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Phase Transitions in Vertebrate and Invertebrate Photoreceptor Membranes

W. T. Mason* and E. W. Abrahamson

Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

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Summary. Phase transitions in the photoreceptor membranes of the vertebrate frog (*Rana catesbiana*) and the invertebrate squid (*Loligo pealii*) have been detected by differential scanning calorimetry in ethylene glycol. Both the intact membranes and the extracted lipids exhibit relatively sharp endothermic transitions, the frog centering at −28 °C and the squid centering at −31 °C. The transition for the lipids is observed to be slightly larger in absolute heat content and shifted somewhat downward in temperatures for the two membranes differ also, the frog membranes thermally denaturing at 58 °C and the squid membranes denaturing at 74 °C. Both denaturations occur identically in buffer, glycol or glycerol.

The significance of thermal phase transitions in biological membranes has become a topic of increasing interest in recent years. Steim has reported that a number of mitochondrial, microsomal and bacterial membranes undergo broad endothermic phase changes with increasing temperature similar to those observed for the extracted lipids of these membranes (Steim, Reinert, Tourtelotte, McElhaney & Rader, 1969; Reinert & Steim, 1970; Ashe & Steim, 1971; Blazyk & Steim, 1972). These phase changes were interpreted as transitions from a gel to a liquid phase. Furthermore, studies by both X-ray diffraction (Gulik-Krzywicki, Rivas & Luzzati, 1967) on thermal broadening of diffraction maxima and electron spin resonance (Hsia, Schneider & Smith, 1970) as a function of temperature support the notion that natural membranes undergo a phase change analogous to a gel-liquid crystal transition. This latter (Ladbrooke, Williams & Chapman, 1968) transition is observed most commonly in synthetic phospholipid membranes.

Recent studies by Papahadjopoulos, Nir and Ohki (1971) have indicated that temperature as well as cholesterol content radically affects the perme-

* *Present address* (and to whom requests for reprints should be mailed): The Physiological Laboratory, Downing Street, University of Cambridge, Cambridge, England.

ability properties of phospholipid membranes. Accordingly, we felt that it would be of considerable interest to study the natural pigment membranes of photoreceptor outer segments for the presence of thermotropic phase transitions. The differences in phospholipid and cholesterol contents of vertebrate and invertebrate photoreceptor membranes reported by Mason, Fager and Abrahamson (1973*a*, *b*) provided a good rationale for this study. Furthermore, known differences in the permeability properties of the two membranes are of significance in the generation of photoreceptor membrane potentials in the visual process (Hagins, 1972). We felt studies of this type might therefore provide some explanation for the differences in the magnitude of the electrical attraction and membrane polarization in vertebrate and invertebrate photoreceptor membranes. Accordingly, we report here the results of studies by differential scanning calorimetry on the vertebrate frog and invertebrate squid photoreceptor membranes.

Materials and Methods

Frog photoreceptor outer segments were prepared and purified by the continuous density sucrose gradient technique of Mason *et al.* (1973*a*). Squid photoreceptor rhabdomeric membranes were prepared by a similar gradient technique described elsewhere (Mason *et al.*, 1973*b*). Both preparations were washed three times with 0.1 M Tris buffer, pH 7.0 and centrifuged for 30 min at 32,000 × *g* in a Beckman Spinco refrigerated ultracentrifuge. Membranes were alternatively resuspended twice in 10% glycerol (v/v) or 50% ethylene glycol (v/v) and recentrifuged as before.

Membrane samples of approximately 125 mg wet weight were sealed in stainless steel calorimetry pans. A Perkin Elmer DSC-1B differential scanning calorimeter was used to study phase transitions in these respective membranes. A sealed sample pan containing the respective suspending media and a small amount of Sephadex G-75, to prevent convention (Blazyk & Steim, 1972) was placed in the reference pot of the instrument. Membrane samples were scanned in the direction of increasing temperature at the rate of 5 deg/min. Linoleic acid, hexane, carbon tetrachloride and water were used as calibration samples.

Membrane lipids were extracted in the presence of N_2 with chloroform/methanol (2:1). After extraction in the cold overnight, the sample was filtered and the solvent evaporated. The lipid residue was resuspended in 50% ethylene glycol and sealed in sample pans as before.

