The Photoreceptors of Diurnal Squirrels: Outer Segment Structure, Disc Shedding, and Protein Renewal[1]

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The photoreceptor outer segments of five species of diurnal squirrels were studied by light and electron microscopy. In all species rod outer segments could be distinguished from cone outer segments by the extent of disc–membrane continuity and by their pattern of protein renewal as determined by autoradiography. Rods showed the characteristic band of radioactive protein within the outer segments, while the cones showed only a diffuse pattern of outer segment labeling. Rod as well as cone outer segments were found to shed packets of discs intermittently. The shedding process appeared to be qualitatively similar in rods and cones. Multiple invaginations of the outer membrane, probably signifying the assembly of new discs, were detected at the base of rod outer segments. Single partially formed invaginations, analogous to those found in rods, also were found at the base of cone outer segments. Thus, cone as well as rod outer segments may assemble and shed discs in the mature animal.

From those interested in the vertebrate visual system, certain species of diurnal squirrels have received uncommon attention primarily because historically they were known as the only mammals to possess pure-cone retinas *(39)*. This generalization lately has been shown to be incorrect due to the discovery of a minority population of rods (from 4–40%) in the retinas of several squirrel species *(8, 15, 20, 40)*. We recently reported that *both* rods and cones in the tree squirrel retina shed outer segment discs *(1, 2)*, although the pattern of protein renewal in these outer segments is identical to that found in other vertebrate photoreceptors where disc shedding has been assumed to occur only in rods *(44)*. In order to augment these findings, we undertook a comparative study of outer segment structure, disc shedding, and protein renewal in five species of arboreal and terrestrial squirrels.

Vertebrate rods continuously engage in both the synthesis and disposal of the discs which comprise the outer segment of the cell. These discs are assembled at the base of the outer segment by a process that includes the invagination of the outer cell membrane which encloses the disc stack *(29, 36)*. The molecular constituents of these discs are synthesized mainly within the myoid region of the inner segment, and then are transported to the outer segment base for final assembly into new membrane *(47)*.

Autoradiographic studies of protein renewal by light and electron microscopy show that a band of radioactive material, oriented at right angles to the length of the photoreceptors, can be identified at the base of rod outer segments within hours after the administration of [³H]amino acids *(42)*. This material is thought to be newly synthesized protein delivered from the myoid region of the cell for use in the assembly of new rod discs *(48)*. In the tree squirrel retina *(Sciurus carolinensis)*, we recently demonstrated that the rod outer segments show this characteristic accumulation of radioactive protein *(2)*. In rods, the band of radioactive protein is progres-

sively displaced from the outer segment base at a rate that varies with the species involved *(22, 42, 44)*, the temperature and illumination levels at which the organism is kept *(42)*, the type of rod involved *(42, 48)*, and with the developmental stage of the organism *(22)*. In tree squirrel rod outer segments, the band of labeled protein is displaced from the outer segment base at the rate of 2.4 μm/day.[2] In the disposal phase of the process, discs that have reached the apex of the outer segment are intermittently shed in packets and are then phagocytosed by the pigment epithelium *(45, 49)* where they are subsequently digested *(17, 37, 49)*.

Similar protein renewal studies in vertebrate cones reveal a pattern of incorporation differing from that in rods. Rather than being localized as a distinct band, the radioactive protein is diffusely distributed throughout the cone outer segment.[3] At no time after the injection of [3H]amino acids was there a band of radioactive protein detected at cone outer segment bases in frog *(43)*, salamander *(44)*, or rhesus monkey *(46)* retinas. This absence was taken as evidence against the production of new discs in cones, and by implication, in the shedding of old discs *(44)*. Moreover, electron microscopic observations of the pigment epithelium in several submammalian species that possess all-cone or predominantly cone retinas showed no ingested disc packets or phagosomes *(5, 17)*. However, we have observed evidence of disc shedding from the cone outer segments of tree squirrel photoreceptors which, after the incorporation of [3H]-amino acids, show the usual diffuse pattern of renewal *(2)*. And Hogan *et al.* *(16)* presented electron microscopic evi-

dence for the phagocytosis of human cone outer segment discs, suggesting that the phenomenon may be present in other vertebrate cones as well.

In this report we present our observations on the photoreceptors of two species of arboreal squirrels (genus *Sciurus*) and three species of terrestrial or ground squirrels (genus *Spermophilus*). These observations lead us to conclude that the outer segments of squirrel cones, like their rod counterparts, may assemble as well as shed outer segment discs.

METHODS

Animals

The tree squirrels used in the investigation included the western gray squirrel *(Sciurus griseus)* and the eastern gray squirrel *(Sciurus carolinensis)*. The terrestrial species included the Mexican *(Spermophilus mexicanus)*, 13-lined *(Spermophilus tridecemlineatus)*, and California *(Spermophilus beecheyi)* ground squirrels. Observations were made on the retinas of two western gray squirrels, four eastern gray squirrels, two California ground squirrels, one Mexican ground squirrel, and one 13-lined ground squirrel.

Procedure

Electron microscopy. All squirrels were anesthetized with a lethal dose of sodium pentobarbital (Nembutal, 50 mg/ml, Abbott). Two methods of fixation were employed. In the first method, the eyes were enucleated and the anterior structures were removed. The posterior portion was immersed in 2.5% glutaraldehyde in sodium cacodylate buffer (0.067 M sodium cacodylate and 0.05% $CaCl_2$ in aqueous solution, pH 7.4) for 1.5 hr. After the initial aldehyde fixation, the tissue was briefly washed in buffer solution and then postfixed in 2% osmium tetroxide in veronal acetate buffer (pH 7.4) for an additional hour. The tissue was then washed in distilled H_2O, dehydrated in a graded ethanol–H_2O series, and transferred to propylene oxide. Tissue specimens of about 5 mm in diameter were embedded in Araldite (Cargille 6005). In the second method, retinas were fixed by intracardiac perfusion of the glutaraldehyde fixative or a mixture composed of 1% glutaraldehyde, 1% paraformaldehyde, and 0.04% picric acid in the same buffer (modified from *18*). Animals were perfused with 300–400 ml of fixative at a pressure of 90 mm Hg. The eyes were then enucleated and immersed in the perfusate for 1.5 hr at 4°C. The rest of the procedure remained unchanged. Fixation by immersion resulted in a

[2] These figures are corrected values for those in Anderson and Fisher *(2)* where the rate of displacement and disc synthesis were erroneously reported to be 1.6 μm/day and 65 discs/day, respectively.

[3] There is a small amount of diffusely distributed radioactive protein in rod outer segments as well *(4)*.

characteristic and highly ordered rippling artifact in cone outer segment discs (see 8, 40), and in the formation of numerous small vesicles at the base of cone outer segments (see Fig. 1). Fixation by perfusion eliminated the rippling artifact, the vesicles, and generally gave superior results.

Sections were cut on a Porter–Blum MT-2B ultramicrotome. One-micron sections were stained with a 1:1 solution of 1% methylene blue and 1% Azure II, or with a saturated aqueous solution of p-phenylenediamine. Thin sections (600–700 Å) were placed on either bar or mesh grids, stained with 1% aqueous uranyl acetate for 20 min, followed by lead citrate for 10 min, and then were examined in either a Siemens Elmiskop IA or 101 electron microscope.

Autoradiography. Two male eastern gray squirrels (500 and 1000 g) and one male 13-lined ground squirrel (300 g) were injected intraperitoneally with 40 mCi of L-[4,5-^{3}H]leucine (sp act 51 Ci/mmole; 389 Ci/mg; Amersham Searle) dissolved in 2 ml of H_2O. At 24 and 72 hr after injection, the gray squirrels were anesthetized and fixed by intracardiac perfusion of 2.5% glutaraldehyde. Forty hours after injection, the 13-lined squirrel was anesthetized and perfused with the aldehyde–picric acid mixture.

Autoradiograms were prepared according to the method described by Young (46). One-half micron sections were placed on precleaned glass slides and dipped into a 1:1 aqueous solution of Kodak Nuclear Track Emulsion (NTB-2) maintained at 40°C. The dipped slides were transferred to light tight boxes and exposed from 8–16 weeks at 4°C. Slides were developed for 2 min in Dektol (17°C) and placed in acid fixer for 5 min. After washing in distilled H_2O, the autoradiograms were stained with p-phenylenediamine.

Due to the small diameter of the photoreceptor outer segments (1–2 μm), the best results for black-and-white reproduction were obtained using phase contrast optics in conjunction with a blue-absorbing filter (Kodak Wratten 15). Ektapan negatives (4 × 5) of autoradiograms were taken using a Zeiss Ultraphot II light microscope.

RESULTS

General Features of the Photoreceptors

In general, the structure of the photoreceptors in different species of squirrels is similar. Figures 1 and 2 show some of the basic characteristics of the photoreceptors in arboreal and terrestrial species. The photoreceptors have short (7–15 μm), cylindrical outer segments, the tips of which extend into or close to the apical surface of the pigment epithelium and its processes. Two types of pigment epithelial processes

can be readily identified in electron micrographs. Villous processes, containing melanin granules oriented parallel to the longitudinal axis of the photoreceptors, extend down from the apical pigment epithelium to interdigitate between the outer segments (Fig. 2). These processes occupy the interreceptor spaces and extend down to the bases of the outer segments (Fig. 2). The other type of processes are more slender extensions, typically without large numbers of pigment granules, lying in close apposition to the outer segments (Figs. 14, 18, 19, 22). The inner segments of the photoreceptors are somewhat larger in diameter than the outer segments. The ellipsoid region is barrel-shaped and filled with mitochondria. Calycal processes from the inner segments extend up along the lateral margins of the outer segments (Fig. 2).

Tree Squirrel Photoreceptors

The photoreceptors of eastern and western gray squirrels are essentially alike. Both species possess the characteristic double-tier arrangement where rods are displaced vitreally from cones (8). This displacement is most noticeable at the level of the inner segments which are segregated into two distinct rows—the rods forming the inner, more vitread row (Fig. 1). Similarly, there are two rows of photoreceptor nuclei, the more vitread row belonging to the rods. The tips of the cone outer segments adjoin the apical membrane of the pigment epithelium, while the rods contact slender pigment epithelial processes which "cap" their tips (Fig. 1). Thus, the segregation of photoreceptor types by tiers appears at the levels of pigment epithelial–outer segment junctions, inner segments, and the receptor nuclei.

