Characterization of Rhodopsin in Synthetic Systems

Wayne L. Hubbell

Department of Chemistry, University of California, Berkeley, California

Received May 13, 1974

The rod cell of the vertebrate retina is a remarkably competent device for the conversion of photons of a specific frequency range into electrical signals. Phototransduction is accomplished with a large current gain, a response time of a few milliseconds, and a dynamic range extending over approximately 5 log units of light intensity (for reviews dealing with the physiology and electrical activity of photoreceptors see ref 1-3). A schematic representation of the anatomy of this cell is shown in Figure 1. The division of labor is striking: the cylindrical rod outer segment is totally dedicated to the transduction process, while the inner segment is concerned with the more usual tasks of cellular metabolism.

Protein accounts for approximately 40% of the outer segment dry weight, of which 85% is the water-insoluble, light-sensitive protein rhodopsin.[4-6] The other predominant molecular species is the phospholipid, which accounts for roughly 40% of the dry weight.[7] Although they are of certainly no less interest, little information is currently available on the carbohydrate and small molecule content of the outer segment. The available chemical characterization with respect to rhodopsin and phospholipids reflects largely the composition of the disc membranes, since the surrounding plasma membrane constitutes only a few per cent of the total membrane mass.

As indicated in Figure 1, the disc membranes appear to enclose a space which is not continuous with the cytoplasmic space surrounding the discs. The intradiscal volume is very small, the spacing between adjacent membranes of a single disc being no larger than 15-20 Å.[8] The disc-to-disc repeat is about 300 Å, while the thickness of an entire disc is about 150 Å,[9-11] giving a single membrane thickness of about 67 Å.[8] The phospholipids in the disc membrane are arranged in a smectic liquid-crystalline phase, the phospholipid bilayer, as is the case for most biological membranes. The rhodopsin molecule is an integral part of the disc membrane and cannot be separated from it without disrupting the entire structure.

Blasie and Worthington[12] have interpreted diffraction from rod outer segments to indicate that the rhodopsin molecule behaves as if it were in a planar fluid, and Cone[13] and Brown[14] have provided convincing experimental evidence for the rotational diffusion of rhodopsin about an axis normal to the membrane surface. Poo and Cone have since demonstrated the translational diffusion of rhodopsin in the plane of the membrane.[15]

The distribution of the rhodopsin mass *within* the bilayer is a matter of current controversy, and it seems that every possible model has been put forth (see ref 7 for a review). Freeze-fracture analysis (to be discussed below) of native rod outer segments[16-19] and vesicles derived from the disc membranes[19] indicates that rhodopsin remains with the outer (cytoplasmic) half of the membrane during fracture. Chen and Hubbell[19] have demonstrated the existence of defects or holes on the *inner* half of the fractured membranes, suggesting that the rhodopsin molecule may span the entire thickness of the membrane. Proof of this latter point, however, must await further chemical and structural analysis. In light of this evidence, only the two general models shown in Figure 1 seem realistic, and they differ in the extent of penetration of rhodopsin into the bilayer. Heller and Lawrence[20] have shown that rhodopsin is a glycoprotein, and the carbohydrate moiety is indicated by "S" in Figure 1. The placement of the carbohydrate is based on experiments by Steinemann and Stryer,[21] but is still open to question.

The anatomy of the outer segment of the rod cell puts strict limitations on possible mechanisms of phototransduction. Penn and Hagins[22] and Korenbrot, Brown, and Cone[8] have shown that the effect of photon absorption in the disc is expressed as a decreased Na^+ conductance in the rod *plasma* membrane. Since the plasma membrane is electrically isolated from the discs,[22] the photon absorption

Wayne L. Hubbell, a native Californian, is Associate Professor of Chemistry at University of California, Berkeley. He studied for his B.S. degree at Oregon State University, and then went on to graduate work at Stanford University, where he received the Ph.D degree in 1970, followed by a year of postdoctoral work with Harden McConnell. His research interests are in structure and regulatory functions of biological membranes and molecular mechanisms of sensory transduction.

