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Formation, Structure, and Spectrophotometry of Air-Water Interface Films Containing Rhodopsin

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Summary. Air-water interface films of cattle rhodopsin and defined lipids are formed without the use of organic solvents by a method in which vesicle membranes consisting of egg phosphatidyl choline and purified rhodopsin are osmotically shocked at the interface. Lipid and protein molecules organize as insoluble films at the interface. The structure of these films varies with the lipid to protein mole ratio of the source vesicle membranes. Electron microscopic observations reveal that films formed with membranes of 150:1 mole ratio consist of nonoverlapping, randomly distributed vesicle membrane fragments separated by a lipid monolayer. These membrane fragments exist as single sheets on the water surface and occupy approximately 35% of this surface. Essentially all the rhodopsin molecules at the interface are spectroscopically intact and are contained within the membrane fragments. The visible absorption spectrum of the interface films is identical to that of suspensions of rod disc membranes. Moreover, flash illumination of rhodopsin in air-dried multilayers formed from the interface films results in the formation of a stable Metarhodopsin *I* intermediate ($\lambda_{max} \simeq 480$ nm) which can be fully bleached by increasing the relative humidity of the multilayers or can be photoconverted into rhodopsin and, presumably, isorhodopsin. Furthermore, rhodopsin is chemically regenerable at the air-water interface. Bleached rhodopsin can generate dark rhodopsin at the interface in the presence of 11-cis retinal in the aqueous subphase. Thus, the spectroscopic structure and the chemical regenerability function of rhodopsin in these interface films are indistinguishable from those exhibited by the protein in intact rod disc membranes.

Rhodopsin is a light-absorbing protein embedded in the disc and plasma membranes of the outer segments in rod photoreceptors (Jan & Revel, 1974; Basinger, Bok & Hall, 1976). Rhodopsin constitutes 85–90% of the protein component of these membranes (Robinson, Gordon-Walker & Bownds, 1972; Heitzman, 1972). In the dark, rhodopsin exhibits a characteristic absorption spectrum in the visible range which results from the interaction of the protein with an 11-cis-12s isomer of retinal (Honig & Ebrey, 1974). Upon absorption of light, the retinal chromophore isomerizes to an all-trans form and the protein proceeds

through a sequence of conformational states (Hubbard, Brown & Kropf, 1959; Kropf, 1972). Each conformational state is characterized by a distinct visible absorption spectrum, and the rate at which any state proceeds to the next one is a function of both temperature (Yoshizawa, 1972) and relative humidity (Wald, Durell & St. George, 1950). The absorption of a single photon by rhodopsin initiates the change in ion permeability of the plasma membrane which results in the electrophysiological response of the rod cell (Hagins, 1972; Montal & Korenbrot, 1976). The mechanism by which rhodopsin mediates this permeability change remains unknown.

An experimental approach to investigate the function of rhodopsin, as has been done with other membrane-bound proteins (Korenbrot, 1977*a*), is to study lipid interface films containing rhodopsin as the only protein. Ideally such interface films would separate two large and easily accessible aqueous compartments and would have chemical and spectroscopic characteristics identical to those of the native membrane. Hong and Hubbell (1972) and Applebury, Zuckerman, Lamola and Jovin (1974) have reported the incorporation of purified rhodopsin, free of disc membrane lipids, into small membrane vesicles of defined lipid composition. Chabre, Cavaggioni, Osborne, Gulik-Krzywicki and Olive (1972) and Darszon and Montal (1976), on the other hand, have reported the incorporation into membrane vesicles of a detergent extract of purified outer segments, rich in rhodopsin and disc membrane lipids. These vesicle membranes are excellent subjects for structural (Chabre *et al.*, 1972; Chen & Hubbell, 1973), tracer flux (Darszon & Montal, 1976) and spectroscopic (Hong & Hubbell, 1972; Hong & Hubbell, 1973; Applebury *et al.*, 1974) studies. Unfortunately, their experimental use is limited because the intravesicular compartment is small and not directly accessible to measurements of solute concentrations or electrical parameters. To complement studies in vesicle membranes, we have recently shown, for the case of bacteriorhodopsin (Hwang, Korenbrot & Stoeckenius, 1977*a*), that air-water interface films are helpful experimental tools, since they can be used for solute transport studies and, furthermore, they can potentially be developed into films separating two large aqueous compartments (Tsofina, Liberman & Babakov, 1966; Takagi, Azuma & Kishimoto, 1965; Montal & Mueller, 1972).

Over the years, several reports have appeared on the formation of air-water interface films containing rhodopsin. These include films formed with fragments of cattle rod disc membranes (Azuma & Takagi, 1966; Bonting & Bangham, 1967), ethanolic extracts of frog rhodopsin

(Hyono, Kuriyama, Tsuji & Hosoya, 1962) and hexane or ether "proteolipids" of cattle rhodopsin (Montal & Korenbrot, 1973; Montal, 1975). These reports, however, have not fully characterized the properties of rhodopsin interface films. In particular, the question of the structure of rhodopsin at the interface and how it compares with the native structure in the disc membrane has not been investigated. 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 functional properties of proteins in interface films must substantiate that the structure of the protein at the interface is intact. We report here on the formation and characteristics of air-water interface films of lipid and spectroscopically intact and chemically regenerable cattle rhodopsin. These films at the air-water interface are formed by spreading membrane fragments of defined lipid composition in which rhodopsin is present as the only protein, using a novel method which does not require organic solvents.

Materials and Methods

Materials

Egg phosphatidyl choline (egg PC) was prepared and purified according to Singleton, Gray, Brown and White (1965). The purified lipid appeared as a single spot on silica gel 60 TLC plates (E. Merck Labs) eluted with chloroform/methanol/acetic acid/water (50:25:8:4). The lipid was stored in benzene under nitrogen at −40 °C. The detergent used, tridecyl trymethyl ammonium bromide (TriTAB) was synthesized by the reaction of anhydrous trimethylamine (Eastman Chemicals) and tridecyl bromide (Columbia Organic Chemicals, Columbia, S.C.) according to the method of Hong and Hubbell (1973). Detergent was recrystallized 2 times from acetone-methanol. 11-cis retinal was the kind gift of P.K. Brown, Harvard University. All inorganic chemicals were analytical reagent grade. Water used throughout was 4 times glass distilled including once from alkaline permanganate and once from sulfuric acid.

Purification of Rhodopsin

Purified, delipidated rhodopsin containing less than 1 mole phosphate per mole rhodopsin was prepared generally following the methods of Hong and Hubbell (1973) and Applebury *et al.* (1974). All procedures involving rhodopsin were carried out under dim red light (General Electric BAS bulbs, 25 W) in an ice bath or a cold room (4 °C). Rod outer segments were isolated and purified from 50–100 frozen cattle retinas (G. Hormel Co., Austin, Minn.). The retinas were homogenized in 100 ml of a 40% sucrose solution in 100 mM phosphate buffer, pH 6.8, with a Teflon-glass homogenizer. The homogenate was centrifuged at $3{,}700 \times g$ for 15 min. The supernatant was collected and diluted with

equal volumes of phosphate buffer and recentrifuged for 20 min at 26,000 × *g*. The resulting pellet was resuspended by homogenization in 50 ml of 1 M sucrose in phosphate buffer and centrifuged 30 min at 26,000 × *g*. The supernatant was collected, diluted with equal volumes of buffer and centrifuged for 15 min at 26,000 × *g*. The resultant pellet was again floated in 1 M sucrose. The final pellet was washed 3 times with 5 mM phosphate buffer. Within 24 hr, rhodopsin was extracted from these membranes by resuspending them in 3 to 5 ml of 5 mM phosphate buffer and adding enough solid TriTAB to make a 300-mM solution. Following 10 to 15 min incubation at room temperature, the suspension was centrifuged for 15 min at 26,000 × *g*. The clear supernatant contained the rhodopsin.

Purification of rhodopsin and removal of phospholipids was carried out by chromatography on hydroxylapatite (DNA-grade Bio-Gel HTP, Bio-Rad Labs, Richmond, Calif.). A 2.5 × 6 cm column was equilibrated with a solution of 100 mM TriTAB, 1 mM dithiothreitol (DTT) in 15 mM phosphate buffer, pH 6.8. The TriTAB extract of rhodopsin was applied in 3 to 5 ml, and the column was eluted with a 300-ml linear gradient of 0 to 0.5 M NaCl in 100 mM TriTAB, 1 mM DTT in 15 mM phosphate buffer. Fractions of 2.5 ml were collected and their absorbance at 278 and 498 nm measured. All fractions with an absorbance ratio A_{278}/A_{498} of 1.8 or less were pooled. Solutions of purified, delipidated rhodopsin were stored in complete darkness at 4 °C for periods never longer than 6 weeks.

Formation of Rhodopsin-Containing Membrane Vesicles

Incorporation of rhodopsin into vesicle membranes was accomplished by removal of detergent by dialysis in the presence of added phospholipids. An aliquot containing egg PC in benzene was first evaporated under a stream of nitrogen, resuspended in hexane, and again evaporated under nitrogen. Purified rhodopsin in 100 mM TriTAB was added to the solvent-free lipid and the mixture was incubated 4–6 hr at 4 °C to obtain a homogenous solution. Detergent was removed by dialysis at 4 °C against a 100-fold excess volume of 5×10^{-5} M DTT in 10 mM MOPS buffer, pH 6.8. The dialysis was carried out for 3–4 days with changes of solution approximately every 12 hr. The resulting multilayered membrane vesicles (Hong & Hubbell, 1972) were collected by centrifugation at 105,000 × *g* for 45 min. The vesicles were resuspended in the standard salt solution consisting of 200 mM NaCl, 10 mM $CaCl_2$ in 10 mM TRIS-buffer, pH 7, pelleted again and finally suspended in 0.5–1.0 ml of the standard salt solution. This final suspension was in the concentration range of 4–8 mg/ml of phospholipid. The vesicle suspension was stored at 4 °C. Rhodopsin in these vesicles is not thermally stable over a long period of time; therefore, the vesicles were never stored longer than 2 weeks, a period over which the spectroscopic properties of rhodopsin did not change.

