INCORPORATION OF PHOTORECEPTOR MEMBRANE INTO A MULTILAMELLAR FILM

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ABSTRACT Multilamellar arrays of photoreceptor membrane up to 50 μm thick have been produced using a new method. Rhodopsin chromophore orientation in the films was studied using optical linear dichroism. The rhodopsin appears to be structurally intact and capable of photobleaching and regeneration. The production of biologically active liquid-crystal films offers a promising new approach to the study of biomembranes.

The inability to crystallize membrane proteins accounts in large part for the lack of information about their structure. As a possible first step towards eventual membrane protein crystallization, we have sought a means of incorporating biological membranes into smectic liquid-crystals (i.e., uniform multilamellar arrays of membranes) (1). Since membrane proteins in a multilamellar array are arranged uniaxially around an axis perpendicular to the membrane plane, the average orientation of membrane protein groups relative to this axis can be probed using a variety of biophysical techniques including x-ray, polarized IR spectroscopy, nuclear magnetic resonance (NMR), and circular dichroism. Further, biologically active multilamellar array films may offer a convenient means to study biomembrane functional properties.

Many methods have been reported for orienting biological membranes. These include the use of electric or magnetic fields (2, 3), the deposition of membrane sheets on a substrate by successive dipping through an air/water interface (4, 5), surfactant coated surface induced orientation (6), drying (7), and centrifugation (8). It is however, sometimes difficult to make quantitative measurements with such membrane preparations due to poor orientation, light scattering, optical inhomogeneities, insufficient thickness of the sample, or thickness variations. It has been reported that it is possible to increase the degree of membrane orientation in an ultracentrifuged multilamellar array by partial dehydration (9, 10). We have recently found that a further improvement on this approach can lead to relatively thick, optically homogeneous multilamellar arrays from biological membranes (11) suitable for a variety of spectroscopic studies. This technique consists of slowly evaporating solvent from a suspension of membrane fragments while simultaneously ultracentrifuging the fragments onto an isopotential surface (isopotential spin-dry method). Consideration of the physical processes involved in the surface induced orientation of membrane vesicles and sheets (11) indicates that ultracentrifugation or drying alone are not sufficient to produce optimally uniform multilamellar arrays. Even fields of up to 100,000 *g* are not effective in overcoming the disorienting influence due to the spontaneous curvature of closed membrane structures or thermal curvature fluctuations in membrane sheets. In contrast, the isopotential spin-dry

method can promote surface induced order in samples up to 50 μm when the starting membrane fragments are >1 μm in diameter. Previous application of this method to purple membrane fragments from *Halobacterium halobium* has been shown to produce highly ordered monodomain smectic liquid-crystals (11).

We report here the production of liquid-crystal films containing >2,000 stacked layers from photoreceptor membrane along with initial evidence that the rhodopsin in these films is highly oriented, structurally intact, and able to function in many respects like native photoreceptor membrane. Previous spectroscopic studies of multilamellar arrays of oriented bilayers containing rhodopsin have been reported on films with <20 stacked membrane sheets (4).

MATERIALS AND METHODS

Photoreceptor membrane was prepared either from freshly excised calf eyes or frozen cattle retinas (Hormel, Austin, Minn.) according to the method of de Grip et al. (12). This method resulted in rod outer segments (ROS) of high purity with a 280-/500-nm absorption ratio of ~2.0. Other controls on purity consisted of polyacrylamide sodium dodecyl sulfate gel electrophoresis, electron microscopy, and infrared (IR) spectroscopy, which has been shown to be sensitive to the presence of impurities (13). To orient the photoreceptor membrane into multilamellar arrays, ROS were osmotically lysed by washing three times in filtered, double distilled, deionized water. This technique produces disk vesicles ~1 μm in diameter (12, 14) as was verified with electron microscopy. The suspensions were then spun at 11,000 *g* for up to 17 h at 4° C in an isopotential centrifugation cell (ICC) (described in detail in reference 11) onto a variety of substrates including glass, polyethylene, AgCl, and aluminum foil. The resulting films appeared to be of uniform thickness (5–50 μm) and were optically clear. In some cases films were removed from glass or polyethylene and suspended in immersion oil or mounted on a thin wire for the purpose of photomicroscopy. Films deposited on quartz or polyethylene could also be mounted in cuvettes filled with buffer for spectroscopic measurements or equilibrated in a sealed chamber with a specific relative humidity. Films deposited on aluminum foil were suitable for electrical measurements as well as x-ray diffraction.