(1) T. Tomita, *Quart. Rev. Biophys.*, **3**, 179 (1970).
(2) G. B. Arden, *Prog. Biophys. Mol. Biol.*, **19**, 373 (1969).
(3) W. A. Hagins, *Annu. Rev. Biophys. Bioeng.*, 1, 131 (1972).
(4) D. Bownds, A. Gordon-Walker, A. C. Gaide-Huguenin, and W. Robinson, *J. Gen. Physiol.*, **58**, 225 (1971).
(5) F. J. M. Daemen, W. J. DeGrip, and P. A. A. Jansen, *Biochim. Biophys. Acta*, **271**, 419 (1972).
(6) H. Heitzmann, *Nature (London), New Biol.*, **235**, 114 (1972).
(7) F. J. M. Daemen, *Biochim. Biophys. Acta*, **300**, 255 (1973).
(8) J. I. Korenbrot, D. T. Brown, and R. A. Cone, *J. Cell. Biol.*, **56**, 389 (1973).
(9) W. J. Gras and C. R. Worthington, *Proc. Natl. Acad. Sci. U.S.A.*, **63**, 233 (1969).
(10) A. E. Blaurock and W. H. F. Wilkins, *Nature (London)*, **223**, 906 (1969).
(11) J. Corless, *Nature (London)*, **237**, 229 (1972).
(12) J. K. Blasie and C. R. Worthington, *J. Mol. Biol.*, **39**, 417 (1969).
(13) R. A. Cone, *Nature (London), New Biol.*, **236**, 39 (1972).
(14) P. K. Brown, *Nature (London), New Biol.*, **236**, 35 (1972).
(15) M. Poo and R. A. Cone, *Nature (London)*, **247**, 438 (1973).
(16) T. S. Leeson, *J. Anat.*, **108**, 147 (1971).
(17) A. W. Clark and D. Branton, *Z. Zellforsch.*, **91**, 586 (1968).
(18) L. Jan and J. P. Revel, Abstracts, 13th Annual Meeting of the American Society for Cell Biology, No. 155a (1973).
(19) Y. S. Chen and W. L. Hubbell, *Exp. Eye Res.*, **17**, 517 (1973).
(20) J. Heller and M. A. Lawrence, *Biochemistry*, **9**, 864 (1970).
(21) A. Steinemann and L. Stryer, *Biochemistry*, **12**, 1499 (1973).
(22) R. D. Penn and W. A. Hagins, *Biophys. J.*, **12**, 1073 (1972).

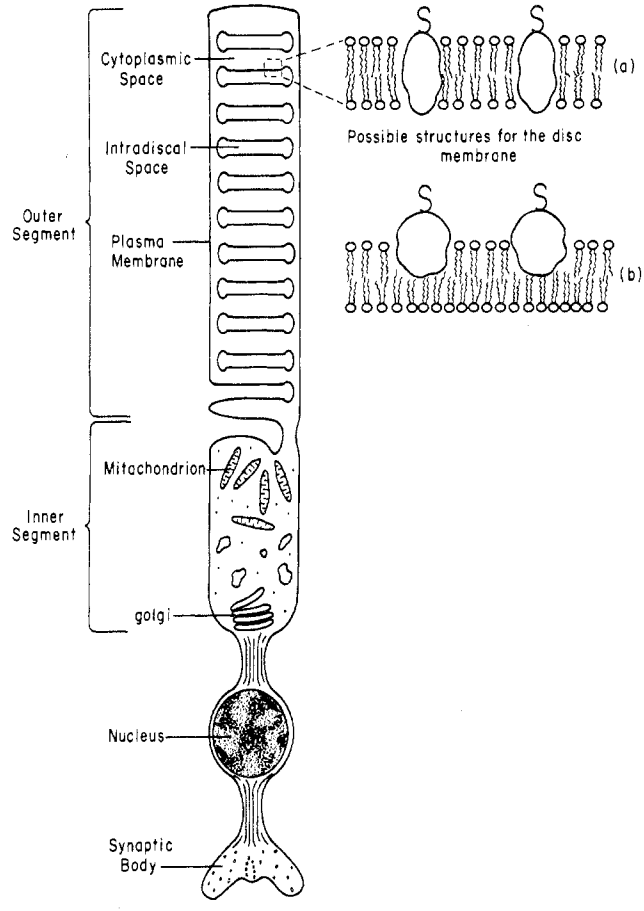


Figure 1. Schematic representation of a vertebrate rod cell. The volume of the intradiscal space is greatly exaggerated, and in the normal bovine outer segment there are approximately 1500 discs within an outer segment 50 μ in length. In the magnified views showing possible structures for the disc membrane, the wiggly lines represent the hydrocarbon chains of the phospholipids, and the open circles the polar head groups. The "S" on the rhodopsin molecule signifies the carbohydrate moiety.

event must be indirectly communicated to the plasma membrane. Fuortes and coworkers[23-25] have suggested that rhodopsin may regulate the level of an internal chemical "transmitter" which in turn regulates the Na^+ conductance of the plasma membrane. Hagins[3] and Cone[26] have recently discussed evidence for this mechanism in vertebrate photoreceptors. Yoshikami and Hagins[27] have suggested that Ca^{2+} may act as a transmitter which is released from the intradiscal space upon photon absorption. In a scheme proposed by Wald,[28] rhodopsin is considered to be a pro-enzyme, activated by light. The active enzyme could turn over a large number of substrate molecules, each one a transmitter, thus accounting for one stage of amplification. A modification of this scheme would involve photolyzed rhodopsin activating an endogenous enzyme which in turn controls the intracellular level of transmitter. Very recently, evidence for a light regulation of the 3′,5′-phosphodiesterase has been described.[29] Possibly, a cyclic nucleotide could act as the transmitter, the nucleotide levels being regulated by the phosphodiesterase whose activity would in turn be mediated through rhodopsin.

Chemical investigation of the molecular events following photon absorption is hampered by the densely packed, two-compartment structure of the native outer segment. Gaining selective access to the compartments and specific components in the intact structure is at best very difficult to achieve. One promising approach to overcome these difficulties involves the disassembly of the photoreceptor structure and characterization of the individual isolated components. This approach is certainly not without precedent in biochemistry. In principle, it is possible to reconstruct the salient features of the transduction process in a synthetic system formed from the isolated components, where the molecular requirements and details of the process could be most easily deciphered.