The values of the egg PC:rhodopsin mole ratio expressed throughout this report were measured after harvesting the vesicle membranes. The concentration of rhodopsin was determined from the absorbance at 498 nm of samples cleared with 100 mM TriTAB, assuming an extinction coefficient of 40,600 (Wald & Brown, 1953). The concentration of egg PC was determined by measuring phosphate concentration by the procedure of Bartlett (1959) and assuming a mol wt of 800 for the lipid molecules.

Preparation of Single Bilayer Vesicles

Single bilayer rhodopsin membrane vesicles were formed by sonication of the above described multilayered vesicles. 100–300 μl aliquots of membrane suspensions always at a concentration of 4 mg egg PC/ml in the standard salt solution were sonicated at room temperature (20–22 °C) in a bath sonicator (Model G-80-80, Laboratory Supply Co., Hicksville, N.Y.). Sonication proceeded until the suspension appeared completely clear under deep

red light, usually 20–30 sec. Suspensions were never sonicated for periods longer than about 1 min, since longer times initiated rhodopsin denaturation as measured by loss of absorbance at 498 nm and increase in absorbance around 370 nm. After sonication a 35-μl aliquot was cleared with detergent and its absorption spectrum recorded. With the use of a radioactive marker, $^{45}CaCl_2$, and using a Sephadex G-50 (Pharmacia, Sweden) column to separate membrane vesicles from the extravesicular salts, we confirmed that the salt solution in which the sonication was carried out became entrapped within the vesicle volume.

Single bilayer lipid vesicles were prepared by bath sonication at room temperature of a 4–8 mg/ml suspension of egg PC in the standard salt solution until the suspension appeared clear, about 20 min.

Surface Balance

The recording surface balance used in these experiments was contained within a plastic box and consisted of a Teflon trough ($10.5 \times 38 \times 1$ 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 in the trough and, therefore, the film area was fed into one axis of an $X-Y$ plotter (Hewlett-Packard 7035B). The other axis recorded surface pressure and thus surface pressure-*vs.*-area isotherms were recorded directly. Surface pressure was measured by the Wilhelmy method (Gaines, 1966). A strip of sand-blasted platinum foil (2.5×0.5 cm), cleaned by flaming before each experiment, was suspended from a sensitive pressure transducer (Statham Instruments Gold Cell U-5 with microscale accessory). The output of the transducer was recorded with a stabilized bridge-balance amplifier. Surface experiments were carried out at room temperature (20–22 °C), and those involving rhodopsin were performed in a darkroom dimly illuminated with deep red safelight (General Electric BAS bulb, 25 W) or in complete darkness.

Spreading of Interface Films

Lipid films at the air-water interface were formed either by spreading the lipid with a hexane solution or by the osmotic method detailed below. Rhodopsin-containing interface films were formed exclusively by the osmotic method. In the osmotic method, a suspension of single bilayer vesicles containing a high salt concentration is gently delivered onto the surface of a subphase containing no salt. Single bilayer vesicles, with or without incorporated rhodopsin molecules, were prepared by sonication always in a 200 mM NaCl, 10 mM $CaCl_2$, 10 mM TRIS buffer, pH 7.0, solution within 2 hr of their being osmotically spread. The subphase in all experiments was a 5 mM solution of MOPS buffer, pH 6.8. The vesicle suspension was applied to the surface by gently delivering it drop by drop with a Hamilton syringe onto a dry, acid-cleaned glass slide partially submerged in the subphase. The glass slide (1×2.2 cm) was cut from coverslip glass (#2 Scientific Products, 0.18–0.22 mm thick), cleaned with dichromate-sulfuric acid and positioned at a 45° angle with respect to the aqueous surface. Interface films formed instantaneously upon delivery of the vesicle membranes. In the case of rhodopsin vesicles, interface films were formed by spreading 50 μl of a membrane vesicle suspension at a concentration of 4 mg egg PC/ml, regardless of the lipid/protein mole ratio. In the case of lipid vesicles, films were formed by spreading 100–200 μl of a membrane vesicle suspension in the concentration range of 4–8 mg/ml. The form of the surface pressure-area isotherm for the rhodopsin films, but not for the lipid films, depends on the surface area initially available for spreading. The efficiency of spreading depends on the position of the glass slide in the trough. In all experiments reported here, the initial surface area was 315 cm^2 and the glass slide was positioned at one end of the trough, with its long axis parallel to that of the trough.

Deposition of Interface Films on Solid Support

The rhodopsin and lipid films were transferred from the air-water interface to a hydrophilic solid support, either glass or mica, by slowly inserting and withdrawing the support through the interface (0.3–1.0 cm/min). Surface pressure was not maintained constant during transfer. The pressure loss associated with a single film transfer was 3–5 $mN \cdot m^{-1}$. The film was mechanically recollapsed between successive trips of the solid support through the interface. Thus, transfer was carried out with films which were within 3–5 $mN \cdot m^{-1}$ of their collapse pressure and success of transfer was monitored by loss of surface pressure. When mica was used as support, it was cleaved immediately before transfer. Glass used as support (#2 coverslip, 0.18–0.22 mm thick, Scientific Products, cut to 0.9×2.2 cm) was cleaned in warm dichromate-sulfuric acid solution and was extensively rinsed in distilled water before use.

Spectrophotometry

Visible and ultraviolet spectra of rhodopsin solution were recorded in a Cary 118 C spectrophotometer (Varian Instruments). Visible absorption spectra of multilayers of rhodopsin interface film were recorded in the same instrument equipped with a scattering transmission accessory. We found that we could not build up more than 10 layers of rhodopsin film in a single glass slide (5 on each side). Absorbance of a 10-layer stack was not detectable even at the highest instrument sensitivity. We overcame this problem by transferring 10 films onto each of 4 to 6 glass slides, and arranging the slides in tandem in the spectrophotometer. These multilayer slides were formed either from the same interface film or from different films prepared from the same vesicle sample. The slides were placed in a holder in which 0.25 mm grooves were machined at 0.25 mm intervals. The baseline of the spectrophotometer was manually adjusted between 650 and 400 nm with clean glass slides in the special holder. The clean slides were substituted with the rhodopsin-multilayer slides and spectra were then recorded.

Electron Microscopy

The physical organization of the rhodopsin and lipid films was studied by electron microscopic observation of single interface films transferred to glass on the withdrawal trip across the interface. Metal replicas of the interface films were prepared by one of two methods. *Shadow-casting*: The rhodopsin-lipid or pure lipid films were transferred to glass slides and allowed to dry. A replica of the surface film was formed by evaporating first platimum-carbon at a 45° angle followed by carbon at a 90° angle at 2×10^{-6} torr, either in an Edwards High Vacuum evaporator (Model 306) or a Balzers freeze-fracture unit (Model 301). In the Balzers unit, the microtome arm was cooled to liquid nitrogen temperature and was placed above the sample to act as a cold trap while the high vacuum was reached. The replicas were floated off the glass in 25% hydrofluoric acid and transferred to Formvar-carbon coated grids for observation. *Freeze-etching*: Rhodopsin films were transferred to a glass slide and the wet slide was immediately frozen in freon-22 and stored in liquid nitrogen. The frozen slide was placed on the sample stage of the Balzers unit precooled to −150 °C. A vacuum was applied, and upon reaching 2×10^{-7} torr, the temperature of the sample stage was raised to −100° for 15–20 min, in order to sublimate the frozen water. A platinum-carbon replica was made and reinforced with carbon. The sample was brought back to room temperature, floated off in hydrofluoric acid and transferred to grids for EM observation.

Autoradiography

Rhodopsin interface films with either the lipid or the protein radioactively labeled were studied by autoradiography. Rhodopsin radioactive labeling was accomplished by reacting sonicated rhodopsin membrane vesicles with ^{3}H-Concanavalin A (^{3}H-Con A) (sp act=45 Ci/mM, New England Nuclear, Bedford, Mass.). The rhodopsin vesicles were incubated with ^{3}H-Con A in a mole ratio of 1:50 Con A monomer to rhodopsin computed from a 27,000 mol wt estimate for the Con A monomer. The incubation was carried out for 1–2 hr in the dark at 4 °C in the presence of 1 mM $CaCl_2$ and 1 mM $MnCl_2$ added to a calcium-free standard salt solution. Under these conditions, Con A has been shown to specifically bind to rhodopsin (Steinemann & Stryer, 1973). In those films in which the lipids were radioactively labeled, purified ^{3}H-dipalmitoyl PC (sp act=10 mCi/mM, Applied Science, College Park, Pa.) was mixed in a 1:100 mole ratio with egg PC. The same lipid mixture was used both to form lipid monolayers and to form rhodopsin membrane vesicles, which were in turn used to form the rhodopsin interface films.

Interface films in which either the lipid or the protein molecules were labeled were transferred in the usual way to a glass slide. A metal-cast replica of the film was made as described above and, without separating it from the glass support, the replica was covered with a monolayer of Ilford L-4 autoradiographic emulsion (Poly Sciences, Warrington, Pa.) by the loop method of Caro, Van Tubergen and Kolb (1962). The emulsion was exposed for 3 weeks at 4 °C and was then developed with Microdol-X (Eastman-Kodak) using standard procedures. The replica and developed emulsion were floated off the glass support in hydrofluoric acid and transferred to grids for observation. All electron microscopic observations were done on a JEM 100B microscope (Jeol, Japan) operated at 60 kV. The fractional area occupied by membrane fragments in the electron micrographs was measured by the stererological method of volumetric point counting (Weibel & Bolender, 1973). Ten randomly selected fields, each about 40 μm^2, were each measured with a square point lattice of 1600 points, and results averaged. Under these conditions, the statistical accuracy of the measurement is 5%.

Light Exposure

Flash illumination of multilayer stacks of rhodopsin on glass support was accomplished with GE flash cubes (General Electric Co.) (30 msec duration) whose output was filtered through a filter combination passing light of wavelengths longer than 510 nm (Corning glass 3–69 and I–75 IR absorbing glass). Continuous illumination was carried out with a quartz-iodide source (100 W), also filtered to pass light of wavelengths longer than 510 nm. To increase the relative humidity of the light-exposed multilayer stacks, the glass slides, while still in the carrier from the spectrophotometer, were placed in a glass beaker containing wet filter paper and covered with Parafilm.

