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Preparation and Properties of Phospholipid Bilayers Containing Rhodopsin

(photoreceptor/membrane/spin-label/retinal rod outer segments)

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ABSTRACT **Purified rhodopsin has been prepared containing less than 1.1 mol of phosphate per mol of protein. The purified rhodopsin has been incorporated into phosphatidylcholine bilayers, and the molecular interactions within the bilayers were investigated by the use of spin-labeled phosphatidylcholines. Rhodopsin appears to inhibit segmental motions of the hydrocarbon chains, an effect similar to that of cholesterol on phospholipid bilayers.**

Photoreceptor membranes are the only nerve membranes from which it has been possible to isolate a specifically labeled membrane protein known to be directly involved in the excitation process. Thus, the rod outer segment (ROS) membranes of vertebrate retina offer a unique opportunity for the study of functional and structural aspects of lipid–protein interactions. The x-ray diffraction results of Blaurock and Wilkins (1), Corless (2), and Gras and Worthington (3) on frog ROS disc membranes strongly support the contention that the phospholipids of these membranes are in a bilayer configuration. About 80–90% of the protein in bovine (4) and frog (5) ROS membranes is rhodopsin, which can be obtained in a relatively high state of purity (5–8).

Cone (9) and Brown (10) have demonstrated a rotational motion of rhodopsin about an axis perpendicular to the ROS disc-membrane surface. Blasie and Worthington (11) conclude from a detailed x-ray diffraction analysis that rhodopsin exists in a two-dimensional fluid in the ROS disc membrane of the frog, observations consistent with the results of Cone and Brown. These data, along with the high content of hydrophobic amino acids (5–8, 12), and the water insolubility of rhodopsin, suggest that the topology of the protein involves appreciable fields of hydrophobic residues, and that these areas are directly solvated by the hydrocarbon chains of the membrane phospholipids. Recently, Blasie (13) has given further support to this model, and suggests that rhodopsin penetrates to about one-third of its diameter within the membrane, assuming the molecule to be spherical.

In order to study the molecular details of the interaction of rhodopsin with phospholipids, and hopefully to elucidate the functional role of the protein, we recombined purified bovine rhodopsin with chemically defined phospholipids. The word "recombination" is used here rather than "reconstitution," since reconstitution implies restoration of function. Since the *in vivo* function of rhodopsin is not definitely known, a functional assay is not available.

Abbreviations: $DodMe_3NH_4Br$, Dodecyltrimethylammonium bromide; ROS, rod outer segment.

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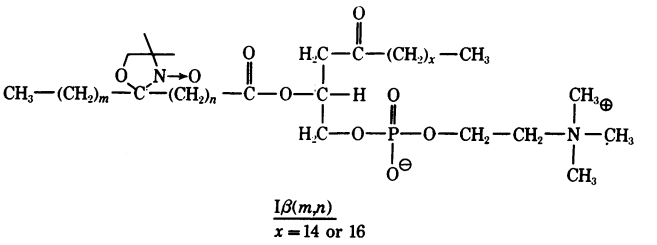
The present report describes the successful recombination of phospholipids and purified, delipidated rhodopsin to produce bilayers with incorporated protein. In addition, we report on preliminary structural studies of the recombinants using the methods of spin-labeling and freeze-fracture electron microscopy. Spin-labeling has recently enjoyed wide application as a method of obtaining structural information about biological membranes and membrane model systems (14–21). Hubbell and McConnell (14), McConnell and McFarland (22), and Griffith (23) have presented quantitative theories relating parameters of the paramagnetic resonance spectra of amphiphilic spin labels to molecular motion, and orientation of these labels in biological membranes and phospholipid bilayers. A study of the structure of membrane lipid regions and phospholipid bilayers with the spin-labeled phospholipids $I\beta(m,n)$ has recently been published (14). In the present



$I\beta(m,n)$
$x = 14$ or 16

study, we use $I\beta(m,n)$ to derive information concerning the orientation and motion of hydrocarbon chains in the rhodopsin–phospholipid recombinants.

