained in liquid nitrogen and then placed on the precooled (−150 °C) specimen stage of a Balzers BA510 freeze-fracture unit. After low pressure had been attained (2×10^{-6} Torr), the thin copper disc was pried off. The exposed fracture face was etched for 5 min at −100 °C and replicated with platinum-carbon at 45°, followed by carbon at 90°. All replicas were viewed in a Siemens 1A electron microscope with 80 kV accelerating voltage.

Lipid Extraction and Thin Layer Chromatography

Total lipids were extracted from purple membrane according to Bligh and Dyer (1959). The extracted lipids were concentrated by evaporation under nitrogen and run on Silica Gel 60 plates (E. Merck Labs) eluted with either chloroform/methanol/acetic acid/water (50:25:8:4) or chloroform/methanol/acetone/acetic acid/water (5:2:1:1:0.5). The chromatograms were developed by spraying with 50% sulfuric acid and charring.

Results

The absorption spectra of purple membrane fragments suspended in basal salt or in the hexane phase immediately after a standard 60 sec sonication are compared in Fig. 1. Both spectra show a single broad absorption band with a maximum at 570 nm in the basal salt and at 565 nm in the hexane, some fine structure near 400 nm, and a second maximum at 278 nm. If we assume that the extinction coefficient of bacteriorhodopsin remains constant upon partition into hexane, the spectra show that over 98% of the protein molecules are spectroscopically intact in the hexane phase. Sonication for longer periods resulted in progressive loss of absorbance at 565 nm and increase in absorbance at 370 nm, indicating denaturation of bacteriorhodopsin. An intact absorption spectrum is a necessary but not a sufficient criterion to establish the structural integrity of bacteriorhodopsin in hexane. Dark-adaptation of bacteriorhodopsin, that is, the reversible blue-shift of λ_{max} from 570 to 560 nm in the dark, seen in water or basal salt (Oesterhelt & Hess, 1973) also occurs in the hexane suspension (Fig. 2). This is a further indication that bacteriorhodopsin does not undergo gross structural changes in the organic phase.

Suspension of the purple membrane fragments in the hexane-soya PC solution resulted in the extraction of lipids from the membrane. A typical thin layer chromatogram of the colorless hexane phase recovered after centrifugation of the purple membrane suspension in hexane

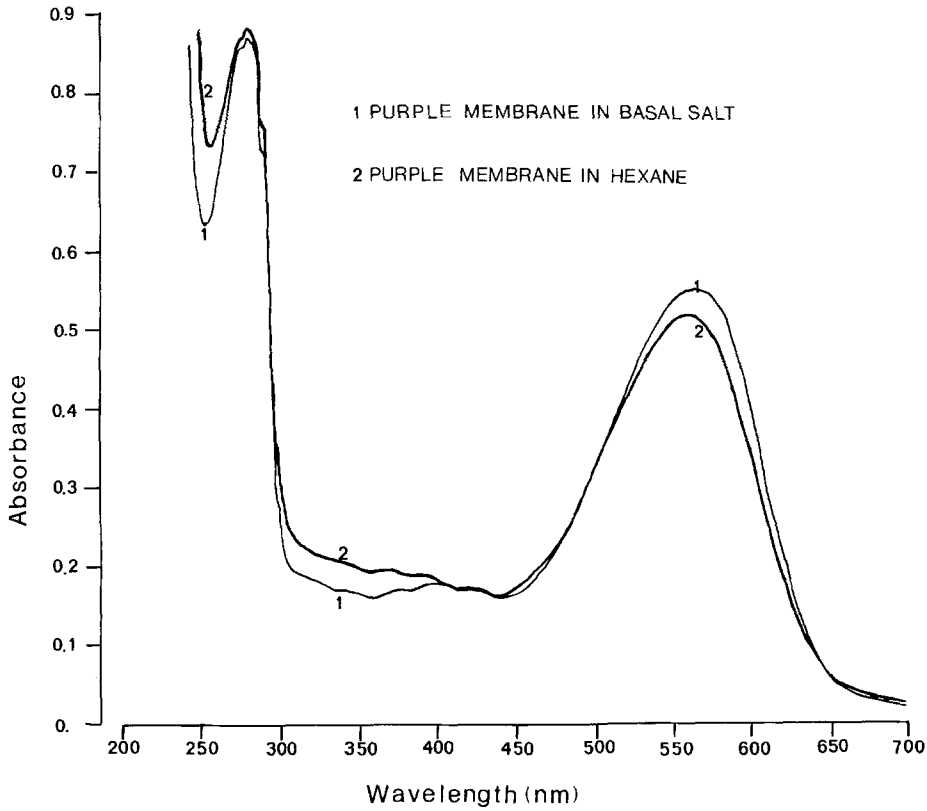

Fig. 1. Absorption spectra, following 60-sec sonication, of a suspension of purple membrane in basal salt solution (*1*) or in a hexane solution of soya PC (*2*). Both spectra show a single broad absorption band in the visible range. The λ_{max} of absorbance is blue shifted in the hexane with respect to the aqueous phase by about 5 nm

is shown in Fig. 3. When this chromatographic pattern is compared with that of a total lipid extract of purple membrane, all spots detected in the total lipid extract are also present in the hexane phase preparation. In addition, the hexane phase pattern shows a large soya PC spot. We thus succeeded in preparing a suspension of purple membrane in hexane in which bacteriorhodopsin remains spectroscopically intact even though some of the purple membrane lipids have been extracted from the membrane, and perhaps replaced by soya PC.