Results and Discussion

A low temperature phase transition was detected for both invertebrate and vertebrate photoreceptor membranes. This transition is entirely endothermic in nature and was detected exclusively in 50% ethylene glycol. The transition for frog photoreceptor membranes centers at −28 °C and extends over a narrow temperature range of some 6 to 8 °C (Fig. 1). The membrane phase transition for squid rhabdomes was detected at −32 °C

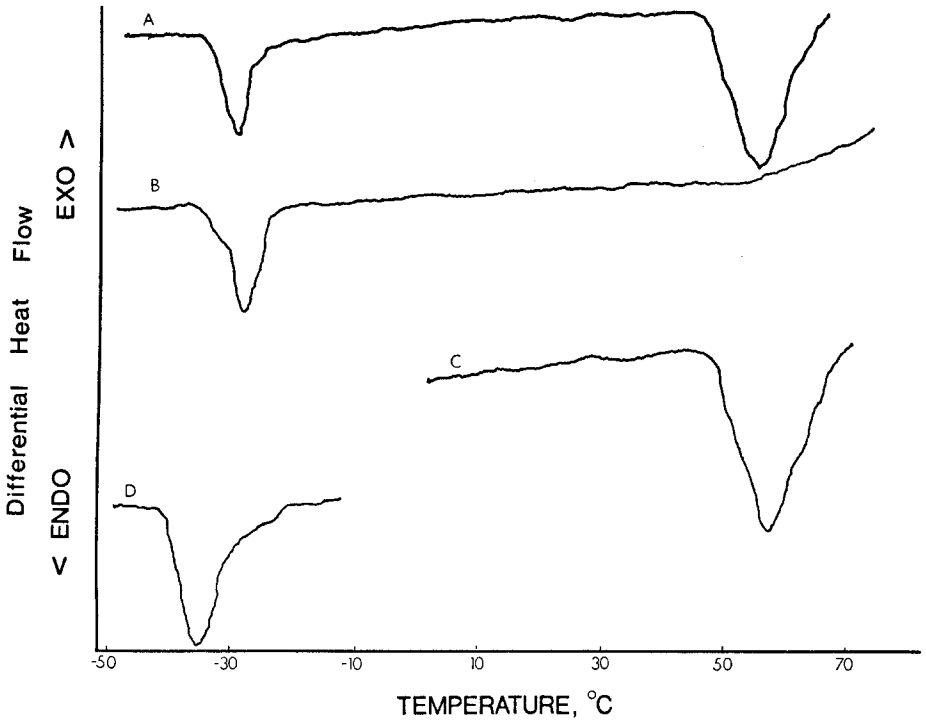

Fig. 1. Thermogram tracings of differential scanning calorimetry of frog photoreceptor membranes. (*A*) Membranes scanned upward in 50% ethylene glycol; (*B*) membranes scanned upward in 50% glycol following irreversible denaturation; (*C*) membranes scanned upward in 100 mM Tris buffer, pH 7.2; (*D*) extracted lipids dispersed in 50% ethylene glycol and scanned upward

and appeared to be only slightly less narrow in temperature range, extending over a 9 to 10 °C temperature range (Fig. 2). The relative breadth of the transitions may be interpreted as an indication of the homogeneity of the lipid milieu.

Photoreceptor membranes scanned upward also undergo irreversible thermal denaturation, the frog membranes at 58 °C and squid membranes at 74 °C. This transition is highly endothermic and some three times larger in absolute heat content than the low temperature membrane transition reported on above. The high temperature transition can most likely be ascribed to denaturation of membrane protein. The large difference in denaturation temperatures of the two membranes may arise from several factors which directly take into account the physical and chemical characteristics of the membrane proteins involved. It has been found that: (1) the squid membrane contains a higher percentage of protein (50%) than does the vertebrate photoreceptor membrane (40%) (Mason *et al.*,