In any scheme for phototransduction, no matter how complex the temporal sequence of events, the action begins with the rhodopsin molecule in the disc membranes. Rhodopsin has now been isolated and purified in a variety of detergents. However, evidence is accumulating from many sources to suggest that the native conformation and function of membrane associated proteins is strongly dependent on the local environment, and in some cases the presence of a particular species of phospholipid appears to be required for function.[30] Thus, as in the water-soluble proteins, the structure of membrane proteins may be determined by interactions with the "solvent," in this case the phospholipids.

In view of the above comments, it is certainly desirable to investigate the isolated protein in a more "native" environment. The methodology is now available to obtain purified rhodopsin, free from native phospholipid, and to reassemble chemically defined membranes containing the protein and synthetic phospholipids. In such systems, the dark- and light-dependent chemistry of rhodopsin as well as the interactions with other membrane components can be carefully studied. The present Account deals with results from this first step in the reconstruction of visual excitation in synthetic systems.

Properties of Rhodopsin in Detergent Systems

In the binary system phospholipid–water, the lamellar phase is stable for compositions with a water content greater than about 20–30% by weight, and anhydrous phospholipids added to excess water will spontaneously form bilayer structures with long-range order.[31] Addition of certain micelle-forming amphiphiles ("detergents") at well-defined concentrations results in the appearance of a mixed micellar phase[32] that is stable with respect to the bilayer saturated with detergent. Increasing the concentra-

(23) M. G. F. Fuortes and H. F. Hodgkin, *J. Physiol.*, **172**, 239 (1964).
(24) A. Borsellino and M. G. F. Fuortes, *Proc. IEEE*, **56**, 1024 (1968).
(25) D. A. Baylor and M. G. F. Fuortes, *J. Physiol.* **207**, 77 (1970).
(26) R. A. Cone in "Biochemistry and Physiology of Visual Pigments," H. Langer, Ed., Springer-Verlag, New York, N. Y., 1973, pp 275–282.
(27) S. Yoshikami and W. A. Hagins, in ref 26, pp 245–255.
(28) G. Wald in "Enzymes: Units of Biological Structure and Function," T. P. Singer, Ed., Academic Press, New York, N. Y., 1956, pp 355–367.
(29) N. Miki, J. Keirns, F. Marcus, J. Freeman, and M. Bitensky, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3820 (1973).
(30) T. Steck and C. F. Fox in "Membrane Molecular Biology," C. F. Fox and A. D. Keith, Ed., Sinauer Associates, Stamford, Conn., 1972, pp 27–75.
(31) V. Luzzati in "Biological Membranes," D. Chapman, Ed., Academic Press, New York, N. Y., 1968, pp 71–121.
(32) Use of the word "phase" to describe the micelle may meet with some objection, but no conclusions will be drawn here which require a strict thermodynamic definition of the term.

tion of detergent results in complete disappearance of the bilayer phase and dilution of the mixed micelle with respect to phospholipid. The structure of the mixed micelle and the location of the phase boundaries depend upon the phospholipid as well as the amphiphile structure. The only complete phase diagram available for detergent–phospholipid–water is that for cholate–phosphatidylcholine–water.[33]

Phospholipid-containing biological membranes respond in a qualitatively similar fashion upon addition of detergents. Although the details of the process may be more complex in these multicomponent systems, mixed micelles containing both phospholipid and detergent form, and the water-insoluble membrane proteins are incorporated within these structures.

Utilizing adsorption chromatography on hydroxylapatite, rhodopsin has been quantitatively separated from the native phospholipids in detergent-solubilized disc membranes.[34] At the same time, resolution of rhodopsin from other proteins is achieved. One class of amphiphilic compounds used to effect this separation are members of the homologous series of *N*-alkyltrimethylammonium bromides, $(H_3C)_3N^+(CH_2)_nCH_3$ (I), where n = 7, 9, 11, 12, 13, 15. The absorption spectrum of rhodopsin isolated in solutions of these detergents is similar to that in the native membrane, with a broad absorption at 498 nm due to the 11-*cis*-retinal chromophore. The pure protein is thermally labile in solutions of the homologs with n = 7, 9, 11, and the 498-nm absorption rapidly decays at room temperature, although it is quite stable at 4°. The thermal stability is increased with increasing detergent concentrations for any particular homolog and increases with increasing chain length in the homologous series.[34b] These effects may be due to the increasing micelle size with increasing concentration and chain length and, in the latter case, increasing micelle stability. The purified protein is unstable in the absence of detergent and undergoes extensive thermal bleaching, even at 4°.

Irradiation of thermally stable rhodopsin at 498 nm in solutions of any of the detergents results in photochemical isomerization of the 11-*cis*-retinal and a loss of the 498-nm absorption ("bleaching"), as is the case for rhodopsin in the native membranes. Subsequent addition of 11-*cis*-retinal does not lead to regeneration of the 498-nm absorption in solutions of the quaternary ammonium bromides, while similar experiments using the native membranes give high regeneration yields. Regeneration yields of rhodopsin bleached in solutions of most detergents are generally very low, with the notable exception of digitonin, which will be discussed in more detail below.