Cone outer segments are approximately 7–10 μm in length. Rod outer segments may reach 15 μm, but average about 10–12 μm. These figures are approximately the same for both species, although the rods may be slightly longer in the eastern spe-
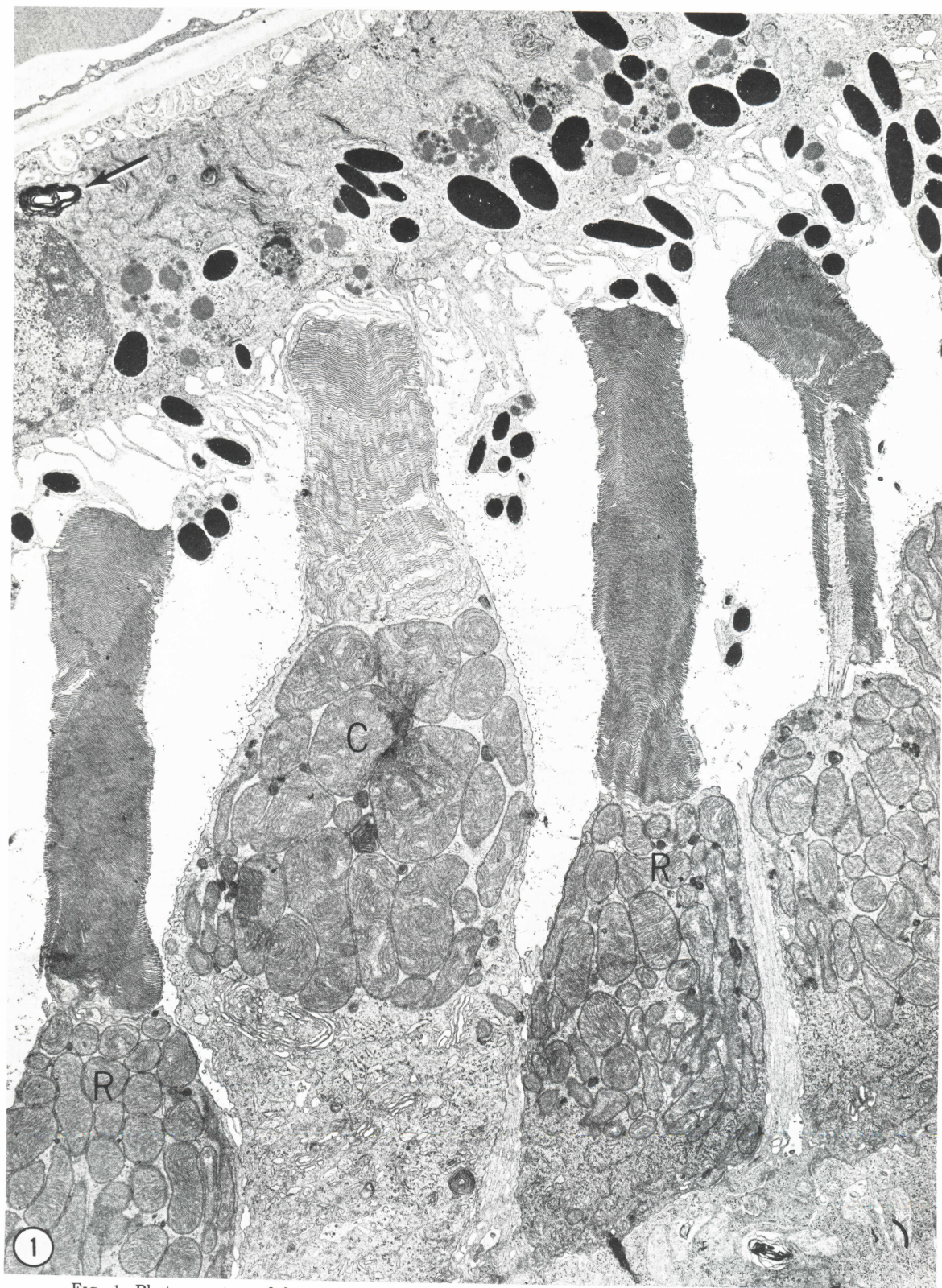
Fig. 1. Photoreceptors of the western gray squirrel. The rods (R) are displaced vitreally with respect to the cones (C). Note the characteristic pigment epithelial contacts by the photoreceptors and the rippling artifact which appears in the cone outer segments fixed by immersion. An electron-dense phagosome (arrow) which has retained its lamellar substructure appears in the basal zone of the pigment epithelium. × 7800.
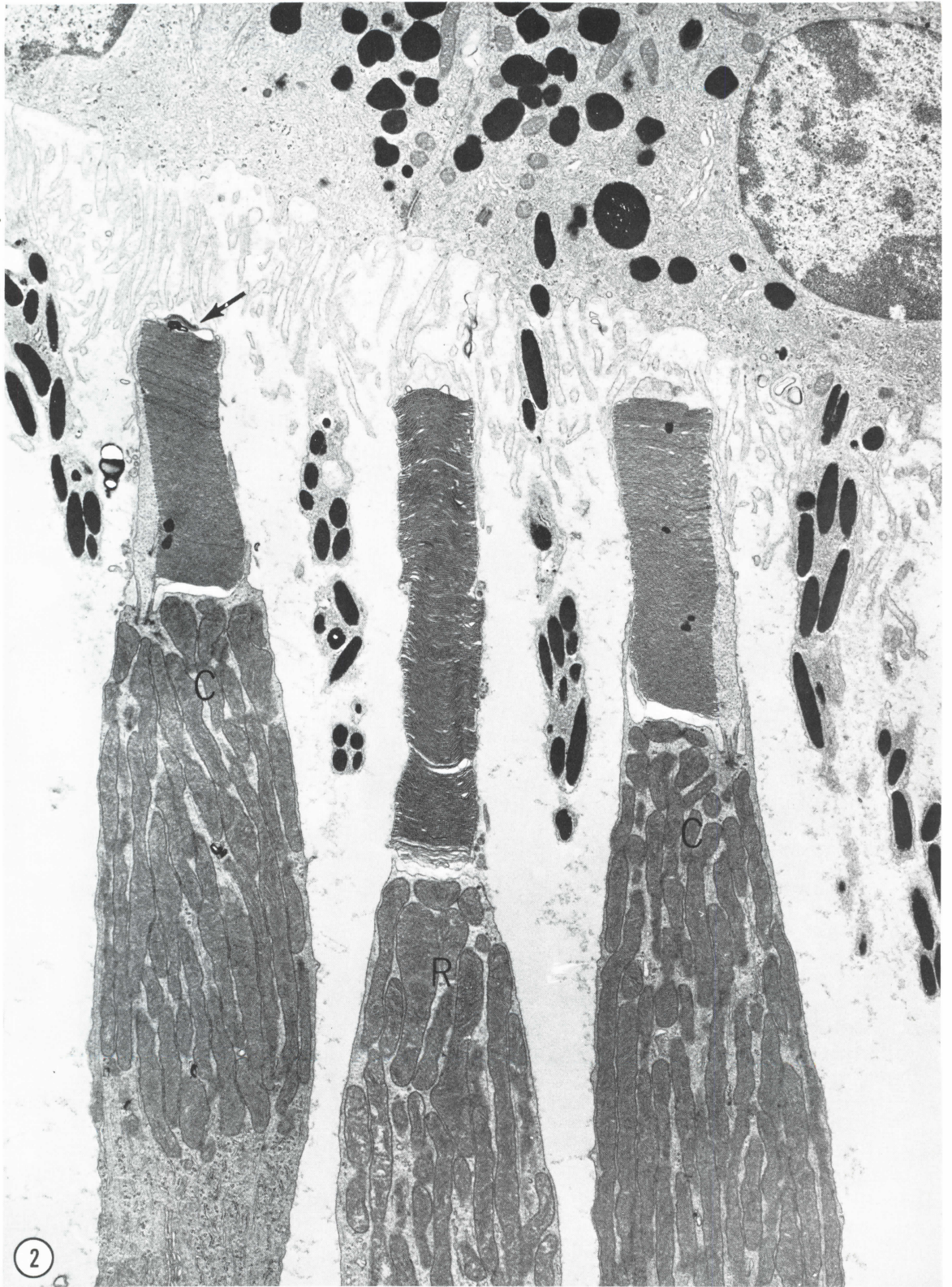
Fɪɢ. 2. Photoreceptors of the 13-lined ground squirrel. The rods (R) are similarly displaced, but not to the same extent as in the gray squirrel. Both rods and cones (C) contact the pigment epithelium in the same manner. Processes filled with melanin granules occupy the interphotoreceptor space. The arrow indicates discs in the initial phase of shedding from a cone outer segment. × 7200.
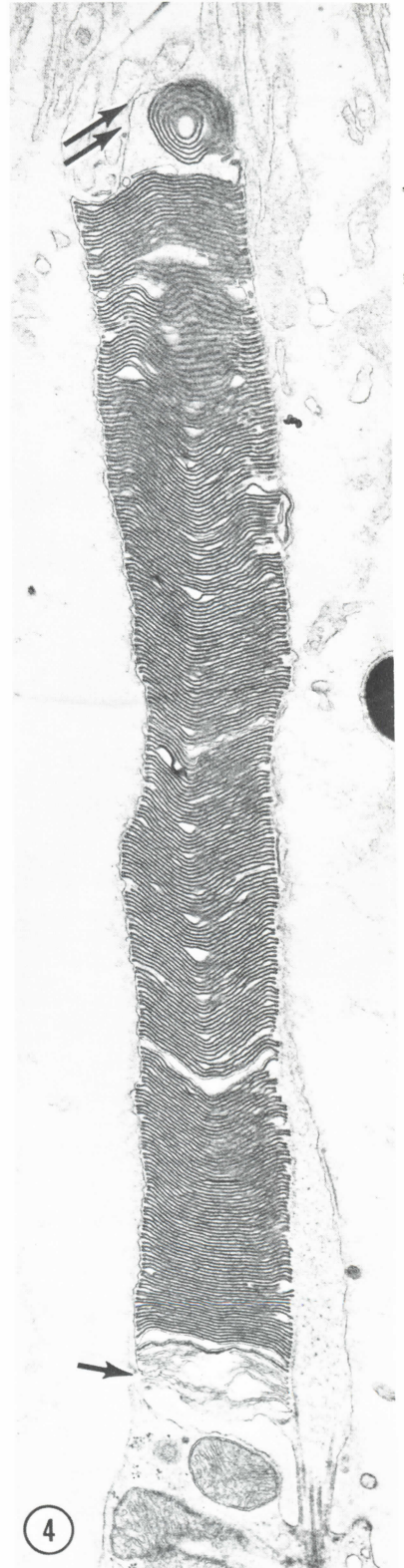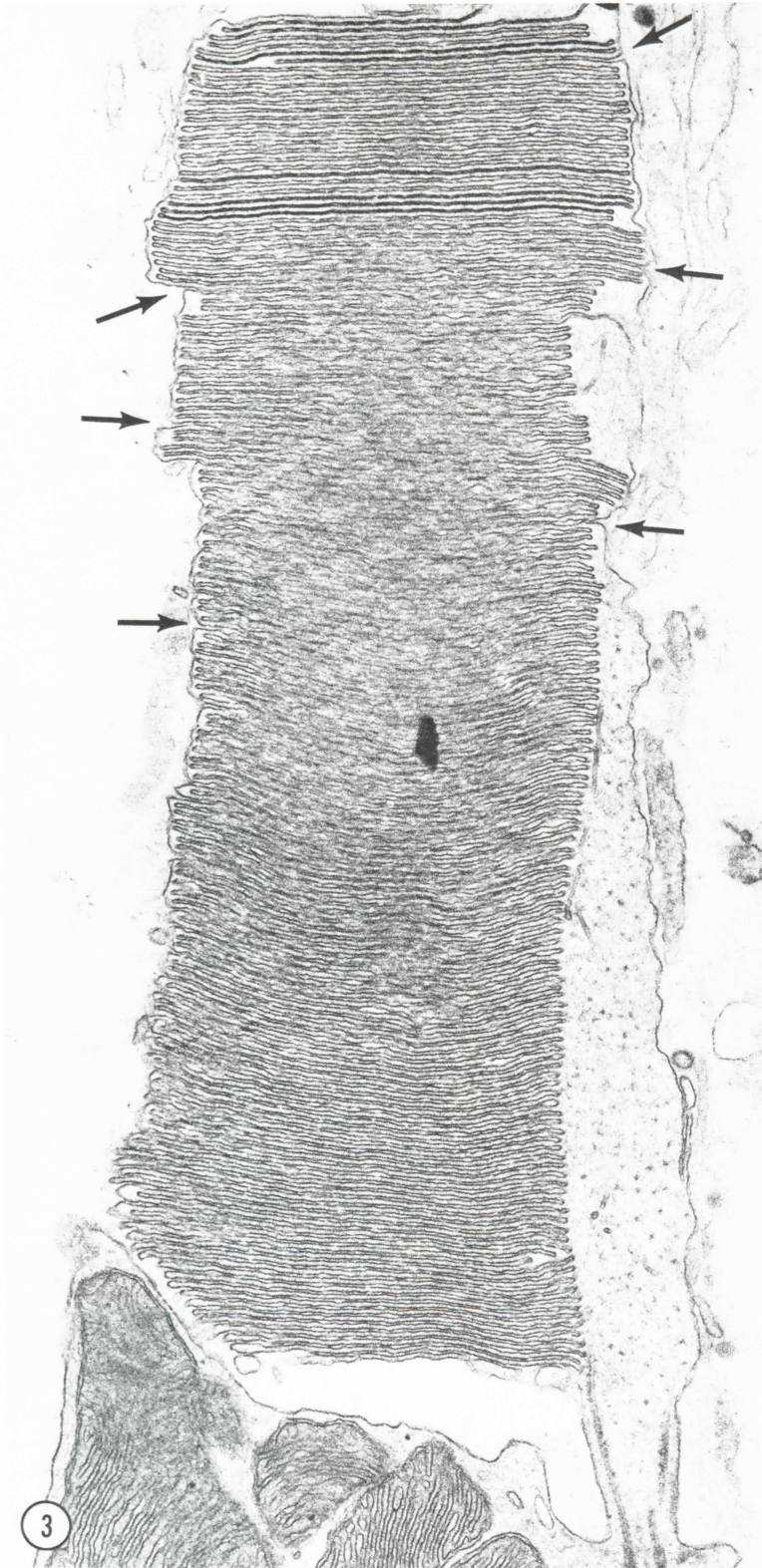
cies. In cone outer segments, continuities between disc membranes and the outer cell membrane are clearly seen in the basal one-third or less of the outer segments, but are not apparent at more distal locations (2, 8). Disc–membrane continuities are not seen in the rods of either species except at the extreme base of the outer segments (Fig. 43).

