Bioenergetics (1974), 6, 205-220

Membrane Fusion in the Rod Outer Segment Disk Membrane

Ralph J. Kessler, Takashi Wakabayashi
and David E. Green

Institute for Enzyme Research
University of Wisconsin
Madison, Wisconsin 53706

Received 28 May 1974

Abstract

The rod outer segment disk membrane of bovine retina has been isolated in a predominantly fused state. The physical and chemical properties of the membrane in the fused state are profoundly different from the corresponding properties of the same membrane in the unfused state. Exposure to light induces the transition of the disk membrane from the fused to the unfused state. Evidence is presented which suggests that the fusion-defusion cycle of the disk membrane is a primary event of photoexcitation and nerve stimulation.

Introduction

Rhodopsin, the visual pigment of the retina, has been shown [1, 2] to be located in the disk membranes of the rod outer segment (ROS). The gross morphology of the retinal ROS obtained by multiple methods of isolation has been documented by electron microscopy [3]. The outer segment consists of an external plasma membrane enveloping a stack of membranous disks. The disks appear, depending upon the isolation

procedure, as flattened spheres with the two sides coming into more or less contact along the centre. An open loop is formed around the edge where the two membranes of the flattened disk come together.

We have found that under the conditions of our isolation procedure, the disk membrane is a fusion membrane. This state was obtained by isolating the disks from fresh bovine retinas as rapidly as possible under isotonic conditions. It occurred to us that this fused state of the ROS disk membrane may correspond to the physiological state of the disk membrane. Many of the standard methods used for the purification of the ROS result in the complete, or all but complete, elimination of the fusion state in consequence of the use of frozen retinas and hypotonic solutions. The assumption is commonly made that the expanded interdiscal space seen under these conditions and in such preparations is normal for the ROS disk.

X-ray diffraction studies [4, 5] on the rod outer segment have been interpreted to indicate that the disk membrane occurs as two separate membranes with a definite free space occurring between them. The extent of this space was found to be sensitive to the sucrose concentration of the incubating medium. This could be interpreted to mean that either there is no fusion in the disk membranes of the frog species studied, or that the fusion state has been lost during the isolation and preparation of the sample.

There is now abundant evidence in the literature that membrane fusion is a widespread phenomenon [6, 7, 8, 9] and is a property intrinsic to most, if not all, membranes. It is our contention that in the physiological state of the retina, the membranes on each side of the flattened disk coalesce to a single fusion membrane. The actual molecular mechanism by which membrane fusion takes place will be described elsewhere. In this report, we shall be concerned primarily with the properties of the fused disk membrane which readily rationalize the behaviour of rhodopsin in the disk membrane.

In all of the current models [10, 11, 12, 13, 14] proposed for the ROS disk membrane, the rhodopsin molecules are assumed to float and spin freely in the membrane. The differences between the various models have to do with assumptions as to the shape of the rhodopsin molecule and the degree to which it is submerged within the lipid of the membrane. The model we are proposing is based on the assumption that the rholopsin molecules are not always free to move within the membrane, but are held together by protein-protein interactions as long as the membrane is in the fused state. However, upon defusion, which can be produced by the absorption of light by rhodopsin, osmotic swelling, freezing or the effect of certain cations, protein-protein interactions are attenuated and the rhodopsin molecules are free to rotate within the membrane.

Methods and Materials

Purification of Fused ROS Disk Membranes

The ROS disk membranes were prepared by dissecting the retinas from bovine eyes obtained from freshly slaughtered cattle. All work was done in the dark or under dim red light at temperatures from 0° to 4° C. The retinas were homogenized in 0.15 M NaCl buffered with 0.01 M Tris-Cl (pH 7.4) by one pass with a loose-fitting Teflon pestle. The homogenate was sedimented at 35,000 × g for 20 min. The pellet was resuspended in 36% w/w sucrose in 0.15 M NaCl at pH 7.4 and spun at 78,000 × g for 45 min. The float was removed, diluted 3 to 1 with the cold saline solution and sedimented at 78,000 × g for 45 min. The pellet was suspended in a quantity of saline solution and layered on to a linear gradient (27% w/w to 36% sucrose in 0.15 M NaCl) and spun at 63,000 × g for 90 min. The upper layer containing the rod segments was removed and diluted 3 to 1 with cold saline solution. The purified ROS was collected by centrifugation at 78,000 × g. The pellet was washed two times in cold saline to remove excess sucrose. The unfused membranes were prepared by washing the fused membranes in 0.15 M NaCl containing 0.01 M $CaCl_2$. Bleaching of the rod outer segments was accomplished by exposure to a 120 watt flood light for 2 min. The solution was kept near 0° C during bleaching by rapid stirring in an ice water bath. In some instances, bleaching was accomplished by exposure to fluorescent light at 3° C.