EXPERIMENTAL

Egg phosphatidylcholine was prepared according to the method of Singleton *et al.* (24). The phosphatidylcholine spin labels $I\beta(m,n)$ were prepared as described (14). Dodecyltrimethylammonium bromide ($DodMe_3NH_4Br$) was prepared by the reaction of dodecylbromide and trimethylamine, and was purified by recrystallization. ^{14}C-labeled $DodMe_3NH_4Br$ was prepared by the reaction of [^{14}C]methyl bromide (New England Nuclear Corp.) with dimethyldodecylamine. 11-*cis* retinal was a generous gift of Paul Brown, The Biological Laboratories, Harvard University.

Rod outer segments were isolated from dark-adapted bovine retinae (Hormel Institute, Austin, Minn.) by a sucrose flotation method (6). All procedures involving rhodopsin were done under dim red light (Kodak series 1 filter, 15-W bulb) or total

darkness at 4° unless otherwise specified. Rhodopsin was purified and delipidated by the following general procedure: lyophilized ROS from 100 retinas was extracted with 2–3 ml of 100 mM $DodMe_3NH_4Br$ in 15 mM phosphate buffer (pH = 7.1) and centrifuged at 45,000 × *g* for 20 min. The optically clear supernatant was applied to a hydroxyapatite column and eluted with a linear gradient of 0–1.1 M NaCl in 100 mM $DodMe_3NH_4Br$. The details of the purification procedure will be described elsewhere.

The extinction coefficient of purified rhodopsin at 498 nm was determined by both the hydroxylamine and thiobarbituric acid procedures (12). All concentration measurements were based on this extinction coefficient.

Detergent was removed from rhodopsin by exhaustive dialysis against distilled water. For recombination of phosphatidylcholine with rhodopsin, the solid phospholipid was added directly to the $DodMe_3NH_4Br$ solution of purified rhodopsin and dialyzed exhaustively against 5 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (Calbiochem) buffer (pH = 6.6). The efficiency of detergent removal was determined with ^{14}C-labeled $DodMe_3NH_4Br$. After dialysis, the recombinants were harvested by centrifugation, and the transparent pellet was resuspended in buffer and layered on a 7.5-cm linear sucrose gradient from 5 to 30% sucrose and centrifuged for 4–12 hr at 100,000 × *g*.

ROS membranes and recombinants were photolyzed by exposure at room temperature to a 300-W flood lamp at a distance of 30 cm for 5 min. After this treatment the suspensions were distinctly yellow in color, and showed no absorption maximum at 498 nm. Rhodopsin was regenerated by addition in the dark of a 2- to 3-fold excess of 11-*cis* retinal to the suspension of photolyzed ROS membranes or recombinants. The suspension was then incubated for 5 hr in the dark at room temperature and, after the addition of hydroxylamine, the absorbance at 498 nm was determined. The regenerated suspension was then photolyzed as before, and the decrease in absorbance at 498 nm used to determine the percentage of regeneration. Phosphate analysis was done according to the method of Bartlett (25). Electron paramagnetic resonance spectra were obtained on a Varian V-4500 X-band spectrometer; a proton probe was used for precise field measurement and a Varian temperature control accessory was used for temperature regulation at 15°.

RESULTS AND DISCUSSION

Properties of rhodopsin–phospholipid recombinants

Rhodopsin purified on hydroxyapatite in 100 mM $DodMe_3NH_4Br$ showed absorption maxima at 498, 350, and 278 nm. The ratio of absorbance at 278 nm to that at 498 nm was typically 1.7, while the ratio at 400 nm to that at 498 nm was 0.19. The extinction coefficient of purified rhodopsin at 498 nm in 100 nM $DodMe_3NH_4Br$ was 42,700 ± 3%.

TABLE 1. *Composition and densities of recombinants*

Approximate mole ratio of phospholipid to rhodopsin before dialysis	Mole ratio of phospholipid to rhodopsin after dialysis and purification	Apparent density in sucrose
100:1	104:1	1.10
200:1	170:1	1.05
400:1	308:1	1.03

Virtually all of the $DodMe_3NH_4Br$ could be removed from purified rhodopsin or rhodopsin–phospholipid mixtures by dialysis against distilled water or 5 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid buffer (pH = 6.6) for 4 days at 4°, as estimated by the use of [^{14}C]$DodMe_3NH_4Br$. At the end of this dialysis period, about one detergent molecule remained for every 10 rhodopsin molecules. Removal of detergent in the absence of phospholipid resulted in extensive thermal bleaching of rhodopsin, although the protein is stable for more than 2 weeks in 100 mM $DodMe_3NH_4Br$ at 4°. At room temperature, however, the rhodopsin in 100 mM $DodMe_3NH_4Br$ undergoes rapid thermal bleaching, with a half-life of about 10 min.

