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Thermotropic Behavior of Retinal Rod Membranes and Dispersions of Extracted Phospholipids

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Summary. High sensitivity, differential scanning calorimetry studies of bovine retinal rod outer segment (ROS) disk membranes and aqueous dispersions of the extracted ROS phospholipids have been performed. ROS disk membranes were found to exhibit a broad peak of excess heat capacity with a maximum at less than about 3°C, ascribable to a gel-to-liquid crystalline phase transition of a fraction of the phospholipids. A similar thermotropic transition was observed for aqueous dispersions of the total extracted and purified ROS phospholipids. Comparison of the results obtained for the dispersion of total ROS phospholipids to those of the purified head group fractions suggests that the thermotropic behavior reflects a gel-to-liquid crystalline transition, leading to lateral phase separation, involving those phosphatidylcholine (PC) molecules containing saturated fatty acyl chains, possibly together with the highest melting ROS phosphatidylethanolamine (PE) and phosphatidylserine (PS) components. The interpretation of the thermal behavior of the ROS disk membranes depends on whether the transition is assumed to derive from the ROS PC and/or PE/PS fractions, and whether the transbilayer arrangement of the ROS phospholipids is assumed to be symmetric or asymmetric. The calorimetric data can be simply explained in terms of an asymmetric distribution of the major ROS disk membrane phospholipids (G.P. Miljanich et al., *J. Membrane Biol.* **60**:249–255, 1981). In this case, the transition would arise from the PE/PS fractions in the outer ROS disk membrane monolayer, and the anticipated transition from the PC in the inner monolayer would be broadened due to interaction with cholesterol. For the ROS membranes at higher temperatures, two additional, irreversible transitions are observed at 57 and 72°C, corresponding to the thermal denaturation of opsin and rhodopsin, respectively.

Key Words differential scanning calorimetry · retinal rod membranes · lipid phase transitions · cholesterol · membrane asymmetry · protein denaturation · rhodopsin · vision

Introduction

The thermotropic behavior of biological membranes can be usefully employed to gain information regarding the organization of lipids and the nature of their interactions with components such as proteins and cholesterol. The retinal rod outer segment (ROS)[1] disk membrane is particularly attractive for such studies, since its composition is simpler than that of many other biomembranes with more diverse functions. Rhodopsin comprises more than 95% of the integral disk membrane protein (Krebs & Kühn, 1977) and the lipid composition has been thoroughly investigated (Daemen, 1973; Hendriks et al., 1976; Miljanich et al., 1979; Stone, Farnsworth & Dratz, 1979). In addition, rhodopsin is stable in detergent solutions (Hubbard, 1958; Knudsen & Hubbell, 1978; Stubbs & Litman, 1978; McCaslin & Tanford, 1981) and can be reconstituted with a variety of synthetic and natural phospholipids (Hong & Hubbell, 1973; Applebury et al., 1974; Stubbs, Smith & Litman, 1976*b*).

In recent years a number of studies of rhodopsin-lipid interactions have appeared (Chen & Hubbell, 1973; Hong & Hubbell, 1973; Lamola, Yamane & Zipp, 1974; Brown et al., 1976, 1977*a*,*b*; Stubbs, Litman & Barenholz, 1976*a*; O'Brien, Costa & Ott, 1977; Watts, Volotovski & Marsh, 1979; Deese et al., 1981*a*,*b*; Litman, Kalisky & Ottolenghi, 1981; Watts et al., 1981; Kusumi & Hyde, 1982). At present, further investigations of the lipid organization of the retinal ROS disk membrane and the possible relationship to photoreceptor function are necessary. In this work, we have studied the thermal behavior of retinal ROS membranes and dispersions

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[1] *Abbreviations used:* ROS, rod outer segment; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; EPR, electron paramagnetic spin resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; EDTA, ethylenediaminetetraacetic acid; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; PDPC, 1-palmitoyl-2-docosahexenoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