The rhodopsin molecule has reactive sulfhydryl groups in solutions of the quaternary ammonium bromide detergents, the number and reactivity depending on the chain length of the homolog and the photochemical state of the protein.[35,36] These reactive groups have been utilized as points of covalent attachment for nitroxide free radicals (spin labels). The electron paramagnetic resonance (EPR) spectrum of such labeled protein molecules yields direct information about the motional freedom of the radical, which in turn depends on the conformation of the local environment in the protein.[37,38] EPR spectra of rhodopsin labeled with a nitroxide maleimide[38] have been obtained in various detergents and indicate that the conformation of the protein depends both on the structure of the detergent and on the photochemical state of the protein.[36] For example, in digitonin solution the nitroxide radical on the protein is strongly immobilized, while much greater freedom of motion is observed for the same labeled protein in solutions of the quaternary ammonium bromides. These observations suggest that rhodopsin in the digitonin micelle is a relatively more compact, tightly folded structure in the vicinity of the label. Bleaching of the rhodopsin in digitonin produces no detectable changes in the local protein conformation, while an additional unfolding of the molecule apparently takes place upon bleaching in solutions of the quaternary ammonium bromides. Heller[39] has observed a substantial increase in the hydrodynamic volume of rhodopsin upon bleaching in hexadecyltrimethylammonium bromide solutions, and the EPR spectral changes are consistent with such an unfolding of the polypeptide chain.

It is interesting to note that rhodopsin is regenerable in digitonin solution but not in quaternary ammonium bromide solutions. The conformational changes observed upon bleaching in these detergents thus may represent an irreversible denaturation. In view of the apparent conformational dependence of rhodopsin on environment, it is desirable to obtain the pure protein in a system whose structure resembles as closely as possible that of the native membrane, for although chemical and structural data obtained on rhodopsin in detergent solutions are of interest in their own right, they may be of doubtful relevance to the native conformation.

Properties of Rhodopsin in Synthetic Membranes

Once phospholipid-free rhodopsin has been obtained in detergent solution, mixed phospholipid–detergent micelles may be added in the desired proportions and the original solubilization reversed by slow removal of the detergent.[34a] Under the appropriate conditions, the reassembly of a phospholipid bilayer containing protein takes place with any of a number of pure synthetic and naturally occurring phospholipids.[34b] During the reassembly process, some rhodopsin is inevitably denatured, resulting in loss of the 498-nm absorption, and recovery of rhodopsin is defined as that percent of the original 498-nm absorbance before reassembly that is recovered in the synthetic membranes. The recovery is high (≥75%) for all of the systems discussed here.

The properties of rhodopsin in the synthetic membranes are very different than those in the detergent solution from which they were formed, and more

(33) D. M. Small, M. C. Bourgès, and D. G. Dervichian, *Biochim. Biophys. Acta*, 125, 563 (1966).

(34) (a) K. Hong and W. L. Hubbell, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 2617 (1972); (b) *Biochemistry*, 12, 4517 (1973).

(35) W. J. De Grip, G. L. M. Van De Laar, F. J. M. Daemen, and S. L. Bonting, *Biochim. Biophys. Acta*, 325, 315 (1973).

(36) Y. S. Chen, K. Hong, and W. L. Hubbell, in preparation.

(37) H. M. McConnell, *Annu. Rev. Biochem.*, 40, 227 (1971).

(38) H. M. McConnell and B. G. McFarland, *Quart. Rev. Biophys.*, 3, 91 (1970).

(39) J. Heller, *Biochemistry*, 7, 2914 (1968).

closely resemble the corresponding properties of the protein in the native membrane.[34b] For example, the thermal stability is greatly increased and the 498-nm absorption seems indefinitely stable at room temperature compared to the brief half-life in solutions of the short-chain detergent homologs. Furthermore, the EPR spectrum of nitroxide maleimide-labeled rhodopsin which has been incorporated into the bilayers shows the radical to be strongly immobilized, in contrast to that found for rhodopsin in the detergent solutions before reassembly. The effect is reversible, and resolubilization again gives a spectrum characteristic of increased mobility. It should be noted here that the EPR spectrum of spin-labeled rhodopsin in the synthetic membranes is essentially identical with that obtained by labeling rhodopsin in the native disc membranes. It is possible that the label is located at the surface of the protein and the immobilization of the radical is due simply to close packing of phospholipids. This is considered unlikely, however, since sharp thermal transitions in the lipid of the membrane are not reflected as such in the mobility of the nitroxide.[36] This supports the earlier suggestion that the radical senses true conformational states of the protein, and apparently a reversible unfolding of rhodopsin takes place when membranes are solubilized in solutions of the quaternary ammonium bromides. Spectral changes are seen after bleaching in some of the synthetic membranes and are currently under study. The extent of the conformational changes observed cannot be inferred from these data, and the changes could involve the entire molecule or be highly localized.