RESULTS AND DISCUSSION

Fig. 1 *a* is a photomicrograph of photoreceptor membrane. The fragment edge can be clearly seen and is ~30 μm thick. If light is passed through the fragment with the polarization oriented in the film plane more light is absorbed (Fig. 1 *a*) than light polarized perpendicular to the film plane (Fig. 1 *b*). A similar effect has been observed in intact ROS (15) and is due to two factors: (*a*) The orientation of the retinylidene chromophore of rhodopsin ~17° from the membrane plane (15). (*b*) The stacked arrangement of the photoreceptor membrane inside the rod outer segment. The photodichroism observed in our films implies a similar multilamellar arrangement of the photoreceptor membrane parallel to the films plane. Birefringence could also be observed in the film by tipping the sample between crossed polarizers (cf. Fig. 1 *c*). This effect is due to anisotropy in the index of refraction of the bilayer (16). The absence of a birefringence effect in the film plane indicates that the sample is optically uniaxial.

The degree of orientation of the photoreceptor membrane in the films can be determined by measuring the linear dichroism at the 498-nm absorption maximum of rhodopsin (4, 7, 17). This is possible by relating the linear dichroism to the orientational parameter p_M, which is the rms average of $(3\cos^2\theta_M - 1)/2$, where θ_M is the angle a normal to each membrane fragment