The suspension of purple membrane in soya PC-hexane applied onto a clean air-water interface forms an insoluble film. The surface pressure-area isotherm of films formed a suspension in which the bacteriorhodopsin to soya PC weight ratio is 7:1 is illustrated in Fig. 4. When this isotherm is compared with that of pure soya PC films (Fig. 4), it indicates

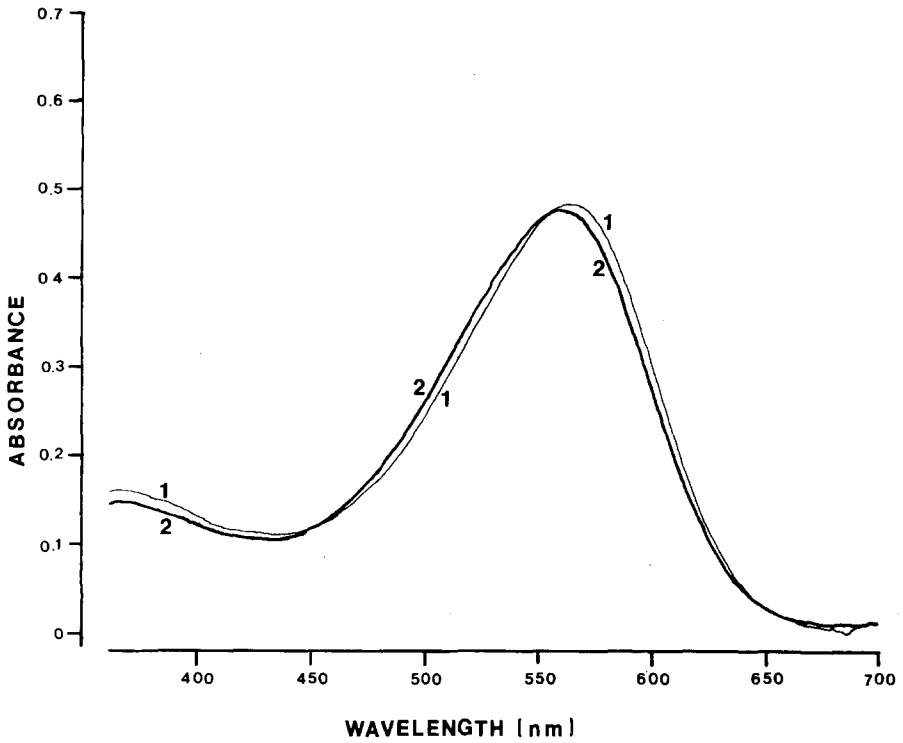

Fig. 2. Dark-adaptation of bacteriorhodopsin in a suspension of purple membrane fragments in hexane. The hexane suspension was kept overnight in the dark and spectrum *2* was then recorded. Following illumination of the sample, spectrum *1* was recorded. The spectra differ in their λ_{max}, which are near 560 nm in the dark sample and 565 nm in the illuminated one. A similar reversible blue-shift upon dark-adaptation is seen in aqueous suspensions of purple membrane

that incorporation of purple membrane fragments produces expanded interface films, more compressible than soya PC monolayers. The collapse pressure, however, is the same for both films, 45 dynes/cm (Fig. 4). The surface potential isotherms illustrated in Fig. 4 also show the different ordering of the two films. In addition, the surface potentials of the bacteriorhodopsin film at collapse is about 100 mV more negative (260 ± 15 mV) than that of the PC film (380 ± 5 mV). This difference in surface potential could be the result of net negative charges being introduced into the film or of the introduction of permanent dipoles or both. Purple membrane lipids and bacteriorhodopsin both have a net negative charge at the pH of the subphase and can both, therefore, contribute to the change in surface potential. Bacteriorhodopsin molecules also have a