Regeneration of Bleached Rod Outer Segments

Bleached ROS were incubated for 2 h at 0° C with an excess of 11-cis retinal. The absorption spectra of isolated rhodopsin in Emulphogene BC720 indicated that bleached rhodopsin had been regenerated to the dark state.

Electron Microscopy

Preparation of the sample for electron microscopy followed the method of Wakabayashi *et al.* [32] except that fixation of the sample was carried out in the dark.

Assay of Cross-linked Protein

The membranes were cross-linked with 0.5% glutaraldehyde in 0.25 M sucrose buffered with Tris-Cl, 0.01 mM (pH 7.4) for 30 min at 0° C. The pellet was spun down and washed twice with water. Protein was extracted by stirring the pellet in 2% Emulphogene BC720 for 2 h at room temperature. The insoluble material was precipitated by centrifugation at 150,000 x g for 45 min. The protein content of the supernatant was determined by the biuret procedure described by Gornall *et al.* [29].

Incorporation of Phospholipid into ROS Membranes

Samples of fused or defused ROS disk membrane containing 10 mg of rod outer sigment protein in isotonic saline buffered with Tris-Cl, 0.01 M (pH 7.4) were incubated with and without phospholipid. The fused ROS disk membranes were prepared as described. Defusion by Ca^{++} was accomplished by washing the ROS membranes in saline containing 0.01 M $CaCl_2$. Bleaching was accomplished by constant exposure to fluorescent light at 3° C. Purified phospholipid prepared from rod outer segments was added in the amount of 21 mg per sample as an aqueous suspension. The samples were stirred for 72 h at approximately 3°C. The samples were then layered on the top of a linear sucrose gradient (10% to 36%) and spun at 63,000 x g for 12 h. The ROS layer was collected by dripping and washed twice with isotonic saline. The protein concentration was assayed by the method of Lowry *et al.* [30] and the phospholipid was determined by the method of Chen *et al.* [31].

Gel Electrophoresis

Approximately 50 μg of material was solubilized in 3% sodium dodecyl sulphate with 5 mM β-mercaptoethanol. Gel electrophoresis was performed and gels were fixed and stained as described by Shapiro *et al.* [28].

Materials

Cattle eyes were obtained from the local abattoir. The eyes were enucleated immediately after slaughtering and placed on ice in a dark container. Emulphogene BC720 was a generous gift from GAF Corporation, New York, New York. All-trans retinal was purchased from

Eastman (Kodak). The 11-cis retinal was prepared according to the method of Brown and Wald [34]. All other materials were reagent grade.

Results

The fused ROS disk membrane is in a different physical and chemical state than the unfused ROS disk membrane as conventionally prepared. A high resolution electron micrograph of the ROS disk membrane prepared in isotonic saline (see Fig. 1) shows the individual disk to be tightly closed and fused along its entire length except for the small loop at each end. This fusion involves more than merely the close association of two individual membranes. The double-tiered structure of the paired single membranes merges into a single triple-tiered membrane. This would involve the rearrangement of the phospholipid and proteins within the newly formed membrane. In the single unfused disk membrane, rhodopsin molecules would be freely floating and spinning along the surface of the lipid bilayer as in the models of Vanderkooi and Sundaralingam *et al.* [10] or Dratz *et al.* [14]. In the fused membrane, we are proposing that the oriented rhodopsin molecules are held rigidly by metal chelate interactions and/or hydrogen bonding. The divalent ion, Mg^{++}, was found to be necessary for the fused state. Presumably, Mg^{++} interacts with charged sites not only on rhodopsin, but also on the polar phospholipid head groups and thus, these head groups can now coalesce around Mg^{++} ions in the centre of the triple-tiered membrane. This would result in a change from a state in which the rhodopsin molecules repulse one another (the state of the unfused membrane) into a state in which strong attractive forces between the molecules of rhodopsin would be operative (the state of the fusion membrane).

The effect of Mg^{++} in stabilizing the fusion membrane can be overcome by addition of Ca^{++} which apparently competes with the naturally occurring Mg^{++} for binding sites and ultimately leads to defusion of the membrane. It is not excluded that in the disks of the ROS, as in myelin, there is a basic protein which plays a key role in the enhancement of the protein-protein interactions that underlie membrane fusion.