Differences in electron density which have been reported to exist between the rods and cones in the eastern gray squirrel retina were not evident in our material (40). However, the photoreceptors from several of the ground squirrel's retinas, fixed by the same techniques, do show differences in cytoplasmic density (20), and these differences could be used to distinguish between rods and cones (Fig. 9).

Ground Squirrel Photoreceptors

In the ground squirrel retinas, both rods and cones are present although the rod/ cone ratio is much lower than in the tree squirrels (see 20, 40). Cone outer segments, about 7–9 μm long and 1.5 to 2 μm wide, are approximately two-thirds the length of rod outer segments. The inner segments are less barrel-shaped than counterparts in the tree squirrels, and there is much less displacement of rod and cone inner segments. Although rod nuclei appear only in the more vitread tier of the outer nuclear layer, cone nuclei occupy positions in both tiers. Unlike the tree squirrels, cone and rod outer segments in the ground squirrels show a similar kind of pigment epithelial contact (Fig. 2). Thus, the segregation of photoreceptors by tiers is much less prominent in the ground squirrels than in the tree squirrels.

Disc–membrane continuities in ground squirrel cone outer segments are more prevalent than they are in tree squirrels. Such continuities occur most frequently in the basal one-half of cone outer segments, but they occasionally can be identified even at the outer segment tip (Fig. 3). The basal discs in cones form an interconnecting network and are continuous with the connecting ciliary membrane (Fig. 3, 46, 53), just as they are in the tree squirrels. At more distal locations from the base there are increasing numbers of independent discs (Fig. 3). Beyond the midpoint of the cone outer segments, the majority of discs are seen as separate from the outer cell membrane in single thin sections.

As is the case in the tree squirrels, rod outer segment discs in the ground squirrels are seen as "free-floating" units (Fig. 4). We have no indication of continuities between the discs and enclosing outer membranes except at the extreme outer segment bases (Figs. 40–42, 44–45).

Protein Renewal in Squirrel Photoreceptors

The photoreceptors of diurnal squirrels conform to the pattern of protein renewal typically found in the rod and cone outer segments of other vertebrates. Rod outer segments in the eastern gray squirrel accumulate radioactive protein at the outer segment bases 24 hr after the administration of [³H]leucine (Fig. 5). After 72 hr the band of radioactive protein is displaced a mean distance of 4.8 μm from the outer segment base, a distance which is close to one-half the length of rod outer segments (Fig. 6). By contrast, cone outer segments are diffusely labeled over their entire length at both 24 and 72 hr (Figs. 5 and 6). The pigment epithelium (and its proc-

FIG. 3. Cone outer segment from the 13-lined ground squirrel. The basal discs appear to consist of one uninterrupted network. At more distal locations many discs are separate from the cell membrane, although occasional disc–membrane continuities occur in the upper one-half of the outer segment (arrows). The connecting cilium is filled with small electron-dense granules of unknown origin or composition. × 36 000.

FIG. 4. Rod outer segment from the 13-lined ground squirrel. The discs are seen as "free-floating" units within an enclosing cell membrane, except at the extreme base of the outer segment (arrow) where the cell membrane folds inward and is continuous with the most basal discs. A folded disc packet is visible at the outer segment tip and is enclosed within the rod cell membrane (double arrows). × 18 000.
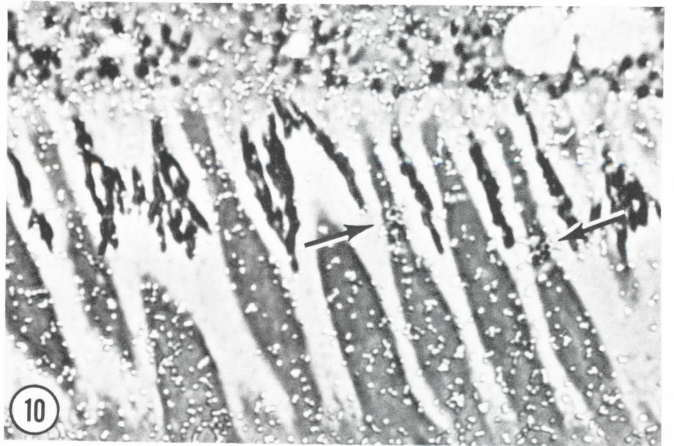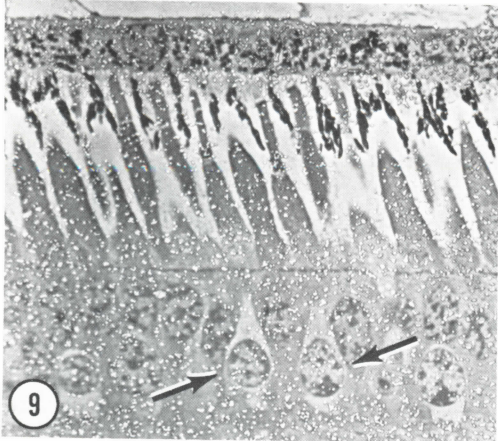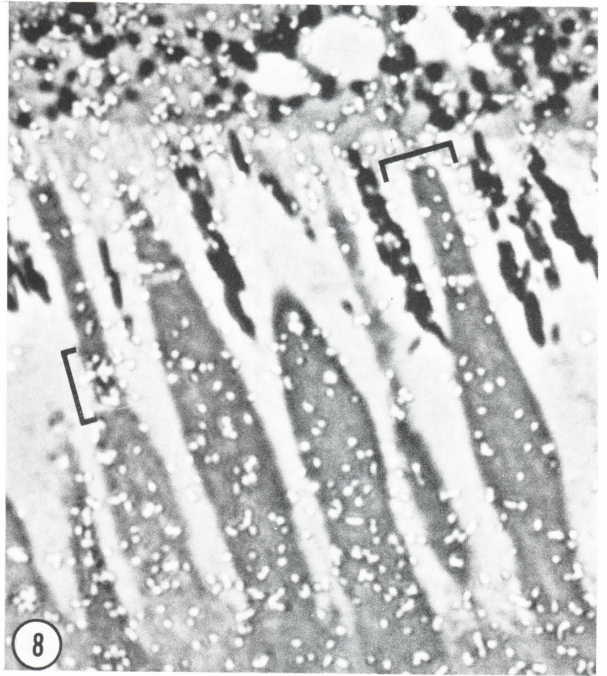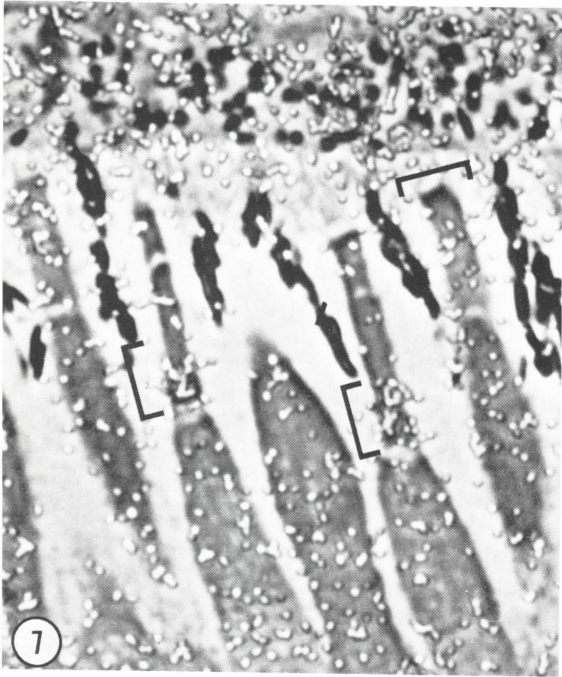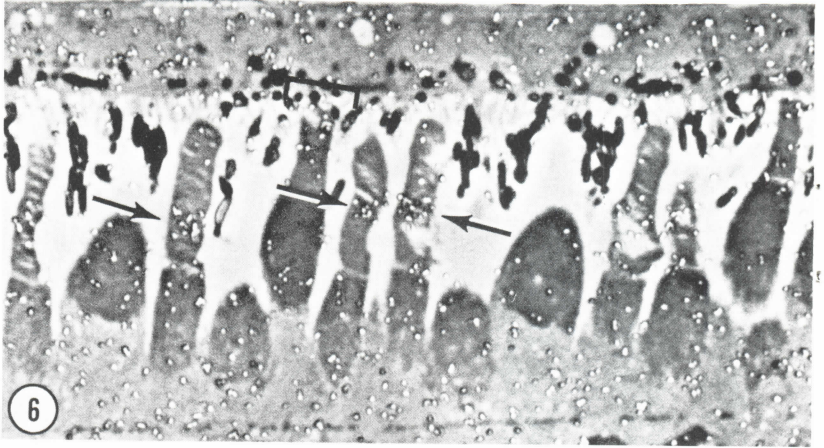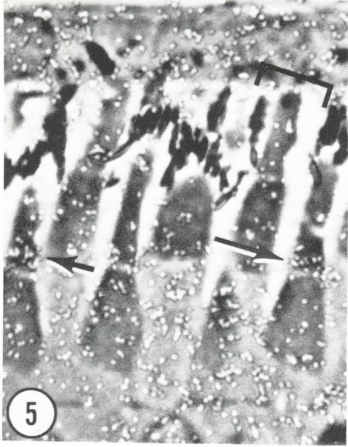
esses), the inner segments, the photoreceptor nuclei, and the terminal cytoplasm are all labeled (Figs. 5–10) as is the rest of the retina. However, no distinct concentration of radioactive material could be found at any other location. Rod and cone outer segments in the 13-lined squirrel also showed the expected labeling patterns. In rod outer segments the band of radioactive protein is displaced 1–2 μm from the outer segment base after an interval of 40 hr (Figs. 7–10). Cone outer segments, on the other hand, are diffusely labeled over their entire extent (Figs. 7 and 8). At distal locations from the base of rod outer segments, there is some evidence of diffuse labeling but its intensity is low. Very little background labeling occurs in the extracellular space between the photoreceptor outer segments.