To bleach all the rhodopsin molecules in the sonicated vesicle membranes, the vesicles suspended in 200 µl aliquots were exposed to 200 msec duration steps of light of wavelengths longer than 540 nm (Corning glass 3–70) and 10^3 erg/cm^2·sec intensity once every 3 sec for 60 min. This treatment bleached over 99% of the rhodopsin molecules, as measured directly in the spectrophotometer.

Results

Cattle rhodopsin purified by chromatography on hydroxylapatite columns in TriTAB solutions exhibits absorption spectra like that shown

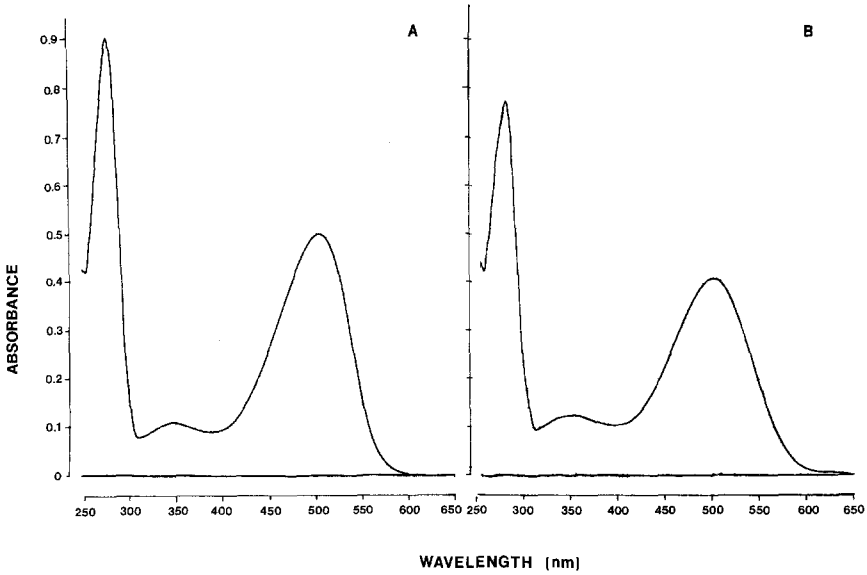

Fig. 1. Absorption spectra of purified cattle rhodopsin. (*A*): Delipidated rhodopsin in 0.1 M TriTAB following purification on hydroxylapatite. (*B*): Rhodopsin membrane vesicles formed by dialysis of detergent in the presence of egg PC. Lipid to protein mole ratio in the membranes is 150:1

in Fig. 1. The average absorbance ratio for *A* 400/*A* 498 was 0.19 (range 0.17–0.22) while for *A* 278/*A* 498, it was 1.71 (range 1.61–1.85). The values for these spectral ratios are characteristic of highly purified rhodopsin and are similar to those obtained with other purification schemes. Hong and Hubbell (1973) and Applebury *et al.* (1974) have shown that hydroxylapatite purification also removes phospholipids from the rhodopsin-detergent micelles leaving less than 1 mole phosphate per mole rhodopsin. Incorporation of this purified and delipidated rhodopsin into egg PC membrane vesicles inevitably results in some protein denaturation: rhodopsin in the recombinant membranes had a typical absorbance ratio for *A* 400/*A* 498 of 0.27 (range 0.23–0.31), while for *A* 278/*A* 498 it was 2.07 (range 1.91–2.10) (Fig. 1). Any vesicle membranes with spectral characteristics outside this range were not used in these experiments. Prolonged sonication results in protein denaturation; therefore, sonication to produce single bilayer vesicle membranes never lasted longer than 1 min and only sonicated vesicles with *A* 278/*A* 498 absorbance ratios within 0.1 units from the unsonicated material were used. These membrane vesicles of defined lipid composition and containing known amounts of purified rhodopsin were used to form the air-water interface films.

Formation of Interface Films

Lipid membrane vesicles are sensitive osmometers, highly permeable to water, but not to alkali cations (Bangham, DeGier & Greville, 1967; Haran & Shporer, 1976). Therefore, when small lipid membrane vesicles containing an impermeant salt solution are gently delivered onto the surface of water containing no salt, the resulting water flux into the vesicles would be expected to produce swelling and bursting of the vesicles. Some of the vesicles might reseal and become resuspended in the subphase; others, however, might remain at the interface, without resealing, as flat membrane fragment sheets. In the case of pure egg PC vesicles, these membrane sheets on the water surface (lipid bilayers) would be thermodynamically unstable structures and would spread immediately into a classical phospholipid monolayer. This is all the more plausible, since it is known that purified phospholipids deposited on a clean water surface in their solid form will spread into a monolayer if the temperature is above their phase transition (Phillips & Chapman, 1968). At 20 °C, egg PC is well above its phase transition (Chapman, 1975). Indeed, we were able to form monolayers by this technique. Fig. 2 compares the surface pressure *vs.* area isotherm of an interface film

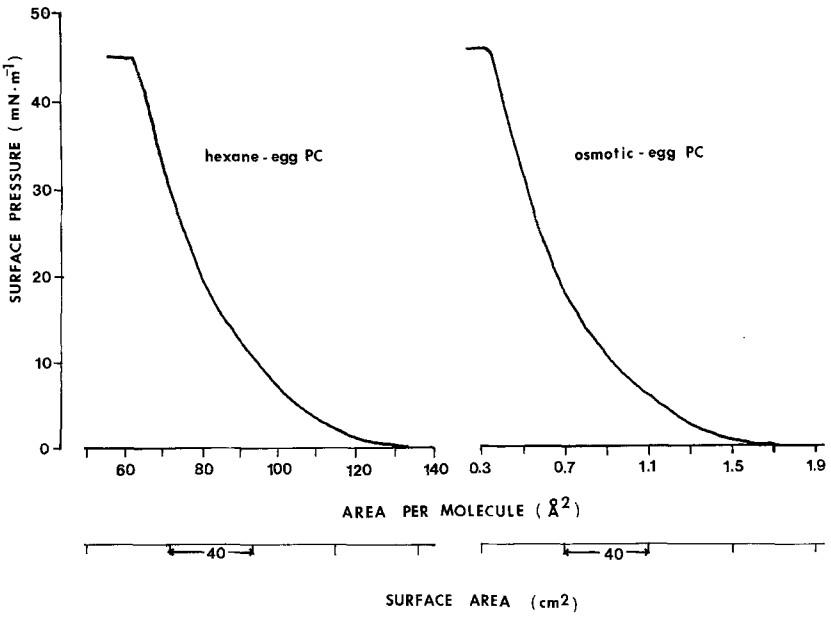

Fig. 2. Surface pressure *vs.* area isotherms of egg PC. On the left is the isotherm obtained by spreading 30 μg of lipid from a hexane solution. On the right is the isotherm recorded by osmotically spreading 2 mg of the lipid in the form of sonicated vesicle membranes. The surface area scale for both isotherm curves is the same. Subphase was 5 mM MOPS buffer, pH 6.8

formed by osmotically bursting egg PC membrane vesicles at the interface, with the isotherm of an egg PC monolayer spread from a hexane solution. The shapes of the isotherms are indistinguishable from each other, and similar to those previously reported by others for egg PC monolayers (Jones, 1975). However, the area per molecule at collapse pressure is very different. For the hexane-spread molecules, the area per molecule is 61 $Å^2$, in agreement with values obtained by others (Jones, 1975). The osmotically spread film, in contrast, shows an area per molecule of about 0.35 $Å^2$. Furthermore, the value of the area per molecule obtained in hexane-spread monolayers is highly reproducible, ± 2 $Å^2$, whereas the value in films spread by the osmotic method is reproducible only to within a factor of 2. Since essentially every egg PC molecule spread from hexane remains at the interface, the large difference in the estimated area per molecule in the monolayers formed by the two methods suggests that a large fraction of the egg PC molecules spread osmotically do not remain at the interface. By varying the intravesicular salt concentration, we found that the apparent area per molecule in osmotically spread films is proportional to osmotic pressure over the range 100–600 mM NaCl. Therefore, even though only a fraction of the lipid molecules may remain at the air-water interface, the osmotic spreading method produces insoluble interface lipid films, the surface pressure-area characteristics of which are indistinguishable from those of monolayers spread with hexane.

A suspension of rhodopsin membrane vesicles can be spread osmotically at an air-water interface to form insoluble films. The surface pressure *vs.* area isotherm of a film formed by spreading rhodopsin membrane vesicles consisting of 150:1 egg PC/protein mole ratio is shown in Fig. 3. For comparison, the isotherm of an osmotically spread egg PC film is also shown. The surface pressure at collapse is similar for both films: 45 $mN \cdot m^{-1}$ for the pure lipid and 47 $mN \cdot m^{-1}$ for the rhodopsin film, but the form of the isotherm is markedly different. Also, the collapse pressure both types of film immediately relaxes to a stable value of about 42 mN/m^{-1} upon stopping the motion of the trough barrier. The form of the isotherm of the rhodopsin films depends on the initial surface area onto which vesicles are spread, but for a given surface area, the isotherms are highly reproducible. All data reported here was obtained from films spread onto an initial area of 315 cm^2. The form of the isotherms reveals that rhodopsin incorporation at the interface results in films which are expanded and more compressible than egg PC monolayers.