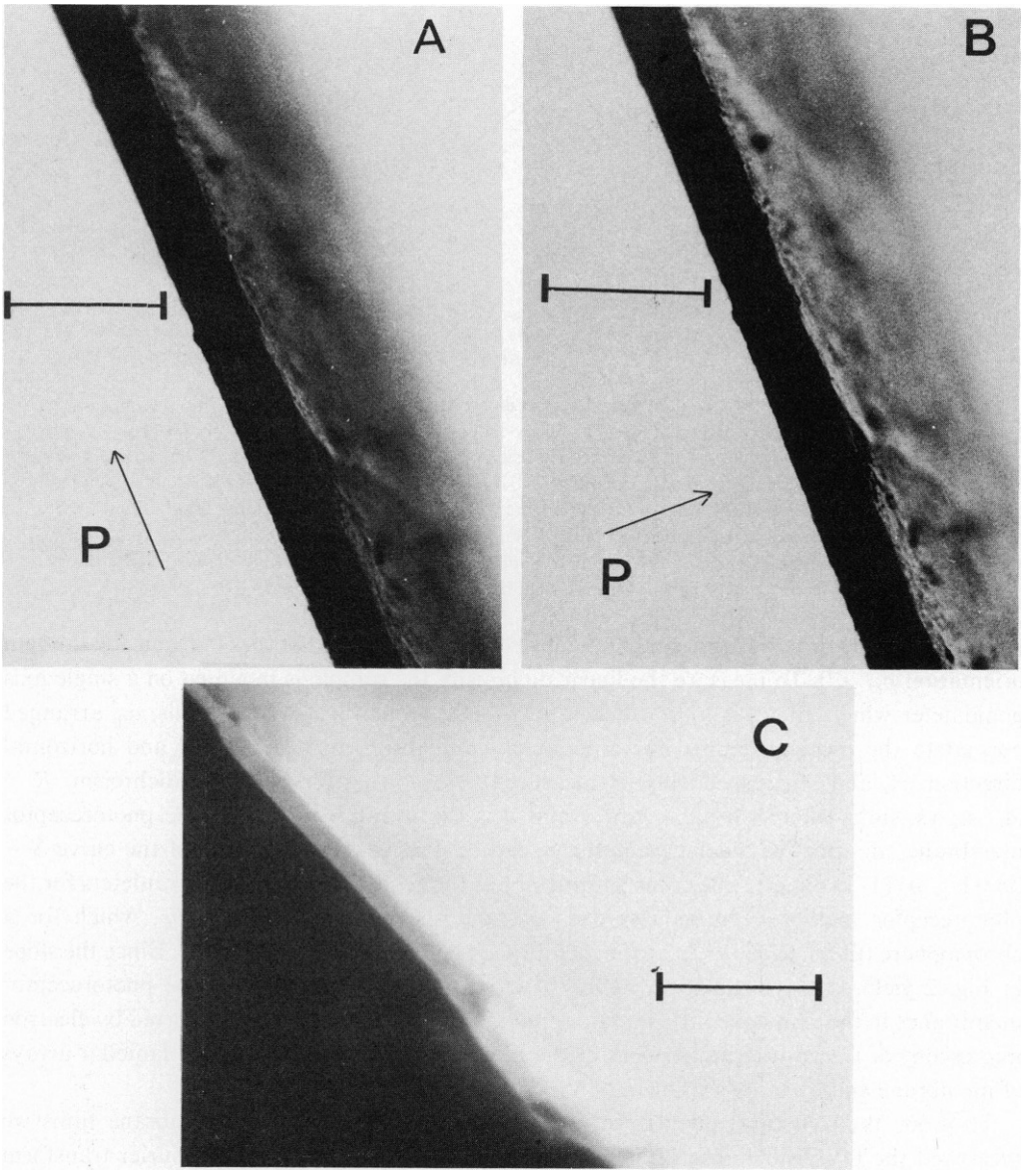


FIGURE 1. Photomicrographs of photoreceptor membrane film fragments removed from a glass substrate. The polarization of the illuminating light was as follows: (*a*) parallel to film plane; (*b*) perpendicular to *a* (arrow); (*c*) film is placed between crossed polarizers and rotated so that the film edge is ~45° to polarization axes. An edge imperfection can be seen. For all three cases the film plane was tilted at ~35° to incident light direction. Film edge thickness was determined to be 30 μm by calibrating photos with a graticule. Bars, 100 μm.

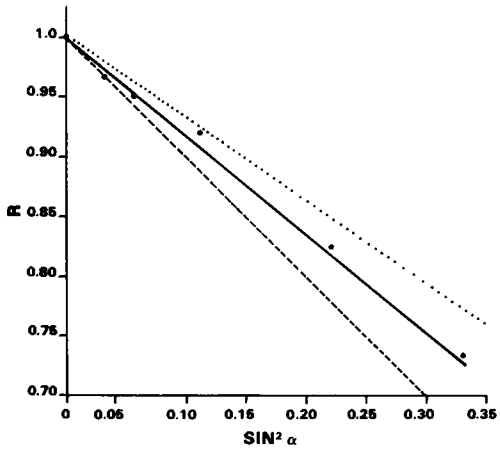
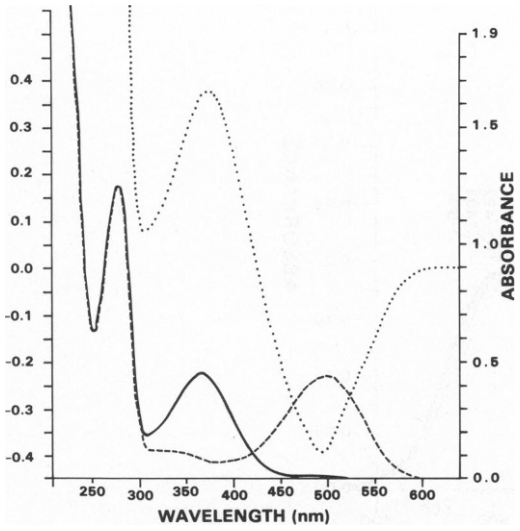