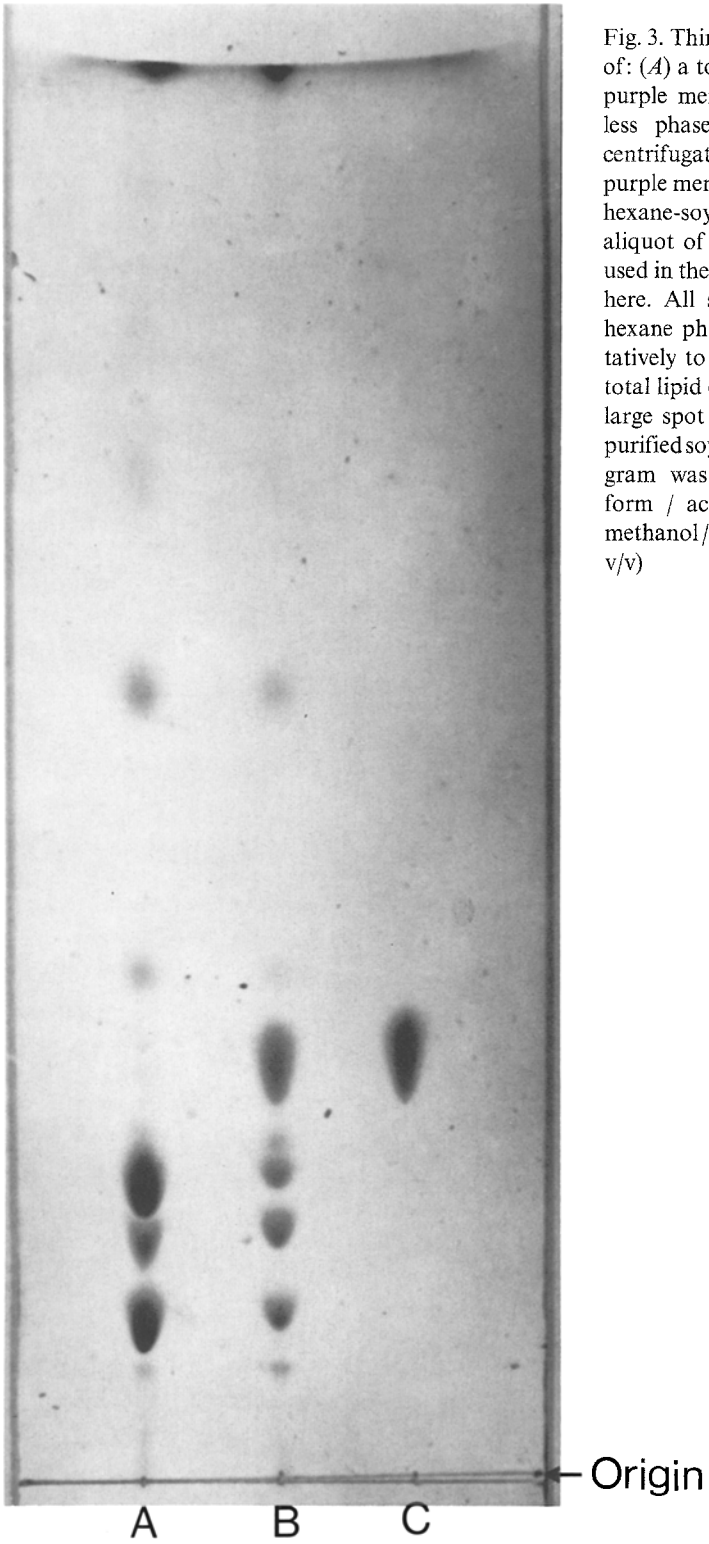

Fig. 3. Thin layer chromatogram of: (*A*) a total lipid extract from purple membrane; (*B*) a colorless phase obtained following centrifugation of a suspension of purple membrane fragments in a hexane-soya PC solution; (*C*) an aliquot of the purified soya PC used in the experiments reported here. All spots detected in the hexane phase correspond qualitatively to those detected in the total lipid extract, except for the large spot corresponding to the purified soya PC. This chromatogram was eluted with chloroform / acetone / acetic acid / methanol/water (5:2:1:1:0.5 v/v)

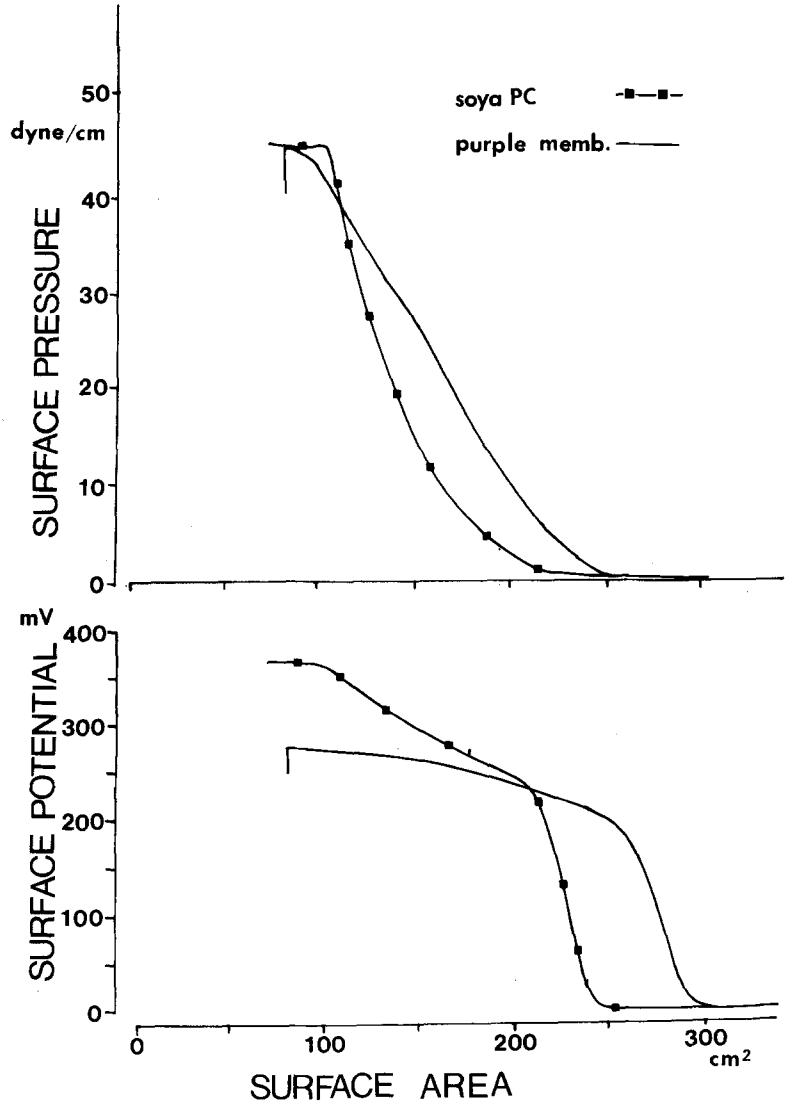

Fig. 4. Surface pressure and surface potential *vs.* area isotherms of purple membrane interface films of 7:1 bacteriorhodopsin/soya PC and soya PC monolayers. Isotherm were recorded at 20° over a subphase of distilled water, pH 5.6–5.8. Surface pressure and surface potential are presented as a function of through area. The isotherms, measured in separate experiments, were redrawn for illustration so that they coincide in area at the collapse pressure

large permanent dipole (Kriebel & Albrecht, 1976) and this could also contribute to the change in potential, if the molecules were oriented at the interface. We shall show below by freeze-fracture electron microscopy that bacteriorhodopsin molecules are indeed highly oriented at the interface.