In the development of synthetic systems, it is necessary to assess the degree to which the protein has maintained its native integrity during the various manipulations involved in the protein purification and recombination with phosphlipids. Clearly, functional activity is the ultimate criterion. At present the *in vivo* function of rhodopsin is not known, and other criteria must be selected. High regenerability is a characteristic of native rhodopsin, and the regeneration yield forms the basis of one such criterion. Purified rhodopsin has been incorporated into bilayers of a number of naturally occurring lipids including phosphatidylcholine and phosphatidylethanolamine from egg yolks, digalactosyl diglycerides from spinach chloroplasts, and the natural mixture of phospholipids from the native disc membranes.[34b] Successful incorporation has also been achieved in a homologous series of symmetrical, saturated, synthetic phosphatidylcholines, ranging from bis(decanoyl)-phosphatidylcholine to bis(hexadecanoyl)phosphatidylcholine.[19] In membranes formed from each of the above-mentioned natural and synthetic lipids, with the single exception of the bis(decanoyl)phosphatidylcholine, the bleaching of rhodopsin is reversible, *i.e.*, the 498-nm absorption band is recovered to a minimum of *ca.* 70% upon the dark incubation of the bleached membranes with 11-*cis*-retinal. This is in contrast to the lack of regeneration in most detergents and is close to the regeneration yields obtained with the native membranes. Under optimal conditions, regeneration yields are as high as 97% in the synthetic membranes.

Apparently, the collection of forces that stabilize the rhodopsin molecule in the bilayer cannot be dominated by specific interactions with the polar head group of the lipid molecules. This is indicated by the fact that stable, regenerable rhodopsin has been obtained in bilayers with a variety of polar head groups, including the neutral diglactosyl diglyceride. Furthermore, a specific type of hydrocarbon chain is not required for successful structural incorporation or high regenerability. It is interesting that the regenerability of the bleached bis(decanoyl)phosphatidylcholine membrane is low, even though the recovery of rhodopsin is high. If rhodopsin is an amphiphilic molecule occupying a specific domain in the membrane interior, the bilayer thickness would be important in providing the appropriate environmental geometry. Thus, the low regeneration yields in this membrane may be the result of a bilayer too thin to provide that particular geometry.

Experiments with the spin-labeled rhodopsin discussed earlier demonstrate that the conformations of rhodopsin in detergent solutions and phospholipid bilayers are quite different. The conformation stabilized by the lipid bilayer allows a high regeneration yield, a characteristic of the native protein, while the yields in detergent are low. This regenerable conformation is apparently stabilized by general structural features of the bilayer, and not by specific interactions with individual lipid molecules.[34b]

In the above discussion it was assumed that the reassembly procedure yields in all cases the lipid bilayer with incorporated rhodopsin. It is possible that rhodopsin–lipid and rhodopsin–rhodopsin interactions could dictate the formation of structures quite unlike those formed by the pure lipids under the conditions of the reassembly, and the method of freeze-fracture electron microscopy has been used to investigate the structure of the synthetic membranes and to derive information concerning the location of the rhodopsin molecule.[19,34] In this technique, the aqueous sample is rapidly frozen by plunging in Freon cooled by a liquid nitrogen bath. When the frozen sample is fractured, membranes and phospholipid bilayers preferentially fracture along the center of the bilayer rather than along the membrane–ice interface, thus exposing extensive face views of the hydrophobic interior of the membrane.[40] The fracture process is performed under high vacuum, and if ice is allowed to sublime after fracture, an additional face is exposed (etch face) corresponding to the outer, polar surface of the membrane. Following the fracture and optional sublimation step, a replica of the surface is formed by the evaporation of a thin film (*ca.* 20 Å) of platinum and carbon, and this replica is viewed in the electron microscope.

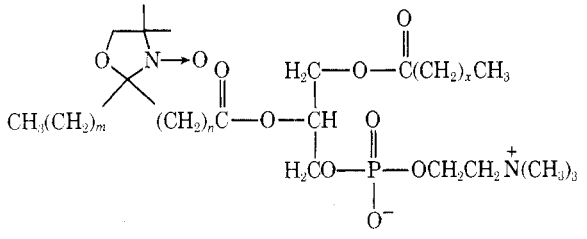
Freeze-fracture images of bilayers formed from naturally occurring mixtures of phospholipids give fracture and etch faces which appear to be smooth, with no discernible structure.[41] This is in contrast to functional biological membranes which have particles of 80–110 Å covering the fracture face, the size and distribution of the particles depending on the particular type of membrane. It has been suggested

(40) D. Branton, *Proc. Natl. Acad. Sci. U.S.A.*, 55, 1048 (1966).
(41) D. W. Deamer, R. Leonard, A. Tardieu, and D. Branton, *Biochim. Biophys. Acta*, 219, 47 (1970).

by Branton that these particles are morphological manifestations of membrane proteins located within the hydrophobic interior of the bilayer.[42] Figure 2 shows representative freeze-fracture electron micrographs of pure lipid bilayers and synthetic membranes prepared with naturally occurring, unsaturated lipids. The fracture face of the synthetic membrane is covered with particles of *ca.* 110-Å diameter, the density of which is proportional to the rhodopsin content. This general appearance is characteristic of all of the synthetic membranes, including those with only one molecular species of phospholipid (to be discussed later).