Phosphate analysis of purified rhodopsin that had been dialyzed against distilled water to remove inorganic phosphate and detergent indicated 1.1 mol of phosphate per mol of rhodopsin. Since bound phospholipid is not dialyzable, purified rhodopsin in $DodMe_3NH_4Br$ solution is assumed to have 1.1 mol or less of bound phospholipid per mol of protein.

If solid egg phosphatidylcholine is added to the $DodMe_3NH_4Br$ solution of rhodopsin in the range of 100–400 mol of phosphatidylcholine per mol of rhodopsin before dialysis, the rhodopsin is stable for an indefinite period after detergent removal, in contrast to its instability in the absence of the phospholipid under these conditions. Furthermore, the thermal stability is greatly enhanced and the absorption at 498 nm appears indefinitely stable at room temperature. We have investigated phospholipid–rhodopsin structures formed by detergent removal from solutions of rhodopsin containing about 100, 200, and 400 mol of phosphatidylcholine per mol of rhodopsin. These structures will be referred to as the 100:1, 200:1, and 400:1 recombinants. Sucrose density gradient centrifugation reveals that in each case most of the rhodopsin-containing material formed during detergent removal has a surprisingly uniform density and forms a sharp band at the apparent density shown in Table 1. Recombinants purified by sucrose gradient centrifugation have been analyzed for phosphate and rhodopsin content, and the mole ratio of phospholipid to rhodopsin is generally lower than that expected on the basis of complete recombination. This is particularly evident for high ratios, as shown in Table 1.

The stability properties of the rhodopsin in the recombinants, and the fact that the structures formed are of an apparently uniform density in sucrose that varies with the phospholipid to protein ratio, suggests that the rhodopsin is incorporated within a lipid structure, rather than simply precipitating as a separate phase upon detergent removal.

Rhodopsin could be regenerated in the photolyzed recombinants to about 90% of the value regenerable in native ROS membranes. The significance of this regeneration will be discussed below.

Structure of the phospholipid regions in the recombinants

The structure of the lipid regions in the recombinants is of interest, since rhodopsin–lipid and rhodopsin–rhodopsin interactions could dictate the formation of structures quite unlike that formed by pure phosphatidylcholine under the conditions of the recombination. The phosphatidylcholine used to form the recombinants has been doped with small amounts of the spin-labeled phosphatidylcholines Iβ(7,4) and Iβ(7,8) [50 to 1 mole ratio of egg phosphatidylcholine to Iβ(*m*,*n*)]. Control samples of doped egg phosphatidylcholine without rhodopsin

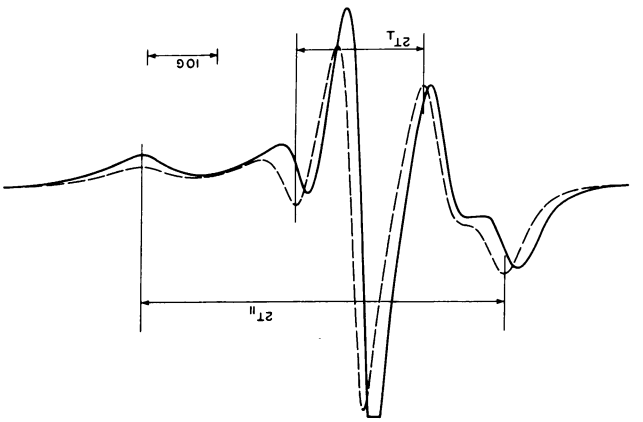


FIG. 1. Paramagnetic resonance spectra obtained from an isotropic distribution of the 100:1 recombinant (*solid line*) and phosphatidylcholine bilayers (*dashed line*) doped with Iβ(7,4). Values of $2T_{\parallel}$ and $2T_{\perp}$ are indicated for the *dashed line* spectrum.

