FREEZE-FRACTURE EVIDENCE FOR THE PRESENCE OF CHOLESTEROL IN PARTICLE-FREE PATCHES OF BASAL DISKS AND THE PLASMA MEMBRANE OF RETINAL ROD OUTER SEGMENTS OF MICE AND FROGS

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ABSTRACT

The freeze-fracture technique was used to examine the membranes of the photoreceptors of mice and frogs. Particle-free patches were found in the plasma membrane and basal disk membranes of the outer segments of both mice and frogs housed at room temperature, but not in frogs kept in a cold room. These patches were shown not to be artifacts of cryoprotection or fixation, and they persisted when fresh isolated outer segments were frozen by an ultrarapid method. They were also found to persist in mouse rods when retinas were incubated and subsequently fixed at temperatures up to 80°C. Cholesterol was implicated as a significant component of the patches by the observation that, in the outer segments, pits, induced by treatment with the sterol-specific polyene antibiotic filipin, were present in and confined to the particle-free patches. That these lesions are not inherently limited to particle-free membrane areas was evident in the apical plasma membrane of the photoreceptor inner segments, where particles and pits were intermixed. Treatment with saponin, a surface-active agent which specifically complexes cholesterol, resulted in the disappearance of the particle-free patches. Patches were found in basal disks of both mouse and frog rods but not in older disks nearer the pigment epithelium, which indicates that changes occur in the composition of disk membranes and/or in the molecular ordering of their protein and lipid components during the early phase of their transit from the base towards the apex of the outer segment.

KEY WORDS freeze-fracture · particle-free patches · rod outer segment membrane · filipin · cholesterol · disk membrane assembly

Vertebrate photoreceptors have a number of characteristics which make the freeze-fracture technique especially useful in studying correlations between their structure and function. Their photoreceptive portions, or outer segments, contain numerous membranous disks, stacked within a plasma membrane. These membranous disks and their enveloping plasma membrane are rich in integral membrane proteins, most of which is photopigment (11, 20, 32, 33, 58). The lipid composition of outer segments has been studied (1-4, 74, 75) as has the fluidity of disk membranes

(12–14, 18, 52, 53, 60–62). Outer segments normally lose apical portions containing plasma membrane and disks which are consumed as phagosomes by cells of the pigment epithelium (77), in a process with circadian aspects (35, 46, 47), while new disks are formed at the base of the outer segments. This establishes a process of fairly continuous membrane renewal, which includes the synthesis of photopigment protein (opsin) in the inner segment of the photoreceptor (10, 15, 32, 57, 59, 77, 78) and its migration by an unknown path to the outer segment where it is incorporated into disks. Moreover, membranes doubtlessly play a role in maintaining the distinctive shapes of rod and cone outer segments (ROS, COS) in the apparent absence of membrane-associated cytoskeletal elements.

These considerations suggested that it would be worth doing a systematic freeze-fracture survey of membranes from the inner and outer segments of photoreceptors of a variety of species, with special attention to possible differences between rods and cones, to disk-forming regions, and to phagosome-forming regions. The literature contains a number of freeze-fracture studies reporting detailed or casual observations on outer segments from a variety of species (7, 17, 19, 39, 44, 48–50, 55, 56, 63), but with a few exceptions these are focused on comparing the *P* and *E* faces of disk membranes. The *P* face of disk membranes was uniformly reported to be fully occupied by closely spaced membrane particles in mature animals, but particle-free patches (PFPs) were observed both during initial photoreceptor differentiation (56) and after prolonged vitamin A deficiency (39). In addition, such PFPs were notable features of the *P* face of the plasma membranes of the murine (39) and bovine (44) outer segment.

We observed these PFPs in a variety of species and decided to investigate their nature before proceeding to other studies. We report here some of our observations and the results of several experiments designed to explore the molecular significance of PFPs in outer segment (OS) membranes of mice and frogs.

MATERIALS AND METHODS

Animals

Mice used in this study were of the C57BL/6 strain and were kept under conditions of cycling light (12 h light/12 h dark [12L/12D]). The frogs used were *Rana pipiens* kept either (*a*) in a cold room at ~6°C with no regular light cycle and no food, or (*b*) in a plastic tub at room temperature (~22°C) under 12L/12D cycling light and were fed crickets. Frogs were obtained commercially (NASCO, Fort Atkinson, Wisc.) and were said to originate in Mexico.

Isolation and Incubation

After animals were decapitated, enucleated mouse eyes were placed in Earle's balanced salt solution, and enucleated frog eyes were placed in Ringer's solution (as modified by Fain [26]) at physiological pH (7.0–7.6) and osmolarity (285–290 mOsmol for mice, 220–225 mOsmol for frogs), and the retinas were rapidly removed under a dissecting microscope and white light (up to 40 fc).

Some retinas were incubated in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid)-buffered versions of the dissecting solutions over a wide range of known temperatures for 30 s before immersion in buffered fixative at the corresponding temperature. Other retinas were incubated in solutions containing the polyene antibiotic filipin (generously provided by Dr. G. B. Whitfield, Jr. of the Upjohn Co., Kalamazoo, Mich.). Fresh stock solutions were made by dissolving 10 mg of filipin in 1 ml of dimethylformamide (DMF) before each experiment. Mouse retinas were incubated for 10 min at 37°C in either 100 μg filipin/ml Earle's (1% DMF) or 1 mg/ml (10% DMF). Frog retinas were incubated for 20 min at room temperature in modified Ringer's containing filipin and DMF as above. To control for the effects of such high DMF concentrations, mouse and frog retinas were incubated in similar solutions lacking filipin.

Some mouse retinas were incubated in solutions containing saponin (Sigma Chemical Co., St. Louis, Mo.; either 2.5 or 0.25 mg/ml in Earle's) for 2.5–15 min at room temperature or for 10 min at 37°C.

Fixation

Unless otherwise stated, all retinas were fixed in a 2.5% glutaraldehyde solution in either 0.16 M cacodylate buffer or Earle's (mouse retinas), or in 0.1 M phosphate buffer or Ringer's (frog retinas). The bulk of fixation generally occurred at 4°C, for 12 h or more, and was sometimes preceded by a brief incubation in fixative at higher temperatures corresponding to a prior incubation.