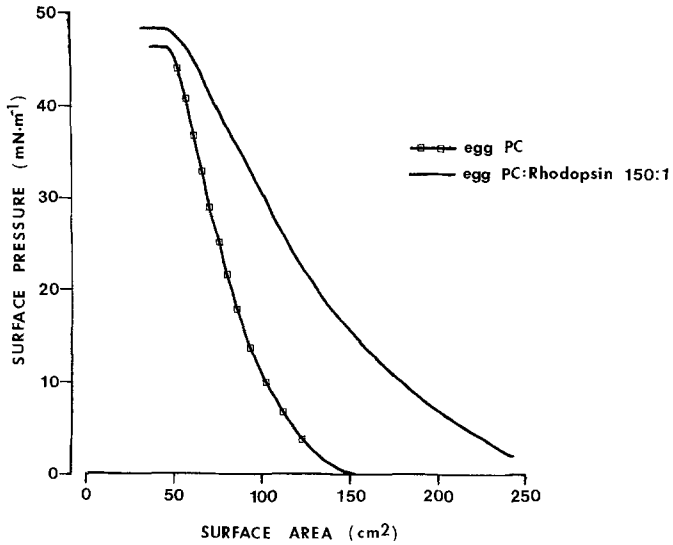

Fig. 3. Surface pressure *vs.* surface area isotherms of egg PC and rhodopsin-egg PC membrane vesicles spread osmotically. The rhodopsin-egg PC membranes are in a 1:150 mole ratio. Isotherms were recorded at 20 °C on a subphase of 5 mM MOPS, pH 6.8. The isotherms, measured in separate experiments, are redrawn for illustration so as to coincide in area at their collapse pressure

Electron Microscopy of Interface Films

Electron microscopy allows direct observation of the organization of the interface film. Fig. 4 is an electron micrograph of replicas of egg PC films formed either by spreading from hexane or by the osmotic method, and transferred to a glass support at their collapse pressure. No distinct feature is seen. In contrast, Fig. 5 is an electron micrograph of a replica of rhodopsin interface films spread from rhodopsin membrane vesicles consisting of 150:1 lipid/protein mole ratio. Membrane fragments are seen which are of irregular shape and random distribution separated by smooth spaces. Furthermore, a survey of a large number of micrographs indicates that at this mole ratio less than 5% of the fragments overlap. The rhodopsin molecules are in the membrane fragments since autoradiographic studies of interface films in which only the rhodopsin molecules are labeled reveal exposed grains only over the membrane fragments (Fig. 6). In contrast, when the phospholipids in the rhodopsin interface film are labeled, the autoradiography electron micrographs reveal exposed grains distributed throughout the interface film, (Fig. 7). The autoradiographic image of a PC monolayer formed with the same batch of lipid present in the rhodopsin films also shows exposed grains

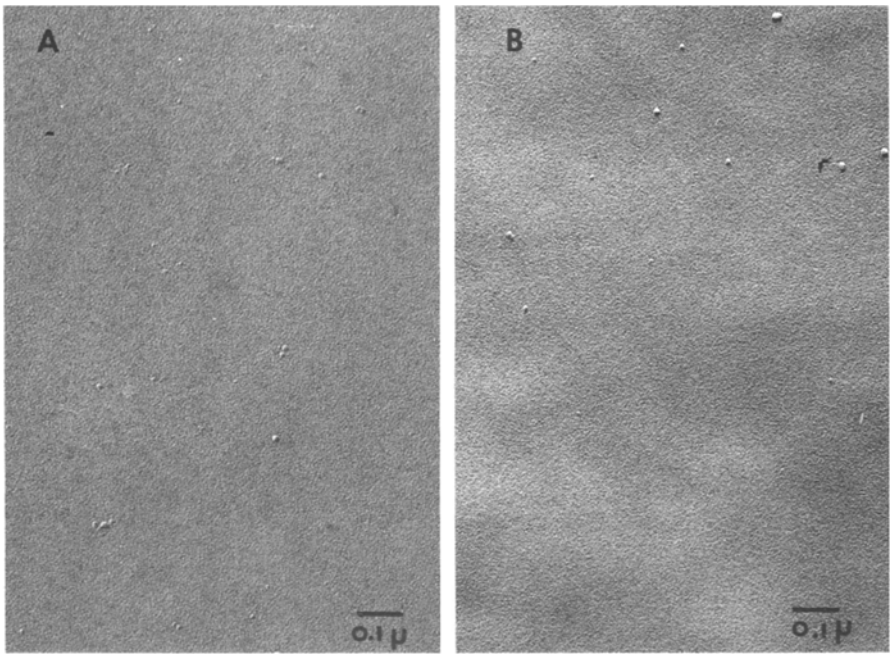

Fig. 4. Electron micrographs of metal replicas of single egg PC monolayers transferred to glass support at their collapse pressure. (*A*): Egg PC monolayer spread from a hexane solution. (*B*): Egg PC monolayer spread osmotically. No distinctive feature is seen in either interface film

distributed throughout the film (Fig. 7). Therefore, these images indicate that the smooth spaces between the rhodopsin membrane fragments at the interface are occupied by lipid monolayer.

The structure of the rhodopsin interface film varies with the lipid to protein mole ratio in the membrane vesicles. Electron micrographs of replicas of rhodopsin films formed from vesicles with 63:1 and 100:1 lipid/protein mole ratios are shown in Fig. 8. The interface films consist of membrane fragments which overlap extensively and form stacks several membranes thick. These images are not the result of artifacts which could be introduced by drying the films on the glass support before shadow-casting, since electron micrographs of replicas produced by freeze-etching methods are nearly identical to those obtained by shadow casting (Fig. 9). Thus, interface films of nonoverlapping rhodopsin membrane fragments can be obtained under our conditions only from membrane vesicles with an egg PC/rhodopsin mole ratio between 150:1 and 170:1, but not less. We have not explored higher mole ratios.

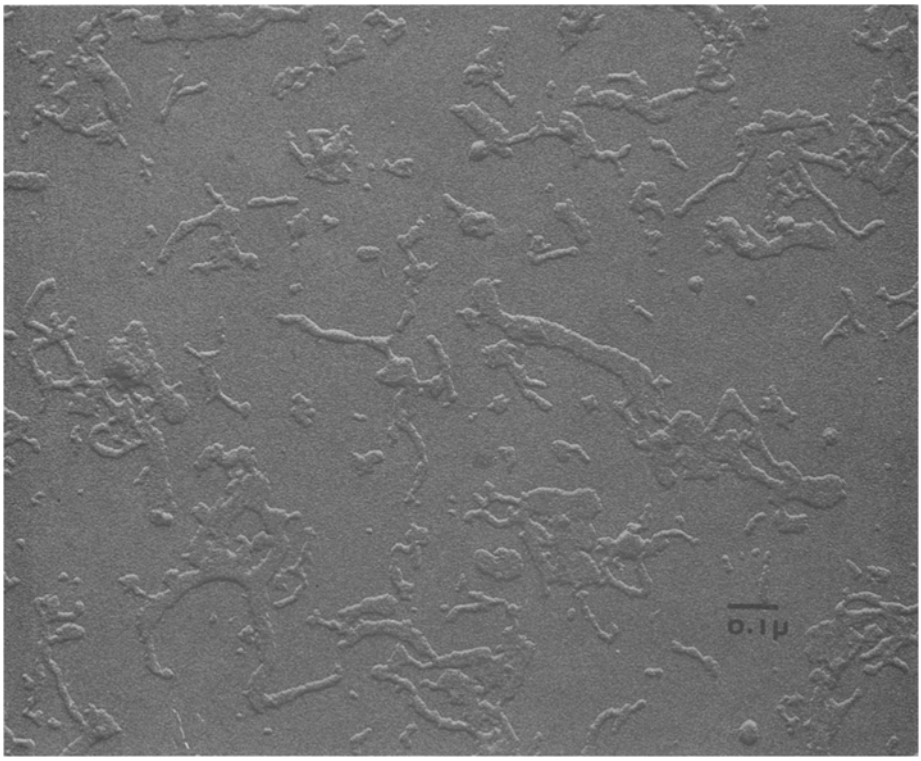

Fig. 5. Electron micrograph of a replica of a single rhodopsin-egg PC interface film transferred to glass at its collapse pressure. The interface film is formed by osmotically spreading membrane vesicles with an egg PC/rhodopsin mole ratio of 150:1. Membrane fragments are randomly distributed on the surface and do not overlap

Spectrophotometry of Interface Films

To investigate whether the molecular conformation of rhodopsin in the interface films is similar to that in disc membranes, we measured the visible absorption spectra of rhodopsin interface films transferred to glass slides and air dried. Rhodopsin films transfer to a glass slide on both withdrawal and insertion through the interface only on the first two trips. The slides emerge wet and are allowed to thoroughly dry between successive trips. On the third trip the film transfers only upon withdrawal and any subsequent transfer results in the film floating off the support. Thus, only 10 layers can be transferred to a single glass slide. By stacking several glass slides, spectra such as those shown in Fig. 10 are obtained. In the dark, a single absorption band with λ_{max} at 498 nm is recorded. This absorption spectrum is stable for at

Fig. 6. Electron micrograph of an autoradiographic metal replica of a single rhodopsin/egg PC film transferred to glass. Rhodopsin is made selectively radioactive by reaction with H^3-Concanavalin A. Exposed silver grains are seen exclusively over the membrane fragment and not in the areas in between. Egg PC/rhodopsin mole ratio in the membrane vesicles was 168:1. The presence of Con A resulted in some aggregation of the membrane fragments at the interface

least 4 weeks. When the slides are exposed to continuous light in a humid environment the absorption band completely disappears. These spectral characteristics are indistinguishable from those of rhodopsin in intact cattle rod disc membranes.

We found the absorbance at 498 nm in multilayer stacks to be linearly proportional to the number of layers in the stack. Therefore, the optical density at 498 nm of a single layer can be estimated by linear extrapolation of the absorbance measured in difference spectrum of multilayer stacks such as that shown in Fig. 10. The relation between single film absorbance at 498 nm and the egg PC/rhodopsin mole ratio in the film forming membranes is shown in Fig. 11. The data in Fig. 11 can be used to demonstrate that the membrane fragments at the interface in the 150:1 ratio films are single membrane sheets, and not collapsed vesicles. The extinction coefficient of rhodopsin, ε, is generally accepted to be about 40,600 $mole^{-1}$ cm^{-1} (Wald & Brown, 1953) and the rhodopsin molecule is now believed to be of an elongated shape with a cross section of about 1350 $Å^2$ at its widest point (Wu & Stryer, 1972; Sardet,