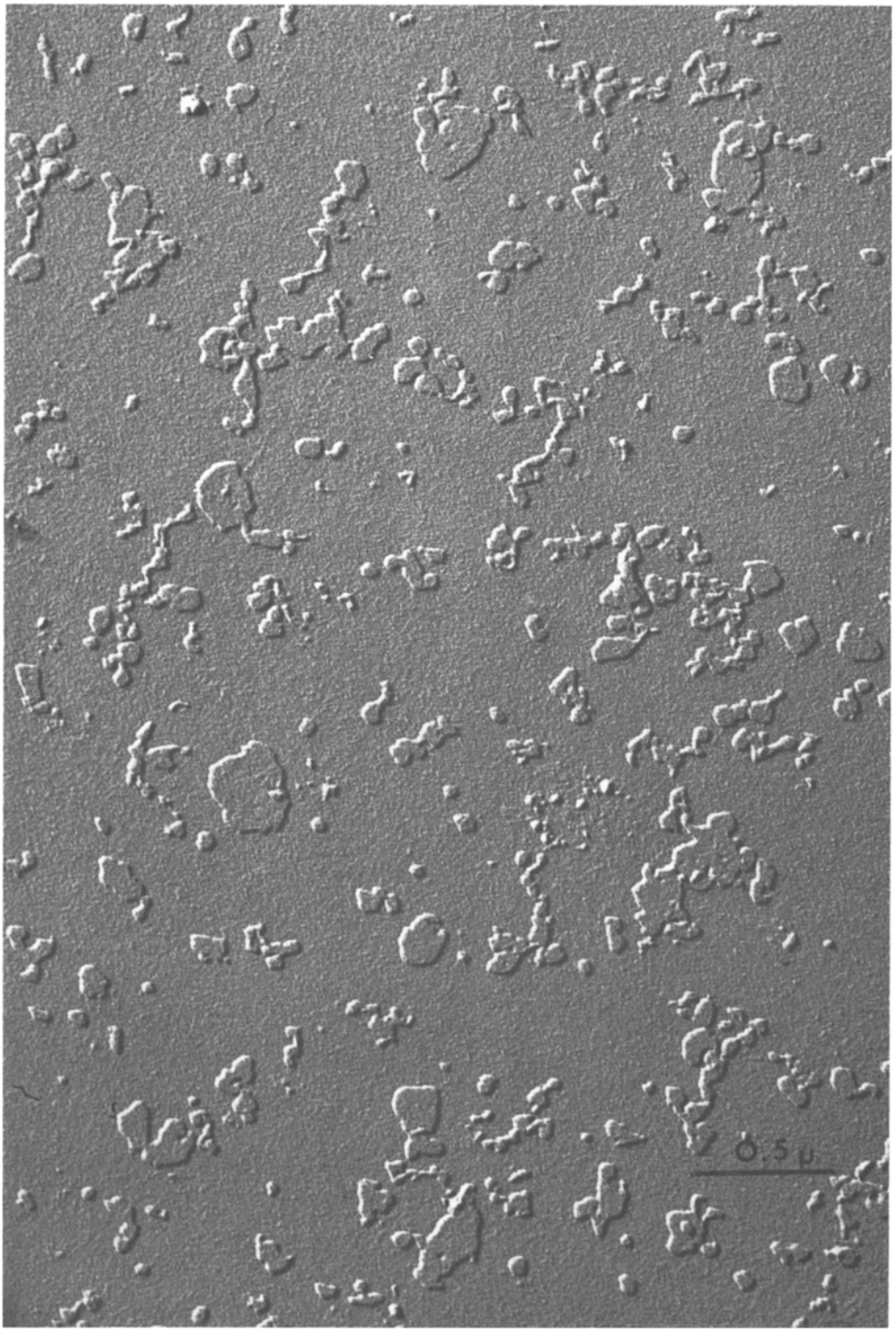

Fig. 5. Electron micrograph of a replica of a 7:1 bacteriorhodopsin/soya PC film on mica support. The membrane fragments are randomly distributed and do not overlap. The areas between membrane fragments are presumably occupied by a monolayer of soya PC and purple membrane lipids

Electron microscopy allows direct observation of the organization of the interface film. Fig. 5 is an electron micrograph of a replica of a film formed with 7:1 bacteriorhodopsin/soya PC weight ratio transferred to mica. The purple membrane fragments, with an average diameter of 0.1 μm, are randomly distributed and separated by smooth spaces presumably occupied by PC and purple membrane lipids in a monolayer. 36% (±5%) of the surface area is occupied by purple membrane fragments under these conditions. The purple membrane fragments in the interface film lack the distinctive cracked surface seen on only one of the faces of replicas of purple membrane fragments sprayed from aqueous suspensions and air-dried (Blaurock & Stoeckenius, 1971). The lack of cracked surface, however, cannot be ascribed to the preferential orientation of fragments at the interface, since these cracks are also absent in purple membrane fragments in which native lipid membranes are replaced by deoxycholate (Hwang & Stoeckenius, 1977). Therefore, we may attribute the structural change in the purple membrane to the partial removal of lipids from the membrane by hexane.

The bacteriorhodopsin/soya PC weight ratio in the spreading suspension affects the structure of of the interface film. Weight ratios greater than 10:1 result in films in which the purple membrane fragments no longer appear as discrete patches, but overlap in stacks several membranes thick. Therefore, only films in the weight ranges of 2:1 to 10:1, in which purple membrane fragments do not overlap at the interface, were used in the experiments described here.

The vectorial nature of the proton transport by bacteriorhodopsin, requires in an adequate model system that molecules exist uniformly, or at least preferentially, oriented and that this orientation be highly reproducible. It is possible to identify the directions of purple membrane orientation in freeze-fracture preparations because the replica of the outer membrane half (*B* face) appears smooth, whereas that of the inner membrane half (*A* face) appears rough with particles arranged in hexagonal lattices (Blaurock & Stoeckenius, 1971; Hwang & Stoeckenius, 1977). A freeze-fracture replica of a purple membrane-soya PC film is shown in Fig. 6. We found that not all purple membrane fragments in the film fracture along their hydrophobic interior. Purple membrane surfaces as well as fracture faces are observed and can be identified by their surface structure and the length of their shadows. Where interior fracture occurred, more than 85% of fracture faces appear rough, that is, corresponding to the inner membrane "half" (*A* face). Because the observed fracture is that of the membrane "half" attached to the glass