Freeze-Fracture Procedures

Cryoprotection was always done in a 25% glycerol solution either in Earle's (mice) or in diluted Earle's or 0.1 M phosphate buffer (frogs), at room temperature for from 1–2 h. Small pieces of tissue were loaded on Balzers specimen carriers (Balzers Corp., Nashua, N.H.), rapidly frozen in Freon 22 near its freezing point of −160°C, transferred to liquid nitrogen, and freeze-fracture was carried out on a Balzers apparatus (BAF

301). Replicas were cleaned by overnight immersion in methanol followed by 5–6% NaOCl at room temperature. After being washed in sterile distilled water the replicas were picked up on electron microscopy grids (either naked or Formvar [Monsanto Co., St. Louis, Mo.] coated) and examined and photographed in an Elmiskop 1A electron microscope.

Observations were made of unfixed retinas by three different procedures. The normal freezing and subsequent freeze-fracture techniques were applied to both cryoprotected and uncryoprotected unfixed mouse retinas, and, in addition, mouse ROS were observed after ultrarapid freezing by the method of Gulik-Krzywicki and Costello (30). ROS were aspirated from the surface of a freshly removed retina and immediately placed on a thin (0.003 in.) copper "hat." ("Hats" were generously provided by Dr. Daniel Branton, Biological Laboratories, Harvard University.) After placing an electron microscope grid over the suspension, a similar hat was inverted and placed on the top. This assembly was frozen in a small drop of liquid Freon 22 in contact with its ice, and then rapidly transferred to liquid nitrogen.

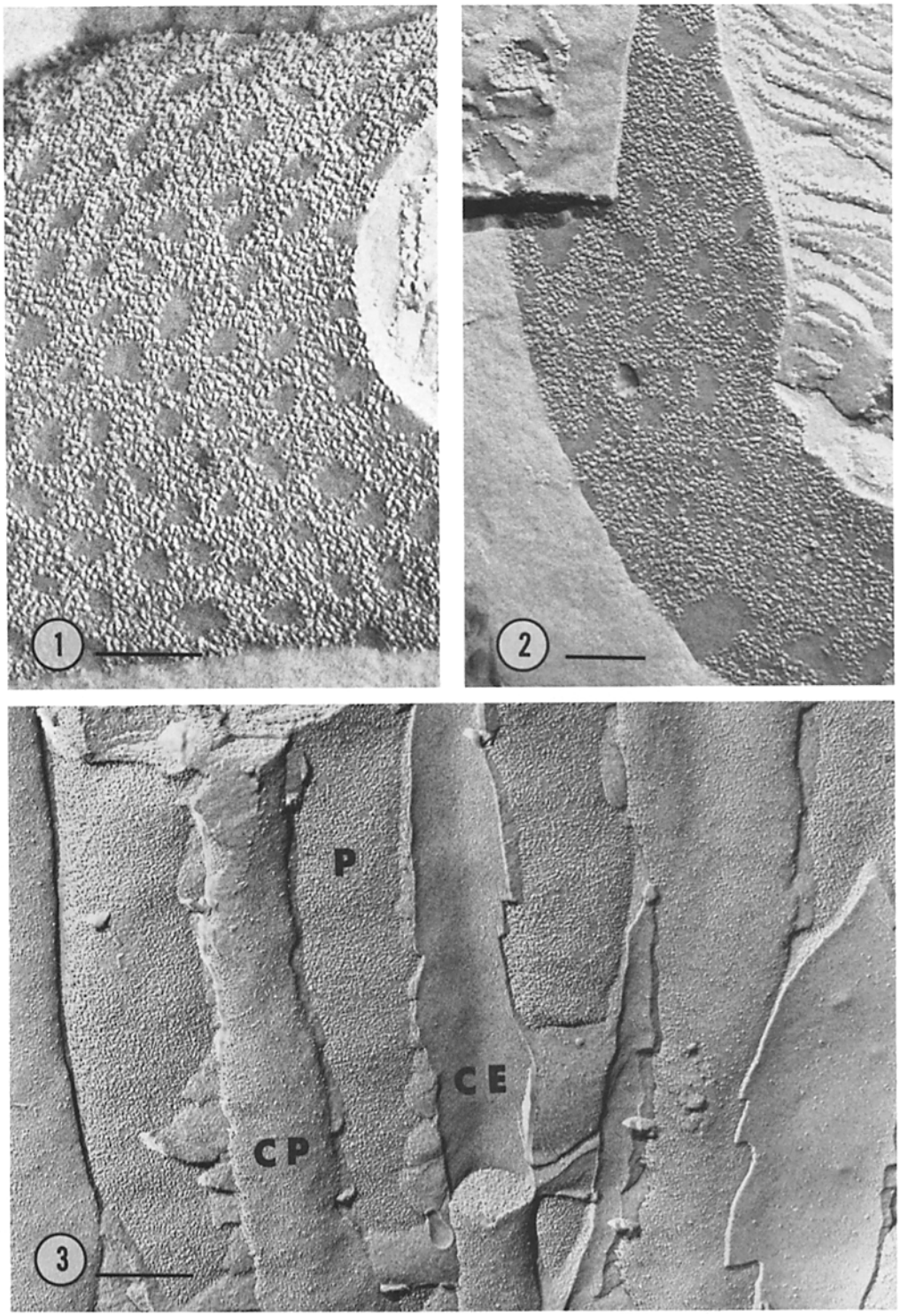
Patch and Membrane Area Measurements

These measurements were made with a compensating polar planimeter. Before a patch was measured, it had to satisfy two criteria: (*a*) its outline had to be clearly defined, and (*b*) the piece of membrane containing it had to appear relatively flat and parallel to the overall plane of the replica. Area values for each patch were averages of 10 measurements. For estimation of the fraction of membrane area covered by patches, membrane regions were found which met the latter criterion and which contained patches that satisfied both criteria. Such areas were rare, and the measurements made correspondingly reflect a statistically small set of membrane regions.