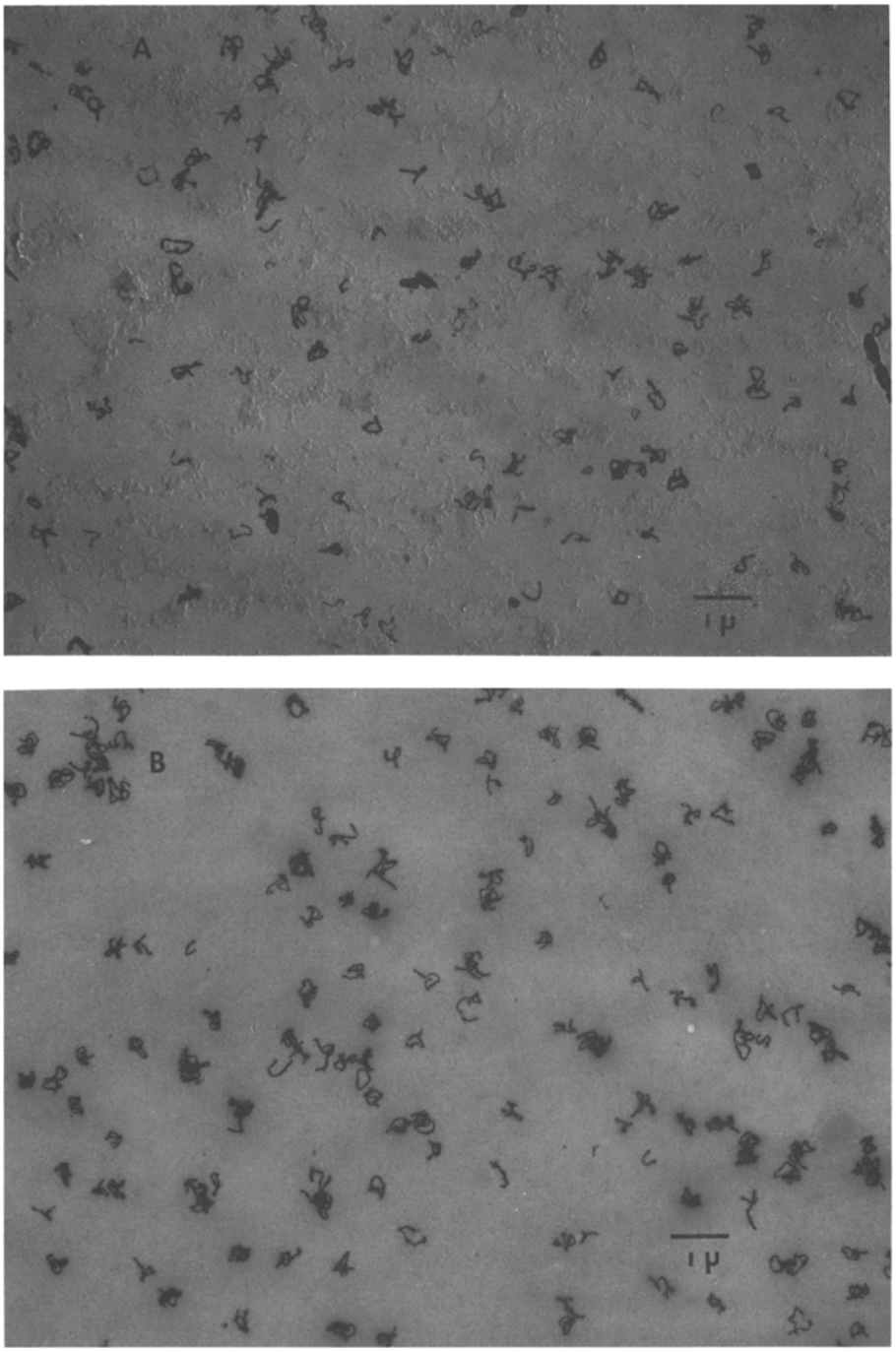

Fig. 7. Electron micrographs of autoradiographic replicas of an egg PC monolayer and a rhodopsin/egg PC interface film transferred to glass. The lipids are made selectively radioactive by mixing egg PC with H^3 labelled dipalmitoyl PC in a 100:1 mole ratio. The lipid monolayer was spread from hexane (*B*). The rhodopsin film was formed from membrane vesicles in a 140:1 lipid/protein mole ratio (*A*). Exposed silver grains are uniformly distributed throughout the interface films in both cases

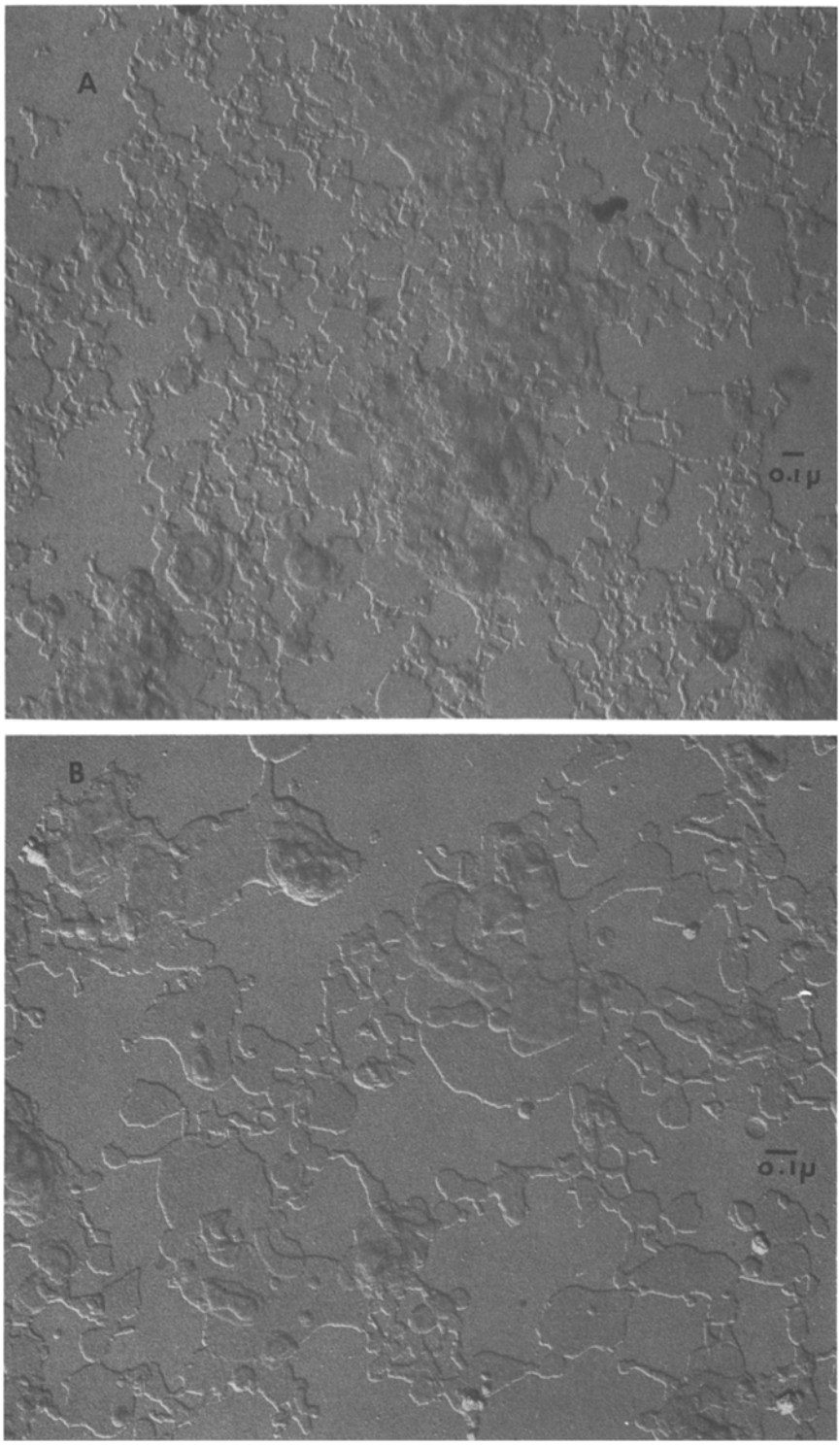

Fig. 8

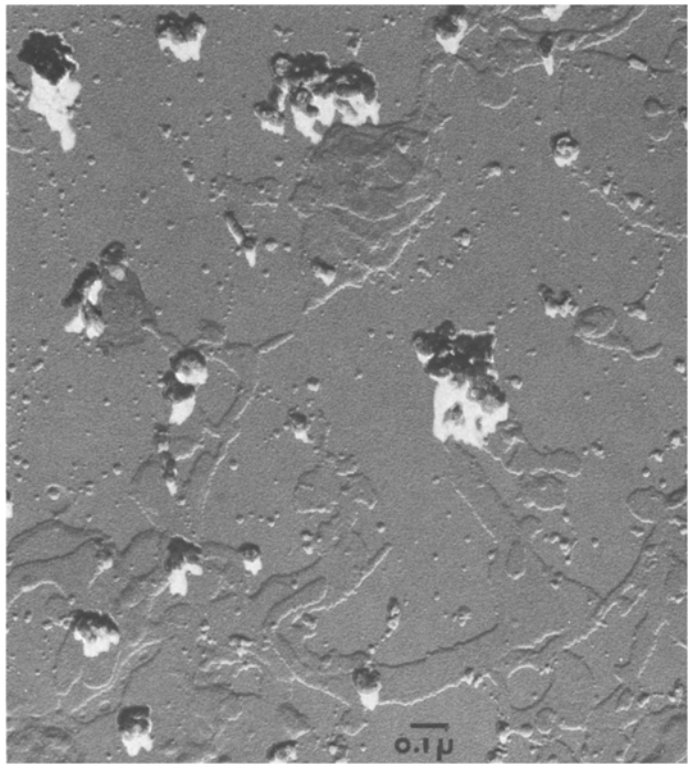

Fig. 9. Electron micrograph of a freeze-etched replica of rhodopsin/egg PC film formed from 1:100 mole ratio membranes. The structure of the film is essentially identical to that seen in shadow-cast replicas of the same film (compare with Fig. 8*B*). Ice crystal artifacts are seen throughout the image

Tardieu & Luzzati, 1976). The cross sectional area of a single egg PC molecule is 61 $Å^2$. These numbers can be used to calculate the fraction of surface area which must be occupied by membrane fragments at 150:1 egg PC/rhodopsin mole ratio as single sheets, double sheets or any multiple of sheets in order to account for the measured single film absorbance. But further, it must also be considered that the extinction coefficient quoted is measured with randomly oriented chromophores, whereas our absorbance measurements are done with chromophores highly oriented in the plane of the membrane fragments and a measuring beam

Fig. 8. Electron micrographs of replicas of rhodopsin/egg PC films transferred to glass support at the collapse pressure. Films were formed by osmotically spreading membrane vesicles of different lipid/protein mole ratio. (*A*): Films of 63:1 mole ratio membranes. (*B*): Films of 100:1 mole ratio membranes. In both cases membrane fragments overlap extensively and what are probably collapsed membrane vesicles can also be seen