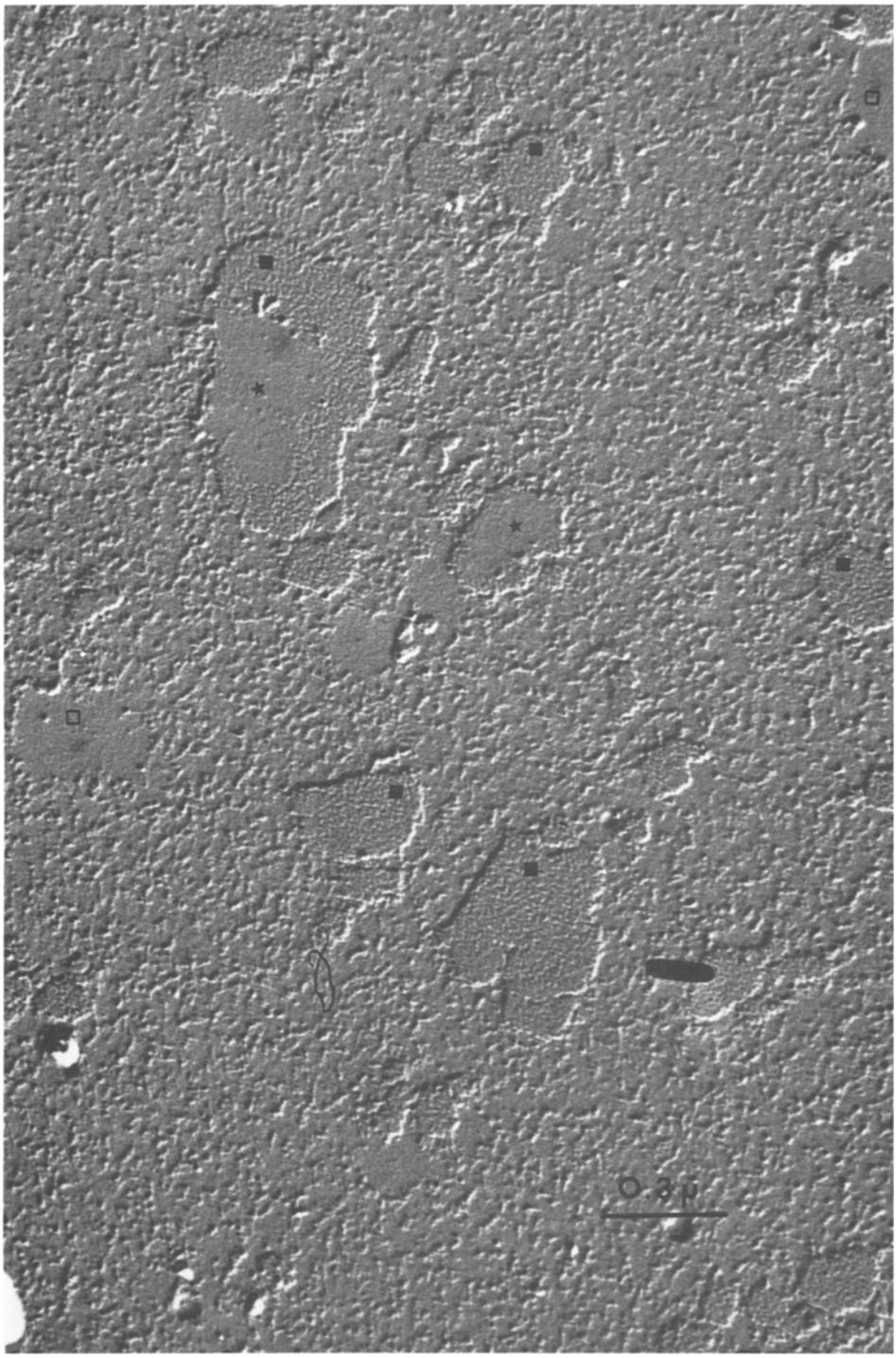

Fig. 6. Freeze-etch electron micrograph of a 7:1 bacteriorhodopsin/soya PC film on a glass support. The fracture face replicated is the one facing away from the water subphase. A few nonfractured fragments can be seen (*). Of the fractured fragments, the great majority show the particle and lattice structure characteristic of the fracture face of the cytoplasmic leaflet of the purple membrane (■) and only a few show the smooth fracture face of the outer leaflet (□)

support, the electron micrographs indicate that, in the interface film, over 85% of the purple membrane fragments are oriented with their cytoplasmic surface towards the aqueous subphase. The orientation may even be better than this, since it was not possible to distinguish a smooth fracture face *B*, from the smooth glass surface replica. On the other hand, no truly reliable quantitative statement can be made, since our estimates are limited to the sampling of those areas of the interface film in which we succeeded in fracturing the membrane. Nevertheless, a high fraction of the membranes are preferentially oriented. Such high degree of orientation is confirmed by the surface potential of the films discussed above and its direction by the detection of light-induced proton transport across the film described in the accompanying paper (Hwang *et al.*, 1977). Furthermore, the orientation obtained in highly reproducible as suggested by the small standard deviations in the values of surface potential and proton transport measured in these films.

To investigate whether the structure of bacteriorhodopsin at the interface is the same as its structure in aqueous suspension, we measured the spectral characteristics in the visible range of purple membrane interface films transferred to glass slides. Purple membrane interface films transfer to the glass support only on withdrawal across the interface. The slides emerge wet and must dry thoroughly before being dipped again (approximately 10–20 min), otherwise the film floats off the support on the downstroke. We prepared films with up to 160 layers, 80 on each side of the glass. The visible absorption spectrum of an 80 layer stack of a 7:1 bacteriorhodopsin/soya PC film (Fig. 7), shows a single absorption band with λ_{max} at 570 nm, and is identical to the spectrum of an aqueous suspension of purple membrane except for the light scattering in the multilayers (*compare* Fig. 7 to Fig. 1). To reduce the scattering contribution, we produced a difference spectrum by "bleaching" the bacteriorhodopsin-soya PC film. In the presence of ether, aqueous bacteriorhodopsin suspensions bleach in strong light, because the lifetime of the photocycle intermediate, M_{412}, increases (Oesterhelt & Hess, 1973). We found that bacteriorhodopsin in the multilayer film also bleaches in the presence of ether vapors when exposed to light absorbed in the 570 nm band. Figure 7 shows the ether-bleached spectrum of the multilayers. The difference spectrum obtained is indistinguishable from that of bacteriorhodopsin in aqueous suspensions of purple membrane or in intact bacteria. However, extreme drying results in a blue-shift of the absorbance peak.