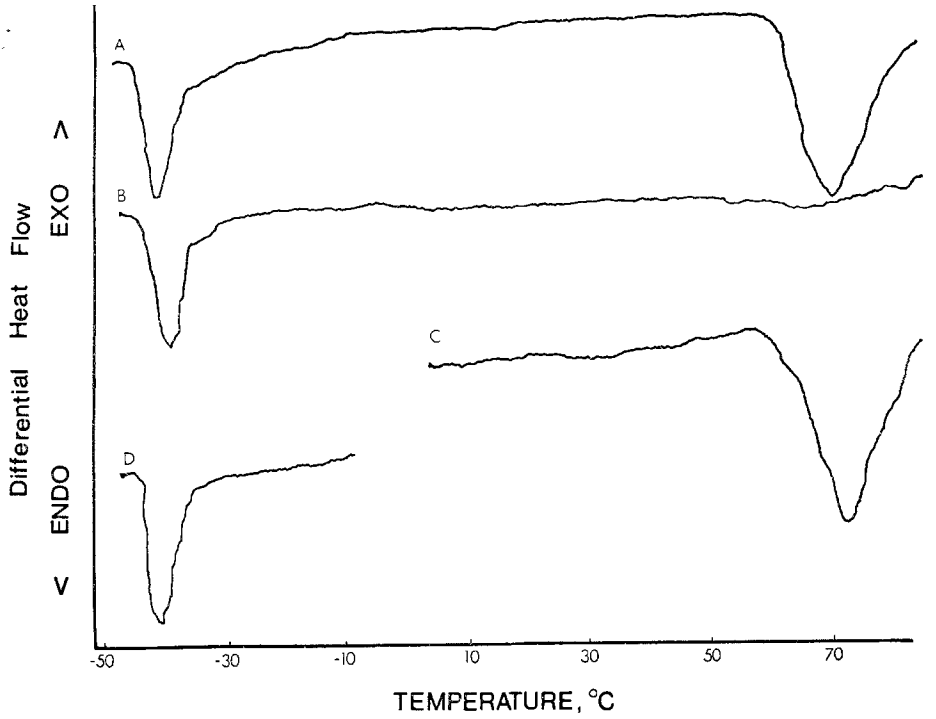

Fig. 2. Thermogram tracings of differential scanning calorimetry of squid rhabdome photoreceptor membranes. (*A*) Membranes scanned upward in 50% ethylene glycol; (*B*) membranes scanned upward in 50% glycol following protein denaturation; (*C*) membranes scanned upward in 100 mM Tris buffer, pH 7.0; (*D*) extract of membrane lipids dispersed in 50% ethylene glycol and scanned upward

1973*a*, *b*), and (2) the vertebrate photoreceptor membrane contains predominately a single protein, namely 80 to 90% rhodopsin (Bownds & Gaide-Huguenin, 1971; Mason *et al.*, 1973*b*) whereas the squid rhabdome appears to contain a wide variety of different proteins and correspondingly less visual pigment (Hagins, 1973; Mason *et al.*, 1973*a*). Although relatively little is known of the proteins involved in these membranes, it was found, in the course of studying the effect of light and dark adaptation of the light-sensitive outer segment membranes on the phase transition, that if one heated dark-adapted vertebrate photoreceptor membranes to some 5° below the temperature where the high temperature endothermic transition peak arising from denaturation appears, they maintained intact their spectrum of native visual pigment, with an absorption maxima at 500 nm. When these same membranes were heated to 2 or 3° beyond this peak temperature, they in turn lost the characteristic 500-nm absorption and reverted to thermally "bleached" membranes absorbing at 465 nm. In a qualitative sense this same effect was also observed in preliminary experiments of the present

paper by heating a membrane suspension with a copper-constantan thermocouple immersed in the suspension. By quickly switching the light on and off at various temperatures, the color of the suspension could be observed by the naked eye to shift suddenly from deep pink to orange as the temperature rose past that region defined more precisely by differential scanning calorimetry to be the denaturation peak. These results indicate that in vertebrate photoreceptor membranes, the thermal denaturation of native rhodopsin is to a large degree responsible for the high temperature endothermic transition. It is well known that different proteins vary in their physical characteristics, such as denaturation temperature, and it may thus be reasonably inferred that the higher temperature of endothermic transition (74 °C) observed for squid rhabdomes is due largely to the characteristics of the protein milieu of these invertebrate outer segment membranes. In spite of this, one cannot discount the participation of protein-lipid interactions as influencing the characteristic denaturation temperature of both membrane systems.