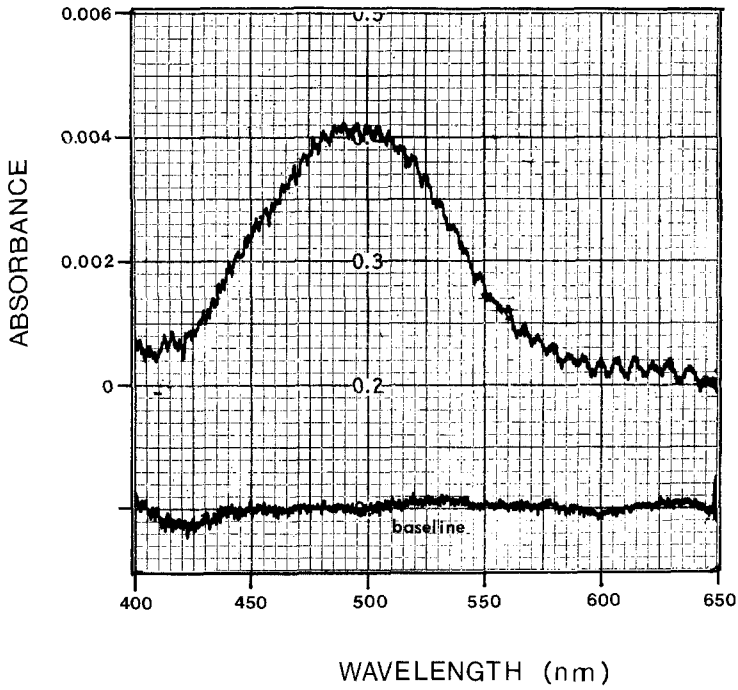

Fig. 10. Absorption spectrum of rhodopsin/egg PC interface films. Shown is the spectrum recorded from a multilayer consisting of 50 layers of 152:1 lipid/rhodopsin interface film. A single absorption band is seen with a maximum absorbance at 498 nm

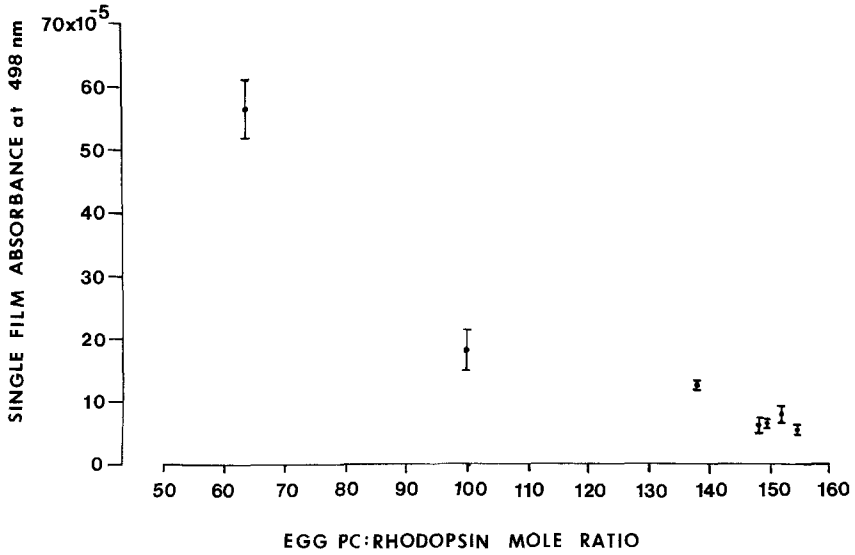

Fig. 11. Single film absorbance at 498 nm of films formed from membrane vesicles at various egg PC/rhodopsin mole ratios. Each point is the average of measurements of 2 to 5 multilayers; the bars indicate the range of values recorded

normal to this plane. Liebman (1962) has extensively discussed this situation, which is analogous to measuring absorbance along the long axis of a rod outer segment. He has shown that the effective extinction coefficient in the oriented sample, ε', is dependent on the angle which the dipole moment of transition of the chromophore in rhodopsin makes with the plane of the membrane, Θ, thus:

$$\varepsilon = \tfrac{3}{2}\,\varepsilon \cos^2 \Theta.$$

Liebman has estimated Θ to be 15° in the disc membrane. Using this value for Θ[1] and the other values quoted above, the single layer absorbance of the 150:1 film measured experimentally to be 6.43 ± 1.50 OD ($\pm$SD ($n=10$ for all films in the ratio range 148 to 155:1) predicts that $38 \pm 6\%$ of the surface would be occupied by single sheet membranes, half as much as by double sheets and so forth. The fraction to the aqueous area occupied by membrane fragments measured directly in electron micrographs is $35 \pm 4\%$. The excellent agreement between these two figures indicates that at 150:1 mole ratio the rhodopsin interface film consists of single sheet membrane fragments. At mole ratios lower than 150:1, the membrane fragments are extensively overlapped and may in some cases consist of collapsed vesicles as is evident from Fig. 8 and from the failure to account for the measured absorbance data by assuming single sheets. The excellent agreement in the occupied area estimates made by electron microscopy and by spectrophotometry indicates that in the 150:1 mole ratio films nearly every rhodopsin molecule at the interface is spectroscopically intact.

The effects of illumination on the spectral characteristics of air-dried rhodopsin multilayer stacks are illustrated in Fig. 12. Data shown are those from films of 150:1 mole ratio; however, similar effects are seen with films at the other lipid/protein mole ratios tested. Following flash illumination, a single absorption band is recorded with its λ_{max} shifted from the original 498 nm to approximately 485 nm. This new absorption band is stable for at least 4 hr, if the glass slides are kept dry in a closed volume with use of P_2O_5. If the slides remain at ambient humidity in complete darkness, the absorbance band slowly declines. The characteristics of the absorption band change by simply incubating the slides in a high humidity environment. In the dark, in a humid environment, the single band in the spectrum becomes progressively smaller and its

[1] The rhodopsin film multilayer stacks are linearly dichroic (Korenbrot & Bogomolni, *unpublished observations*). The dichroic ratio in the multilayers is consistent with a 15° value for θ in the rhodopsin films.

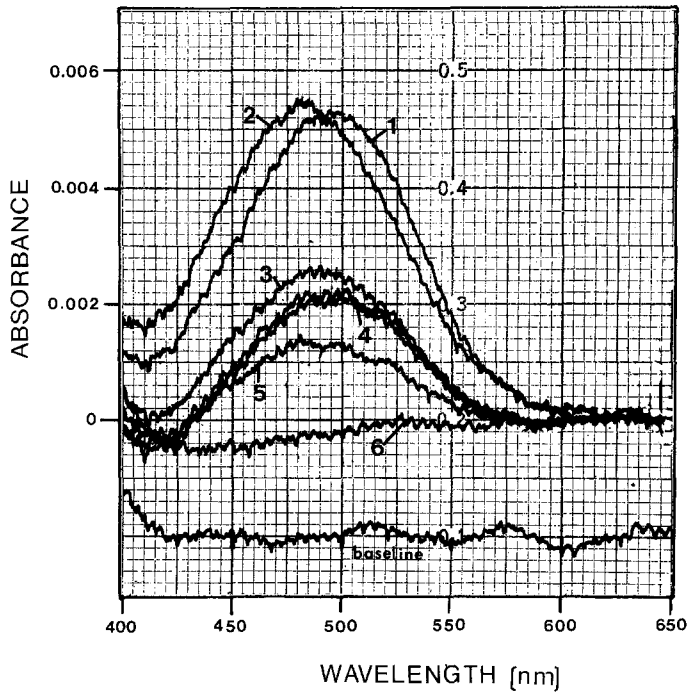

Fig. 12. Effect of illumination on the absorption spectrum of rhodopsin/egg PC interface films. Shown are the spectra of a multilayer consisting of 60 layers of 152:1 egg PC rhodopsin film. Spectrum *1* is recorded in the dark, its λ_{max} is about 498 nm. Following a single intense flash of wavelength longer than 510 nm, spectrum *2* is recorded, its λ_{max} is about 485 nm. While being kept in constant darkness, the multilayers are exposed to a high humidity environment and spectra *3* and *4* are recorded with a 15 min interval. Spectrum *4* is stable for at least 3 hr. The λ_{max} of spectrum *4* is about 495 nm. If the multilayers are again exposed to an intense flash, spectum *5* results with a λ_{max} at about 485 nm. Spectrum *6* is obtained by keeping the multilayers in high humidity for several hours

λ_{max} red shifts. A steady-state is reached in which a band with small absorbance and a λ_{max} around 495 nm is recorded. This band is stable for many hours, and can be again converted to the 485 absorption band by flash bleaching. These spectroscopic results are essentially identical to those first reported by Wald, Durrell and St. George in 1950 for rhodopsin embedded in dry gelatin films. They also agree with the spectral changes seen following flash illumination of rhodopsin solutions at -20 °C (Yoshizawa, 1972), a temperature at which Metarhodopsin *I* ($\lambda_{max}=480$ nm) is thermally stable. In the dry state, as in the cold, the rhodopsin photoreaction cycle proceeds only to the Meta *I* intermediate which has a λ_{max} at 480 nm. The photoreaction cycle can be completed only by providing water or by raising the temperature. Therefore, the

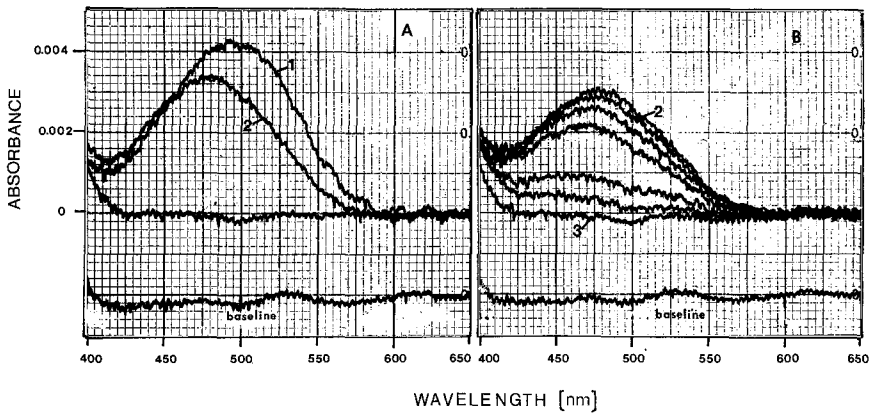

Fig. 13. Effect of illumination on the absorption spectra of rhodopsin/egg PC interface films. Shown are the spectra of a multilayer consisting of 60 layers of 152:1 egg PC/rhodopsin film. (*A*): Spectrum *1* is recorded in the dark; its λ_{max} is near 498 nm. Following flash illumination spectrum *2* is recorded; its λ_{max} is near 480 nm. (*B*): The effect of incubating the multilayers in high humidity environment under continuous illumination. Spectra shown are sequentially recorded at approximately 3-min intervals. The absorption band completely disappears with a slight, but progressive, blue shift of its maximum absorbance

spectroscopic data shown indicate that in the dry multilayers, flash illumination produces a stable photo steady-state mixture of rhodopsin, Meta-rhodopsin *I*, and possibly photoregenerated isorhodopsin, resulting in the observed shift in λ_{max}. Wetting the film in the dark results in the completion of the photoreaction cycle of those molecules which reached the Meta *I* state, and reveals the presence of unbleached and photoregenerated rhodopsin and isorhodopsin.