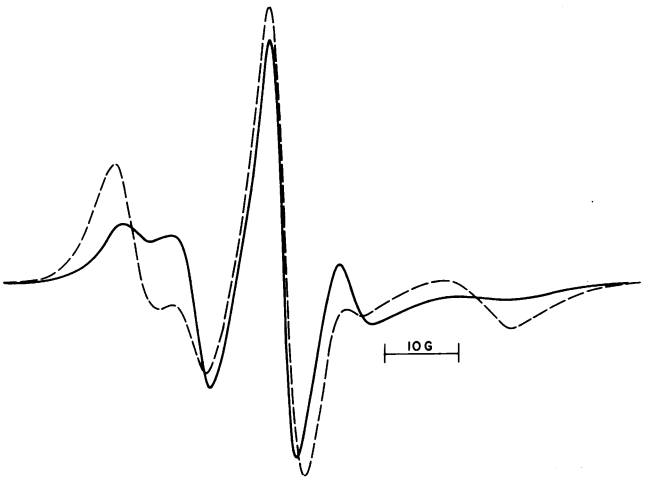


FIG. 2. Paramagnetic resonance spectra obtained from a sheared film of the 200:1 recombinant containing Iβ(7,4) with the magnetic field perpendicular (*dashed line*) and parallel (*solid line*) to the shear plane.

were prepared by an identical procedure of solution in 100 mM $DodMe_3NH_4Br$ followed by exhaustive dialysis. The properties of the controls prepared in this way were indistinguishable, from the standpoint of the paramagnetic resonance spectra, from those prepared by directly dispersing the phospholipid in water with sonication. Fig. 1 shows the paramagnetic resonance spectra obtained from the 100:1 recombinant doped with Iβ(7,4), and the corresponding spectrum of the control without rhodopsin. In each case, the spectra of the controls and the recombinants show resolved hyperfine extrema. This line shape has been previously observed in the paramagnetic resonance spectra of phospholipid bilayers containing the I$\beta(m,n)$ and can be interpreted in terms of a rapid, anisotropic diffusion about an effective long axis of the hydrocarbon chain (14). The resolved spectral splittings, $2T_{\parallel}$ and $2T_{\perp}$, are related to the probability of bond rotational isomerizations in the hydrocarbon chains, and thus to local fluidity within the bilayers. An order parameter, S_n, has been derived from $T_{\parallel}$ and $T_{\perp}$, and is a convenient, quantitative measure of the fluidity of the hydrocarbon-chain region in phospholipid bilayers and biological membranes (14). The subscript n indicates the dependence of the order parameter on n for the different I$\beta(m,n)$ (14).

The recombinants have many properties in common with dispersions of pure phospholipids. Most revealing of these similarities is the orientation of the nitroxide group produced by shearing a concentrated sample of the doped recombinant between two flat quartz plates. For example, Fig. 2 shows the paramagnetic resonance spectra of the sheared 200:1 recombinant containing Iβ(7,4) with the magnetic field parallel and perpendicular to the shear plane. Since the recombinants have been purified by gradient centrifugation, there is no excess phospholipid present, and the membranes that contain the Iβ(7,4) also contain the rhodopsin. The large changes in the relative intensities of the hyperfine extrema show clearly that the magnetic symmetry axis of the nitroxide fatty acid chain is preferentially oriented perpendicular to the shear plane, the orientation observed for I$\beta(m,n)$ in shear-oriented phospholipid bilayers (22).

Comparison of the resonance spectra of the I$\beta(m,n)$ in the recombinants reveals an increasing configurational freedom (decreasing order parameter) with increasing n, characteristic of associated hydrocarbon chains in a liquid–crystalline state (14). These data alone may not distinquish the phospholipid bilayer from a bilayer in which one half was composed of pure rhodopsin and the other half of pure phospholipid. This possibility can be eliminated, however, since the phospholipid to rhodopsin mole ratio can be experimentally varied, while the above type of structure would have a nearly constant ratio of protein to lipid. From these data it is concluded that the lipid phase containing the incorporated rhodopsin is in the bilayer configuration.

Rhodopsin–bilayer interactions

Freeze-fracture electron microscopy exposes to view interior surfaces of lipid bilayers and biological membranes, as suggested by Branton (26) and convincingly demonstrated by Pinto da Silva and Branton (27). Freeze-fracture micrographs prepared by W. Stoeckenius of the 100:1 recombinant are shown in Fig. 3*a* and *b*, unetched and etched, respectively. Densely clustered particles are clearly visible in the fracture plane of the specimens, while the bilayer outer surfaces exposed by the etching process appear quite smooth. A control sample of egg phosphatidylcholine dispersion prepared in the same way shows completely smooth fracture surfaces. These data demonstrate the incorporation of rhodopsin and indicate that a respectable fraction of the molecular mass of the rhodopsin in the recombinants is located within, rather than on, the phospholipid bilayer.