Shedding of Outer Segment Discs

Rod outer segments. In all of the species examined, discs are shed in packets from the rod outer segment tips. Packets of up to 30 discs typically curl upward at their lateral edges toward the apical pigment epithelium (Figs. 11–13). Occasionally we observed one or two discs in the process of curling but such observations were the exception rather than the rule. Disc packets may curl upward from one side of the outer segment, in which case the discs are seen as folded over upon themselves (Figs. 12 and 15), or the curled discs may form a U-shaped structure with the open end facing the pigment epithelium (Figs. 13 and 16). In a few instances, a group of discs curled toward rather than away from the main body of the outer segment — such curling appears to take place before any major deformations can be seen in the enclosing outer membrane (Figs. 11 and 13).

By a process which is still not understood, the folded disc packets detach from the rod outer segments, and photoreceptor cytoplasm fills in the space formerly occupied by the disc's lateral edges (Figs. 11 and 13). The cell membrane may then invaginate to separate the folded discs from the rest of the outer segment, or alternatively, invagination of the cell membrane may be initiated by inward penetration of the pigment epithelial processes which then pinch off the folded discs (37). Once the disc packet detaches from the rest of the outer segment, it is enclosed within a membrane which is presumably formed from the rod cell membrane. In tree squirrel rods, detached disc packets can sometimes be identified within the processes which compose the pigment epithelial "cap" (Figs. 18 and 19). It is these processes which presumably transport the detached packets from the rod outer segment tips to the pigment epithelial interior. At this stage the detached packets may appear compressed and/or electron dense (Figs. 17 and 18), but they usually do not show the distortion of the disc membranes which

Figs. 5–10. Light microscope autoradiograms of squirrel photoreceptors. Phase contrast optics.

Fig. 5. Eastern gray squirrel photoreceptors 24 hr after the injection of [^{3}H]leucine. A band of radioactive protein is visible at the base of rod outer segments (arrows). Cone outer segments (bracket) are diffusely labeled over their entire length. × 950.

Fig. 6. Eastern gray squirrel photoreceptors 72 hr after injection of [^{3}H]leucine. The band of radioactive protein is displaced about 5 μm from the rod outer segment base (arrows); cone outer segments (bracket) remain diffusely labeled. × 950.

Figs. 7, 8. Autoradiograms of 13-lined ground squirrel 40 hr after the injection of [^{3}H]leucine. A band of radioactive protein appears about 1–2 μm from the base of rod outer segments (vertical brackets). Cone outer segments are diffusely labeled (horizontal brackets). × 1700.

Fig. 9. Low power view of the photoreceptors shown in Fig. 7. Rod outer segments shown in Fig. 7 connect with terminals containing the "pale" cytoplasm (arrows) that identifies them as rods (see text and 38). × 350.

Fig. 10. Photoreceptors from the 13-lined ground squirrel. A slightly displaced band of radioactive protein is visible in two more rod outer segments (arrows). × 950.
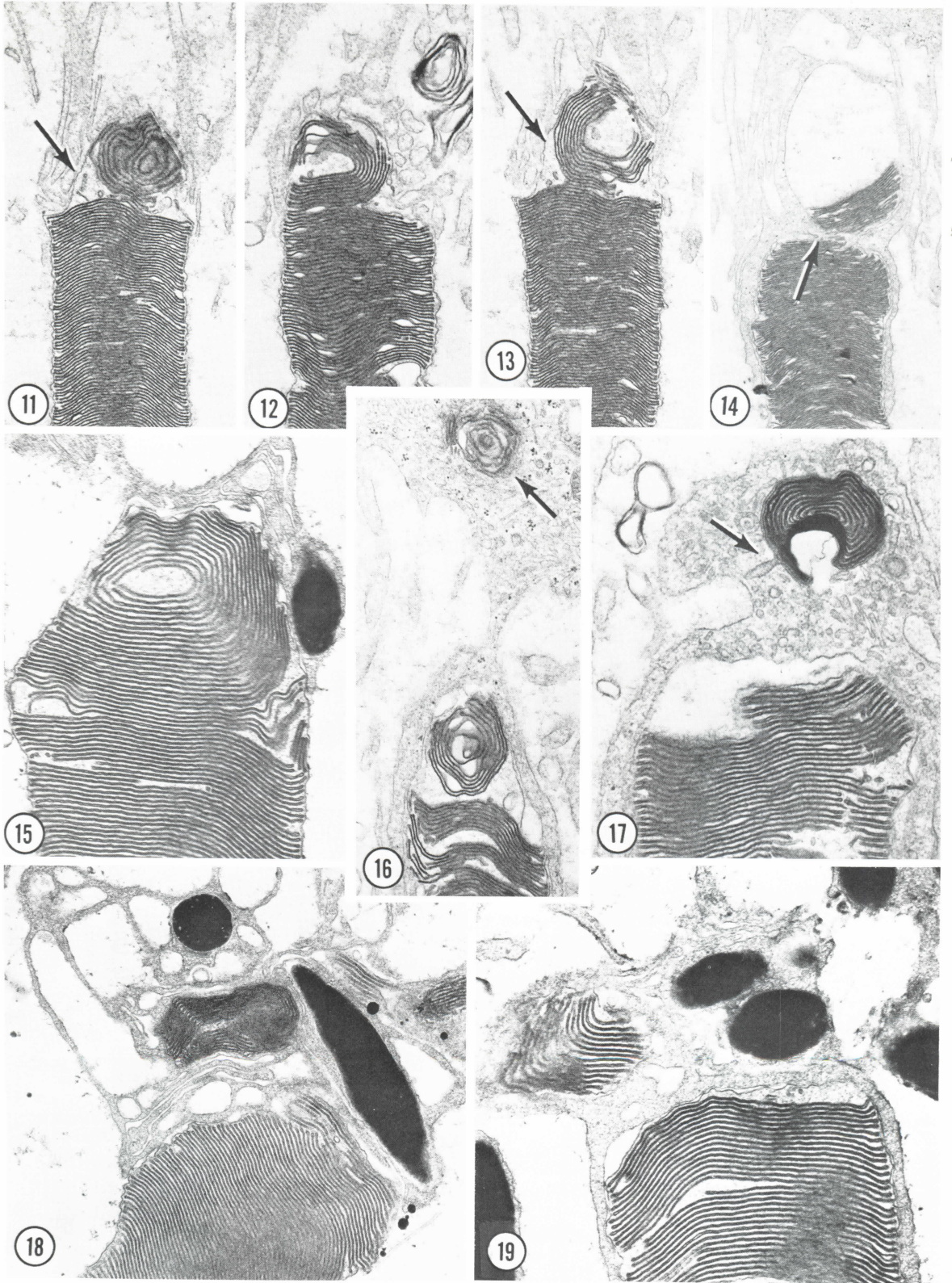
typifies the later stages of digestion within the midzone or basal pigment epithelium (Figs. 17–19).

A substantial majority of rod outer segments show no indication of disc shedding, for in only a minority of rods is it clearly evident that a group of discs is in some phase of the shedding process. Observations that show a group of curled discs at the outer segment tip or within the apical pigment epithelium immediately above a rod outer segment occur most often, but observations depicting a packet of rod discs embedded within pigment epithelial processes which cap the rods are rare.

Cone outer segments. Disc packets are shed from cone outer segments in a manner which does not differ significantly from that in rods. The disc structure at most of the cone outer segment tips appears unperturbed. In a few cases, one or two discs at the extreme tip are irregularly oriented (Fig. 2). In other instances, small packets of cone discs are folded over in a manner which is reminiscent of the shedding process in rods (Figs. 20, 22, 28, and 29). Sometimes the disc packets show evidence of membrane distortion and may appear as electron-dense whorls overlying the cone outer segment tip (Figs. 21 and 27). In still other cases, small disc packets are arranged as concentric lamellae at the tip of cone outer segments (Figs. 30 and 34–36), a condition which appears most frequently in the ground squirrel retinas and quite infrequently in the retinas of the tree squirrels. The reason for this difference is not known.