On the basis of these observations, the possibility that the particles are due to aggregation of specific lipids in a heterogeneous mixture can be excluded, and the particles must be direct manifestations of the incorporated rhodopsin molecules. The molecular weight of rhodopsin is approximately 40,000, and assuming the usual density for protein, the diameter for a spherical rhodopsin molecule would be about 40 Å. The larger size of the freeze-fracture particles is certainly due in part to the thickness of the platinum shadow but could also be due to associated forms of rhodopsin as well as the possibility of associated lipid molecules. In these samples, a second, smooth face is exposed on etching, characteristic of the bilayer fracture mode. The most straightforward interpretation of these results suggests that a substantial fraction of the protein is located *within* the hydrophobic interior of a phospholipid bilayer.

An independent approach to investigating the structure of the synthetic membranes is provided by spin-labeling the lipid components themselves. In some experiments, the phosphatidylcholines used to form the recombined membranes have been doped with small amounts of spin-labeled phosphatidylcholines having the general structure



$I\beta(m,n)$

where the indices m and n define the position of the nitroxide ring on the hydrocarbon chain. The EPR line shape observed for $I\beta(m,n)$ in synthetic membranes and phospholipid bilayers has been interpreted in terms of a rapid, anisotropic diffusion about an effective long axis of the hydrocarbon chain.[34a,43] The resolved spectral splittings are related to the probability of bond rotational isomerizations in the hydrocarbon chains, and thus to local fluidity within the bilayer. An order parameter, S_n, can be derived from the spectral parameters, and is a convenient quantitative measure of the fluidity of the hydrocarbon chain region in phospholipid bilayers and biological membranes.[43,44] The subscript n indicates the

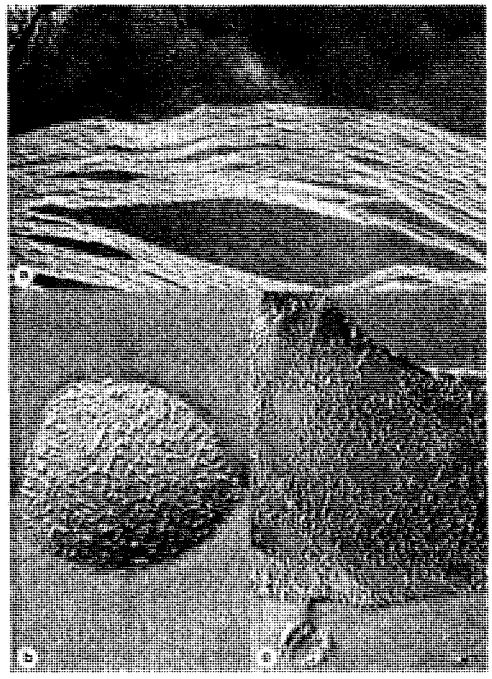


Figure 2. Freeze-fracture electron micrographs of (a) fully hydrated digalactosyl diglycerides, ×52,500; (b) digalactosyl diglyceride–rhodopsin synthetic membrane, mole ratio 100:1 respectively, ×67,500; (c) phosphatidylcholine–phosphatidylethanolamine–rhodopsin synthetic membrane, mol ratio 50:50:1 respectively, ×45,000. The smooth patches devoid of particles may be due to a liquid–liquid phase separation in this three-component membrane system. All samples are frozen from 25°.

dependence of the order parameter on n for the different $I\beta(m,n)$. From the point of view of the $I\beta(m,n)$, the synthetic membranes have many properties in common with a phospholipid bilayer. Most revealing of the similarities is the orientation of the nitroxide group produced by shearing a concentrated sample of the doped membrane between two flat quartz plates. The spectral dependence on the orientation of the film with respect to the magnetic field shows clearly that the magnetic symmetry axis of the nitroxide fatty acid is preferentially oriented perpendicular to the shear plane, the orientation observed for the $I\beta(m,n)$ in shear-oriented phospholipid bilayers.[34a]

Comparison of the resonance spectra of the various $I\beta(m,n)$ in the synthetic membranes reveals an increasing configurational freedom (decreasing order parameter) with increasing n, characteristic of the $I\beta(m,n)$ in phospholipid bilayers.[43] Figure 3 shows the dependence of the order parameters S_4 and S_8 on the measured mole fraction of rhodopsin in the membranes, and indicates an increasing order both near the surface and within the interior of the bilayer with increasing rhodopsin content. This increased ordering of the phospholipid chains resembles the so-called condensing effect of cholesterol[43,45-48] and could be due to a specific interaction of the hydrocarbon chains with the protein surface or a general interference with long-range cooperative flexing modes of the chains by the rigid body of the protein.