The absorbance at 570 nm in multilayers is linearly proportional to

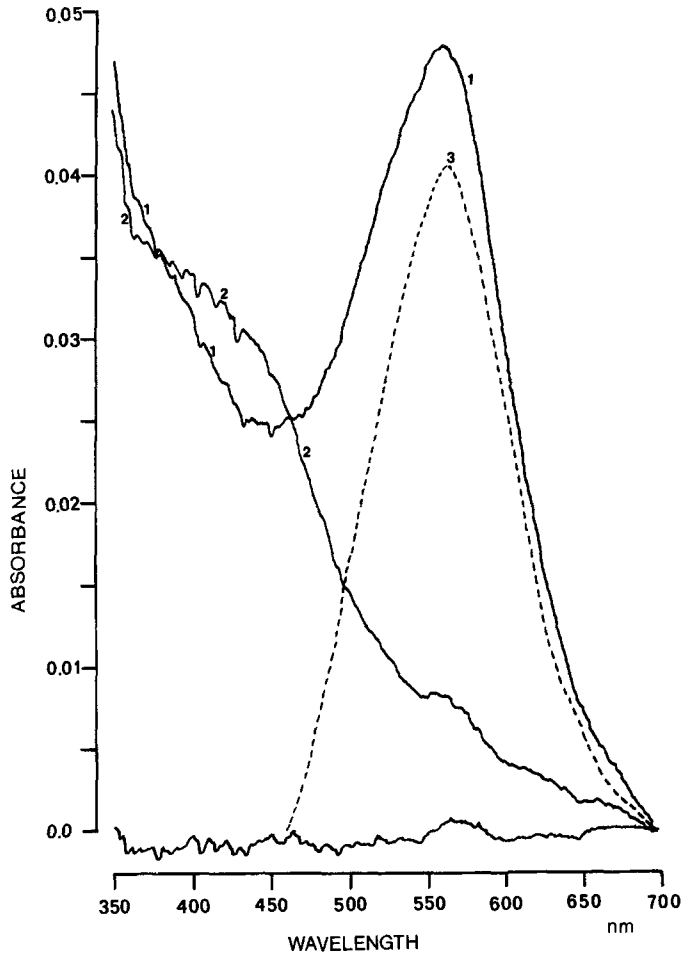

Fig. 7. Visible absorption spectrum of a multilayer consisting of 80 layers of a 10:1 bacteriorhodopsin/soya PC film (spectrum *1*). The contribution of light scattering was corrected for by obtaining a "bleached" spectrum by illumination in the presence of ether vapor (spectrum *2*). The difference spectrum (curve *3* = 1–2) is indistinguishable from the absorption spectrum of bacteriorhodopsin in aqueous suspension

the number of layers in the stack. Therefore, the optical density of a single layer can be estimated from extrapolation of the absorbance at 570 nm in multilayer spectra such as those in Fig. 7. We can thus measure the single film absorbance for films spread at various protein to lipid weight ratios from their respective multilayer absorbance. The relation between absorbance at 570 nm and bacteriorhodopsin concentration applied to form the film is linear (Fig. 8). This suggests that over the range tested the films have the same general structural characteristics detailed above. The absorbance at 570 nm of a single purple membrane-

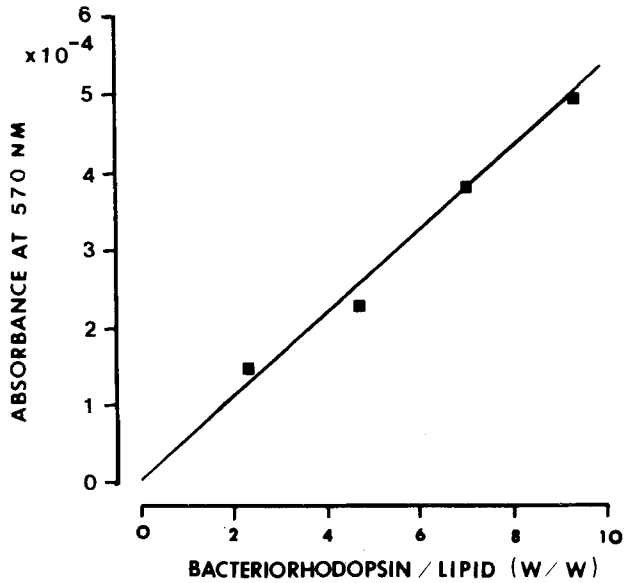

Fig. 8. Single film absorbance at 570 nm of films formed from hexane suspensions of various bacteriorhodopsin/soya PC weight ratios. The single film absorbance was calculated by linear extrapolation of the absorbance of 80 layer multilayers after correction for light scattering

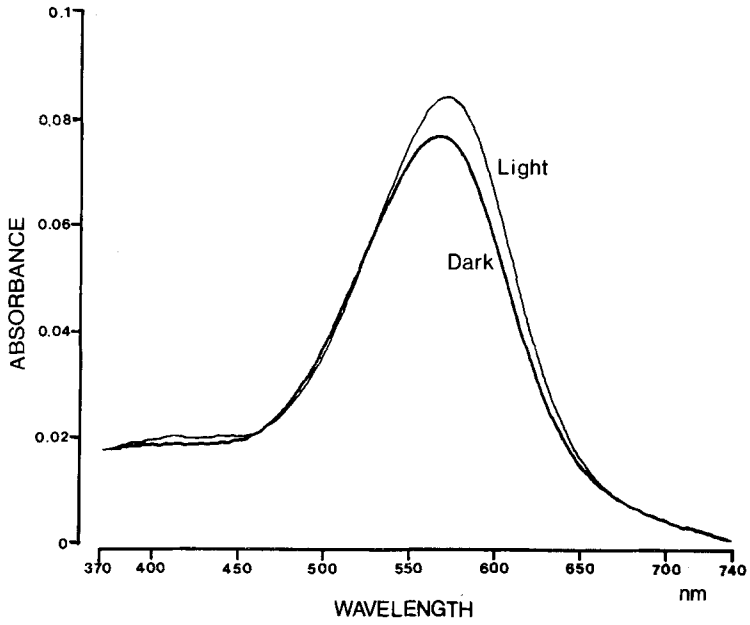

Fig. 9. Dark-Adaptation of 120 layer multilayer of 7:1 bacteriorhodopsin/soya PC interface film. The dark spectrum was recorded following two days of dark-adaptation at room temperature. The multilayer was illuminated with a filter which passed wavelengths longer than 540 nm and the light spectrum was immediately recorded. The spectra demonstrate the characteristic small blue shift and absorbance change of bacteriorhodopsin upon dark-adaptation