of the extracted and purified ROS phospholipids using high sensitivity, differential scanning calorimetry (DSC). DSC is a completely nonperturbing technique which is firmly grounded in classical thermodynamics. In contrast to the rather large number of investigations of model phospholipid bilayer systems, however, relatively few DSC studies of native biomembranes and their isolated components have been reported. Here we show that by using DSC as a nonperturbing "probe," one can begin to infer details of the mixing of the ROS phospholipid classes as a function of temperature, which is in turn related to their lateral and transbilayer distribution in the native photoreceptor membrane (Miljanich et al., 1978). The DSC results are consistent with similar studies employing parinaric acid fluorescent probes (Sklar et al., 1979*a,b*; Sklar & Dratz, 1980), and with ^{31}P NMR studies (Deese, Dratz, & Brown, 1981*a*; Mollevanger & DeGrip, 1984). The results obtained using DSC are compared to inferences drawn from chemical labeling experiments regarding the transmembrane distribution of the ROS disk membrane phospholipids (Raubach, Nemes & Dratz, 1974*b*; Smith, Fager & Litman, 1977; Crain, Marinetti & O'Brien, 1978; Drenthe et al., 1980*a,b*; Miljanich et al., 1981). Additional, useful information regarding the thermal stability (Hubbard, 1958; Cooper & Converse, 1976) of the integral protein components of the ROS disk membranes, *viz.* rhodopsin and opsin, is also obtained.

Materials and Methods

Purification of ROS Membranes and ROS Phospholipids

Bovine rod outer segment (ROS) membranes were prepared from locally obtained eyes as described (Raubach, Franklin & Dratz, 1974*a*). The purified ROS membranes had A_{280}/A_{500} absorbance ratios in the range 2.2–2.6, before regeneration, and were typically ≲25% bleached. The extraction and purification of the total ROS phospholipids using silicic acid chromatography has been described (Brown et al., 1976; Brown, Deese & Dratz, 1982). These procedures separate the total ROS phospholipids, comprising 39 ± 2 mol% PC, 42 ± 2 mol% PE, 16 ± 1 mol% PS, and 2 ± 1 mol% PI, with little sphingomyelin present, from other lipids such as cholesterol, retinal, vitamin E, diglycerides, and free fatty acids (Miljanich et al., 1979). The ROS phospholipids were fractionated according to head group class by diethylaminoethyl cellulose column chromatography, and were characterized by thin layer chromatography and gas-liquid chromatography (Miljanich et al., 1979). Whenever possible, manipulations were performed under an argon atmosphere (Brown et al., 1982) to minimize oxidative damage to the polyunsaturated ROS phospholipids.

Preparation of Samples

ROS membrane samples were prepared for differential scanning calorimetry by osmotic shock in water followed by resuspension in 0.1 M borate containing 0.1 M KCl and 0.1 mM EDTA at a pH of 7. Samples of the extracted and purified ROS phospholipids were evaporated to dryness from a chloroform solution under a stream of argon, followed by exposure to high vacuum for several hours, hydration, and dialysis against 0.1 M borate containing 0.1 M KCl and 0.1 mM EDTA at pH 7. Codispersions of the ROS PC fraction with cholesterol were prepared by evaporating a chloroform solution to dryness, followed by hydration and dialysis as described above. The dialysate provided the reference blank for the calorimeter. Following dialysis, small aliquots of each of the samples were removed for phosphate analysis (Miljanich et al., 1979). Additional aliquots of the ROS membrane suspensions were taken for spectrophotometric analysis of the rhodopsin and opsin concentrations (Raubach et al., 1974*a*).

Differential Scanning Calorimetry

DSC thermograms were obtained using a Privalov-type calorimeter as described (Mabrey & Sturtevant, 1976; Mabrey, Mateo & Sturtevant, 1978). All curves are heating scans, usually at a rate of 1 K min^{-1}. In some cases, scan rates as low as 0.1 K min^{-1} were employed.

Results and Discussion

Thermal Transitions of ROS Disk Membrane Phospholipids

Figure 1 summarizes the results of a series of high sensitivity DSC studies of bovine ROS disk membranes and their extracted lipid constituents. Each of the thermograms depicted in Fig. 1 was found to be reversible upon a subsequent cycle of cooling and reheating. The excess heat capacity curve obtained for unbleached, osmotically shocked ROS disk membranes (Fig. 1, curve *a*) reveals the tail of a broad, endothermic transition, with a maximum at less than about 3°C, i.e., below the easily accessible range of the calorimeter employed for these studies. (Ethylene glycol was not added to the samples.) No effect on the low temperature thermal transition of the ROS membrane preparations was observed upon exposure of the sample to light. The peak of excess heat capacity appears to correspond to a gel-to-liquid crystalline (L_α) phase transition of a fraction of the ROS disk membrane phospholipids, as previously reported by Chabre (1975) in a low angle X-ray diffraction study. Similar conclusions have been reached by Rothschild et al. (1976) from nonresonance Raman studies. Assuming an average value of about 30 kJ (about 7 kcal) for the molar enthalpy change (Mabrey & Sturtevant, 1976; Al-

bon & Sturtevant, 1978; Wilkinson & Nagle, 1981), it can be roughly estimated that more than about 10% of the total ROS phospholipids would be involved in the phase transition, consistent with the results of Chabre (1975).