(42) D. Branton, *Phil. Trans. Roy. Soc. London, Ser. B*, **261**, 133 (1971).
(43) W. L. Hubbell and H. M. McConnell, *J. Am. Chem. Soc.*, **93**, 314 (1971).
(44) J. Seelig, *J. Am. Chem. Soc.*, **92**, 3881 (1970).
(45) R. A. Demel, W. S. M. Geurts Van Kessel, and L. L. M. Van Deenen, *Biochim. Biophys. Acta*, **266**, 26 (1972).
(46) D. Ghosh and J. Tinoco, *Biochim. Biophys. Acta*, **266**, 41 (1972).
(47) V. Cogan, M. Shinitzky, G. Weber, and T. Hishida, *Biochemistry*, **12**, 521 (1973).
(48) E. Oldfield, D. Chapman, and W. Derbyshire, *FEBS Lett.*, **16**, 102 (1971).

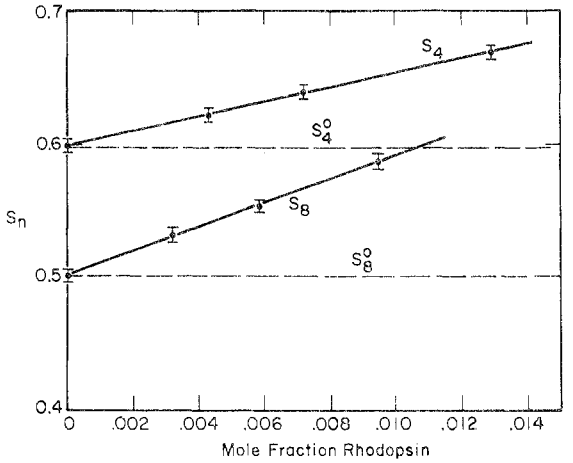


Figure 3. Plot of the order parameters S_4 and S_8 as a function of mole fraction of rhodopsin in egg phosphatidylcholine synthetic membranes. The lines S_4^0 and S_8^0 indicate the value of the order parameters in bilayers of phosphatidylcholine containing no rhodopsin.

These results are in accord with the freeze-fracture data and demonstrate solvation of the protein surface by the hydrocarbon chains of the bilayer. It is worth noting that the native membranes have a much higher order than bilayers formed from the extracted lipids alone, consistent with the results obtained with the synthetic membranes.

Phase Separation in Synthetic Membranes

Pure hydrated phospholipids can undergo a thermal phase transition between a "gel phase," where the hydrocarbon chains are rigid and well ordered, and a smectic liquid crystalline phase where the chains are more fluid.[43,49] In mixed systems containing two phospholipid components, complete solidification of the hydrocarbon chains takes place over a wide temperature range, in which two-dimensional phase separations occur.[50,51] Shimshick and McConnell have determined phase diagrams for several systems with two lipid components and find either complete miscibility in all states or solid-state immiscibility, depending on the structural similarities of the two lipids.[50]

In the preceding section, evidence was presented to indicate that some fraction of the rhodopsin surface is directly solvated by the hydrocarbon chains of the phospholipid bilayers in synthetic membranes. Since rhodopsin is an amphiphilic molecule,[20] it likely occupies a domain in the bilayer structurally similar to the phospholipids, and it is reasonable to expect two-dimensional phase separations to occur similar to those observed by Shimshick and McConnell. Since the incorporated protein is clearly discernible in freeze-fracture micrographs of synthetic membranes, this technique has been chosen to monitor the topological distribution of rhodopsin. From this distribution it is possible, in some cases, to identify the phases present under the desired experimental conditions.[19]

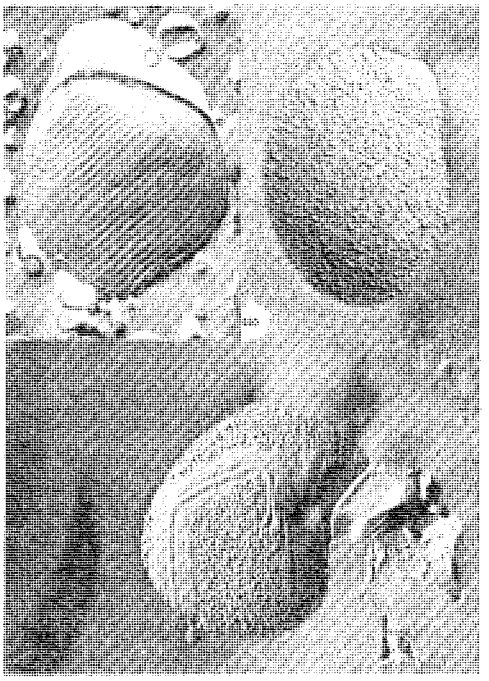


Figure 4. Freeze-fracture electron micrographs of (a) fully hydrated bis(tetradecanoyl)phosphatidylcholine bilayers prepared at 20°, ×35,000; (b) bleached bis(tetradecanoyl)phosphatidylcholine–rhodopsin membranes frozen from 20°, ×45,000; (c) unbleached bis(tetradecanoyl)phosphatidylcholine–rhodopsin membranes prepared at 20°, ×37,500.