As can be seen from the example in Fig. 1, the presence of incorporated rhodopsin in phosphatidylcholine bilayers doped with I$\beta(m,n)$ is reflected in changes in the spectral splittings, $2T_{\parallel}$ and $2T_{\perp}$, and thus in the order parameter, S_n. The general features of the line shapes, however, are conserved, and a detailed comparison of the controls without rhodopsin and the recombinants by the order parameter is valid. Due to the rapid longitudinal diffusion of phospholipids in a fluid bilayer (29), the resonance spectra of phospholipids like the I$\beta(m,n)$ are expected to contain information on the time-average environment(s) in the system. In structurally inhomogeneous systems such as those considered here, the I$\beta(m,n)$ are not

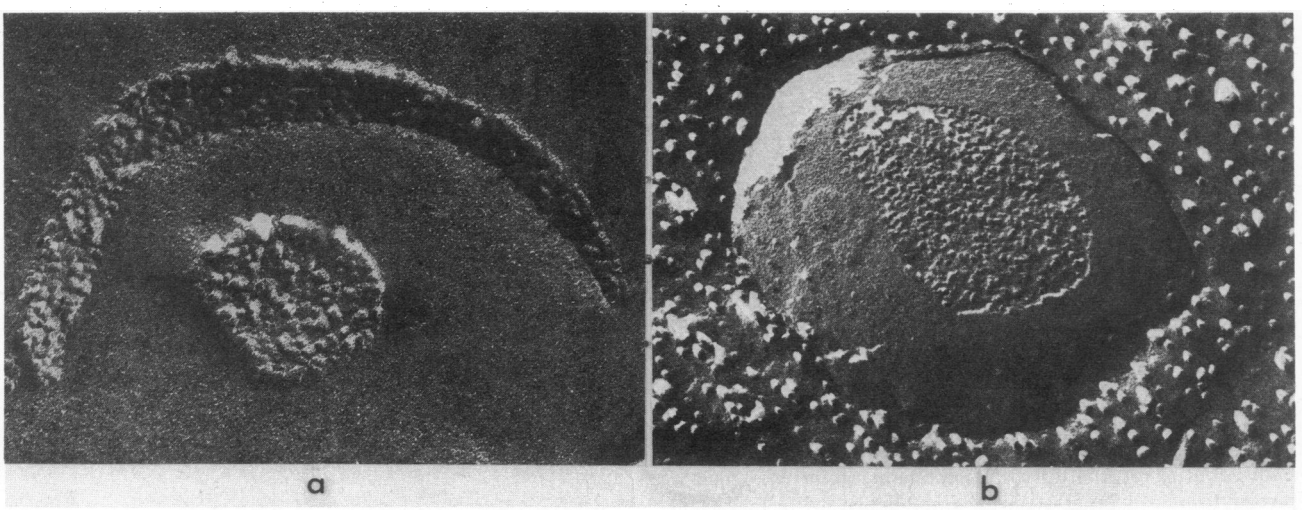


FIG. 3. (*a*) Freeze-fracture micrograph of the 100:1 recombinant ×90,900. (*b*) Etched freeze-fracture micrograph of the 100:1 recombinant ×48,000.

expected to sample all possible environments with equal probability if the presence of the nitroxide group excludes the molecule from regions involving close molecular packing or specific molecular interactions. Such steric exclusion of the I$\beta(m,n)$ would not alter our conclusions, but would decrease the observed effect of rhodopsin.

Fig. 4 shows the dependence of the order parameters S_4 and S_8 on the measured mole fraction of rhodopsin in the recombinants, and indicates an increasing order both near the surface and within the interior of the bilayer with increasing rhodopsin content. This observation further supports the contention that rhodopsin is indeed incorporated in the bilayer, rather than forming a separate phase. In the present experiments, the spin concentration is such that the probability of an I$\beta(m,n)$ occupying any particular site in the bilayer is quite small, and the effect observed must represent a fairly long-range interaction of rhodopsin with the phospholipids. Taken together with the freeze-fracture microscopy, the paramagnetic resonance data strongly suggest that regions of the rhodopsin surface are solvated by the hydrocarbon chains of the phospholipids in the recombinants.