RESULTS

Plasma Membrane

The ROS of both normal C57BL/6 mice (Fig. 1) and frogs acclimated to room temperature (Fig. 2) possess plasma membranes with densely packed intramembrane particles evident on the *P* face which are interspersed with numerous rounded PFPs, but PFPs were not observed in the ROS plasma membrane of frogs kept for some weeks in a cold room at ~6°C (Fig. 3). These observations were verified in ~40 mice and in at least four frogs in each group. The initial observations of these PFPs in pilot studies of the mouse were all obtained after fixation at 4°C, and it was suspected that they represented artifacts of the low fixation temperature because of their resemblance to the PFPs obtained experimentally as a result of similar cooling in a variety of natural and artificial membranes (16, 23, 29, 31, 34, 38, 43, 51, 65, 66, 69, 71–73, 76). The conclusion reached in each of these studies was that the PFPs were the result of temperature-induced lateral phase separations in the component lipids of the membranes examined. In view of the demonstrated presence of photopigment in the outer segment plasma membrane (6, 22, 39), the most compelling similarity was between our results and the PFPs observed in the study by Chen and Hubbell (16) of rhodopsin-containing artificial membranes. The hypothesis that the PFPs observed in ROS plasma membranes were caused by temperature-induced lateral phase separations of membrane lipids led to a series of expériments in which mouse retinas were incubated and initially fixed at temperatures ranging from 4° to 80°C. Fixation with glutaraldehyde has been shown to immobilize rhodopsin in disk membranes (14, 18, 53, 62). The result of these experiments, verified for at least four mice at each temperature, was that PFPs could still be found in the ROS plasma membrane at all the temperatures studied (Fig. 4). Changes in particle distribution and patching behavior were noted, in that a less dense but still random particle distribution was evident at the highest temperatures, and the PFPs displayed an increasing size and change in shape which suggested progressive PFP confluence with increasing temperature. Fixation at 60°C of retinas from frogs acclimated to 6°C also resulted in the appearance of a less densely particulate ROS plasma membrane, but no PFPs were evident. Determination either of the average PFP size or the fraction of the membrane they occupied was impossible to estimate reliably at incubation and fixation temperatures above 37°C, both because the largest PFPs were often only partly revealed by the fracture plane and because the smallest patches seen at the lower temperatures could not have been seen among the more dispersed particles observed at the higher temperatures. However, estimates of the PFP fraction of total membrane area were obtained from photographs of favorably fractured and replicated membrane areas of outer segments incubated and fixed at 4° and 37°C and were found to be 11% and 16%, respectively. Because (*a*) considerable variability among different outer segments was noted and (*b*) very few outer segments fractured so that large areas of their plasma membranes were pre-



FIGURES 1–3 Freeze-fracture replicas of rod outer segments of a mouse (Fig. 1) and frogs (Figs. 2–3). Retinas were fixed at 4°C. Fig. 1 shows the *P* face of the plasma membrane from a mouse and illustrates numerous particle-free patches. Bar, 0.2 μm. × 78,600. Fig. 2 shows the *P* face of the plasma membrane from the retina of a frog acclimated to room temperatures for at least 4 wk. Note numerous particle-free patches. Bar, 0.2 μm. × 59,500. Fig. 3 illustrates several aspects of a photoreceptor from a frog kept in a cold room. *P* and *E* faces of calycal processes (*CP* or *CE*) from an inner segment are shown, as well as the *P* face of the plasma membrane of the rod outer segment (*P*). Note absence of particle-free patches in the outer segment membrane. Bar, 0.4 μm. × 36,000.

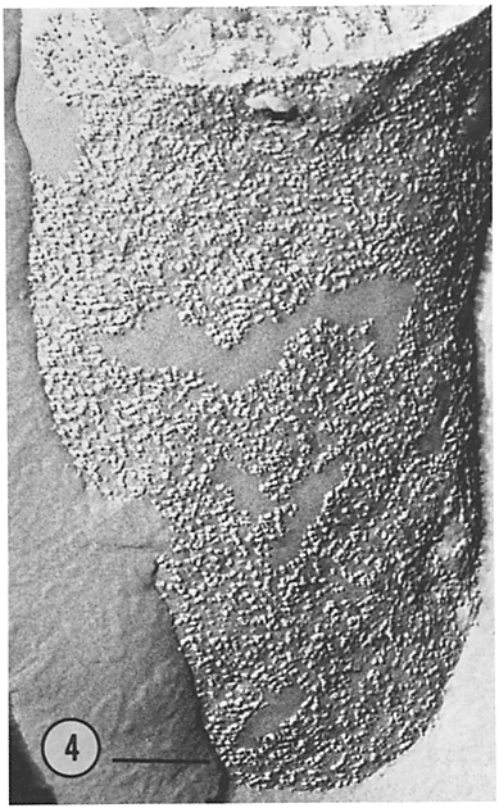


FIGURE 4 Freeze-fracture replica of the *P* face of the plasma membrane of mouse ROS. The retina was incubated at 68°C and prefixed at the same temperature. Note persistence and apparent confluence of particle-free patches. Bar, 0.3 μm. × 43,000.

sented at the proper orientation to the direction of shadowing for the production of replicas from which measurements could be made, these numbers merely reflect the absence of an obvious reduction in overall patch area as a result of increasing the temperature from 4° to 37°C. PFPs were evident in the ROS plasma membrane of room-temperature-acclimated frogs whether fixation was carried out either at 4°C or at room temperature (~22°C); observations at higher temperatures were not made. The attempt to count particles was not pursued because they were extremely heterogeneous in size, to such an extent that no clear demarcation between a "particle" and a mere surface textural irregularity was evident. The problems involved in counting particles in general have been recently discussed (54).

Although glutaraldehyde prefixation has been shown in a number of other membranes to prevent the development of PFPs as a result of subsequent cooling (34, 43, 76), cryoprotection, or the usual freezing technique (34), it was deemed desirable to ensure that it did not artifactually produce PFPs in ROS plasma membrane. In unfixed mouse retinas, this membrane continued to display PFPs after each of the following treatments: (*a*) cryoprotection followed by the usual freeze-fracture techniques; (*b*) normal freeze-fracture of freshly dissected, uncryoprotected retinas; and (*c*) rapid freezing of isolated, uncryoprotected ROS by the method of Gulik-Krzywicki and Costello (30) followed by fracturing and replication.

Another possible source of artifact was the exposure of the retinas to fairly intense white light during isolation. Although bleaching results in rhodopsin dispersal in artificial membranes (16), it is conceivable that the opposite effect might obtain *in situ*. Control experiments involving retinas isolated under dim red light from animals dark-adapted for >1 h revealed no qualitative difference in the appearance of the plasma membranes of ROS of mice incubated at either 37° or 60°C, or of frogs incubated at 22°C.

Disk Membrane

ROS disk membranes of mice and frogs were similar in that the *P* face contained closely packed intramembrane particles, whereas the *E* face was generally smooth and contained very few particles. Visual inspection of micrographs of equal magnification clearly demonstrates the basic qualitative similarity of the fracture faces produced by these membranes (Fig. 5).