The initial curling of the cone discs takes place in the absence of any discernible pigment epithelial involvement (Figs. 20, 28, 29, 34, and 35). In the detachment phase of the process, cone disc packets are often enshrouded by slender pigment epithelial processes. In only a few instances did we observe disc packets which looked like they were being pinched-off from the outer segment by pigment epithelial processes (Fig. 22). After a disc packet detaches from the cone outer segment tip, it is often identifiable within the apical pigment epithelium immediately above the outer segment from which it probably originated (Figs. 23 and 31–33); these phagosomes often possess an internal structure which bears a striking resemblance to the disc structure of the cone outer segment. In retinas fixed by perfusion of the aldehyde–picric acid mixture, a few of the discs in each cone outer segment show a collapsed intradisc space which could be identified in electron micrographs by their high electron density (Figs. 31–33). Some phagosomes directly overlying cone outer segments contain a few of these electron-dense discs (Fig. 32).

The highly ordered rippling which occurs in immersion-fixed cone outer segments (Figs. 21–23) occasionally can be identified in some phagosomes within the pigment epithelium (Fig. 24), provided they have not undergone extensive degra-

Figs. 11–19. Electron micrographs of disc shedding from rod outer segments in terrestrial and arboreal squirrels.

Figs. 11–14. Curling of disc packets from rod outer segments in the 13-lined ground squirrel. The curling of the packets takes place before any significant deformations of the outer cell membrane are obvious (arrows, Figs. 11, 13). In Fig. 14 pigment epithelial processes (arrow) have segregated a small disc packet from the rest of the outer segment. Figs. 11–13, × 15 000; Fig. 14, × 18 000.

Fig. 15. A large packet of curled discs at the tip of a western gray squirrel rod outer segment. The curling of the discs appears to take place before any deformation of the outer cell membrane. × 20 000.

Fig. 16. A curled disc packet at the tip of a rod in the 13-lined ground squirrel, and another packet (arrow) within the pigment epithelium immediately above the outer segment. × 15 000.

Fig. 17. A packet of discs within the pigment epithelial processes which contact a rod outer segment in the 13-lined ground squirrel. A small vesicle (arrow) appears to be fused to the phagosome. × 30 000.

Figs. 18, 19. Disc packets within the pigment epithelial processes which cap the rod outer segment tips in the western gray squirrel. × 20 000.
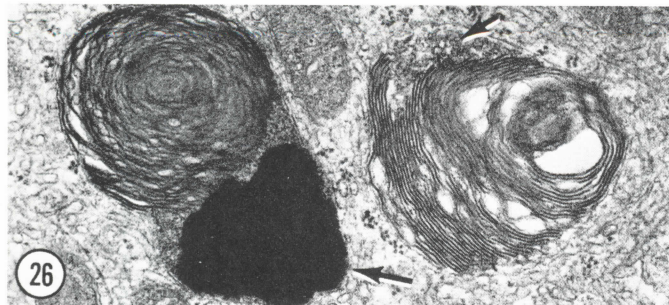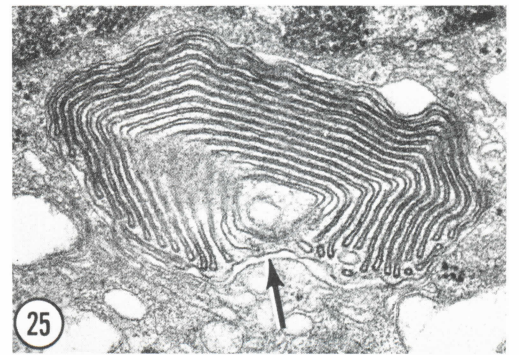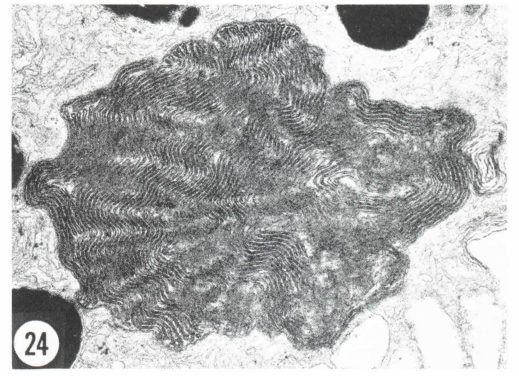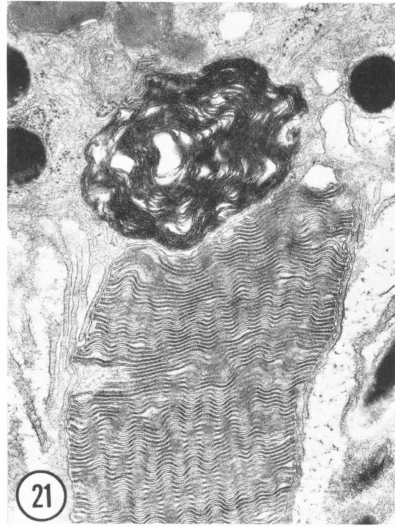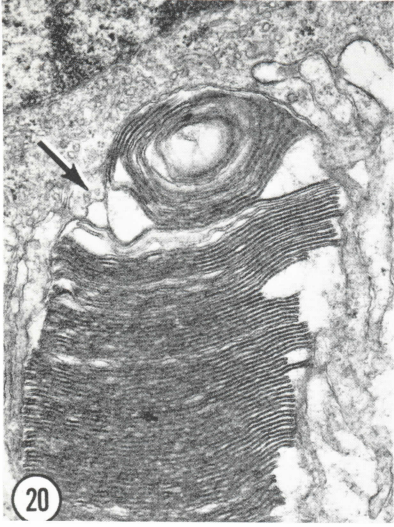
dation. Since we have not seen such rippling in rod outer segments or in phagosomes adjacent to rod outer segments, we presume that these rippled phagosomes arise from cone outer segments. The rippling in cone discs is readily distinguishable from the irregularities in disc structure which accompany partial digestions of phagosomes (Fig. 26).

Digestion within the Pigment Epithelium

Phagosomes take on a variety of forms within the pigment epithelium. In general, they are most easily identified within the apical zone of the pigment epithelium where their internal structure strongly resembles the disc structure of intact outer segments (Fig. 25). In the mid and basal zones of the pigment epithelium, the task of identification becomes progressively more difficult because the membranes within phagosomes may appear distorted, convoluted, or compressed. The membranous, internal structure of some phagosomes may be partially or totally obliterated (Figs. 26 and 39). A granular matrix may be found in place of, or in combination with, membranous material within a phagosome (Fig. 26); and some phagosomes may eventually appear as dense granules

within the pigment epithelium (Figs. 26 and 39).

Electron-opaque organelles, somewhat smaller than the largest phagosomes, are frequently encountered in the western gray squirrel pigment epithelium. They possess a circular, sometimes scalloped perimeter and a homogeneous internal structure (Fig. 23). Often they appear adjacent or possibly fused to presumptive phagosomes. Their specific role in the digestion process, if any, has not been determined.

Phagosomes are less frequently found in ground squirrel than in gray squirrel pigment epithelium. Although phagosomes arising from both rod and cone outer segments can be seen in the apical zone of the pigment epithelium immediately above the outer segments (Figs. 16, 17, and 31–33), only a few can be identified in the mid or basal zones of the pigment epithelium. The reason for this is not clear. In the 13-lined ground squirrel, and to a lesser extent in the other ground squirrels, the pigment epithelium contains numerous dense granules which possess little or no internal structure. Some of these granules are probably melanin granules and are identical with those which protrude between the outer segments (Fig. 2). Other larger, and

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FIG. 20. A packet of curled discs at the tip of a cone in the eastern gray squirrel. In the gray squirrel retinas, cone outer segments are easily distinguished from rod outer segments by their close proximity to the pigment epithelium. As noted above, the curling of the discs appears to have occurred without involvement of the pigment epithelial cell processes (arrow). × 20 000.

FIG. 21. A large electron-dense phagosome at the tip of a western gray squirrel cone. × 14 000.

FIG. 22. A small group of curled discs in a western gray squirrel cone. In this case, and in a few others, pigment epithelial cell processes (arrow) appear to be active in the detachment of the disc packet from the outer segment. × 20 000.

FIG. 23. A cone outer segment and overlying pigment epithelium in the western gray squirrel retina. A presumptive cone phagosome (arrow) overlies the outer segment. In the pigment epithelium, electron-opaque inclusions (asterisk) are often associated with lamellar inclusions which may be partially digested phagosomes. × 16 000.

FIG. 24. Cone phagosome, western gray squirrel pigment epithelium. Note the highly ordered rippling artifact (see text and Figs. 1, 21–23). × 28 000.

FIG. 25. A cone phagosome in the apical epithelium of the western gray squirrel. The phagosome lies immediately above a cone outer segment (not shown) and is surrounded by a membrane (arrow). × 38 000.

FIG. 26. Phagosomes in the pigment epithelium of the eastern gray squirrel. In the later stages of digestion, phagosomes may become partially or wholly electron dense (lower arrow). A granular matrix (upper arrow) can be identified within some phagosomes at this stage. × 30 000.

FIG. 27. An irregularly shaped phagosome above a cone outer segment in the western gray squirrel. × 15 000.
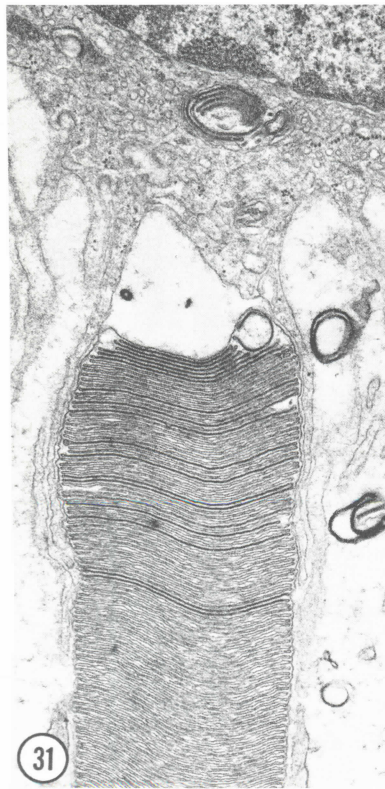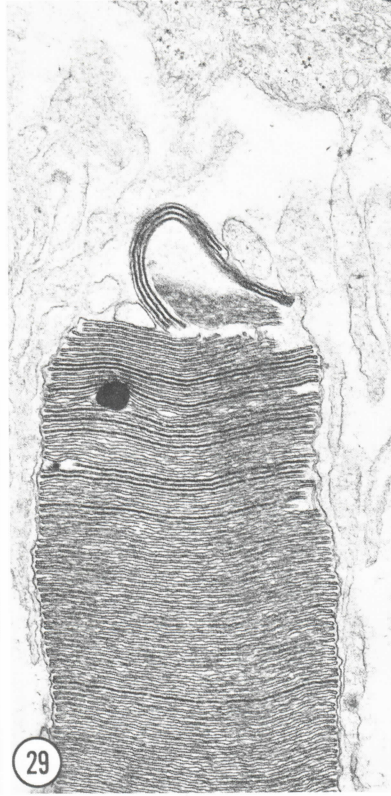
more irregularly shaped granules are also prominent. Whether these granules are actually partially digested phagosomes or whether they are structures whose function is unrelated to the phagocytic process is not known.