FIGURE 2. Plot of unbleached photoreceptor film dichroism, R, at 500 nm vs. $\sin^2\alpha$ for the angle α_0 = 0,15,30,45 and 60°. Measurements were made by mounting the sample on a single axis goniometer. Background due to wavelength dependent transmission of the polarizers and film scattering artifacts was corrected for by subtracting the spectrum of the film fully bleached and recorded under identical conditions to the unbleached film. All measurements were made at room temperature and humidity. The solid line is least mean square fit through data. A slope of $S = -0.7886$ was obtained which compares to $S = -0.813$ expected for $\theta_C = 17°$, the chromophore angle to the membrane plane. Dotted line is theoretical curve for $\theta_C = 17°$ and $p_M = 0.8$. Dashed line is theoretical curve for $\theta_C = 0°$, and $p_M = 1$.

makes with the normal to the sample plane. For perfect orientation $p_M = 1$ and for random orientation $p_M = 0$. To measure the linear dichroism, the sample is mounted on a single axis goniometer which allows it to be tilted to any angle around a horizontal axis, α_0, arranged normal to the incident beam. The amount of light absorbed in a vertical and horizontal direction, A_v and A_h, respectively, is measured. By plotting the resulting dichroism, $R = A_v/A_h$, vs. $\sin^2\alpha$ where $\sin\alpha_0 = n\sin\alpha$ and n is the refractive index of the photoreceptor membrane, an effective order parameter, p, can be derived from the slope of the curve $S = 3p/(1 - p)$ (17) (Fig. 2). The order parameter p is the product of the order parameters for the photoreceptor membrane mosaic spread, p_M, and the chromophore tilt, p_C, which for a chromophore tilt angle of 17° from the membrane plane gives a $p_C = -0.3717$. Since the slope in Fig. 2 yields a $p = -0.355$, p_M must be close to unity and therefore the photoreceptor membranes in the film have little mosaic spread. This conclusion is also supported by electron microscopy of freeze-fracture replicas of the film which reveal characteristic lamellar arrays of membrane and by x-ray scattering.

To probe the structural integrity of rhodopsin in the photoreceptor membrane films we measured the UV-visible absorption spectrum under different conditions. Fourier transform infrared absorption and circular dichroism measurements were also made and are reported in an accompanying article (18) and elsewhere (19). As shown in Fig. 3 *a* the spectrum of rhodopsin suspended in Ammonyx-LO detergent compares closely with the film absorption spectrum in high humidity (Fig. 4 *a*). There is, however, a 1.5 times enhancement of the 498-nm peak of the film spectrum relative to solution resulting in a reduced 280-/500-nm absorption ratio of ~1.4. A similar increase in the effective extinction coefficient of the 498-nm absorption has also been observed in rhodopsin gelatin films (20) and is due to the preferential orientation of the chromophore in the film plane.



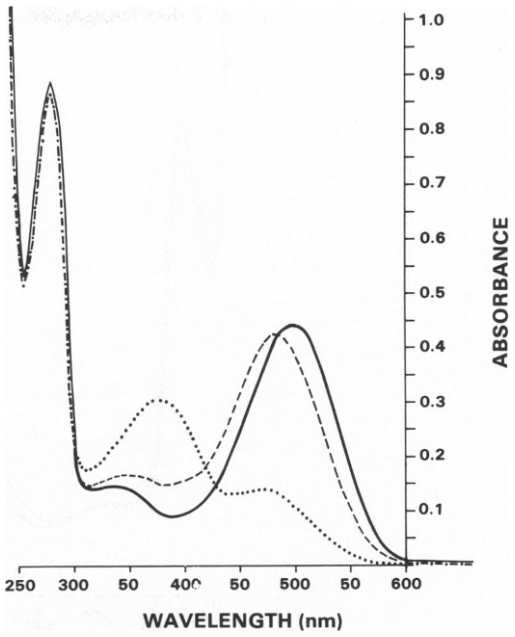


FIGURE 3

FIGURE 4

FIGURE 3. Suspension of ROS in 5% Ammonyx-LO. (*a*) (---) Absorption spectrum of unbleached sample (ordinate on right). (*b*) (—) Absorption spectrum of sample bleached with one flash of Honeywell No. 700 flash gun filtered with No. 12 Wratten filter. (*c*) (· · · ·) Difference spectrum of *a* and *b* (ordinate on left). All measurements were made on a Cary 219 UV-Vis spectrophotometer, slit width, 0.1 nm; scanning speed, 2 nm/s.