PFPs like those of the plasma membrane were observed in the *P* face of some ROS disk membranes in both mice and frogs. In mice (Fig. 6), this occurred only in regions of the replicas also containing cross-sections of rod inner segments, which suggests that the disks displaying patches were close to the base of the outer segment ("basal disks"). Basal disk patches were observed in animals sacrificed either in the light period or at the end of the dark period. In frog retinas, PFPs were found in disks more removed from the layer of inner segments. Because of the shortness of the connecting cilium in frog rods, many fracture planes revealed part of the inner segment and the basal ~100 disks of the same rod. In these cases, the location of any favorably fractured and replicated patch-bearing disks could be directly ascertained (Fig. 7), and thus is was found that in frogs,

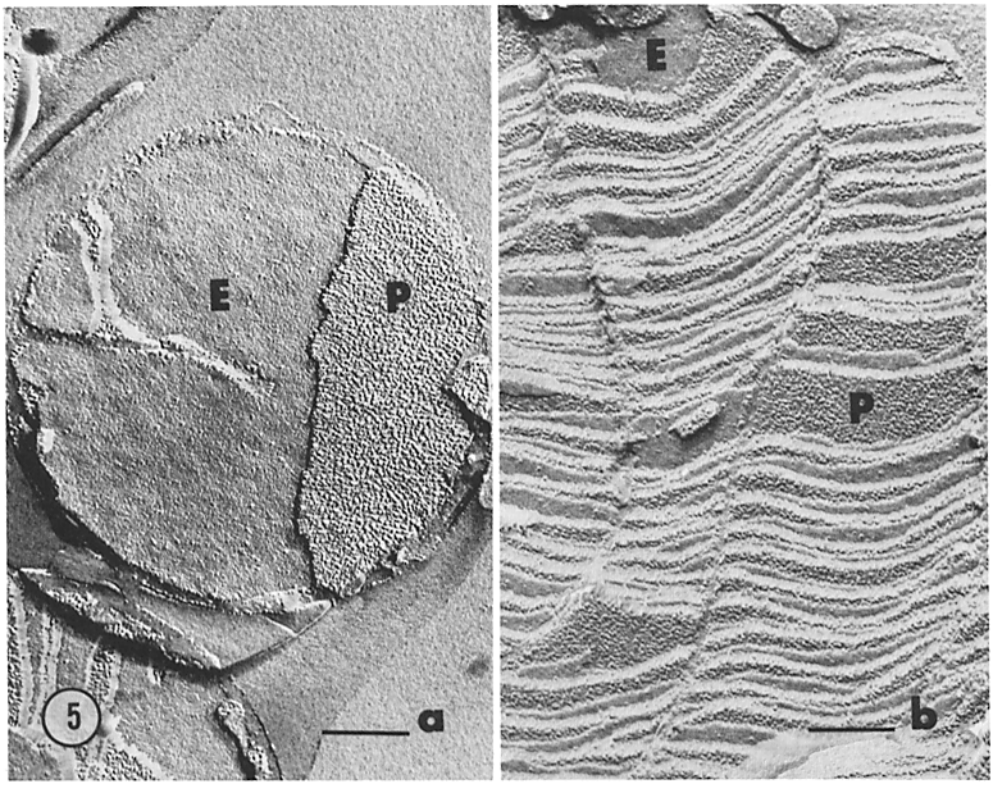


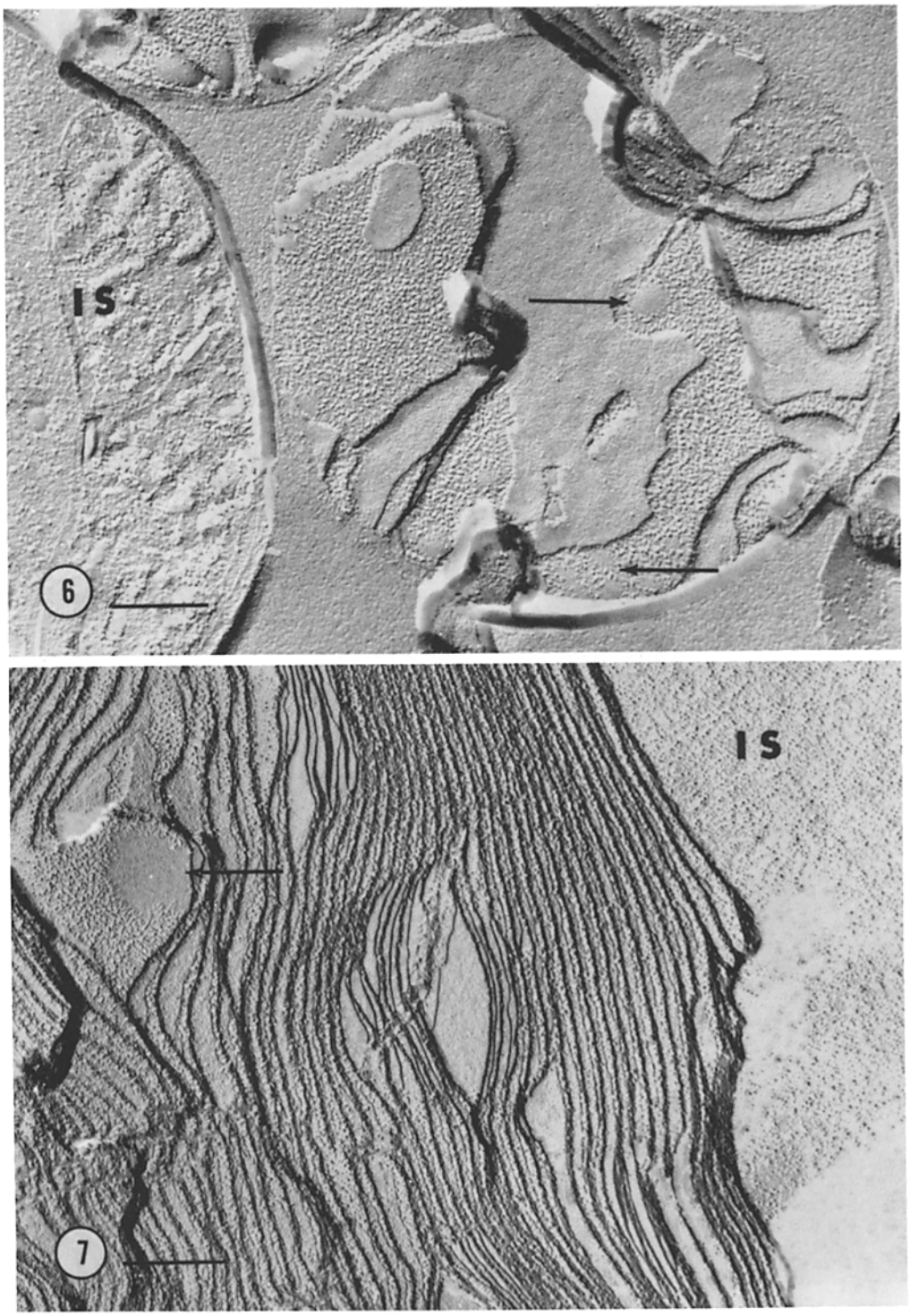
FIGURE 5 Freeze-fracture replicas of (*a*) normal mouse and (*b*) frog ROS disks, showing the highly particulate *P* faces (*P*) and sparsely particulate *E* faces (*E*). Bar, 0.3 μm. × 38,000.