New Disc Assembly

Electron micrographs from the level of the connecting cilium show some interesting differences between rod and cone outer segments in the retinas of both arboreal and terrestrial squirrels. In both photoreceptor types, the single connection between outer and inner segments is the connecting cilium. We observed none of the cytoplasmic bridges which have been previously reported to provide a connecting link between inner and outer segments in ground squirrel photoreceptors *(33)*. The connecting ciliary membrane is continuous with the basal disc membranes in both rod and cone outer segments (Figs. 42–43 and 46–53).

In rod outer segments, the most basal discs are clearly distinguishable from those at more distal locations. Up to a dozen or so of the basal discs appear to be incompletely formed (Figs. 40–45). Here the paired disc membranes are not closely apposed as at more distal locations, thus the intradisc space is somewhat expanded and nonuniform. The buttonlike edges of the basal discs may be absent or indistinct. In well-preserved material there were no indications of vesiculated membranes at the base of the outer segments.

Our observations suggest that new rod discs are formed from the enclosing outer membrane by a process of inward invagination at the outer segment base. The enclosing outer membrane opposite the connecting cilium, and presumably at other locations around the outer segment perimeter, folds inward and traces a path toward the connecting cilium parallel to the existing disc stack (Figs. 40–45). In some instances, the infolding extends from one lateral margin of the outer segment to the other border (Figs. 41 and 43–45). In other cases, the infolding is seen as a partial invagination (Fig. 40). Slight, inwardly directed deformations of the enclosing outer membrane are sometimes observed, as are other instances where the infolding appears to be pinching-off from the rest of the outer membrane, thereby forming a new "free-floating" disc (Fig. 42). In rod outer segments, multiple invaginations of the outer membrane are often observed in favorably oriented sections. In cone outer segments, our observations of thin sections suggest that the most basal discs (perhaps as much as the basal one-third to one-half) form one uninterrupted network of paired membranes. An enclosing outer membrane, as it exists in rod outer segments (Fig. 4), appears only in the distal portion of cone outer segments; and as previously mentioned, it is interrupted by continuities with the disc membranes. The lack of an enclosing outer membrane at the outer segment base invariably distinguishes cone from rod outer segments in diurnal squirrels. In well-preserved material, none of the irregularities in disc structure which occur at the base of rod outer segments are present at the base of cone outer segments (Figs. 46–53).

At the level of the connecting cilium of cones, we have consistently observed a single, partially formed invagination (Figs.

FIGS. 28–30. Curling of discs at the tips of cone outer segments in the 13-lined ground squirrel. Fig. 28, × 15 000; Figs. 29 and 30, × 25 000.

FIG. 31. A small phagosome immediately above a cone outer segment, 13-lined ground squirrel. The cone cell membrane has apparently reformed over the outer segment tip after shedding of the disc packet occurred. × 15 000.

FIG. 32. Phagosome immediately above a cone outer segment, 13-lined ground squirrel. The phagosome contains one of the dense discs (arrow) characteristic of cones (see text). × 15 000.

FIG. 33. Phagosome immediately overlying a cone outer segment, 13-lined ground squirrel. A second group of discs may be curling at the cone tip. × 15 000.
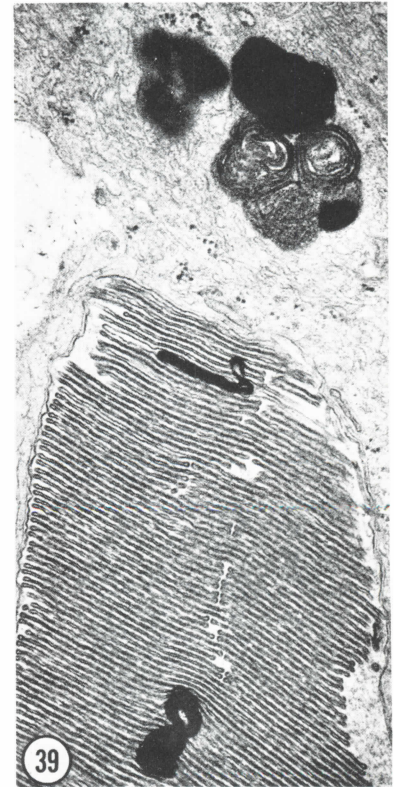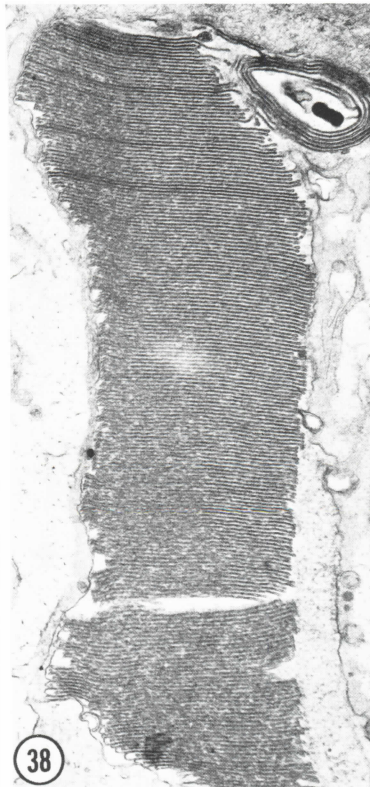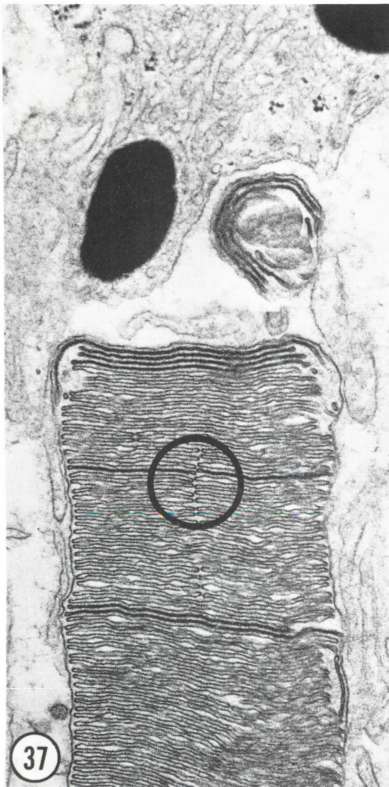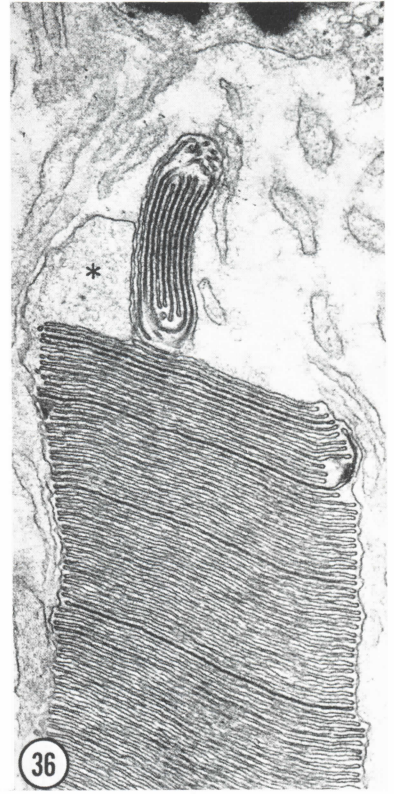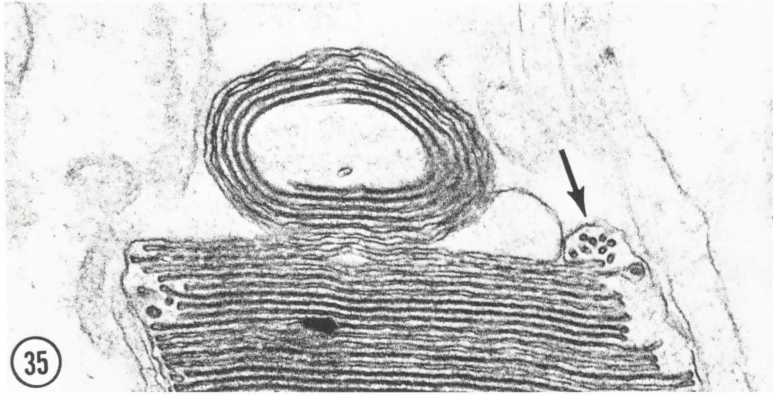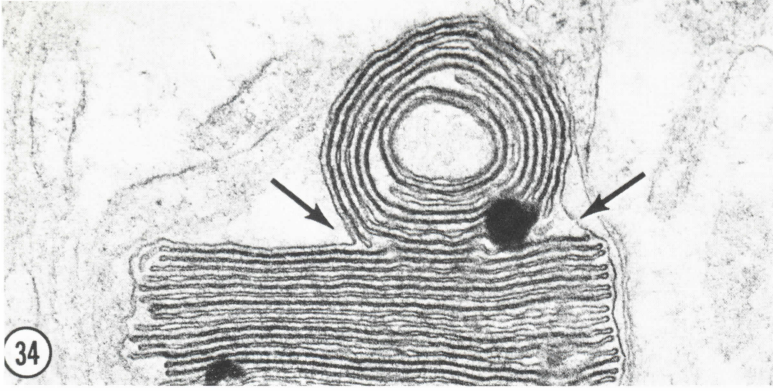
46–53). These partial invaginations are clearly analogous to the multiple invaginations which occur at the base of rod outer segments. As is the case in rod outer segments, the partial invagination in cones traces a path from a point opposite the connecting cilium to a point adjacent to the microtubules which are contained within the cilium.

DISCUSSION

The Classification of Squirrel Photoreceptors

In all of the retinas examined in the present study, two morphologically distinct photoreceptor types were recognized, corresponding closely to those described in the gray squirrel retina by Cohen (8) and with those more recently described in the retinas of terrestrial species (20, 40). On the basis of either outer segment structure or the pattern of protein renewal, the classification of these photoreceptors into either the rod or cone categories is relatively straightforward. The majority-photoreceptor-type shows numerous disc–membrane continuities similar to those in the cone outer segments of other mammals (7, 9). In addition, the majority type in two of the five species studied shows the diffuse pattern of protein renewal characteristically found in the cone outer segments of other vertebrates (43, 44, 46). In the minority-photoreceptor-type, the vast majority of discs appear in electron micrographs as "free-floating" units contained within an enclosing outer membrane—a

characteristic which is shared with rod outer segments in numerous other vertebrates (10). Furthermore, outer segments of this cell type in the two representative species show the characteristic accumulation of radioactive protein which is found in other vertebrate rods (42, 44, 46).