All the rhodopsin molecules in the multilayers can be fully bleached. When flash illuminated multilayers are incubated in a high humidity environment under continuous illumination, the absorption spectra recorded at successive time intervals become progressively smaller with only a very small blue shift in λ_{max}, until all material is completely bleached (Fig. 13). The small blue shift presumably results from Meta *I* enrichment of the photomixture. In all these experiments, the end product of bleaching is presumably free retinal with a maximum absorbance around 370 nm. Unfortunately, we could not record spectra below 400 nm because of the absorbance of the glass supporting the multilayers. The spectral characteristics of the rhodopsin film multilayer stacks in the dark and the effects of illumination are essentially identical to those which have been described for rhodopsin in intact disc membranes, and

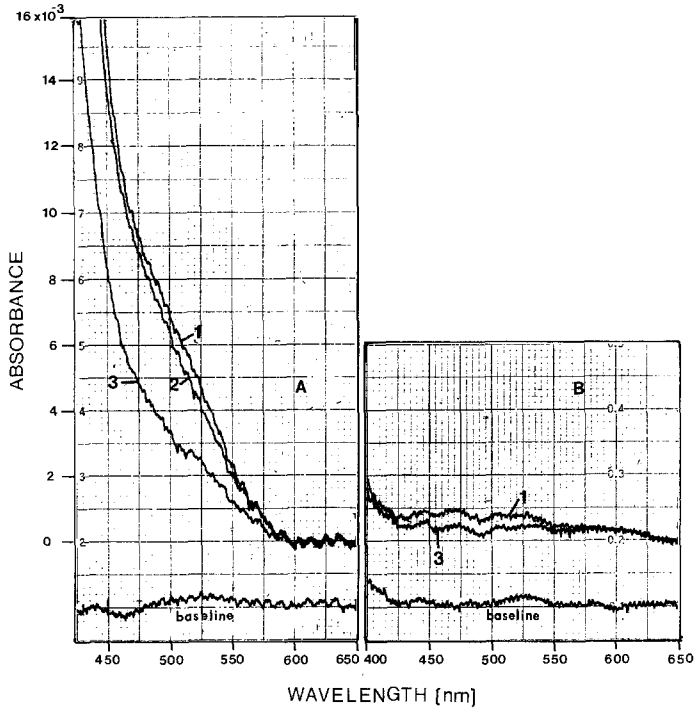

Fig. 14. Chemical regeneration of rhodopsin at the air-water interface. (*A*): The absorption spectrum of a 60-layer array of initially bleached rhodopsin/egg PC film (1:150) transferred to glass from a subphase containing 11-cis retinal. Curve *1* was recorded in the dark, and curve *2* was recorded following flash illumination of the multilayers. Curve *3* was recorded following incubation of the multilayers in a high humidity environment under continuous illumination. (*B*): The absorption spectrum of a 60-layer array of the same initially bleached rhodopsin/egg PC film transferred to glass from a retinal free subphase to which only the vehicle used to add retinal, 0.1% v/v ethanol, is added. Curves *1* and *3* are recorded under the same conditions as in *A*

indicate that rhodopsin in the interface films has a structure similar to that in intact membranes.

A more demanding criterion that rhodopsin structure in the interface films is like that in disc membranes is that of chemical regenerability: that is, the ability of bleached rhodopsin molecules to react with 11-cis retinal to regenerate dark rhodopsin. Membrane vesicles containing over 99% bleached rhodopsin molecules were spread osmotically on a subphase containing approximately 10^{-7} moles of 11-cis retinal. After 6–7 hr multilayer stacks of the interface film were formed on glass. Over this time course the surface pressure of the film decreased by about 10 $mN \cdot m^{-1}$, perhaps because of the finite solubility of the film in water. As usual, the film was brought back to its collapse pressure before

transfer commenced. Since the films transferred to the glass slides emerge wet from the subphase, the resulting multilayers contain free 11-cis retinal. Fig. 14 illustrates the absorption spectrum of the multilayers. The spectrum exhibits an absorbance shoulder around 500 nm superimposed on the larger absorbance band of retinal. 11-cis retinal shows a maximum absorbance near 370 nm, but does not absorb above 500 nm (Brown & Wald, 1956). The absorbance shoulder near 500 nm shifts towards the blue following flash illumination and completely disappears by incubating the slides in a humid environment under continuous illumination. The dark difference spectrum after water incubation (curve 1–3) peaks near 500 nm, whereas the flash-illumination difference spectrum after water incubation (curve 2–3) peaks near 485 nm. These data indicate that the spectrum measured in the multilayers is the sum of the spectra of 11-cis and of photosensitive rhodopsin. Comparison of dark difference spectra obtained from spectra such as that in Fig. 14 with that obtained from spectra of multilayers formed from films of the same vesicle membranes but in which rhodopsin is not bleached indicate that $82 \pm 3\%$ ($\pm$SEM, average of 10 experiments) of the rhodopsin molecules at the interface are chemically regenerable under our conditions. In contrast, we detect less than 10% regeneration in films of bleached rhodopsin spread over a retinal-free subphase (Fig. 14). The chemical regenerability of rhodopsin in films on the water surface and the similarity of the spectral characteristics of rhodopsin multilayer stacks with those of disc membranes establish that the spectroscopically defined structure of rhodopsin is essentially intact in the air-water interface films described here.

Discussion

We have described the formation and the structure and spectrophotometric properties of air-water interface films consisting of lipid and rhodopsin. In these films rhodopsin molecules form a monomolecular layer under some conditions, but they are not dispersed at the interface; they exist organized in membrane fragments of defined lipid composition. In these fragments, rhodopsin molecules are spectroscopically intact and chemically regenerable. The membrane fragments appear to be separated by a continuous phospholipid monolayer. The organization of the lipids in the interface membrane fragments, although presumably a bilayer, has not been determined. Whatever the lipid organization might be,

however, it is stabilized by the presence of the protein, since films formed from lipid vesicles free of protein do not produce interface membrane fragments. Since the lipid molecules which occupy the spaces between membrane fragments in the interface films must come from the vesicle membranes and since a relatively large excess of lipid over protein is necessary to avoid overlapping of the membrane fragments (150:1 to 170:1), we suggest that only at these high lipid/protein mole ratios do regions exist in the membrane in which lipid molecules are far enough from the protein not to remain structurally restricted by it at the interface. These lipid regions would unfold at the interface, much as pure lipid bilayer vesicles do, and form the monolayer which separates the membrane fragments. There is, therefore, a strong reciprocal relationship in the effects of rhodopsin and phospholipid on each other's structure. Hong and Hubbell (1972) have also found that rhodopsin can directly affect lipid structure: spin labels introduced in rhodopsin membrane vesicles exhibit order parameters consistent with a more rigid environment than that found in membrane vesicles free of rhodopsin but of identical lipid composition. Hence, rhodopsin in the interface films stabilizes the structure of the lipid molecules, and these, in turn, support a structural conformation of rhodopsin similar to that in the rod disc membrane.

Air-water interface films containing rhodopsin have been formed previously from rod disc membrane fragments (Azuma & Takagi, 1966; Bonting & Bangham, 1967), ethanolic extract of rhodopsin (Hyono *et al.*, 1962), or hexane or ether proteolipid of rhodopsin (Montal & Korenbrot, 1973; Montal, 1975). Only in films formed from rod disc membranes has the conformation of rhodopsin been directly investigated: Azuma and Takagi (1966) found that in the dark the spectrophotometric characteristic of films formed from intensely sonicated cattle role disc membranes are similar to those of intact disc membranes. The effect of illumination on the spectra of these films and the regenerability of rhodopsin at the interface was not investigated, nor was the structure of the films. Nonetheless, preservation of the dark absorption spectrum suggests that in these films rhodopsin did not grossly denature at the interface. Verger and Pattus (1976) have recently shown that the enzymatic activity of a membrane-bound aminopeptidase is preserved in air-water interface films formed from fragments of the intestinal brush border membrane to which the enzyme is bound. In addition, Verger and Pattus (1976) also found, as we have, that depositing single bilayer lipid vesicles at the interface forms interface films whose surface pressure isotherms are

indistinguishable from those of monolayer films spread from an organic solvent. Finally, Hwang, Korenbrot and Stoeckenius (1977*a, b*) have recently shown that bacteriorhodopsin, the light-activated proton pump of *H. halobium*, is spectroscopically and functionally intact in interface films formed from purple membrane fragments. All these data suggest that it may be possible in general to form air-water interface films of structurally intact membrane bound proteins by spreading membrane fragments. The osmotic method described here might be one procedure to form these films. In all cases, however, the structural state of the protein at the interface should be directly ascertained and the organization of the film investigated.