A similar thermotropic transition, centered near about 6°C, is observed for the total extracted and purified ROS phospholipids dispersed in excess buffer (Fig. 1, curve *b*). It should be noted that ^{1}H and ^{31}P NMR studies (Brown et al., 1976, 1977*b*; Deese et al., 1981*a*) show that aqueous dispersions of the total ROS phospholipids favor the lamellar (gel or liquid crystalline) phase at temperatures below about 35–45°C under the conditions employed for these studies (*cf. also* De Grip et al., 1979). This is in spite of the rather substantial content (42 mol%) of highly unsaturated phosphatidylethanolamine (PE), which has been demonstrated in other systems to undergo a polymorphic lamellar to hexagonal II phase transition in the temperature range 10–60°C (Cullis & De Kruijff, 1978; Gally et al., 1980). Thus, the thermotropism observed for the total ROS phospholipid dispersions most likely represents a gel-to-liquid crystalline phase transition, as is the case for the ROS membranes. Further DSC, X-ray (Chabre, 1975; Gruner, Rothschild & Clark, 1982), and ^{31}P NMR (De Grip et al., 1979; Deese et al., 1981*a*; Marsh et al., 1982; Albert & Yeagle, 1983) studies are desirable, however. The observation of endothermic phase transitions for both the ROS disk membranes and multilamellar dispersions of the total extracted ROS phospholipids is qualitatively consistent with earlier spectroscopic studies employing ^{1}H NMR (Brown et al., 1976, 1977*b*).[2] Such thermal transitions are also seen in fluorescent probe studies employing parinaric acid derivatives (Sklar et al., 1979*a*; Sklar & Dratz, 1980), but not using the dye 1,6-diphenyl-1,3,5-hexatriene (DPH) (Stubbs et al., 1976*a*).

ROS Phospholipid Fractions

In an attempt to identify the origin of the observed calorimetric transitions, the ROS phospholipids were chromatographically separated by head group class (Miljanich et al., 1979). The purified ROS PE dispersed rather poorly at room temperature, forming large aggregates that would not remain in aqueous suspension even after prolonged sonication.

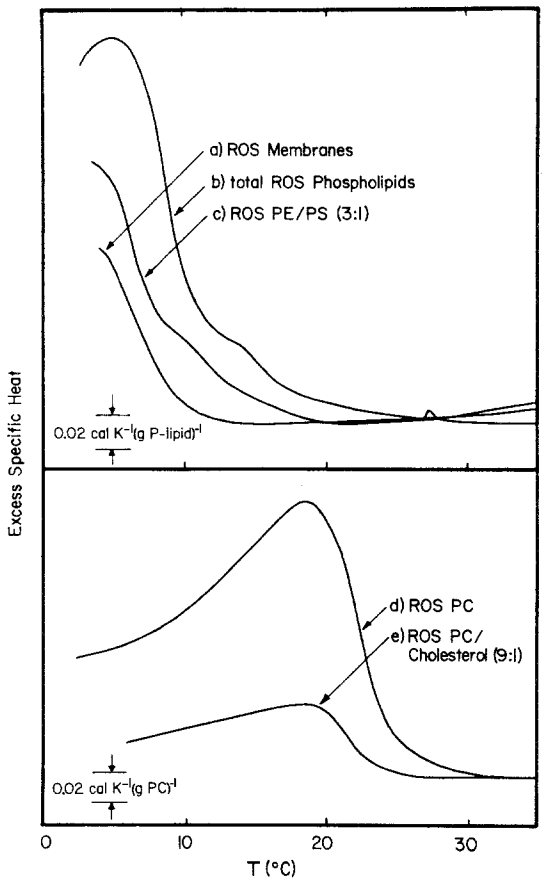

Fig. 1. DSC thermograms of (*a*) ROS membranes not previously exposed to light; (*b*) dispersion of total purified ROS phospholipids; (*c*) 3 : 1 (mol/mol) codispersion of purified ROS PE and ROS PS; (*d*) dispersion of purified ROS PC; and (*e*) 9 : 1 (mol/mol) codispersion of purified ROS PC plus cholesterol. Thermograms *a–c* represent 14.5 μmol or about 12 mg of total phospholipid; thermograms *d* and *e* represent 9.1 μmol or about 7.8 mg of PC