The membrane denaturation portion of the thermogram is observed in both buffer and 10% glycerol as well as in 50% glycol. In all media the transition shows nearly the same temperature and heat content. After heating the membranes past their denaturation temperature and scanning downward from 90 °C or upward from −50 °C the low temperature transition was observed to be fully reversible, but the protein denaturation was irreversible, as one might expect from numerous observations on the relative irreversibility of protein denaturation.

The significance of the low temperature transitions may best be discussed in terms of the lipid and fatty acid compositions of the two membranes. In previous reports we have found the compositions of the vertebrate and invertebrate photoreceptor membrane to differ widely in several important aspects. The first of these is the relative unsaturated fatty acid content of the two membranes, invertebrate photoreceptors containing highly saturated short-chain fatty acids and vertebrate membranes containing highly unsaturated long-chain fatty acids. In addition, vertebrate photoreceptor membranes contain little or no cholesterol ($\ll 2\%$) whereas invertebrate photoreceptors possess some 10 to 12% cholesterol.

The role of the phospholipid compositions in this regard is unclear, but marked differences do exist. The squid membranes contain a 1.5:1 molar ratio of phosphatidyl ethanolamine/phosphatidyl choline; vertebrates contain a 1:1.5 molar ratio of the same. Phospholipid membranes of similar composition but lacking unsaturated fatty acid side chains are known to undergo transitions in the temperature range of 10 to 30 °C (Blazyk &

Steim, 1972). Thus, the present studies strongly suggest that the low temperature transition may be attributed to highly unsaturated fatty acid side chains and cholesterol content of the membranes. For instance, the predominant fatty acid of vertebrate photoreceptor membranes is docosahexanoic acid (22:6), a fatty acid which shows a sharp transition at −60 °C. Other long-chain unsaturated fatty acids undergo similar low temperature phase transitions. Thus, the fatty acids in these membranes may interact in such a way to yield an "average" low temperature transition around −28° C.

As for the squid rhabdomes, the low temperature transition may best be explained on the basis of fatty acid unsaturation of the phospholipids, and more importantly, cholesterol content. In a relative sense, the squid rhabdomere fatty acids are shorter chain and more highly saturated than fatty acids of vertebrate photoreceptor membranes (Mason *et al.*, 1973*b*). Nevertheless, the fatty acids of the invertebrate photoreceptor membranes studied here show evidence of a large extent of unsaturation in the lipid milieu. This unsaturation may be one factor which contributes significantly to the low temperature lipid transition. The phospholipid content alone must also contribute somewhat to the transition, as many reports exist in the literature concerning moderately low temperature phase transitions for pure phospholipids (around 0 °C). As for the small difference of 4° between the observed transition in vertebrate and that of invertebrate photoreceptors, assignation of a single factor giving rise to this effect is difficult, and at best, speculative.

The influence of cholesterol on the physico-chemical properties of model membranes has been studied extensively and it is known that cholesterol, while tending to "rigidify" mixtures of saturated and unsaturated phospholipids, may lower the transition temperature of a pure saturated phospholipid with which it is combined, as well as significantly diminish the heat of transition of that membrane. On the basis of fatty acid composition alone, one might have predicted a higher temperature of transition for the squid membranes, but apparently the cholesterol of these membranes plays a significant role in lowering the transition temperature slightly. However, cholesterol may have the opposite effect upon combination with unsaturated phospholipids as well. Thus, the net effect of cholesterol may be at best ambiguous in this instance; however, as has been pointed out, the squid is a great deal more saturated in the lipid moiety than comparable vertebrate photoreceptors, and one might therefore expect cholesterol in the squid rhabdome to exert an effect relatively comparable in direction, if not in magnitude, to that observed with saturated phospholipids, namely to lower slightly the transition temperature.

Papahadjopoulos *et al.* (1971) and others have also reported that cholesterol tends to liquefy membranes, probably by altering the molecular packing within the bilayer. Corroborative evidence has been obtained by Ladbrooke *et al.* (1968) who observed by X-ray diffraction that addition of cholesterol to model lipid membranes gives rise to a diffuse wide angle spacing of 4.45 Å rather than the sharp 4.2 Å spacing in lipid membranes without cholesterol. These results also suggest that cholesterol may, under favorable conditions, tend to lower the transition temperature of membranes. These various effects could explain the slightly lower transition for rhabdomeric membranes than for vertebrate rod photoreceptors. Though such an effect induced by cholesterol may account for the difference in transition temperatures from −28 to −32 °C, it does not account entirely for the low temperature nature of the thermal transitions observed here. The vertebrate membranes being nearly cholesterol-free, phospholipid and fatty acid surely play a major role in determining the low temperature of the transition in rod outer segment membranes.