Refusion of the unbleached ROS disk membrane can be accomplished by the replacement of the Ca^{++} ions by Mg^{++} ions (Fig. 2). Murakami and Packer [15] have found analogous results in their studies of the fused membranes of spinach chloroplasts. Electron micrographs showed that Mg^{++} or similar divalent ions caused close packing, stacking and

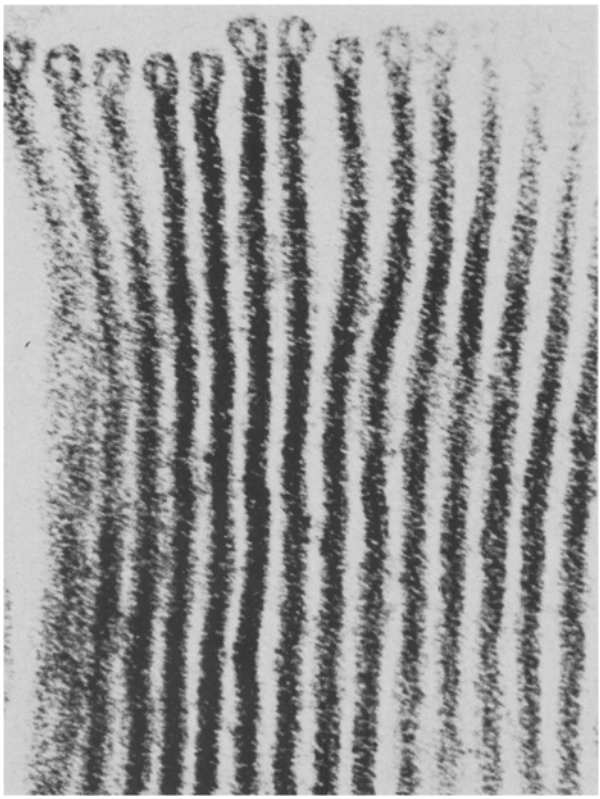
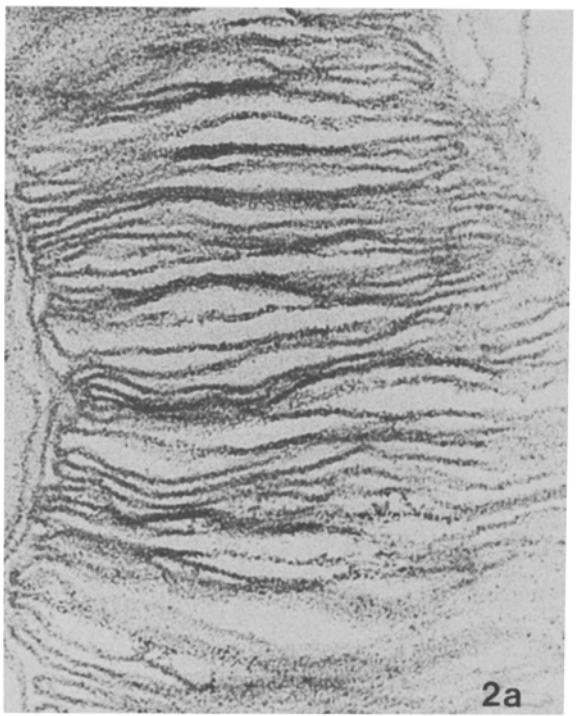


Figure 1. Fused ROS disk membranes isolated in isotonic saline. × 200,000.

reordering of the fused membranes of the grana after these had been defused by exposure to distilled water. Complete fusion has also been achieved in Ca^{++}-treated ROS disk membranes by the introduction of the basic protein, protamine, which has been shown to be a powerful inducer of fusion [16].

The effect of light on the unbleached, fused ROS membrane is most dramatic. Bleaching the membrane at 0° C followed by fixation with glutaraldehyde makes it possible to visualize the effects of the generation of charged intermediates in the fusion membrane. As can be seen in Fig. 3, bleaching produces massive defusion of the membrane. This effect of illumination is not unlike that reported by Deamer *et al.* [17] for chloroplasts and is consistent with the light-induced changes in the retinal rod reported by Chabre and Cavaggioni [18].



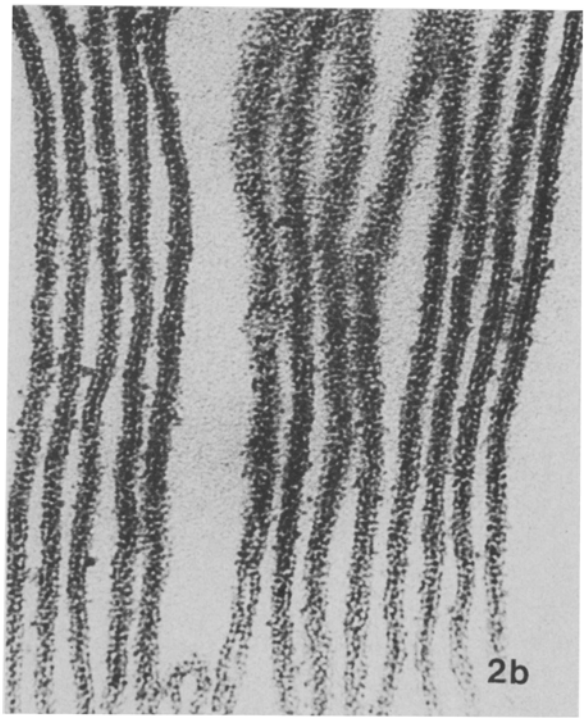


Figure 2. Effect of divalent ions on fusion in the ROS membrane.

a. Rod outer segment membranes prepared by the method given in the legend to Figure 1, followed by washing in 0.15 M NaCl containing 0.01 M $CaCl_2$.

b. Aliquot of the unfused membrane shown in "A" after washing two times in 0.15 M NaCl containing 0.01 M $MgCl_2$.