This behavior may result from the inability of the ROS PE to form bilayers, but rather to favor the inverted hexagonal II phase, as observed for other unsaturated phosphatidylethanolamines of natural and synthetic origin (Cullis & De Kruijff, 1979). Results suggestive of hexagonal II phase formation have been obtained using ^{31}P NMR for an aqueous dispersion of the extracted ROS phospholipids enriched in the ROS PE (M.F. Brown et al., *unpublished*). Aqueous dispersions of saturated or unsaturated PS, on the other hand, favor the lamellar phase (Browning & Seelig, 1980). A 3 : 1 (mol/mol) codispersion of the ROS PE and ROS PS, which mimics the native ROS PE/PS ratio (Miljanich et al., 1979), was prepared and found to exhibit a transition with a maximum of excess specific heat at less than 3°C (Fig. 1, curve *c*). Similar results are

[2] More recent ^{1}H correlation NMR studies, performed at 360 MHz, show clearly that the intensities of the sharp phospholipid resonances of the ROS membrane vesicles decrease below about 20°C; at 5°C only a broad, unresolved spectrum remains (G.P. Miljanich et al., *unpublished*).

obtained for a 2.7 : 1 (mol/mol) ROS PE/PS dispersion using *trans*-parinaric acid methyl ester as a fluorescent probe (Sklar et al., 1979*a*). In this case, ^{31}P NMR spectra are not available and are required before firm conclusions can be reached. However, ^{31}P NMR studies of a similar 4 : 1 (mol/mol) codispersion of egg yolk PE and bovine brain PS (Cullis & Verkleij, 1979) show that incorporation of acidic phospholipids may stabilize the lamellar phase of unsaturated phosphatidylethanolamines. Therefore, the transition observed in the 3 : 1 ROS PE/PS sample may represent a gel to liquid crystalline phase transition; further work is required. The presence of such a transition has been suggested (Sklar et al., 1979*a*) to involve asymmetric phospholipids containing one saturated and one unsaturated acyl chain. Chemical analysis has shown that such mixed chain saturated-unsaturated species make up 70% of the combined total ROS PE and PS (Miljanich et al., 1979).

Dispersions of the purified ROS PC fraction in excess buffer were observed to give rise to a broad, thermotropic transition centered at about 19°C (Fig. 1, curve *d*). Comparison to results for other synthetic and natural phosphatidylcholines suggests that the observed peak of excess heat capacity represents a gel to liquid crystalline phase transition (Chapman, 1975). If the ROS PC fraction is mixed and codispersed with cholesterol (9:1, mol PC/mol cholesterol), then the maximum of the ROS PC transition is still centered near about 19°C (Fig. 1, curve *e*); the apparent enthalpy is substantially reduced, however, consistent with studies employing synthetic phosphatidylcholines (Mabrey et al., 1978; Estep et al., 1978). The above observations are in good agreement with steady-state fluorescence depolarization studies employing *trans*-parinaric acid (Sklar et al., 1979*a*).