It is of interest to note that mitochondria of the retinal inner segment were removed from the density gradient purification procedure and treated with similar buffers as before. These membranes were not highly purified, but they were found to undergo a rather broad low temperature phase transition as well, centering around −20 to 25 °C for both the squid and frog. The similarity in transition temperature between photoreceptor and mitochondrial membranes may imply that similar lipids are contained in the two membranes, and thus, because both organelles reside within a single cell type, that a common metabolic origin for these lipids exists. This is not surprising in light of the fact that many workers have shown the receptor cell inner segment to be the center of synthetic function for the cell, both in development and in turnover (Berger, 1964; Meller & Breipohl, 1965; Young, 1971).[1] Thus, lipids found in the cellular organelles probably arise from a similar source although they are destined for somewhat different locations within the cell. Similar characteristics are therefore found for photoreceptor and mitochondrial membranes. This must, however, be speculation due to the absence of information in the literature concerning the lipids and fatty acid content of receptor cell mitochondria.

One should note that Blasyk and Steim (1972) have found transition temperatures of 0 °C for mitochondrial and microsomal fractions of the rat liver. This transition is exceptionally broad relative to the purified photoreceptor membranes studied here, spanning some 20° in temperature (−10

1 *Also* Mason, W. T. and Bighouse, K. J. Ultrastructure of the developing chick retina. (*Submitted for publication*).

to 10 °C). The extracted mitochondrial lipids show a similar effect in breadth and temperature of the transition. The phase transitions of mitochondria of the rhabdome photoreceptors studied here are also somewhat broader than the corresponding transition for membranes of the vertebrate rod outer segment. The reasons for these differences are unclear at the present time; however, mitochondria of the photoreceptors studied here contain a vastly greater variety of protein than do the receptor cells. This difference between the transition of mitochondria of the inner segment and membranes of the receptor cell outer segment may be interpreted to mean that interactions between protein, lipid, and other membrane components play at least some role in the breadth of the low temperature transitions.

One point which surely bears on this discussion is that the detection of a thermal phase transition in the photoreceptor membrane probably implies the existence of a membrane bilayer. Other evidence for bilayers in vertebrate photoreceptor membranes is not lacking; various workers studying a variety of vertebrate outer segments have, on the basis of X-ray diffraction measurements, postulated electron density profiles indicative of lipid-protein bilayers (Blasie & Worthington, 1969; Gras & Worthington, 1969; Worthington, 1971). Although calorimetric measurements such as those reported here do not yield direct information as to the extent or nature of the bilayer, they do in fact provide experimental confirmation for the more mathematically oriented data treatment required in synthesizing an electron density profile from X-ray measurements.

We have observed that lipid extracted from these membranes undergoes a transition several degrees lower in temperature and 25% larger in heat content than the native membranes. The relative values of heat content for each transition were evaluated by direct measurement of the thermograms, with the ratio of areas under the endotherm serving as the point of comparison. Lipid content was determined for all samples following differential scanning calorimetric measurements; this was done by drying in a desicator the chloroform/methanol (2:1) extract in preweighed aluminum sample pans. Since all measurements were relative rather than absolute, the baseline of the thermogram was interpolated identically for all samples, and area measurements made. The areas for different samples were standardized on the basis of dry weight, and three samples, all from different cell preparations, were utilized for each determination. The error between measurements on the three extracts was on the order of 5 to 8% deviation from the average.

Because of the differences in heat content of intact membranes and their extracted lipid, it appears that the presence of protein and the interaction of protein with lipid in the membrane may act in such a way as to decrease

the total interaction energy of the membrane components. Clearly, the excessively low temperature of the transition observed in both extracted lipids and native membranes implies that at normal physiological temperatures of the organism, the receptor membranes are well above their transition temperatures and therefore highly fluid in nature.