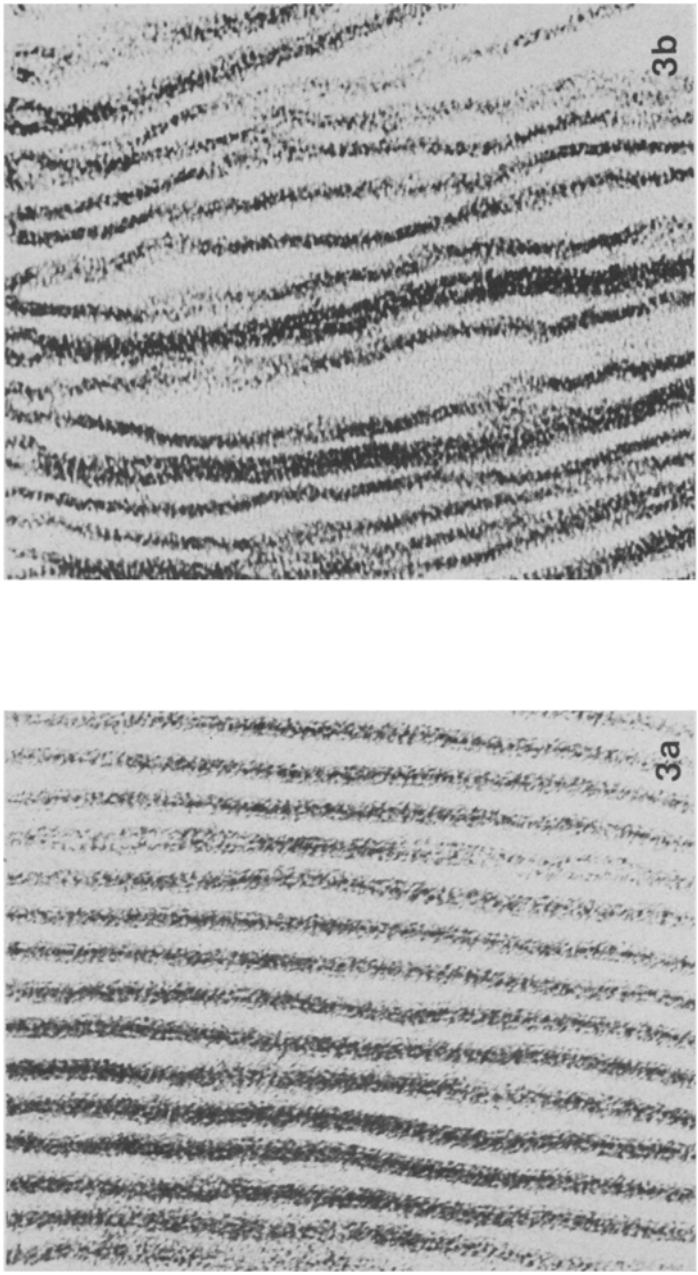
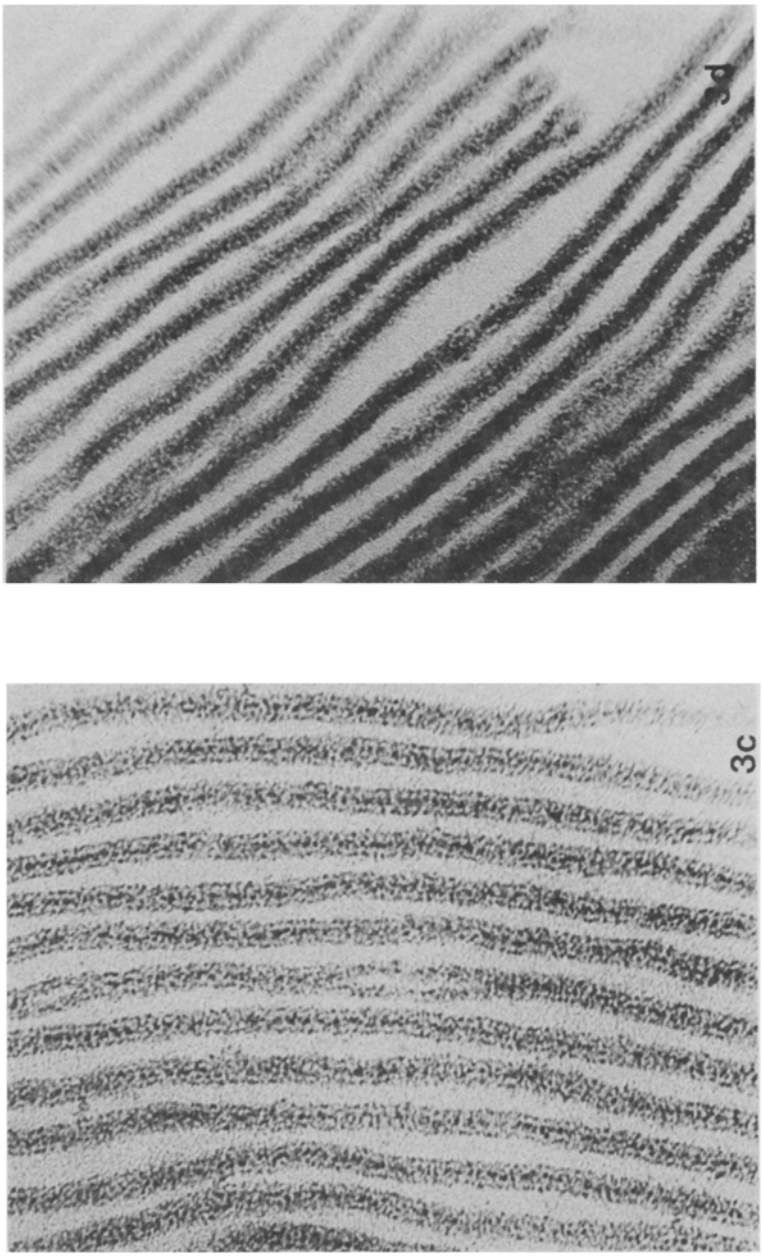