The presence of a phase transition in dispersions of the ROS PC fraction can be explained in terms of the known fatty acyl chain composition (Miljanich et al., 1979). The ROS PC fraction contains 18–27 mol% of species having two saturated fatty acyl chains (disaturated PC), largely as 1,2 - dipalmitoyl - *sn* - glycero - 3 - phosphocholine (DPPC). The remainder of the ROS PC is predominantly composed of species containing two polyunsaturated fatty acyl chains (dipolyunsaturated PC) and asymmetric (mixed chain) saturated-polyunsaturated species (Miljanich et al., 1979). The thermotropic transition observed at about 19°C in the dispersion of purified ROS PC (Fig. 1, curve *d*) most likely represents a gel-to-liquid crystalline transition involving primarily the disaturated PC fraction. Binary mixtures of DPPC or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and an unsaturated PC with a gel-to-liquid crystalline phase transition temperature well below 0°C, such as, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or 1-palmitoyl-2-docosahexenoyl - *sn* - glycero - 3 - phosphocholine PDPC), would be expected to approximate the thermal behavior of the ROS PC fraction over the temperature range investigated here. The thermal transition midpoints, T_M, of DOPC and PDPC in excess water are −22°C (Phillips, Ladbrooke & Chapman, 1970) and about −25°C (Sklar et al., 1979*b*), respectively. Multilamellar binary mixtures of DPPC and DOPC, DSPC and DOPC, and possibly DPPC and PDPC all appear to exhibit monotectic, or close to monotectic, behavior due to the large differences in the transition temperatures of the individual components (Phillips et al., 1970; De Kruyff et al., 1973; Sklar et al., 1979b). That is, at temperatures above the phase transition of the highest melting component, both PC species are miscible and coexist as a homogeneous, but presumably nonideal, liquid crystalline phase. As the temperature is decreased, the higher melting, disaturated PC component favors a gel or solid phase which is laterally separated from the fluid phase enriched in the lower melting, unsaturated PC. At still lower temperatures, two coexisting, largely immiscible solid phases are formed. In each case, the onset temperature of the gel-to-liquid crystalline transition, as given by the fluidus line of the equilibrium phase diagram (Mabrey & Sturtevant, 1976), is lowered relative to that of the pure disaturated PC species. For the case of an 8 : 2 (mol/mol) binary phospholipid dispersion containing PDPC and DPPC (which should behave similarly to the ROS PC), a broad transition with a midpoint, T_M, near 15–17°C is observed in parinaric acid fluorescence depolarization studies (Sklar et al., 1979*a*,*b*). This value is in good agreement with the T_M of about 19°C determined for the ROS PC fraction using DSC. Taking an average value of about 30 kJ (about 7 kcal) for the molar latent heat of transition would yield that roughly about 30% of the total ROS PC is involved. This estimate is in reasonable agreement with the 18–27 mol% of the ROS PC which is disaturated as determined by chemical analysis (Miljanich et al., 1979), and is similar to the value of 25% estimated from parinaric acid fluorescence depolarization experiments (Sklar et al., 1979*a*).

The peak of excess specific heat observed for dispersions of the total ROS phospholipids near about 6°C (Fig. 1, curve *b*) would then appear to represent a gel-to-liquid crystalline phase transition involving the highest melting, disaturated PC fraction, possibly together with the highest melting ROS PE/PS components (Sklar et al., 1979*a*). Since the purified ROS PC melts at about 19°C, the shift to

lower T_M could represent mixing of the ROS PC with lower melting PE/PS components. As a result, a largely homogeneous mixture of the total ROS phospholipids in the lamellar, liquid crystalline phase would exist above about 20°C, consistent with ^{31}P NMR studies (Deese et al., 1981*a*).

ROS Membranes

For the ROS membranes, the thermotropism observed could derive from either or both of the PE/PS and PC fractions, depending on whether or not these phospholipid classes can undergo lateral mixing, as presumably occurs in dispersions of the total extracted ROS phospholipids. The above is in turn related to (i) whether rhodopsin interacts strongly and specifically with one or more of the phospholipid head group classes, and/or (ii) the assumed transbilayer distribution of lipids within the ROS disk membrane. At present, we are unable to firmly assign the observed lipid phase transition in the ROS membranes based on DSC data alone. In view of the spin-label EPR results of Watts et al. (1979), would appear improbable that rhodopsin specifically associates with or binds particular phospholipid head group classes. Reports to the contrary have appeared, however (Albert & Yeagle, 1983). In this regard, the DSC data render it very unlikely that rhodopsin is strongly interacting with or preferentially binding the PE/PS fractions. The observed peak in the ROS membranes would then be largely due to the PC fraction and would be expected to shift to a midpoint, T_M, intermediate between that observed for dispersions of the total ROS phospholipids ($T_M \sim 6$°C), containing 39 mol% PC, and the purified ROS PC ($T_M \sim 19$°C). The above is not seen (*cf.* Fig. 1, curve *a*). However, specific interactions of rhodopsin with the ROS PC fraction would not be ruled out, *prima facie*, by the presently available DSC results (*vide infra*).