ROS disk patches can occur in any of at least the ~75 most basal disks.

Disk and Plasma Membranes after Treatment with Filipin

The persistence of some PFPs in the plasma membrane of ROS of mice at all the temperatures tested, and the suggestion of an increase in membrane area represented by patches at 37°C relative to 4°C, led to further experiments designed to probe their molecular nature. Incubations of mouse and frog retinas in solutions containing the polyene antibiotic filipin were done to test the hypothesis that the PFPs observed in photoreceptor membranes are correlated with the presence of cholesterol. This hypothesis was suggested by Hubbell's (37) reference to unpublished observations of the formation of PFPs as a result of the inclusion of cholesterol in rhodopsin-containing artificial membranes. Filipin has been found to produce characteristic membrane lesions, observable by freeze-fracture, resulting from a specific reaction with sterols in membranes containing them (67, 69). We reasoned that this reaction specificity should apply to sterols possibly localized in particular membrane regions, and therefore might be valuable in testing the above hypothesis. In experiments involving mice and frogs (the latter acclimated to room temperature), filipin-induced "pits" were found in and were confined exclusively to the PFPs of both the disk and plasma membranes of their ROS (Fig. 8). In the most successful mouse experiment, pits were found in virtually every plasma membrane PFP; however, many of the disk patches were free of pits in all experiments. No obvious change was evident in the number, size, or disposition of the PFPs as a result of the exposure to filipin. In control experiments involving equivalent DMF concentrations but no filipin, nothing corresponding to the pits induced by filipin was evident.

The results of the experiments employing filipin incubations were found to be very variable. The initial experiment was done at a concentration of 100 μg/ml and produced a nearly uniform reac-



FIGURES 6 and 7 Freeze-fracture replicas of mouse (Fig. 6) and frog (Fig. 7) ROS basal disks. In both figures, arrows point to particle-free patches in the *P* face of disk membranes. The approximate level of the mouse disks is established by the nearby cross-fracture of an inner segment (*IS*) while the precise level of the frog disks is established by their relation to the junction of the outer segment and inner segment (*IS*). Fig. 6: Bar, 0.3 μm, × 48,000; Fig. 7: Bar, 0.3 μm, × 54,000.