Figure 3. Effect of light on fused ROS membranes in the dark state.
a. Electron micrograph of fused rod outer segments prepared as described in Methods.
b. Similar rod outer segments after bleaching for two minutes under a 120 watt flood light. The solution was kept near 0° C during the bleaching by rapid stirring in an ice water bath.



c. Bleached ROS (as shown in "B") after incubation for 2 h at 0° C with excess 11-cis retinal.
d. Bleached ROS after incubating at 37° C in the dark for 2 h.

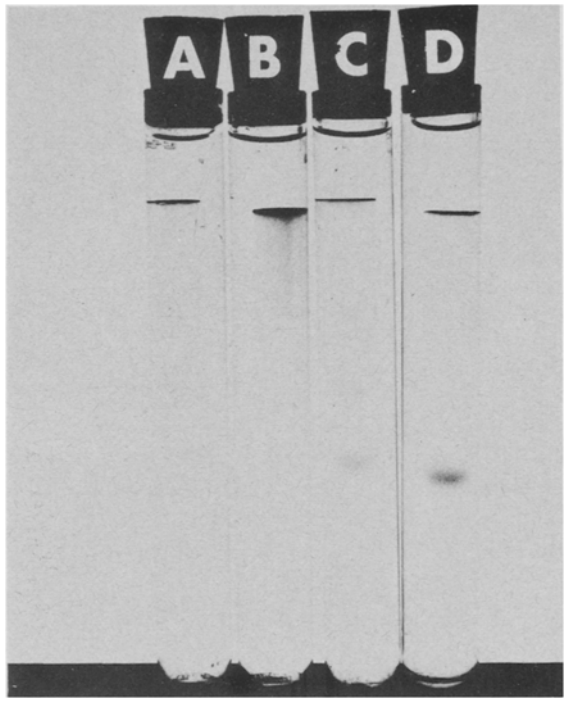


Figure 4. The cross-linking of rhodopsin induced by glutaraldehyde as visualized in 5% acrylamide gels.

A. Fused disk membranes without glutaraldehyde.
B. Fused disk membranes reacted with glutaraldehyde.
C. Unfused disk membranes without glutaraldehyde.
D. Unfused disk membranes reacted with glutaraldehyde.

The magnitude of cross-linking in the fused disk membrane (gel B) is shown by the dark staining at the top of the gel and also by the absence of the rhodopsin band seen in the other gels. Samples B and D were reacted with 0.5% glutaraldehyde for 30 min at 0° C (as described in Methods).

Incubation of the bleached ROS membrane in the dark at 0° C in the presence of Mg^{++} will not produce complete refusion, presumably due to the fact that the bleached rhodopsin is trapped at the metarhodopsin II stage of the photochemical process. Complete refusion of the bleached membrane can be accomplished in two ways. First, interaction of the bleached membrane with 11-cis retinal at 0° C, which reconstitutes rhodopsin from the apoprotein, can induce total fusion of the disk membrane (Fig. 3). Second, the membrane can be refused by incubation at 37° C which allows the metarhodopsin II to continue the photochemically induced reaction to the stage of all-trans retinal with concomitant release of the chromaphore from the protein and discharge of the photoactivated state.