Finally, it is of interest to examine the extent to which the low temperature lipid phase transition may influence the kinetics of intermediate processes in the photolysis of rhodopsin. The only data available for comparison is that of Rapp (1970). He studied the lumirhodopsin$_{497} \rightarrow$ metarhodopsin$_{480}$ process in bovine discs over a wide range of temperatures, varying from -50 to $+36$ °C. Treating the reaction as first order, he found in the range of -40 to -50 °C that the kinetic activation parameters were $\Delta H^{\#} = 25 \pm 1$ kcal/mole and $\Delta S^{\#} = 70 \pm 5$ cal/mole deg, but in the range $+3$ to 36 °C the parameters were $\Delta H^{\#} = 3.5 \pm 0.5$ kcal/mole and $\Delta S^{\#} = -5.8 \pm 1.0$ cal/mole deg. This represents a vast difference in kinetic behavior and although measurements were not made just below and above the lipid phase transition temperature, which is near that of frog discs, it is clear that this difference can largely be ascribed to the low temperature transition in the lipid bilayer phase of the outer segment disc membranes.

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References

Ashe, G. B., Steim, J. M. 1971. Membrane transitions in gram positive bacteria. *Biochim. Biophys. Acta* **233**:810

Berger, E. R. 1964. Mitochondria genesis in the retinal photoreceptor inner segment. *J. Ultrastruct. Res.* **11**:90

Blasie, J. K., Worthington, C. R. 1969. Planar liquid-like arrangement of photopigment molecules in frog retinal receptor disk membranes. *J. Mol. Biol.* **39**:417

Blazyk, J. F., Steim, J. M. 1972. Phase transitions in mammalian membranes. *Biochim. Biophys. Acta* **266**:737

Bownds, D., Gaide-Huguenin, A. C. 1971. Rhodopsin content of rod outer segments. *Nature, New Biol.* **235**:114

Gras, W. J., Worthington, C. R. 1969. X-ray analysis of retinal photoreceptors. *Proc. Nat. Acad. Sci.* **63**:233

Gulik-Krzywicki, T., Rivas, E., Luzzati, V. 1967. Structure et polymorphisme des lipides: Etude par diffraction des rayons X du systeme soune de lipides de mitochondries de ceour de beouf et d'eau. *J. Mol. Biol.* **27**:303

Hagins, F. 1973. Purification and partial characterization of the protein component of squid rhodopsin. *J. Biol. Chem.* **248**:3298

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

Hsia, J. C., Schneider, H., Smith, I. C. P. 1970. A spin label study of the effects of cholesterol in liposomes. *Chem. Phys. Lipids* **4**:238

Ladbrooke, B. D., Williams, R. M., Chapman, D. 1968. Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry. *Biochim. Biophys. Acta* **150**:333

Mason, W. T., Fager, R. S., Abrahamson, E. W. 1973*a*. Characterization of the lipid composition of squid rhabdome outer segments. *Biochim. Biophys. Acta* **306**:67

Mason, W. T., Fager, R. S., Abrahamson, E. W. 1973*b*. Lipid and fatty acid composition of frog photoreceptor outer segments. *Biochemistry* **12**:2147

Meller, K., Breipohl, W. 1965. Die Feinstruktur und Differenzierung des inneren Segmentes und des Paraboloids der Photorezeptoren in der Retina von Huhnenembryonen. *Z. Zellforsch.* **66**:673

Papahadjopoulos, D., Nir, S., Ohki, S. 1971 Permeability properties of phospholipid membranes: Effect of cholesterol and temperature. *Biochim. Biophys. Acta* **266**:561

Rapp, J. 1970. Photolytic Intermediates of Rhodopsin. Ph. D. Thesis. Case Western Reserve University, Cleveland, Ohio

Reinert, J. C., Steim, J. M. 1970. Calorimetric detection of a membrane lipid phase transition in living cells. *Science* **168**:1580

Steim, J. M., Reinert, J. C., Tourtelotte, M., McElhaney, R. N., Rader, R. L. 1969. Calorimetric evidence for the liquid-crystalline state of lipids in a biomembrane. *Proc. Nat. Acad. Sci.* **63**:104

Worthington, C. R. 1971. Structure of photoreceptor membranes. *Fed. Proc.* **30**:57

Young, R. W. 1971. Rod and cone outer segment renewal. *J. Cell Biol.* **49**:302