FIGURE 4. Absorption spectrum of photoreceptor membrane film prepared from osmotically lysed ROS. (*a*) (—) Unbleached film at <50% relative humidity. (*b*) (---) Film after exposure to flash at same humidity. (*b*) (· · · ·) After exposure to 100% humidity for 1 h in dark. The 280-/500-nm absorption ratio was determined by subtracting scattering background which was visually estimated. Scanning parameters were same as for Fig. 3.

Fig. 4 shows the effects of light on the photoreceptor membrane film. Normally, rhodopsin upon absorption of a photon will undergo in the dark a series of spectrally well-defined bleaching transitions (21). It has been found, however, that in the case of partially dried photoreceptor membrane or delipidated rhodopsin (22–24) the bleaching sequence is blocked at an intermediate similar to Meta I, which absorbs near 480 nm. In the case of the photoreceptor membrane film, a similar block is found at the "Meta I" intermediate (Fig. 4 *b*). Exposure to aqueous medium or high relative humidity, however, leads to unblocking and bleaching to a Meta II state at 380 nm (Fig. 4 *c*). We also observe a transition with a half-time of ~5 min from Meta II to a new state absorbing near 455 nm (Meta III). At lower humidity a slowing of the bleaching sequence from an earlier intermediate similar to lumirhodopsin absorbing at 490 nm with an increased extinction coefficient (Fig. 5 *a–c*) is observed.

An important criterion of rhodopsin integrity is its ability to regenerate in the presence of 11-*cis* retinal subsequent to bleaching (21). Regeneration ability is very sensitive to rhodopsin's environment, for example rhodopsin is able to regenerate only in a few nonionic detergents such as digitonin. To check for irreversible change of rhodopsin structure we

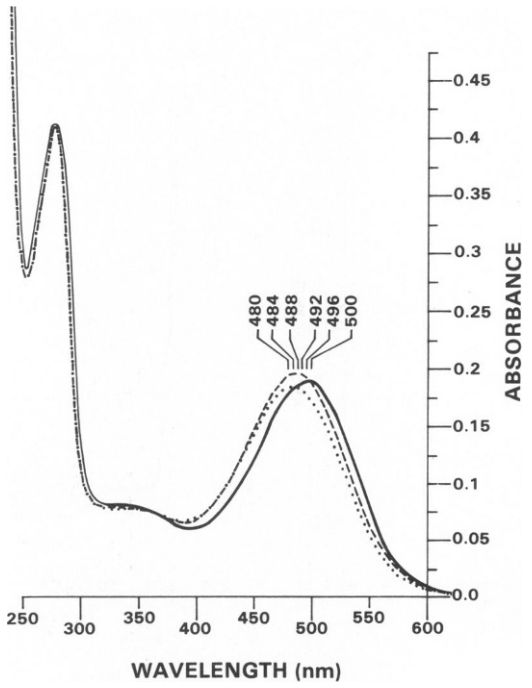


FIGURE 5. Absorption spectra of photoreceptor membrane film at room humidity. (*a*) (—) Unbleached film. (*b*) (---) Film spectrum recorded ~1 min after exposure to light flash. (*c*) (· · · ·) Film 30 min after flash. Film was kept in dark during the time between measurements. Scanning parameters were the same as in Fig. 3.

resuspended the film in digitonin and compared the regeneration activity to untreated photoreceptor membrane. The results indicate that rhodopsin regeneration activity remains at close to 100% after film formation. It was possible to regenerate rhodopsin in the film state by including a five-fold molar excess of 11-*cis* retinal in the suspending buffer before isopotential spin drying (Fig. 6). The film was mounted inside of a cuvette, the cuvette was then filled with either 50 mM phosphate buffer, pH 6.8, or distilled water. The initial absorbance at 500 nm was monitored for 30 min and then the film was exposed to one flash from a Honeywell No. 700 flash gun, filtered by a No. 12 Wratten filter and also a heat-absorbing glass filter (Honeywell, Inc., Denver, Colo.). As shown in Fig. 6, the absorption of the film at 500 nm subsequent to a single flash recovers its original level with a half-time of ~5 min. Complete recovery occurs after additional flashes, although with a slower half-time, until the 11-*cis* retinal pool is exhausted. In contrast, films without 11-*cis* retinal display only 40–50% recovery after the first bleaching flash and no recovery after the second flash. These results indicate that rhodopsin in these films is fully active with respect to chemical regeneration. Furthermore, the kinetics of the rhodopsin regeneration in the film are comparable to those measured for isolated ROS (25).