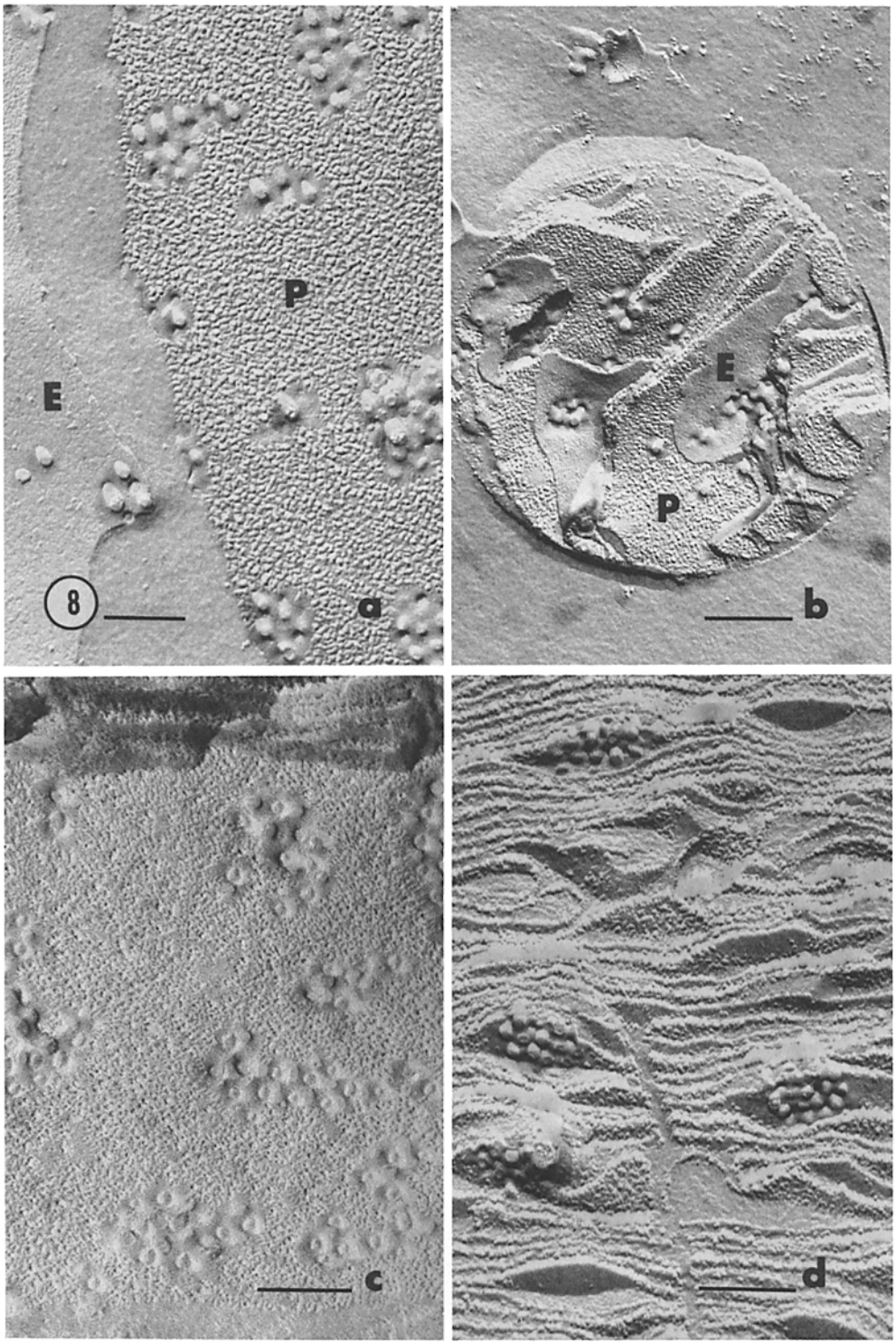


FIGURE 8 Freeze-fracture replicas illustrating the appearance of filipin-induced pits in the disk and plasma membranes of the outer segments of mouse and frog rods. In (*a*) and (*b*) are shown the plasma and disk membranes, respectively, of mouse outer segments, and in (*c*) and (*d*) those of a frog. In (*a*) and (*b*) note that pits are in both *P* and *E* membrane fracture faces (*P*, *E*) and that in all photographs *P* face pits are confined to particle-free patches. The *P* and *E* faces illustrated in (*a*) are from adjacent cells and are separated by extracellular space. (*a*) Bar, 0.2 μm, × 60,000; (*b*) bar, 0.3 μm, × 43,000; (*c*) bar, 0.2 μm, × 70,000; (*d*) bar, 0.3 μm, × 48,000.

tion in the photoreceptors throughout the random portions of the mouse retina examined. Attempts to repeat this experiment and to extend it to other species, including frog, were unsuccessful at this filipin concentration. As a result, higher concentrations (necessitating higher DMF concentrations) were tried and gave good results and much less variation between experiments, but there was still a variation among retinal areas, suggesting real differences in the access of filipin to photoreceptors, possibly because of an irregular persistence in these detached retinas of a gel which is known to surround the outer and inner segments of these cells in at least some species (27). Sources for variation between experiments include problems of solvation, because the filipin solutions were usually cloudy, and problems caused by filipin instability. Filipin is not stable in air, and the sample we received (200 mg) was stated to be from an 11-y-old supply stored under nitrogen at very low temperature. Our storage conditions may have been insufficiently protective, such that upon use the drug was partially degraded.

Plasma Membrane after Treatment with Saponin

Saponin, like filipin, is also a sterol complexing agent (42) which has been shown by negative staining to make lesions specifically in cholesterol-containing membranes (5). Incubating mouse retinas in saponin solutions (2.5 mg/ml) for 10 or 15 min at room temperature or for 10 min at 37°C led to the disappearance of most or sometimes all of the PFPs normally found in the OS plasma membrane (Fig. 9). Sometimes, PFPs much reduced in size and of irregular appearance are evident. An irregular reduction in patching resulted from shorter incubations. In addition, the overall appearance of the OS was typically degraded, as indicated by a swelling of the disks and general deformation of the outer segments. However, some OS appear normal and patch disappearance occurs here as well, indicating that it is not a function of the degree of distortion of the outer segments. This is also supported by the high temperature studies, in which patches persisted despite OS degradation of similar appearance.

DISCUSSION

In this study, we have attempted to investigate the significance of the PFPs observed in the *P* face of various membranes of photoreceptor outer segments. That these were probably not artifacts of fixation, cryoprotection, or freezing was indicated by their persistence in control experiments involving freeze-fracture of unfixed retinas examined after either the usual freezing technique or the rapid-freeze method of Gulik-Krzywicki and Costello (30).

The interpretation of the experiments employing filipin depends upon two assumptions. The first is that if filipin encounters similar local concentrations of cholesterol in photoreceptor membranes, it will react with the same intensity and specificity for cholesterol as was established in other systems (42). This assumption seems reasonable and implies that the presence of the filipin-induced lesions in the PFPs described here indicates the presence of cholesterol at these sites. The second assumption is that nothing about photoreceptor membranes will prevent either the interaction of filipin with any cholesterol present or the subsequent perturbations of the membrane,

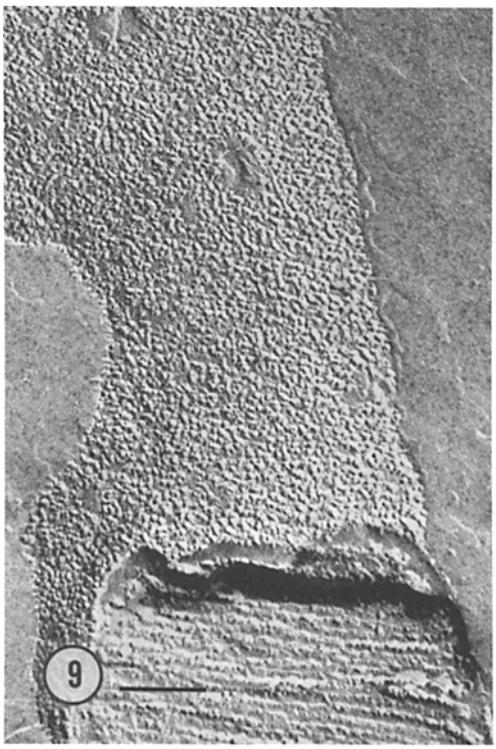


FIGURE 9 Freeze-fracture replica showing the *P* face of the plasma membrane of a mouse rod outer segment from a retina which had been exposed to saponin. Note the absence of particle-free patches. Bar, 0.2 μm. × 57,000.