The fused ROS disk membranes have been shown to be distinguishable from the unfused membranes when subjected to sucrose gradient centrifugation. The density of the fused disks is higher than that of the unfused disk, presumably due to the filling of the intradiscal space with the lower density solution upon defusion.

It has been reported by Brown [19] that glutaraldehyde reacts very slowly with the rhodopsin molecules in the frog ROS membrane. We have found that the rate of the reaction of glutaraldehyde with unfused bovine ROS membranes can be assayed either by examination of the mixture by electrophoresis on 5% acrylamide gels or by quantitating the fraction of protein extractable by a nonionic detergent. The results were quite similar by either method and in agreement with the conclusion of Brown that there is minimal cross-linking of protein in the unfused disk membranes. However, in experiments with fused ROS disk membrane, we observed cross-linking of the majority of the protein as determined by gel electrophoresis (see Fig. 4) or by determination of extractable protein (Table I).

TABLE I. Extractable Protein after Cross-Linking

	Percent Protein Extracted
Fused ROS Disk Membranes	28
Unfused ROS Disk Membranes	63

The fused ROS disk membranes were prepared as described in the legend of Fig. 1. The unfused membranes were prepared by washing the fused membranes in 0.15 M NaCl containing 0.01 M $CaCl_2$.

Hong and Hubbell [20] previously have shown that purified rhodopsin can be incorporated into phosphatidylcholine bilayers in varying amounts and this would indicate that protein-protein interactions are absent in such membranes. However, the state of the purified rhodopsin could be quite different from that of rhodopsin in the native membrane. In order to investigate this possibility, the capability of the ROS disk membrane to incorporate additional native phospholipid was determined. Fused disk membranes were mixed with an aqueous suspension of native phospholipid which had previously been extracted and purified from ROS membranes. Similar experiments were carried out on disk membranes which had been defused by either bleaching or by treatment with Ca^{++} ions. After 72 h, the mixtures were separated on sucrose gradients to remove unbound lipid, and the phospholipid-to-protein ratio of the membrane was measured. As shown in Table II, the fused membrane failed to incorporate additional lipid to a significant degree. The disk membranes as initially prepared were found to have a phospholipid-to-protein ratio of 0.78. The variation from this value after incubation of the fused membranes with and without lipid can be

TABLE II. Incorporation of Phospholipid into ROS Membranes

Treatment of Membrane	State of Membrane		Exposure to Phospholipid	Phospholipid Protein mg/mg
	Initial	Final		
No Treatment	Fused	Fused	0	0.78
Exposed to light 72 hours at 3° C	Fused	Defused	0	0.53
Ca^{++} washed, exposed to light 72 hours at 3° C	Fused	Defused	0	0.42
72 hours at 3° C	Fused	Fused	0	0.50
Exposed to light 72 hours at 3° C	Fused	Defused	+	2.76
Ca^{++} washed, exposed to light 72 hours at 3° C	Fused	Defused	+	2.50
72 hours at 3° C	Fused	Fused	+	0.96

explained by the small fraction of unfused membrane that appears in the initial preparation. This unfused material, which accounts for approximately 10% of the membrane, presumably is generated by bleaching which occurs during the time-span between slaughtering of the animal and enucleation of the eyes. The small loss or incorporation of lipid seen with the fused membrane can be attributed to the portion of unfused material present in the preparation. However, in the unfused state, the phospholipid-to-protein ratio increases dramatically.

Discussion

When the rod outer segment disk membranes are isolated from bovine retina under conditions which prevent swelling and loss of Mg^{++}, the membranes are found to be in the fused state and electronmicroscopically indistinguishable from the same observed *in vivo*. In the fused state, the disk membrane shows a triple-tiered structure in high resolution micrographs of positively stained thin sections in contrast to the double-tiered structure of the unfused membrane. That the fusion-defusion transition entails a major reorganization of both the protein and lipid moieties of the disk membrane is borne out by the changes in physical and chemical properties which accompany this transition. In the fused state of the disk membranes, rhodopsin is rapidly and almost completely cross-linked at low concentrations of glutaraldehyde; in the unfused state the degree of protein cross-linking under the same experimental conditions is greatly reduced. Extra phospholipid can be incorporated into the unfused membrane to an unlimited extent but no such incorporation can be observed with the fused membrane. These observations suggest that rhodopsin exists in structured networks within the disk membrane in the fused state.