The classification of squirrel photoreceptors on the basis of their synaptic terminal organization is not quite so straightforward (20, 40). The shape and synaptic organization of the rod terminals departs somewhat from the organization of rod spherules in primates and cats (6, 14, 27) but this is by no means a unique feature in the squirrel's retina, as is shown by a comparison of rod terminals in frogs and primates (13, 30).

In light of the prevailing evidence, it now seems appropriate to dispense with the "rodlike" and "conelike" terminology when referring to squirrel photoreceptors. These terms were introduced for the photoreceptors of the eastern gray squirrel when the structural and functional evidence for a duplex retina was conflicting (8). In our judgment the ambiguities and conflicts which formerly existed have now been satisfactorily resolved. The anatomical properties of the outer segments correspond closely with those found in other mammalian photoreceptors. Electroretinographic evidence confirms the presence of a scotopic component in the retinas of both terrestrial and arboreal species (15, 20), and the available behavioral evidence is consistent with the conclusion that at least

FIG. 34. A concentric arrangement of discs typical of cone phagosomes in the ground squirrels. The outer segment membrane clearly encloses the phagosome (arrows). 13-lined ground squirrel. × 40 000.

FIG. 35. Phagosome at the tip of a cone outer segment. Mexican ground squirrel. The arrow indicates a disc which is continuous with the cone cell membrane. × 40 000.

FIG. 36. An elliptical-shaped phagosome at the tip of a cone. The cone cell membrane folds over at the top of the outer segment, enclosing an area of cytoplasm (asterisk) and forming a disc. 13-lined ground squirrel. × 30 000.

FIG. 37. A detached cone phagosome appears to be *en route* to the apical border of the pigment epithelium. Note the disc incisures (circle) in the cone outer segment, a feature recently described in cat cones (38). 13-lined ground squirrel. × 30 000.

FIG. 38. Phagosome at the tip of a cone outer segment in the California ground squirrel. × 18 000.

FIG. 39. Presumptive phagosome above a cone outer segment in the California ground squirrel. The phagosome retains some lamellar substructure; however, it also contains granular electron-dense material. The very dense structure adjacent to the phagosome may be a partially digested phagosome. × 30 000.
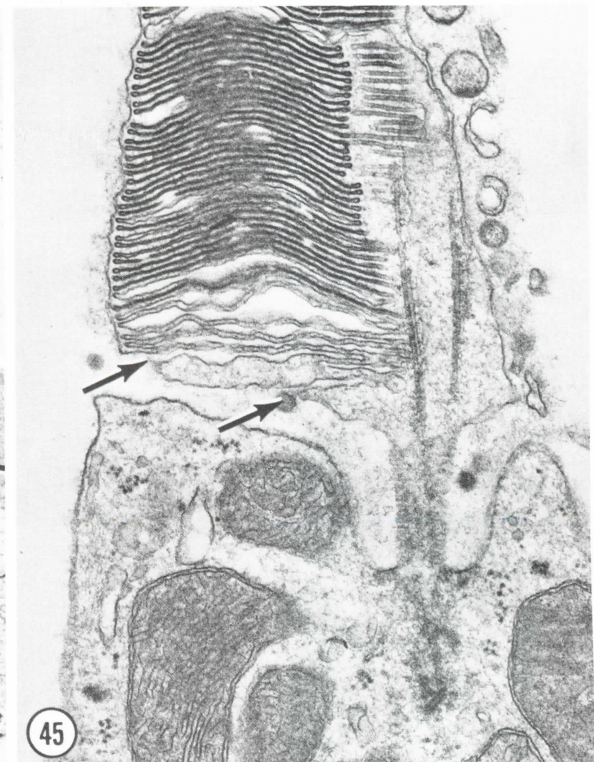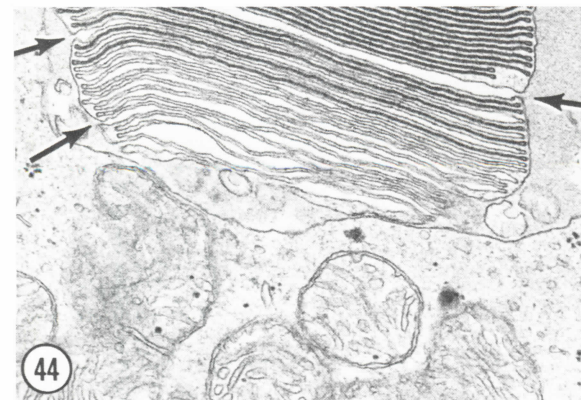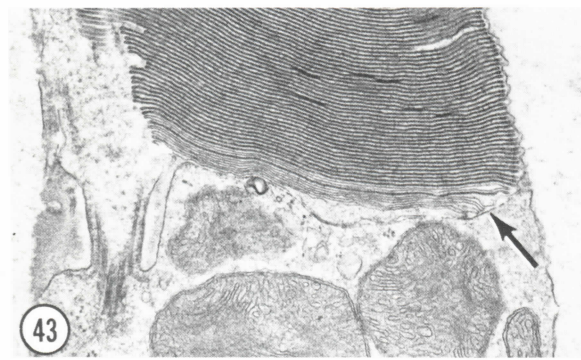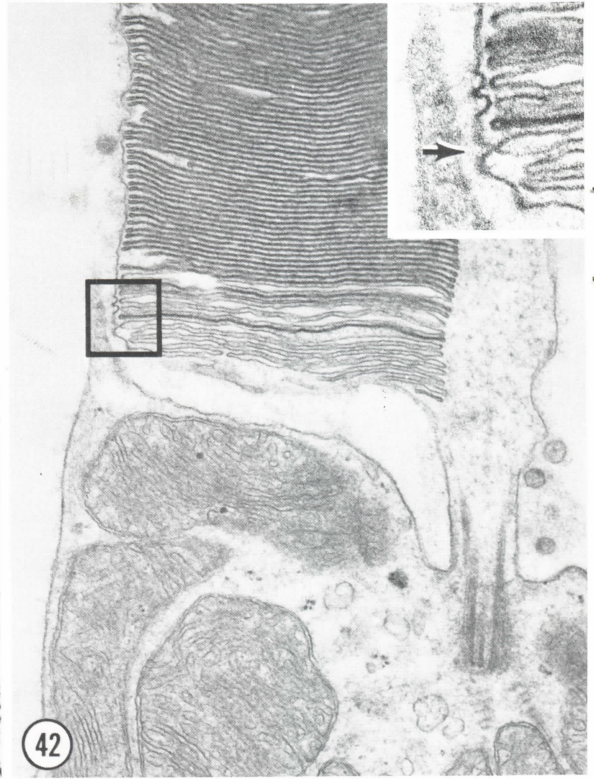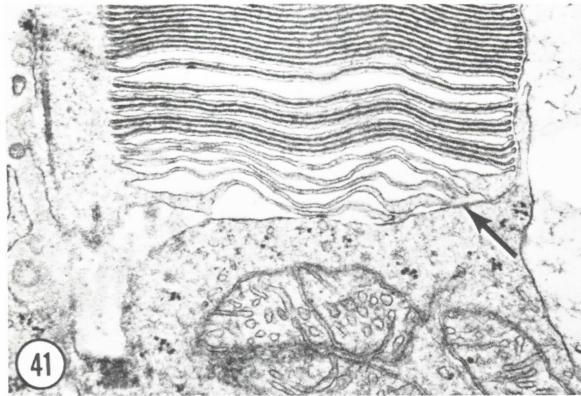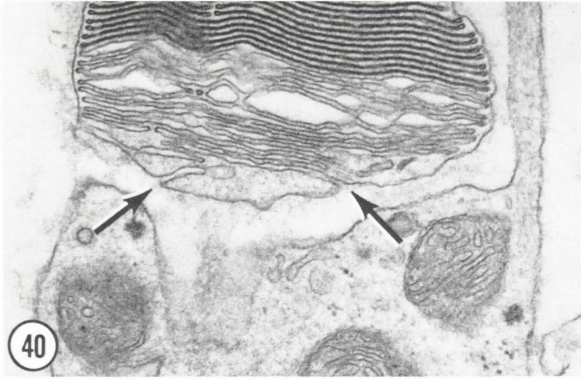
the arboreal species have a viable scotopic contribution to their visual behavior *(19, 34, 41)*. An anatomical distinction between squirrel photoreceptors based upon the structure of the outer segments and their pattern of protein renewal is, in our view, most appropriate, and conforms to the suggestion of Cohen that explicit criteria be used when describing rods and cones in different species of vertebrates *(9)*.

Disc Shedding from Squirrel Photoreceptor Outer Segments

In the rods of diurnal squirrels, disc packets are intermittently shed from the outer segment tips. The dynamics of the shedding process in the various species of squirrels is not qualitatively different and does not seem to differ significantly from descriptions of the shedding process in other species of vertebrates *(45)*. Disc packets of varying size initially curl at their lateral edges toward the apical surface of the pigment epithelium. The space formerly occupied by the edges of the curled discs becomes occupied by photoreceptor cytoplasm. The curling of the discs takes place before any significant deformations of the enclosing outer membrane occur, thus suggesting that the curling is intrinsically triggered from within the cell itself. The slender pigment epithelial processes which usually border the lateral margins of the outer segments assist in the transport of the disc packets to the apical

pigment epithelium. However, it is unclear whether such processes "pinch-off" the disc packets, as has been described in human rod outer segments *(37)*, or whether the active agent in effecting detachment is the photoreceptor cell membrane which invaginates inward and fuses — thereby isolating the disc packet from the remainder of the outer segment *(5, 45)*.

An as yet unanswered question is whether the frequency of disc shedding differs in the rods of different squirrel species. We have estimated the rate of disc turnover in eastern grey squirrel rods at about 100 discs/day, a rate sufficient to turn over the entire disc complement in about 5 days (± 1 day) *(2)*. Work now in progress should determine the turnover rate in the 13-lined squirrel, and whether it departs measurably from the grey squirrel estimate.

Indications of disc shedding from cone outer segments were seen in all of the species examined but, as in the rods, in a clear minority of the outer segments. The shedding of cone discs occurs in packets of varying size, with groups of cone discs initially curling at their lateral margins as in rods. In the cone outer segments of the ground squirrels, disc packets at the outer segment tip and immediately above it showed a surprisingly regular, concentric arrangement (Figs. 34 and 35) which was not as pronounced in the cone outer seg-

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Figs. 40–45. Rod outer segment bases in various species of diurnal squirrels.

Fig. 40. Eight to ten discs at the outer segment base are structurally different from those at more distal locations. Invaginations indicated at arrows likely signify the formation of new discs. 13-lined ground squirrel. × 30 000.

Fig. 41. Multiple invaginations of the rod cell membrane seen at the outer segment base in the California ground squirrel. × 30 000.