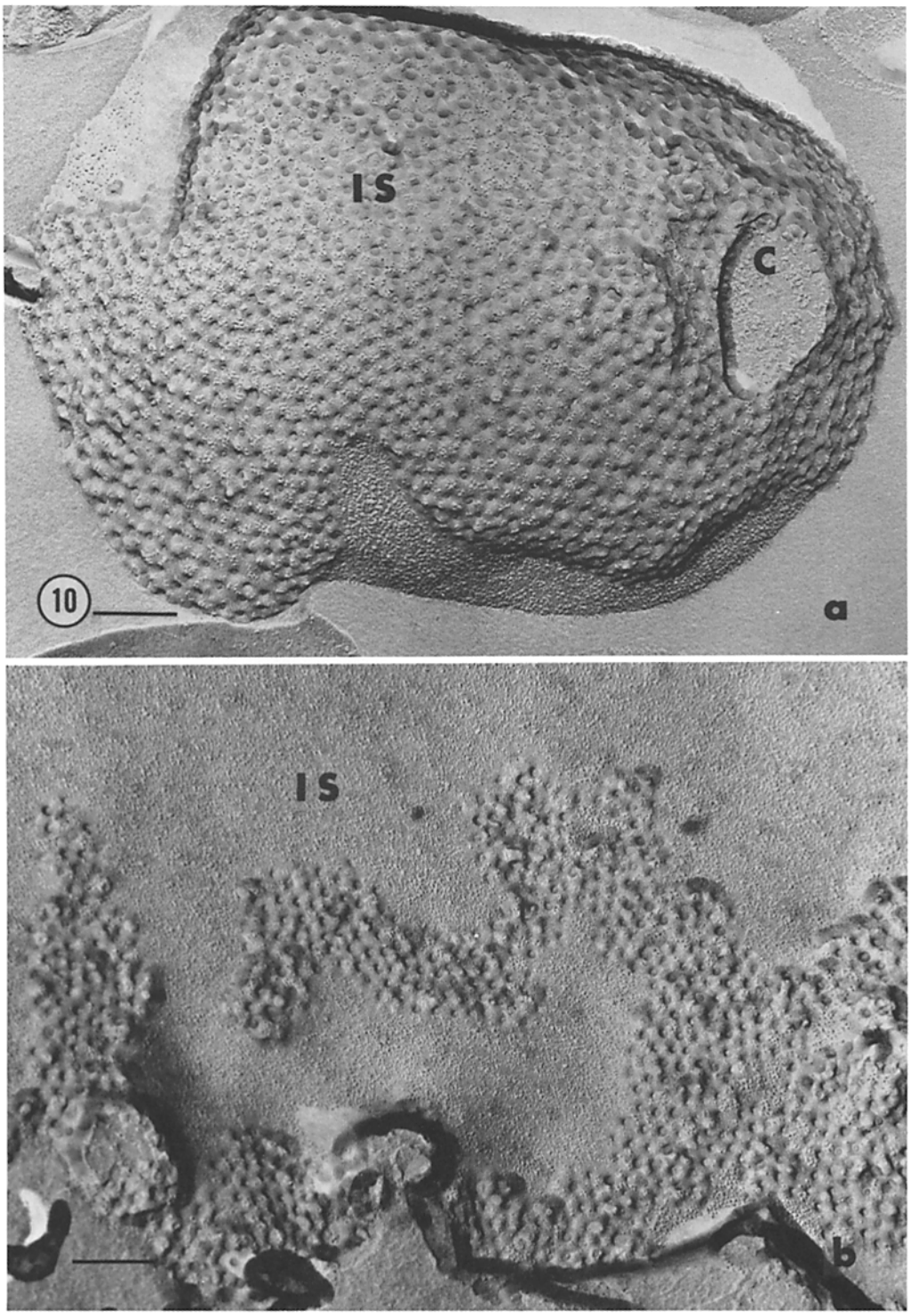


FIGURE 10 Freeze-fracture replicas of the apices of rod inner segments (*IS*) of a (*a*) mouse and (*b*) frog from retinas exposed to filipin. Note the cross-fracture of the cilium (*C*) in the somewhat oblique view of the mouse IS. It may be seen that in both cases membrane particles are interspersed among filipin-induced pits. Bars, 0.3 μm. × 42,000.

identifiable in replicas as filipin-induced pits. That this series of events is not in general confined to particle-free membrane areas is demonstrated by the observation of intercalated filipin-induced pits and membrane particles (which may not be identical to those in the OS) in the inner segment plasma membranes of both mouse and frog rods (Fig. 10). While steric or other restrictions of filipin reactivity confined to the densely particulate areas of outer segment membranes cannot be ruled out, if the second assumption is true then it implies that the confinement of filipin-induced pits to PFPs in mouse and frog ROS indicates a preferential concentration of cholesterol at these sites. This conclusion must be regarded as tentative at this time. One cannot be certain that filipin had access to the isolated mature disks whose membrane is not exposed to extracellular space.

Before starting the current investigation, we knew that particle-free patches occur in artificial membranes containing purified rhodopsin in a number of circumstances: (*a*) if the membrane lipid only consists of phosphatidylcholines (PCs) and is brought below its phase transition temperature before freeze-fracture (16) (this is an example of the widely reported, temperature-induced, lateral phase separation type of patching); (*b*) if the membrane contains *E. coli* phosphatidylethanolamines (PEs) as well as PCs (36); (*c*) if the recombinant membrane lipid is the presumably cholesterol-free polar lipid extracted from bovine ROS (36); and (*d*) if cholesterol is added to 1-stearoyl-2-oleoylphosphatidylcholine (mole ratio 3:1 phospholipid:cholesterol) as the lipid components of the membrane (the latter component by itself produces a random particle distribution) (37).

Persistence of PFPs in mouse retinas incubated and fixed at up to 80°C does not exclude the possibility that they represent a separate phase composed, in part, of (gelled) phospholipids below their phase transition temperature. If the lipid composition of mouse ROS closely resembles that of rat ROS, as seems likely, then examination of data on the latter (3) leads to the expectation that ~1% of the phospholipids present could be distearoylphosphatidylethanolamine (di 18:0 PE), which has a melting temperature of ~85°C (45). R. E. Anderson has failed to detect any di 18:0 PE in frog ROS (personal communication), but he points out that as much as 0.5% could be present and have escaped detection. Because the plasma membrane represents only ~3% of the total membrane of the mouse ROS, and because PFPs represent ~16% of the *P* face of the plasma membrane (at 37°C), then patches comprise only ~0.5% of the total ROS membrane. Saibil et al. (64) estimate from their neutron diffraction data that protein accounts for from 20 to 35% of the hydrophobic volume of OS membrane and is distributed about equally in the inner and outer leaflets, so the maximum estimated proportions of di 18:0 PE possibly present and the area of OS membrane represented by PFPs are roughly comparable. However, insofar as the behavior observed in simplified model systems may be applied to lipids in complex biological membranes, the presence of cholesterol in PFPs as indicated by the filipin binding can be used as an argument that the PFPs are unlikely to simply represent areas of gelled di 18:0 PE. First, as discussed above, disk lipids of outer segments are predominantly in a highly fluid state, and this is likely to be true for the highly particulate regions of ROS plasma membrane. Second, by differential scanning calorimetry (68), cholesterol has been shown to preferentially partition in the fluid portion of artificial membranes composed of either various PCs or PEs. Further, in mixtures of PEs and PCs, cholesterol showed clear preference for PCs in that it prevented the phase transitions of PCs whether, because of their fatty acids, PCs were the mixture component with the highest or lowest melting point. Because ROS membranes contain 40–50% PCs (1), gelled di 18:0 PE would not represent an environment in which cholesterol would be likely to be found.