In conclusion, we have developed a method of producing highly oriented smectic type liquid crystal multilamellar films from photoreceptor membrane. These films possess many of the properties of native rhodopsin including its orientation in the membrane, photo-induced

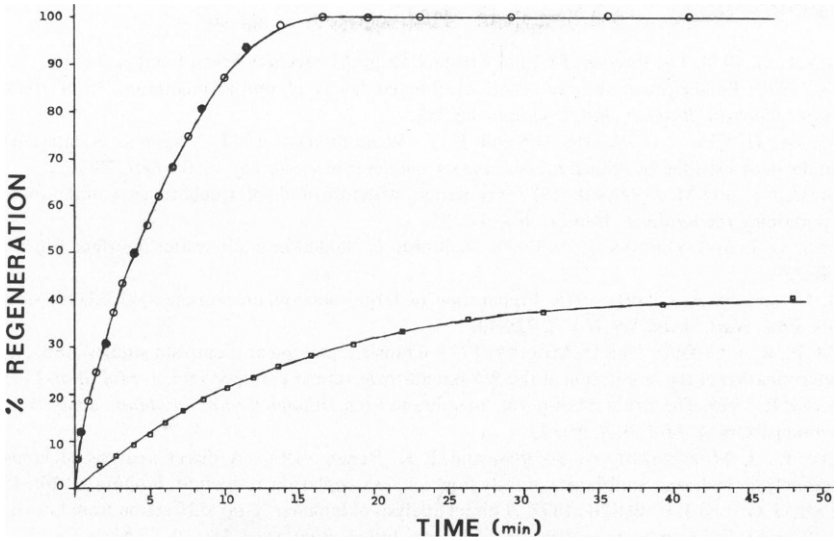


FIGURE 6 Regeneration of photoreceptor membrane film with 11-*cis*-retinal. (○) Film prepared with a five-fold molar excess of exogenous 11-*cis*-retinal in the suspending buffer. Absorption at 500 nm is shown as a function of time subsequent to flash. All curves are normalized to the maximum absorption prior to flash. 100% recovery was found after a single flash illumination. Full recovery occurred after subsequent flashes until excess retinal was used up. (□) Film prepared in the normal manner without 11-*cis*-retinal. 40% recovery is found after the first flash illumination. Further flashing led to a decrease in absorption at 500 nm with no subsequent recovery.

bleaching, and ability to regenerate with 11-*cis* retinal. It is unknown at present, however, if the disk membranes have fused to form large macroscopic sheets or are merely overlapped. In addition, the possibility exists that the protein lipid interaction in the film is different from the native membrane, due to for example possible phase separation (9). It will be extremely important for further studies that the ultrastructure of these films be characterized using x-ray scattering and freeze-fracture electron microscopy. It is possible that a richness of protein-lipid-water phases will be found as in lipid-water systems (26, 27).

Since the films are thick enough for the application of a variety of structural probes including x-ray scattering, circular dichroism and polarized infrared spectroscopy structural information can be obtained. Initial results from the last two methods have recently provided direct evidence of extensive transverse alpha-helices in rhodopsin (18). Functional information about biomembranes ia also obtainable using liquid-crystal films. In particular, transport and electrical measurements can be made by mounting the film between two aqueous compartments. Hence, the isopotential spin-dry method of biomembrane liquid-crystal film formation may offer a useful new approach to the study of biomembrane structure and function as well as providing a means of incorporating selected biomembrane properties such as active transport or energy transduction into liquid-crystal films.

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