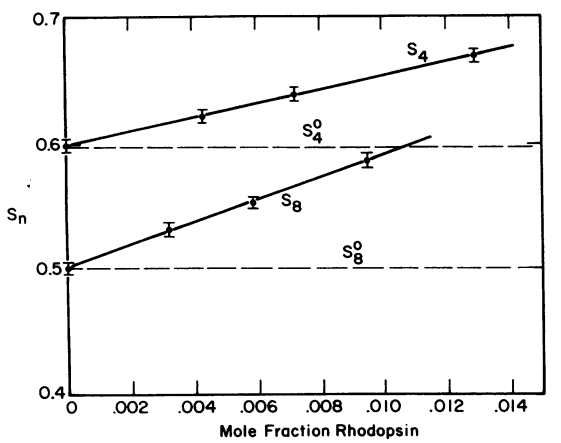


FIG. 4. Plot of the order parameters S_4 and S_8 as a function of mole fraction of rhodopsin in the recombinants. The lines S_4^0 and S_8^0 indicate the values of the order parameters in bilayers of phosphatidylcholine containing no rhodopsin.

The ordering of the phospholipid chains by rhodopsin resembles the so-called condensing effect of cholesterol (30, 31). The hydrocarbon chains in the egg phosphatidylcholine bilayer, while being mobile relative to a solid phase, possess a definite degree of order. For a given set of conditions, the degree of order of the hydrocarbon chains within a bilayer is related to the maximum number of van der Waals contacts that can be made consistent with the favorable conformations of each chain, as well as steric and electrostatic interactions of the polar head groups. We assume here that nonpolar interactions dominate the condensing effect of rhodopsin, since the degree of order of the hydrocarbon chains in the recombinants is nearly independent of ionic strength and pH. Under these circumstances, the inclusion of a molecule into a bilayer will lead to increased ordering if the structure of the molecule is complimentary to the existing structure in such a way as to increase the maximum possible number of van der Waals contacts. The increase in order within the bilayer produced by rhodopsin suggests that the surface structure of the protein is in this sense complementary to the hydrocarbon chains. It is possible that the surface of rhodopsin, and other membrane proteins, is constructed for maximal interaction with a specific species of hydrocarbon chain. It is often observed that bilayers formed from membrane lipids are more fluid than the lipid regions in the membranes themselves (17). The results obtained here suggest that this effect may be due to the penetration of proteins into the hydrocarbon-chain region of the membrane.

General discussion

The role of the detergent in the experiments presented here must not be overlooked. Like water-soluble proteins, the structure of membrane proteins must depend to a large extent on the nature of solvent interactions. Thus, the structure and properties of a detergent-solubilized membrane protein depend on the properties of the detergent, and its choice is important. In recombination studies the detergent must meet several requirements: (*a*) it must readily solubilize the protein and not result in irreversible denaturation; (*b*) it must be possible to purify the protein in its presence, resulting in complete displacement of lipids; (*c*) it must be easily removable under

nondenaturing conditions. The detergent $DodMe_3NH_4Br$ appears to meet these requirements for bovine rhodopsin.

A point to be emphasized about the present experiments is that the protein was purified and essentially devoid of native lipid. Thus, a recombination of individual molecular components has been accomplished, rather than a recombination of membrane units.

In this study, we have no unequivocal assay for assuring that the protein is incorporated within the bilayer in a conformation entirely equivalent to the native structure. There are several assays for the native rhodopsin–membrane structure: (*a*) absorption of light at 498 nm; (*b*) regenerability after photolysis; (*c*) thermodynamic and kinetic details of the dark reactions after photolysis; and (*d*) the circular dichroism spectra of membrane fragments (32). The absorption maximum at 498 nm appears to be quite insensitive to perturbation of the native structure. On the other hand, the regenerability after photolysis seems to be very sensitive to structural and chemical perturbations (12, 33, 34).