The above argument, however, which emphasizes evidence that rhodopsin (16) and cholesterol (68) preferentially partition into relatively fluid regions of artificial membranes under a variety of circumstances, means that the indicated presence of cholesterol in PFPs which are putatively less fluid than their surround requires some special explanation. There are two hypotheses, one suggested by Hubbell's work (37), which propose mechanisms to explain the presence of cholesterol in less fluid PFPs.

The first hypothesis is that when cholesterol molecules enter the membrane they form complexes with phospholipid molecules (perhaps one or two per cholesterol), and, further, that these complexes aggregate and exclude protein, thus forming PFPs. This hypothesis is given some support by the observation that the PFPs of mouse ROS plasma membranes can be made to disap-

pear by exposure to saponin. The influence of cholesterol on the formation of PFPs has been examined in other systems, and has been found to differ greatly. Verkleij et al. (70) found that rounded PFPs appeared in erythrocyte plasma membranes after fusion with an abnormal serum lipoprotein (LP-X) containing equimolar cholesterol and lecithin (and only ~6% protein). Duppel and Dahl (23), however, found that the addition of cholesterol prevented the appearance of PFPs otherwise observed in rat liver microsomes precooled before freeze-fracture.

The second hypothesis is that cholesterol merely partitions into PFPs rather than participates importantly in their formation. This might be expected if it is assumed that PFPs are especially rich in sphingomyelin, because sphingomyelin is found in ROS membranes (1) and cholesterol apparently has an affinity for sphingomyelin even greater than that for relatively fluid membrane areas. This is suggested by the observations of Demel et al., using differential scanning calorimetry (21) that cholesterol abolished the phase transition of sphingomyelin in preference to other components in lipid mixtures in which sphingomyelin was the component with the highest melting temperature.

The observation of PFPs in the basal disks of outer segments in rods of mice and frogs, and of their absence in older disks farther from the inner segment, indicated that in at least some species these disk membranes undergo compositional changes as they begin their displacement toward the pigment epithelium from the site of their assembly at the base of the outer segment. The increase in the proportion of disk area represented by densely packed particles and the progressive elimination of particle-free patches together suggest an increase in the diskal protein:lipid ratio in the process of disk formation in mature animals. This parallels the model of the initial development of disk structure formulated by Olive and Recouvreur (56) based upon their observation of an increase in particle density and a decrease in bare areas within the disks of developing photoreceptors of postnatal mice. However, Besharse and Pfenninger (7) did not report seeing any particle-free patches in either forming disks or the plasma membrane of the outer segments of rods of *Xenopus*. Remodeling the protein-lipid distribution of the disk requires that the former PFP-associated components are either (*a*) dispersed among the particles, (*b*) displaced out to the plasma membrane at points of continuity, possibly as particles are inserted, or (*c*) replaced in favor of particle-associated lipid components. Such remodeling processes are reasonable to hypothesize because lipid components have been shown to exchange freely in and out of ROS membranes (8, 9). However, as noted previously, Hong and Hubbell (36) observed PFPs in artificial membranes composed of purified rhodopsin recombined with polar lipids extracted from isolated bovine ROS. Because this is primarily diskal lipid, their observation supports the hypothesis that dispersed within the normal disk are components capable of forming PFPs. It is quite possible that all three processes of dispersal, displacement, and replacement of PFP components are involved in eliminating the particle-free patches as disks move up the outer segments with time. Kaplan et al. (40) have reported birefringence measurements in isolated frog ROS which are consistent with the results presented here. Their data were interpreted as indicating a decrease in the fraction of the ROS volume that is membrane with increasing distance from the inner segment. In conjunction with a calculated increase in bulk membrane refractive index, they hypothesized that a loss of lipids occurs with disk "aging"; this could correspond to the patch displacement and/or replacement hypothesized above. They observed a decrease in intrinsic birefringence with increasing distance from the inner segment, with most of the change occurring in the basal $^1/_3$–$^1/_2$ of the OS. The change in membrane volume fraction was too small to account for all of this, and, among possible sources they suggested for the remainder, they included "concentration gradients of membrane constituents such as cholesterol or retinal." This would concur with the lipid remodeling hypothesized above.

The ability to study possible segregations of lipid components in the plane of the membrane would appear to be particularly valuable in view of the growing appreciation of the functional importance of lipid physical state in regulating membrane proteins (41). In attempting to establish correlations among lipid composition, lipid physical state, and membrane-associated enzyme activity, it is clear that attention must be directed toward increasing the resolution of this information beyond whole membranes to defined membrane regions. Freeze-fracture observations of lesions induced by filipin have several attractive features in this regard. With freeze-fracture, in

contrast to thin sections, large areas of membrane can be observed at somewhat higher resolution than with current scanning electron microscopy. Filipin has high reaction specificity and produces such easily recognized lesions that positive results are readily interpretable. Two abstracts have recently appeared describing the independent development of techniques employing filipin and saponins to localize membrane cholesterol (24, 28). A recent paper from the same group describes the use of digitonin and freeze-fracture to identify cholesterol-containing regions of various membranes (25). While the use of saponins suffers the relative disadvantages that saponins are somewhat less specific than filipin and, as pointed out by the authors and suggested by our results, tend to solubilize the membrane cholesterol being studied, it has the important advantages of the saponins' ready availability and stability.

This study was funded by grant EY-00258 (A. I. Cohen) of the National Institutes of Health. Lary Andrews was supported by the above grant, the Division of Biology and Biomedical Sciences of Washington University, and the Program for Training in Vision Research (EY-00092) of the National Institutes of Health.

Received for publication 28 August 1978, and in revised form 27 November 1978.

Note Added in Proof: When *R. pipiens* from Mexico were seasonably unavailable we recently examined freeze-fracture replicas of retinas from *R. pipiens* of northern U.S. origin and from *Bufo marinus* from the U.S. southwest. While in both cases PFPs were observed in basal but not mature disks, they were not found in the ROS plasma membrane. Preliminary results nevertheless revealed filipin binding in both densely particulate plasma membranes. Filipin binding is also observed in disks, but we have not yet established with certainty if this is confined to basal disks and to their PFPs.

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