Initially, the observation of phase separations is important in establishing the validity of the two-dimensional solution model for the rhodopsin–lipid membranes; ultimately, such data can be used to gain insight into the nature of rhodopsin–lipid interactions and the structure of rhodopsin–lipid membranes. Rhodopsin has been incorporated into bilayers formed from a number of pure, synthetic phosphatidylcholines and the topological distribution of protein studied as a function of temperature and photochemical state at a fixed mole ratio of 100:1 phospholipid–rhodopsin.[19] In these experiments, the sample is brought to equilibrium at the desired temperature, and then rapidly frozen by plunging directly into Freon 22 cooled by a liquid nitrogen bath. An example of the type of behavior observed is shown in Figure 4 for rhodopsin in ditetradecanoylphosphatidylcholine bilayers. Bilayers of the pure phospholipid melt sharply at about 23°,[50] while the bilayers containing rhodopsin melt over a broader range, with the onset of melting being close to 23°. Figure 4a shows the fracture face appearance for bilayers of the pure lipid rapidly frozen from 20°. The pronounced series of parallel ridges are not observed when the sample is prepared above 23°, and are apparently characteristic structures of the gel phase of many pure phospholipids.[51-53] When bilayers containing rhodopsin are frozen from 20° a dramatic separation of phases in the plane of the membrane is observed, as shown in Figure 4c. The identity of the particle-free phase is established as the pure solid lipid by the characteristic ridge pattern, while the other phase is rich in rhodopsin. When the same

(49) D. Chapman, R. M. Williams, and B. D. Ladbrooke, *Chem. Phys. Lipids*, 1, 445 (1967).
(50) E. J. Shimshick and H. M. McConnell, *Biochemistry*, 12, 2351 (1973).
(51) E. J. Shimshick, W. Kleeman, W. L. Hubbell, and H. M. McConnell, *J. Supramol. Struct.*, 1, 285 (1973).
(52) P. Pinto da Silva, *J. Microsc., (Oxford)*, 12, 185 (1971).
(53) A. J. Verkleij, P. H. J. Ververgaert, L. L. M. Van Deenen, and P. F. Elbers, *Biochim. Biophys. Acta*, 288, 326 (1972).

sample is frozen from 30°, the separation of phases is not observed and the particles are uniformly distributed (appearance similar to Figure 4b). This is reversible, and if a sample at 30° is cooled to 20° and frozen, the phase separation is again observed. The phase diagram for this system has not yet been determined, but will involve at least partial immiscibility in the solid state, the rhodopsin being excluded from the closely packed phospholipids in the gel phase.

Bleaching of the rhodopsin at 30° and cooling to 20° before freezing results in the uniform particle distribution shown in Figure 4b, with no evidence of phase separation. This dispersed state is maintained even when the bleached sample is frozen from 5°, where the membrane is surely in the solid state, and the phase behavior now appears to be characterized by complete miscibility in the solid state. Thus the phase behavior is dependent on the photochemical state of the protein. No redistribution of rhodopsin takes place when bleaching is conducted at 5°, and only when the bleached membrane is transiently warmed to temperatures greater than 23° and returned to 5° is a uniform distribution of rhodopsin obtained. This is expected, since the mixing of components requires lateral diffusion, which in turn requires the hydrocarbon chains to be in a fluid state.

Similar behavior to that described above is observed for rhodopsin incorporated in bilayers of bis(hexadecanoyl)phosphatidylcholine.[19]

At present, the molecular changes in rhodopsin leading to the altered phase behavior with bleaching have not been identified. Blasie,[54] on the basis of X-ray diffraction, has suggested that frog rhodopsin sinks deeper with the interior of the membrane following bleaching, and such a change could be the basis of the phenomena described here, although other possibilities are currently being investigated.

The phase separations described above are thermally induced in two-component membrane systems. Similar separations can be observed at a constant temperature by introduction of a third component, cholesterol. Cholesterol is a normal constituent of many membranes, and has been shown to result in decreased fluidity of the hydrocarbon chains in fluid phospholipid bilayers.[43,45-48] Cholesterol may be readily incorporated into 1-stearoyl-2-oleoylphosphatidylcholine bilayers containing rhodopsin (3:1 mole ratio phospholipid–cholesterol), and freeze-fracture analysis reveals the presence of extensive smooth zones devoid of rhodopsin in the fracture plane. In controls without cholesterol, the rhodopsin distribution in the fracture plane is random.[55] Apparently the rhodopsin is excluded from the less fluid, more ordered cholesterol-containing regions, just as it is excluded from the rigid, ordered gel phase in phospholipid bilayers in the solid state.

The observation of phase separations in the chemically defined synthetic membranes establishes the two-dimensional solution nature of the structure and opens the possibility of investigating the nature of rhodopsin–liquid interactions through the determination of phase diagrams in multicomponent membranes.

I am grateful to Dr. Joan Tinoco, Mr. Keelung Hong, and Mr. Yong Chen for allowing me to quote from unpublished results. I also wish to thank Professor Harden McConnell for many valuable discussions.

(54) J. K. Blasie, *Biophys. J.*, 12, 191 (1972).

(55) J. Tinoco, and W. L. Hubbell, unpublished observations.