We have not made use of the dark reactions or circular dichroism spectroscopy, and tentatively rely on regenerability as a criterion for the native rhodopsin–membrane structure. It is possible to regenerate rhodopsin in the photolyzed recombinants to within 90% of the maximum value regenerable for photolyzed native ROS membranes. It is clear that the presence of phospholipid affects the stability, structure, and properties of rhodopsin. Furthermore, we anticipate that the composition of the phospholipids will influence the properties or rhodopsin, and do not expect bilayers of pure egg phosphatidylcholine containing rhodopsin to duplicate the native membrane. Clearly a great deal more work must be done to establish the biological relevance of this system; however, it represents a system of physical–chemical interest in its own right, and it offers the possibility of gaining insight into the nature of lipid–protein coupling mechanisms.

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1. Blaurock, A. E. & Wilkins, M. H. F. (1969) *Nature* **223**, 906–909.
2. Corless, J. M. (1972) *Nature* **237**, 229–231.
3. Gras, W. J. & Worthington, C. R. (1969) *Proc. Nat. Acad. Sci. USA* **63**, 233–238.
4. Heitzmann, H. (1972) *Nature New Biol.* **235**, 114.
5. Robinson, W. E., Gordon-Walker, A. & Bownds, D. (1972) Nature New Biol. **235**, 112–114.
6. Shichi, H., Lewis, M., Irreverre, F. & Stone, A. (1969) *J. Biol. Chem.* **244**, 529–536.
7. Heller, J. (1968) *Biochemistry* **7**, 2906–2913.
8. Shields, J. E., Dinovo, E. C., Henriksen, R. A., Kimbel, R. L. & Millar, P. G. (1967) *Biochim. Biophys. Acta* **147**, 238–251.
9. Cone, R. A. (1972) *Nature New Biol.* **236**, 39–43.
10. Brown, P. K. (1972) *Nature New Biol.* **236**, 35–38.
11. Blasie, J. K. & Worthington, C. R. (1969) *J. Mol. Biol.* **39**, 417–439.
12. Zorn, M. & Futterman, S. (1971) *J. Biol. Chem.* **246**, 881–886.
13. Blasie, J. K. (1972) *Biophys. J.* **12**, 191–204.
14. Hubbell, W. L. & McConnell, H. M. (1971) *J. Amer. Chem. Soc.* **93**, 314–326.
15. Tourtellotte, M. E., Branton, D. & Keith, A. D. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 909–916.
16. Hubbell, W. L., Metcalfe, J. C., Metcalfe, S. M. & McConnell, H. M. (1970) *Biochim. Biophys. Acta* **219**, 415–427.
17. Rottem, S., Hubbell, W. L., Hayflick, L. & McConnell, H. M. (1970) *Biochim. Biophys. Acta* **219**, 104–113.
18. Griffith, O. H., Libertini, L. J. & Birrell, G. B. (1971) *J. Phys. Chem.* **75**, 3417–3425.
19. McFarland, B. G. & McConnell, H. M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1274–1278.
20. McConnell, H. M., Wright, K. L. & McFarland, B. G. (1972) *Biochem. Biophys. Res. Commun.* **47**, 273–281.
21. Seelig, J. & Hasselbach, W. (1971) *Eur. J. Biochem.* **21**, 17–21.
22. McConnell, H. M. & McFarland, B. G. (1970) *Quart. Rev. Biophys.* **3**, 91–136.
23. Jost, P., Libertini, L. J. & Griffith, O. H. (1971) *J. Mol. Biol.* **59**, 77–98.
24. Singleton, W. S., Gray, M. S., Brown, M. L. & White, J. L. (1965) *J. Amer. Oil Chem. Soc.* **42**, 53–56.
25. Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468.
26. Branton, D. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 1048–1056.
27. Pinto da Silva, P. & Branton, D. (1970) *J. Cell Biol.* **45**, 598–605.
28. Deamer, D. W., Leonard, R., Tardieu, A. & Branton, D. (1970) *Biochim. Biophys. Acta* **219**, 47–60.
29. Kornberg, R. & McConnell, H. M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2564–2568.
30. Demel, R. A., Geurts Van Kessel, W. S. M. & Van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* **266**, 26–40.
31. Ghosh, D. & Tinoco, J. (1972) *Biochim. Biophys. Acta* **266**, 41–49.
32. Shichi, H. (1971) *Photochem. Photobiol.* **13**, 499–502.
33. Shichi, H. (1971) *J. Biol. Chem.* **246**, 6178–6182.
34. Albrecht, G. (1957) *Science* **125**, 70–72.