Proof that the conformation of a protein in a model system is identical to that in the native membrane has generally been based on the preservation of protein function in the model system. In the case of rhodopsin, however, other criteria must be used to establish this identity, since the function of rhodopsin remains under investigation. Listed below are several criteria which have been used to ascertain the structural integrity of rhodopsin and which allow comparison of the interface films described here with native disc membranes: (i) *Dark absorption spectrum.* Rhodopsin in the interface films exhibits visible absorption spectra in the dark identical to those in disc membranes. (ii) *Formation of photoproducts.* Illumination of rhodopsin in air-dried multilayer films results in the formation of Meta *I* in photo steady-state equilibrium with photoregenerated rhodopsin and isorhodopsin. Identical results are obtained when the photo-cycle of rhodopsin is arrested at the Meta *I* state by low temperatures (Yoshizawa, 1972) or drying (Wald et al., 1950). (iii) *Kinetics of photoproduct formation.* Since spectral measurements are carried out here in air-dried films, the kinetics of photoproduct formation are limited by the uncontrolled availability of water. Determination of these kinetic parameters, therefore, must await measurement of spectral properties of the interface films on the water surface. (iv) *Photoregeneration.* Rhodopsin photoregenerates in the multilayer films, just as it does in disc membranes. (v) *Chemical regeneration.* This is a particularly strong criterion since rhodopsin is in a chemically regenerable configuration only under limited conditions. Thus, in some detergents rhodopsin exhibits its characteristic dark absorption spectrum and changes with illumination, yet it does not regenerate (Hubbell, 1975; Stubbs, Smith & Litman, 1976). Rhodopsin is chemically regenerable in the interface films. Furthermore, regeneration occurs on the water surface indicating that rhodopsin is structurally intact at the interface itself and that spectral

characteristics in the multilayer films do not arise from possible conformational changes in the protein upon transfer to the solid support.

Other spectroscopic criteria, such as IR absorption and circular dichroism and optical rotary dispersion spectra have not yet been applied to the interface films because to date we have been unable to form multilayers with enough optical absorbance. Hence, although no single test is proof of structural integrity, the combination of all criteria applied to date suggest that the conformation of rhodopsin in the interface films is indistinguishable from that in the rod disc membrane.

The interface films of nonoverlapping membrane fragments of intact rhodopsin are not only useful for investigation of the optical properties described here. We have also used them to investigate the binding of calcium ions to rhodopsin (Korenbrot, 1977*b*), and they constitute a fundamental step towards the possible organization of oriented interface films separating two large aqueous compartments, an ideal system in which to study the mechanism of rhodopsin function.

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References

Applebury, M.L., Zuckerman, D.M., Lamola, A.A., Jovin, T.M. 1974. Rhodopsin purification and recombination with phospholipids assayed by the Metarhodopsin I-Metarhodopsin II transition. *Biochemistry* **13**:3448

Azuma, K., Takagi, M. 1966. Rhodopsin film at air-water interface. *Annu. Rep. Biol. Works Fac. Sci. Osaka Univ.* **14**:1

Bangham, A.D., DeGier, J., Greville, G.D. 1967. Osmotic properties and water permeability of phospholipid crystals. *Chem. Phys. Lipids* **1**:225

Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466

Basinger, S., Bok, D., Hall, M. 1976. Rhodopsin in the rod outer segment plasma membrane. *J. Cell Biol.* **69**:29

Bonting, S.L., Bangham, A.D. 1967. On the biochemical mechanism of the visual process. *Exp. Eye Res.* **6**:400

Brown, P.K., Wald, G. 1956. The neo-b isomer of vitamin A and retinene. *J. Biol. Chem.* **222**:865

Caro, L.G., VanTubergen, R.P., Kolb, J.A. 1962. High-resolution autoradiography. I. Methods. *J. Cell. Biol.* **15**:173

Chabre, M., Cavaggioni, A., Osborne, H.B., Gulik-Krzywicki, T., Olive, J. 1972. A rhodopsin-lipid-water lamellar system: Its characterization by X-ray diffraction and electron microscopy. *FEBS Lett.* **26**:197

Chapman, D. 1975. Phase transitions and fluidity characteristics of lipids and cell membranes. *Q. Rev. Biophys.* **8**:185

Chen, Y.S., Hubbell, W.L. 1973. Temperature and light-dependent structural changes in rhodopsin-lipid membranes. *Exp. Eye Res.* **17**:517

Darszon, A., Montal, M.1976. Light increases the ion permeability of rhodopsin phospholipid bilayer vesicles. *In:* Mitochondria-Bioenergetics, Biogenesis and Membrane Structure. A. Gomez-Puyou and L. Packer, editors. Academic Press, New York

Gaines, G.L., Jr. 1966. Insoluble Monolayers at Liquid-Gas Interfaces, Interscience, New York

Hagins, W.A., 1972. The visual process: Excitatory mechanisms in the primary receptor cells. *Annu. Rev. Biophys. Bionengineer.* **1**:131

Haran, N., Shporer, M. 1976. Study of water permeability through phospholipid vesicle membranes by ^{17}O NMR. *Biochim. Biophys. Acta* **426**:638

Heitzman, H. 1972. Rhodopsin is the predominant protein of rod outer segment membranes. *Nature New Biol.* **235**:144

Hong, K., Hubbell, W.L. 1972. Preparation and properties of phospholipid bilayers containing rhodopsin. *Proc. Nat. Acad. Sci. USA* **69**:2617

Hong, K., Hubbell, W.L. 1973. Lipid requirement for rhodopsin regenerability. *Biochemistry* **12**:4517

Honig, B., Ebrey, T.G. 1974. The structure and spectra of the chromophore of the visual pigments. *Annu. Rev. Biophys. Bioengineer.* **3**:151

Hubbard, R., Brown, P.K., Kropf, A. 1959. Action of light on visual pigments. Vertebrate lumi and Metarhodopsins. *Nature (London)* **183**:442

Hubbell, W.L. 1975. Characterization of rhodopsin in synthetic systems. *Acc. Chem. Res.* **8**:85

Hwang, S.-B., Korenbrot, J.I., Stoeckenius, W. 1977*a*. Proton transport by bacteriorhodopsin through an interface film. *J. Membrane Biol.* **36**:137

Hwang, S.-B., Korenbrot, J.I., Stoeckenius, W., 1977*b*. Structural and spectroscopic characteristics of bacteriorhodopsin in air-water interface films. *J. Membrane Biol.* **36**:115

Hyono, A., Kuriyama, S., Tsuji, K., Hosoya, Y. 1962. Monolayer film of rhodopsin at the air-water interface. *Nature (London)* **193**:679

Jan, L.Y., Revel, J.P. 1974. Ultrastructural localization of rhodopsin in the vertebrate retina. *J. Cell. Biol.* **62**:257

Jones, M.N. 1975. Biological Interfaces. Elsevier Scientific, Amsterdam

Korenbrot, J.I. 1977*a*. Ion transport in membranes: Incorporation of biological ion translocating proteins in model membrane systems. *Annu. Rev. Physiol.* **39**:19

Korenbrot, J.I. 1977*b*. Some considerations on the ion transport properties of rod disc membranes. *Biophys. Struct. Mechan.* **3**:135

Kropf, A. 1972. Structure and reactions of visual pigments. *In:* Handbook of Sensory Physiology VII/vol. 2, p. 239. M.G.F. Fuortes, editor. Springer-Verlag, Berlin

Liebman, P.A. 1962. In situ microspectrophotometric studies on the pigments of single retinal rods. *Biophys. J.* **2**:161

Montal, M. 1975. Rhodopsin in experimental membranes: An approach to elucidate its role in phototransduction. *In:* Molecular Aspects of Membrane Phenomena. H.R. Kaback, H. Neurath, G.K. Radda, R. Schwyzer and W.R. Wiley, editors. Springer-Verlag, Berlin

Montal, M., Korenbrot, J.I. 1973. Incorporation of rhodopsin proteolipid into bilayer membranes. *Nature (London)* **246**:219

Montal, M., Korenbrot, J.I. 1976. Rhodopsin in cell membranes and the process of phototransduction. *In:* The Enzymes of Biological Membrane. A. Martonosi, editor. Plenum, New York

Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Nat. Acad. Sci. USA* **69**:3561

Phillips, M.C., Chapman, D. 1968. Monolayer characteristics of 1, 2-diacyl phosphatidylcholines (lecithins) and phosphatidyl ethanolamines at the air-water interface. *Biochim. Biophys. Acta* **163**:301

Robinson, W.E., Gordon-Walker, A., Bownds, D. 1972. Molecular weight of frog rhodopsin. *Nature New Biol.* **235**:112

Sardet, C., Tardieu, A., Luzzati, V. 1976. Shape and size of bovine rhodopsin: A small angle X-ray scattering study of a rhodopsin-detergent complex. *J. Mol. Biol.* **105**:383

Singleton, W.S.,Gray, M.S., Brown, M.L., White, J.L. 1965. Chromatographically homogeneous lecithin from egg phospholipids. *J. Am. Oil Chem. Soc.* **42**:53

Steinemann, A., Stryer, L. 1973. Accessibility, of the carbohydrate moiety of rhodopsin. *Biochemistry* **12**:1499

Stubbs, G.W., Smith, H.G., Litman, B.J. 1976. Alkyl glucosides as effective solubilizing agents for bovine rhodopsin. A comparison of several commonly used detergents. *Biochim. Biophys. Acta* **426**:46

Takagi, M., Azuma, K., Kishimoto, V. 1965. A new method for the formation of bilayer membranes in aqueous solution. *Annu. Rep. Biol. Works Fac. Sci. Osaka Univ.* **13**:107

Tsofina, L.M., Liberman, E.A., Babakov, A.V. 1966. Production of bimolecular protein-lipid membranes in aqueous solution. *Nature (London)* **212**:681

Verger, R., Pattus, F. 1976. Spreading of membranes at the air/water interface. *Chem. Phys. Lipids* **16**:285

Wald, G., Brown, P.K. 1953. The molar extinction of rhodopsin. *J. Gen. Physiol.* **37**:189

Wald, G., Durell, J., St. George, R.C.C. 1950. The light reaction in the bleaching of rhodopsin. *Science* **17**:179

Weibel, E.R., Bolender, R.P. 1973. Stereological techniques for electron microscopic morphometry. *In:* Principles and Techniques of Electron Microscopy. M.A. Hayat, editor. Van Nostrand Reinhold, New York

Wu, C.W., Stryer, L. 1972. Proximity relationships in rhodopsin. *Proc. Nat. Acad. Sci. USA* **69**:1104

Yoshizawa. T. 1972. The behaviour of visual pigments at low temperature. *In:* Handbook of Sensory Physiology VII. Vol. 1, p. 146. H.J.A. Dartnall, editor. Springer-Verlag, Berlin