The published values for the percentage of phospholipid in bovine ROS membrane vary from 30% [21] to 59.8% [22]. We have found that our highest percentage of phospholipid occurred in the fused ROS membrane and that on defusion, the membrane was susceptible to loss of lipid. This observation, taken in conjunction with the data presented in Table II, shows that when in the fused state, the ROS membrane neither loses nor takes up lipid in contrast to the ready incorporation of lipid into unfused membrane. This high stability of the fused membrane can be accounted for by our model in which the attractive forces between the protein molecules stabilize and increase the rigidity of the membrane.

The ability of the fused ROS disk membrane to react quickly with the protein cross-linking reagent, glutaraldehyde, as compared to the unfused membrane shows that the rhodopsin molecules are sufficiently close to undergo cross-linking readily. It has been shown by Dratz and others [14, 23, 24] that rhodopsin is accessible at the surface of the ROS membrane to labelling reagents. Hence, the slowness of cross-linking in the unfused membrane cannot be due to the inaccessability of rhodopsin to the glutaraldehyde. The speed of cross-linking in the fused membrane therefore must be due to the clustering of the rhodopsin achieved by protein-protein interactions so that they can be more easily cross-linked.

Cone and others [25, 19] have shown a transient photodichroism in the rod outer segment indicating that rhodopsin in the dark phase is free to rotate. This experimental evidence is not incompatible with our model. The rhodopsin molecules contained in any part of the disk which is in the fused state would be incapable of rotation. However, upon defusion of that area by means of the photoexcitation of one or more rhodopsin molecules, the interaction between the rhodopsins would be lost and all the rhodopsin molecules within the defused area would be able to rotate. This rotation would include both the bleached rhodopsin and the rhodopsin which was still in the dark phase. The extent of the area defused by the bleaching of a single rhodopsin molecule is not known; however, it would presumably involve a large cluster of rhodopsin molecules.

Cone has measured the transient photodichroism in the frog retina and estimated that rhodopsin has a relaxation time of 20 μsec. These results were obtained by partially bleaching the retina with polarized light. This partial bleaching and concomitant defusion of the membrane would set the stage for the unbleached rhodopsin molecules within the immediate area of defusion to rotate. Hence, photoexcitation, defusion and rotation of the molecules are all intimately related. This process would by necessity be quite rapid. The gross defusion shown in the electron micrographs may not occur under physiological conditions, since refusion could take place once the rhodopsin has passed through the photoexcited state.

In the presence of calcium ions in the dark, the flattened disk membrane will defuse and open up; this process is reversed by magnesium ions and by basic proteins such as protamine. It has been shown electron microscopically that calcium ions produce defusion of the disk membrane, whereas magnesium ions induce the refusion of unfused membranes. The effect of light is precisely the same as that of calcium in respect to the induction of an unfused membrane.

In mitochondria, energization of the inner membrane compels the defusion of fused inner membranes, from which it has been deduced that the generation of charged species in the membrane is incompatible with the fused state of the membrane. Since light exerts the same effect in the disk membrane as does energization in the inner mitochondrial membrane, we are postulating that light generates a charge-separated state of rhodopsin and this state is incompatible with the stability of the fusion state, the requirement for which is apparently a low density of charge.

At the end of the fusion-defusion transition induced by light, the prosthetic group of rhodopsin is apparently released into the medium, since incubation in the dark of bleached disk membranes at 4° C with 11-cis retinal leads to the regeneration of the fusion state obtained prior to bleaching. Without 11-cis retinal, the membrane in the dark and cold will remain indefinitely in the unfused state.

The fused state can exist only so long as the electrostatic charges on the protein are at a minimum. It has been shown with beef heart mitochondria [16] that concomitant with the transition from the non-energized to an energized state, the fused inner membrane becomes defused. We are suggesting that absorption of light by rhodopsin would generate charged species and that the introduction of charge would lead to localized defusion of the membrane and thus, loss of attractive forces between rhodopsin molecules. This would allow free rotation of the rhodopsin molecules in this area of defusion.

The effects of a fused membrane would be twofold. First, it would act to stabilize the highly fluid disk membrane through protein interactions, since only a fraction of total membrane would be defused at one time. Second, the release of ions in the defused area could act as a transmission device. In this way, the absorption of a single photon of light by a rhodopsin molecule can trigger the release of a messenger ion.

Recently it has been demonstrated by Mason *et al.* [33] that the retinal rod membranes from both frog and bovine will release Ca^{+2} as a consequence of visual pigment bleaching in the ratio of one mole of Ca^{+2} released per mole of rhodopsin bleached. It was further shown that the disk membranes can actively accumulate Ca^{+2} ions. The fact that bleaching of rhodopsin causes both defusion of the membrane and release of Ca^{+2} ions indicates that these two processes are inter-related.