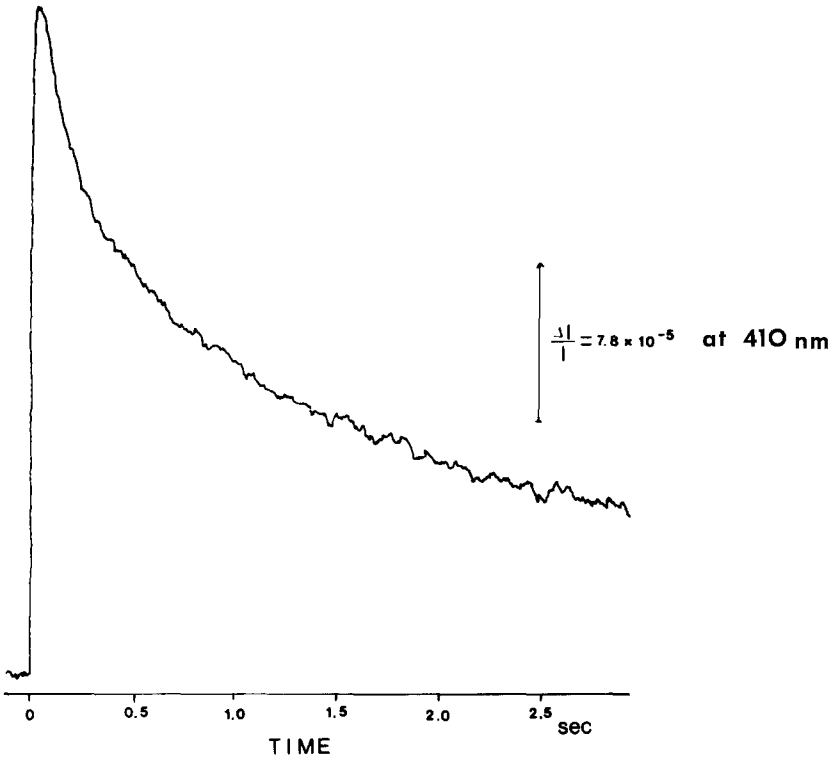

Fig. 10. Transient absorbance increase of a multilayer at 410 nm following flash illumination with 575 nm light. The rise and decay of the 412 intermediate are recorded. The multilayer consists of 120 layers of a 7:1 bacteriorhodopsin/soya PC film. The kinetics of decay of the intermediate are slower than in aqueous suspensions of purple membrane.

film spread from a 7:1 suspension has an average value of 3.63×10^{-4} O.D. (range 3.20 to 4.0). Because each unit cell in the purple membrane lattice contains 3 bacteriorhodopsin molecules and measures $6.3 \times 6.3 \times 5.0$ nm, it is possible to calculate from the absorbance and the molar extinction coefficient the fraction of the surface area occupied by purple membrane. Reported values for the bacteriorhodopsin extinction coefficient range from 54,000 (Oesterhelt & Stoeckenius, 1971) to 63,000 $M^{-1}\,cm^{-1}$ (Oesterhelt & Hess, 1973) and yield values for the fraction of the area occupied by purple membrane area of 53% to 45%, respectively. This number must be corrected, however, because the molar extinction coefficients are measured in preparations with randomly oriented chromophores, whereas our measurements are done with chromophores oriented in the plane of the purple membrane and a measuring light beam normal to this plane (see Liebman, 1962, for detailed discus-

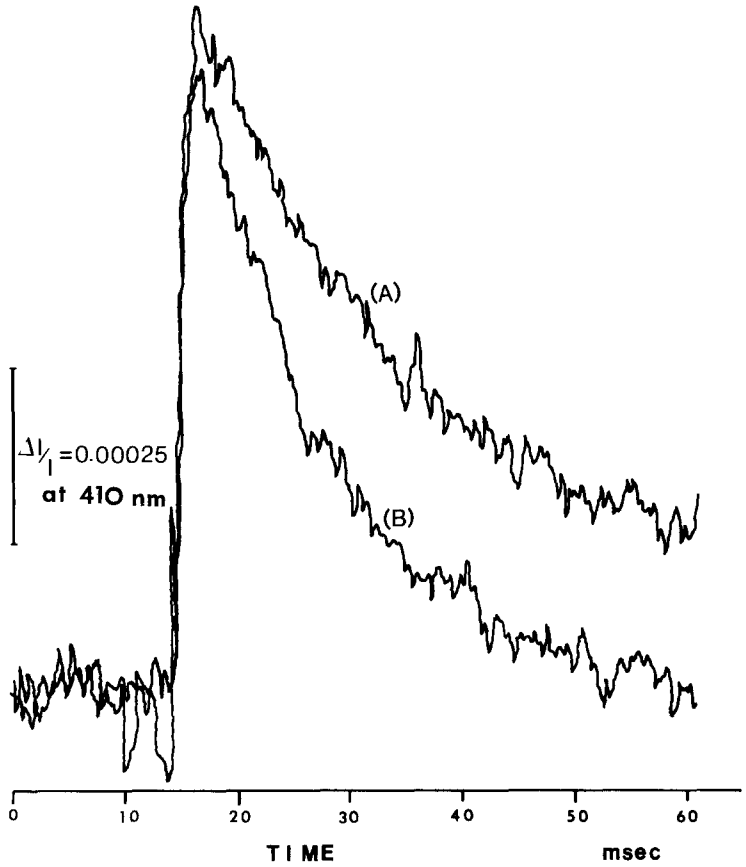

Fig. 11. Effect of water on the kinetics of the M_{412} photointermediate in the bacteriorhodopsin photoreaction cycle in multilayers. Transient absorbance increase at 410 nm after a 1 μsec flash of 575 nm light were measured in a multilayer immersed in water. The rise and decay of the 412 intermediate were recorded. After 6 hr immersion, (*A*) half-time of decay of the intermediate accelerated from an original of about 1 sec to about 33 msec. After 1 week immersion, (*B*) the half-time of decay was about 10 msec, similar to the value obtained in aqueous suspensions of purple membrane. The rate of formation of M_{412} was not affected. The multilayer consisted of 120 layers of a 7:1 bacteriorhodopsin/soya PC film