If the lipid phase transition observed in the ROS membranes is assumed to derive from the PC fraction, then this would imply that the phospholipid organization in the ROS disk membranes is similar to that of aqueous dispersions of the total extracted and purified ROS phospholipids. That is, the transbilayer distribution would be random, or *symmetric* in both cases (*cf.* Drenthe et al., 1980*a,b*), and any broadening or differences in the transition temperature or calorimetric enthalpy of the ROS membranes *vis-à-vis* the total extracted ROS phospholipids could be due to the presence of rhodopsin, or possibly cholesterol. However, a number of chemical labeling studies suggest the possibility of an *asymmetric* transbilayer arrangement of the phospholipid head group classes of the ROS disk membranes (Raubach et al., 1974*b*; Smith et al., 1977; Crain et al., 1978; Miljanich et al., 1981). If the PC fraction is presupposed to reside largely in one monolayer of the ROS disk membrane, and if the ROS PC fraction gives rise to the observed thermotropic behavior, then one would again expect the transition temperature for the ROS disk membranes to lie between that of the total ROS phospholipids and the purified ROS PC fraction—contrary to experimental observation. Here, we have presumed that the thermal properties of the ROS membranes correspond approximately to a superposition of those of the two disk membrane monolayers (Chapman, 1975; Sklar & Dratz, 1980). Thus, the transition is probably not due to the ROS PC, or else the transbilayer distribution of phospholipids is symmetric in conflict with the results of Miljanich et al. (1981).

On the other hand, the thermotropic transition observed for the ROS membranes could derive from the PE/PS fraction, as suggested by a comparison of the heat capacity curves in Fig. 1. One is then left with the problem of explaining the absence of a transition from the PC in the ROS membranes. If the transbilayer distribution of phospholipids is assumed for the moment to be *symmetric* (Drenthe et al., 1980*a,b*), then rhodopsin could preferentially bind the PC fraction and reduce or eliminate its contribution. Little precedent exists for such an explanation, however, which would also conflict with the results of Watts et al. (1979). Alternatively, cholesterol could preferentially interact with the ROS PC, broadening and reducing its calorimetric enthalpy, such that the remaining phase transition in the ROS membranes would largely represent the ROS PE/PS fractions. Cholesterol has been shown to favor PC over PE in mixtures of phospholipids exhibiting monotectic behavior, irrespective of whether PC is the higher or lower melting component (Van Dijck et al., 1976; Cullis et al., 1978). However, cholesterol does not appear to preferentially interact with PC or PE in mixtures not showing monotectic behavior (Blume, 1980). If the transbilayer phospholipid distribution is assumed, rather, to be largely *asymmetric* (Miljanich et al., 1981), then the effective separation of the PE/PS and PC fractions across the plane of the ROS disk membrane bilayer could account for the observed calorimetric properties. The thermotropic behavior would then arise from the PE/PS components (in the outer monolayer of the ROS disks; i.e., the cytoplasmic side), and the absence of a phase transition from the ROS PC near 19°C (in the inner disk monolayer) could be explained by preferential interaction of PC with cholesterol (Van Dijck et al., 1976). Cholesterol is believed to comprise *ca.* 10 mol% of the ROS lipids