It has recently been reported by Oesterhelt and Stoeckenius [26] that the purple membrane of Halobacterium halobrium which contains the single protein, bacteriorhodopsin, generated a proton gradient when illuminated. Racker [27] has reconstituted this bacteriorhodopsin in phospholipid vesicles and demonstrated a light-driven uptake of protons. We investigated the effect of light on proton translocation in fused bovine ROS disk membranes in order to determine whether any large-scale proton movements could be detected upon bleaching rhodopsin in the rod outer segment membrane. Initial studies indicate that only very small proton movements accompany photolysis of the rhodopsin in the fused ROS disk membrane. Under conditions which led to complete bleaching of rhodopsin, the total uptake of protons was of the magnitude of 6 ng atoms H^+ per mg of protein, and this uptake was not reversed in the dark phase.

Acknowledgements

We wish to acknowledge the expert technical assistance of Ms. Nina Mattarella.

Bovine eyes were generously supplied by Oscar Mayer and Company.

This investigation was supported by Program Project Grant 12847 from the National Institute of General Medical Science (U.S.P.H.S.).

References

1. M. M. Dewey, P. K. Davis, J. K. Blasie and L. Barr, *J. Mol. Biol.*, 39 (1969) 395.
2. J. K. Blasie, C. R. Worthington and M. M. Dewey, *J. Mol. Biol.*, 39 (1969) 407.
3. H. Fernandez-Moran in: *The Structure of the Eye* (G. K. Smelsen, ed.), Academic Press (1961) 521.
4. A. E. Blaurock and M. H. F. Wilkins, *Nature*, 237 (1972) 313.
5. J. M. Corless, *Nature*, 237 (1972) 229.
6. C. C. Ramsen, F. W. Valois and S. W. Watson, *J. Bacteriol.* 94 (1967) 422.
7. R. A. Dilley, D. W. Krogman, S. Berg and S. Dodge, *Plant Physiology, Suppl. 4a*, (1972) 27.
8. U. W. Goodenough, and L. A. Staehelin, *J. Cell Biol.* 48 (1971) 594.
9. J. D. Robertson, in: *Handbook of Molecular Cytology* (A. Lima-de-Faria, ed.), North-Holland, Amsterdam, (1969) Chapter 52, 1404.
10. G. Vanderkooi and M. Sundaralingam, *Proc, Nat. Acad. Sci U.S.A.*, 67 (1970) 233.
11. C. R. Worthington, *Fed. Proc.*, 30 (1971) 57.
12. A. E. Blaurock and M. H. F. Wilkins, *Nature*, 223 (1969) 906.
13. J. K. Blasie, *Biophysical J.* 12 (1972) 191.
14. E. A. Dratz, J. E. Gaw, S. Schwartz and W. Ching, *Nature New Biology*, 237 (1972) 99.
15. S. Murakami and L. Packer, *Arch. Biochem. Biophys.*, 146 (1971) 337.
16. D. E. Green, *Ann. N.Y. Acad. Sci.*, 195 (1972) 150.
17. D. W. Deamer, A. R. Crofts and L. Packer, *Biochim. Biophys. Acta*, 131 (1967) 81.

18. M. Chabre and A. Cavaggioni, *Nature New Biology*, **244** (1973) 118.
19. P. K. Brown, *Nature New Biology*, **236** (1972) 35.
20. K. Hong and W. L. Hubbell, *Proc. Nat. Acad. Sci. U.S.A.*, **69** (1972) 2617.
21. F. D. Collins, R. M. Love and R. A. Morton, *Biochem. J.*, **51** (1952) 669.
22. S. Fleischer and D. G. McConnell, *Nature*, **212** (1966) 1366.
23. C. W. Wu and L. Stryer, *Proc. Nat. Acad. Sci. U.S.A.*, **69** (1972) 1104.
24. H. Heitzman and F. M. Richards, *Biophys. Soc. Abst.* (1973).
25. R. A. Cone, *Nature New Biology*, **236** (1972) 39.
26. D. Oesterhelt and W. Stoeckenius, *Proc. Nat. Acad. Sci. U.S.A.*, **70** (1973) 2853.
27. E. Racker, *Biochem. Biophys. Res Commun.*, **55** (1973) 224.
28. A. L. Shapiro, E. Vinuela and J. V. Maizel, *Biochem. Biophys. Res. Commun.*, **28** (1967) 815.
29. A. G. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.*, **177** (1949) 751.
30. O. H. Lowry, N. J. Rosenbough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193** (1951) 265.
31. P. S. Chan, T. Y. Troibara and H. Warner, *Anal. Chem.*, **28** (1956) 1756.
32. T. Wakabayashi, E. F. Korman and D. E. Green, *Bioenergetics*, **2** (1971) 233.
33. W. T. Mason, R. S. Fager and E. W. Abrahamson, *Nature*, **247** (1974) 562.
34. P. K. Brown and G. Wald, *J. Biol. Chem.*, **222** (1956) 865.