sion). The angle between the chromophore transition moment and the plane of the purple membrane has been independently determined by several investigators and found to be 19–22° (Kriebel & Albrecht, 1976; Bogomolni *et al.*, 1977; Mao *et al.*, 1977). Using 20° for the value of this angle and an extinction coefficient of 63,000, the value now widely accepted, the area coverage calculated from the absorbance data is 35%, a value in satisfactory agreement with the purple membrane area coverage

measured in the electron micrographs. This quantitative agreement indicates, furthermore, that essentially all bacteriorhodopsin molecules at the interface are spectroscopically intact.

Dark-adaptation and photoreaction cycles of bacteriorhodopsin are also preserved in the interface films. The absorption spectrum of multilayers kept in the dark for over 24 hr has a λ_{max} near 560 nm. Following illumination, the maximum absorbance increases and shifts to 570 nm (Fig. 9). Illumination of intact bacteria and purple membrane suspensions at room temperature and physiological pH causes a transient absorbance increase at 412 nm. This is due to the formation of the M_{412} intermediate of the photoreaction cycle which decays in about 10 msec (Lozier *et al.*, 1975). In air-dried multilayers we observe upon flash illumination a transient increase in absorbance at 410 nm which reaches its peak in less than 100 μsec and half-decays to its original level in about 1 sec (Fig. 10). Apparently, bacteriorhodopsin undergoes a slower photoreaction cycle in the air-dried multilayers than in aqueous suspension. We attribute this slowdown to the drying of the film, since the cycle accelerates when the multilayers are immersed in water. The acceleration increases with increasing time of immersion (Fig. 11), and the rate of decay is nearly the same as in aqueous suspensions after a week of immersion. The kinetics of the photoreaction cycle of bacteriorhodopsin on the water surface may therefore be comparable to those in aqueous suspensions of purple membrane. The spectroscopic results establish that bacteriorhodopsin spread from hexane at an air-water interface preserves spectroscopic characteristics essentially identical to those seen in intact bacteria and aqueous suspensions of purple membrane.

Discussion

We have described the preparation and the structural and spectroscopic characteristics of air-water interface films containing bacteriorhodopsin. In these interface films bacteriorhodopsin is not molecularly dispersed. It exists organized in fragments of purple membrane. The membrane fragments are highly oriented at the interface and the bacteriorhodopsin molecules are spectroscopically intact. We have used hexane as a volatile spreading solvent for the purple membrane fragments. We formed films by spreading from an organic solvent, rather then by simply depositing fragments of purple membrane at the interface, a method which will also succeed in forming a surface film (*unpublished observations*) because the use of solvent allowed preferential orientation of purple

membrane at the interface. Interface films of purple membrane have also been formed by adsorption of purple membrane from the aqueous subphase into a hydrocarbon-water interface (Boguslavsky *et al.*, 1975). While these films may be similar to those described here, their structural or spectroscopic characteristics have not yet been reported. A singular experimental problem when forming interface films of membrane fragments is to avoid membrane overlap. The formation of a lipid monolayer between membrane fragments allowed us to find empiric conditions under which no membrane overlap occurred. Presumably under these conditions the interfragment distance is large compared with the effective range of membrane-membrane attractive forces.

The high degree of orientation of membrane fragments at the interface could be the result of a difference in the charge density of the two surface of the purple membranes. At a clean air-water interface, a large voltage gradient exists between air and water. Such a large field would be expected to effectively orient structures with dipolar characteristics. However, this possible charge asymmetry could have been introduced in our experiments by the extraction of lipids and our observations cannot be used as evidence of charge asymmetry in intact purple membrane.

In the air-dried multilayer films, the kinetics of decay of at least one intermediate in the photoreaction cycle of bacteriorhodopsin are dependent on the extent of hydration of the film. A similar dependence of photoreaction kinetics on the extent of hydration occurs in vertebrate rhodopsin: At room temperature, the photocycle of Rh stops at the Meta I intermediate in the absence of water (Wald, Durell & St. George, 1950). Both vertebrate rhodopsin and bacteriorhodopsin are known to bind protons in the reaction step which follows Meta I and M_{412} intermediate, respectively. The need of water molecules as donors for the required proton may be a common explanation for the slowing of the kinetics in the relative absence of water.

The highly oriented purple membrane-soya PC interfacial films are not only useful for investigation of the optical properties described here. We have also used them to investigate light driven proton translocation (Hwang *et al.*, 1977) and for the determination of chromophore orientation and its changes during the photoreaction cycle (Bogomolni *et al.*, 1977). Film multilayers placed between metal electrodes produce light-induced potential changes reflecting charge rearrangements during the photoreaction cycle (Hwang & Korenbrot, *in preparation*). Finally these films constitute a fundamental step toward the organization of oriented interfacial films separating large aqueous compartments (Tsofina, Liber-

man & Babakov, 1966; Montal & Mueller, 1972), an ideal system in which to study the mechanism of bacteriorhodopsin function.

We thank Knute A. Fisher for advice and help with freeze-fracture of the films, Richard H. Lozier and Roberto Bogomolni for the flash spectroscopy. We are particularly grateful to Jon Goerke for the use of the surface balance and for his guidance and thoughtful advice. Research supported by grants EY-01586 and HL-06285 from NIH and NASA grant NSG-7151.

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