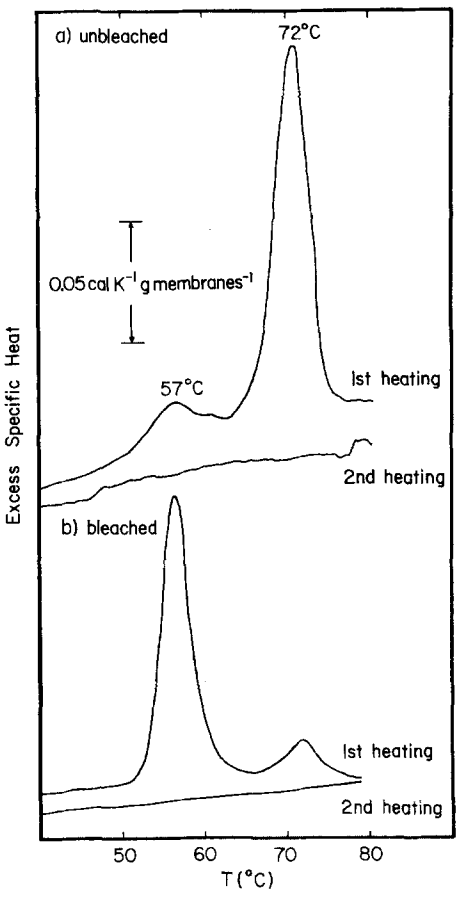

Fig. 2. DSC thermograms of ROS membranes. (*a*): Sample exposed only to dim red light after excision of eyes and membrane isolation, containing *ca.* 4.2 mg rhodopsin and 1.4 mg opsin. Actual experimental traces are shown and illustrate the signal/noise and baseline stability of the instrument. (*b*): Sample exposed to room lights prior to loading into calorimeter, containing *ca.* 14.8 mg combined total rhodopsin plus opsin; the traces have been scaled to a protein concentration equivalent to that of *a*. (Second heatings were obtained after first scans followed by subsequent cooling and are arbitrarily offset on vertical axis)

(Hendriks et al., 1976); if it were localized predominantly to the inner PC monolayer of the ROS disk membranes, then from the results of Fig. 1 it can be inferred that the PC thermal transition might be broadened beyond detection. Such an asymmetric distribution of cholesterol has also been postulated to exist in the case of the erythrocyte plasma membrane (Fisher, 1976; Van Dijck et al., 1976; Demel et al., 1977; Cullis et al., 1978).

While at present one cannot distinguish unequivocally among the various models, the present DSC results, taken together with the spin-label EPR results of Watts et al. (1979) and the chemical labeling studies of Miljanich et al. (1981), appear consistent with an asymmetric transbilayer distribution of the PE/PS and PC/cholesterol fractions in the ROS disks, with little or no specific interactions with rhodopsin being detectable. Thus, the observed lipid phase transition of the ROS disk membranes may arise largely from the PE/PS fraction, and preferential interactions with cholesterol could explain the lack of a contribution from the ROS PC fraction. The disposition of the visual protein rhodopsin, which is believed to span the disk membrane bilayer (Fung & Hubbell, 1978; Nemes et al., 1980), also appears to be asymmetric, with its carbohydrate moiety exposed on the intradiskal side (Fung & Hubbell, 1978). Such a transbilayer distribution of lipid and protein is consistent with the known mechanism of biogenesis of the ROS disks by endocytosis from the rod cell plasma membrane (Young, 1974). Thus, the orientation of the lipid and protein constituents may be inverted relative to that of the plasmalemma (Op den Kamp, 1979; Sklar et al., 1979*a*; Miljanich et al., 1981).

Thermal Stability of Rhodopsin and Opsin

In the ROS membranes, two additional, irreversible peaks of excess heat capacity are observed at higher temperatures, as depicted in Fig. 2. Irreversible decreases in regenerability and 500 nm absorbance are known to occur as a function of temperature for rhodopsin in ROS membranes and in micellar detergent solutions (Hubbard, 1958; Stubbs et al., 1976*b*; Knudsen & Hubbell, 1978). In contrast to the reversible lipid transition observed at lower temperature (*cf.* Fig. 1), the assignment of the irreversible transitions at higher temperatures is relatively unequivocal. ROS membranes that have not been exposed to light at any time following dissection of the eyes (except dim red light) exhibit a large peak of excess specific heat centered at about 72°C and a smaller peak at 57°C (Fig. 2*a*). ROS membranes that have been exposed to room light prior to scanning exhibit the converse behavior—a large peak at 57°C and a much smaller one at 72°C (Fig. 2*b*). Neither peak is observed upon cooling and reheating. The transition at 57°C appears to correspond to the thermal denaturation of opsin, and that at 72°C to the thermal denaturation of rhodopsin. Bovine ROS membranes typically contain 15–25% bleached material, thereby accounting for the presence of the smaller opsin peak in the unbleached sample (Fig. 2*a*). Upon bleaching (in the absence of hydroxylamine), most of the rhodopsin is converted to opsin, with a small fraction of unbleached photopigment remaining (Fig. 2*b*). The lower denaturation temperature of opsin relative to rhodopsin appears to be due to the absence of the stabilizing

influence imparted by the prosthetic group, 11-*cis* retinal. The observed temperatures of denaturation are in excellent agreement with those obtained by Hubbard (1958) from measurements of the regenerability of opsin to rhodopsin after exposure of opsin or rhodopsin to various temperatures.

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