Fig. 42. Rod base in 13-lined ground squirrel. The area outlined shown in inset. Two small indentations of the cell membrane may represent newly forming discs. One disc appears to be pinching off from the cell membrane (arrow). × 30 000; inset, × 80 000.

Fig. 43. A single invagination of the cell membrane (arrow) at the base of a rod in the eastern gray squirrel. × 24 000.

Fig. 44. Multiple invaginations (arrow) of the cell membrane at the base of a California ground squirrel rod. × 34 000.

Fig. 45. The connecting ciliary membrane is continuous with two invaginations (arrows) at the base of a rod in the 13-lined ground squirrel. × 36 000.
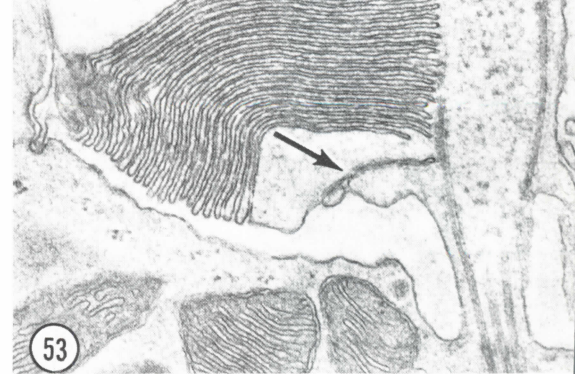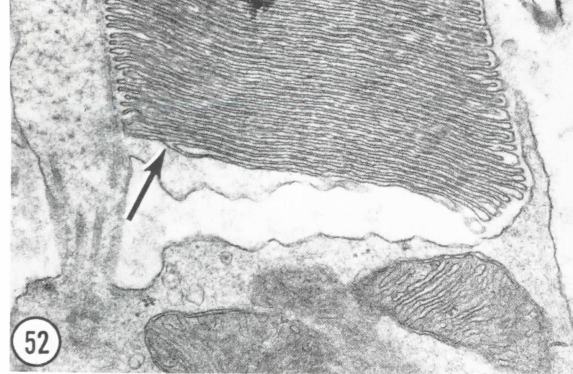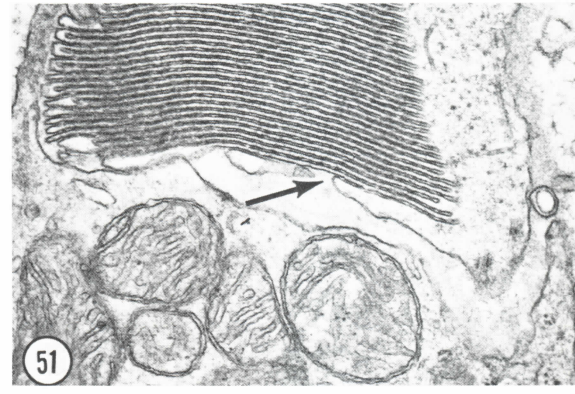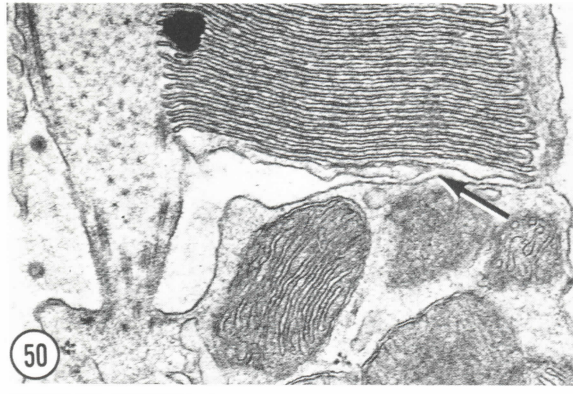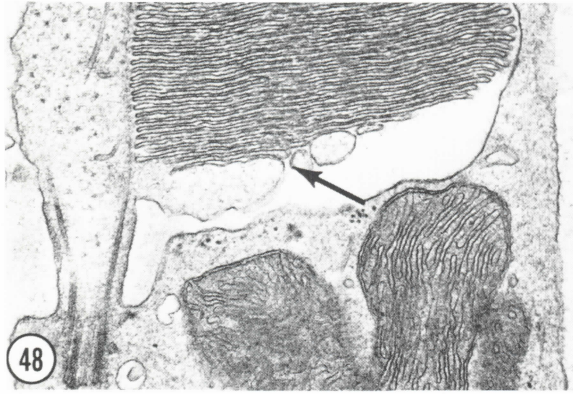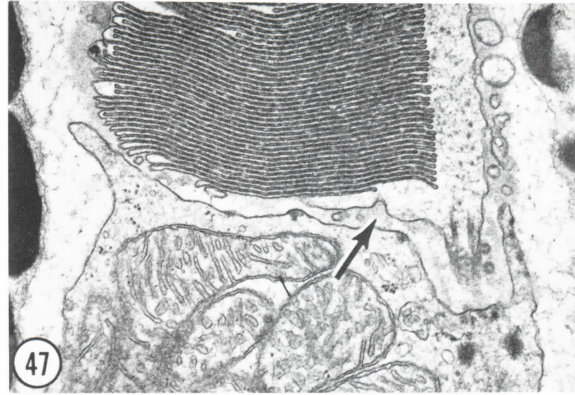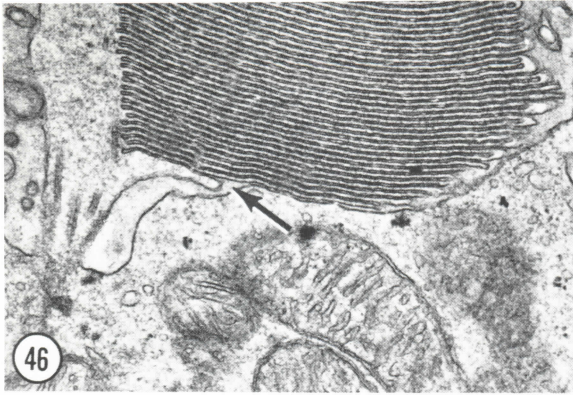
ments of the arboreal species. It is not known whether these observations reflect some underlying difference in the disc shedding process between arboreal and terrestrial squirrels, or whether they are related to differences in the fine structure of their respective cone outer segments.

Renewal in Cone Outer Segments

In vertebrate rods there is ample evidence that the shedding of outer segment discs is merely part of an ongoing renewal process whereby the shedding of old discs is balanced by the addition of new discs at the outer segment base *(44)*. Rod outer segments have thus been described as undergoing renewal by membrane replacement *(47)*. Do some or all cone outer segments engage in a similar kind of renewal process, that is, is the shedding of cone discs also balanced by the addition of new discs to the outer segment?

The present evidence of disc shedding from squirrel cone outer segments and the recent report of phagocytosis of human cone outer segment discs *(16)* has several possible interpretations. First, it is possible that disc shedding from cone outer segments occurs only rarely, in response to unknown metabolic or environmental factors, or as a result of advancing age. As such, it may not be a manifestation of a continuous renewal process as much as it may reflect a process of deteriorative change unbalanced by the assembly of new cone discs. Unfortunately, there is as yet no specific information available on the frequency of disc shedding from cone outer segments, or whether shedding occurs only under some conditions and not others. Attempts to infiltrate cone outer segments with an appropriate marker molecule and to follow its disposition over time may

yield an answer to this important question. Secondly, it is possible that cone outer segments in some species undergo renewal by membrane replacement as well as by molecular replacement – the mechanism suggested by Young *(47)*. The most compelling evidence against this interpretation has been the failure to observe a band of radioactive protein at the base of cone outer segments at any time after the administration of [^{3}H]amino acids *(44)*. However, it is now recognized that a band of radioactive protein is never seen in cone outer segments of the salamander even when new cone discs are assembled during initial development *(12)*. The reason for this is not clear, but it does suggest that the absence of a band of radioactive protein in mature cone outer segments does not necessarily indicate the absence of new disc synthesis. In fact, there is some evidence that mature mammalian cones retain the capacity for new disc synthesis. Kroll and Machemer *(21)* have shown that the cone outer segments of rhesus monkeys regenerate after surgical detachment and reattachment of the retina to the pigment epithelium, although their recovery rate is slower than for rod outer segments. Remé and Young *(32)* have recently described a regenerative proliferation of cone discs in 13-lined squirrels without any autoradiographic evidence of a band of radioactive protein after the cone outer segments had shortened during hibernation. Furthermore, in the present study there is indirect evidence that new cone discs are assembled at the base of the outer segment in a manner which is very much like the disc assembly process which occurs during initial development *(29, 36)*.

In the squirrel retina, we have emphasized that multiple invaginations of the

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Figs. 46–53. Cone outer segment bases in various species of diurnal squirrels.

Figs. 46, 47, 49, 51, 53. A single, partially formed invagination (arrows) is found at the base of the cones. California ground squirrel. × 30 000.

Figs. 48, 50, 52. A single, partially formed invagination (arrows) is found at the base of the cones in the 13-lined ground squirrel. These invaginations are thought to be the cone analogs of the multiple immature discs found at the rod base (Figs. 40–45). Fig. 48, × 26 000; Figs. 50 and 52, × 30 000.

outer cell membrane can be identified at the base of rod outer segments and that only a single, partial invagination appears at the base of some cone outer segments. It is conceivable that this may reflect a difference between squirrel rods and cones in the rate or mode of new disc assembly. If new cone discs are assembled at a slower rate than their rod counterparts, the frequency of disc shedding from cone outer segments also should be less than from rod outer segments provided that the assembly and shedding phases are in equilibrium. A low frequency of disc shedding in the cone outer segments of squirrels may help to explain the scarcity of identifiable phagosomes in the pigment epithelium of the ground squirrels whose retinas contain 95% cones. In that regard, the slower rate of recovery of cone versus rod outer segments in surgically reattached rhesus monkey retinas is particularly interesting as are the observations by Marshall and Ansell (25) that phagosomes are less frequent in the area of highest cone density in the pigeon retina. It seems more likely that our observations reflect differences in the mode of disc assembly in adult squirrel rods and cones. Membrane assembly and shedding from mammalian cones may not be contradictory to the pattern of diffuse labeling seen in autoradiograms (see 10, 11, 45). Our observations are consistent with either the hypothesis that the cone is composed of a continuously folded, fluid membrane through which molecules can diffuse after addition at the base (31, 35) or with the hypothesis that the cone is able to produce new discs along its entire length (12). There is, however, little evidence that mammalian cone outer segments are composed of a continuously folded membrane; rather, present data suggest that isolated discs occur in the upper one-half of the outer segment (9, 10). Furthermore, Liebman and Entine (24) have shown that visual pigment molecules are capable of lateral, but not longitudinal, diffusion within the outer seg-

ment membranes of mud puppy cones. The presence of occasional discs continuous with the cell membrane in squirrel cones (Fig. 3) may represent newly assembled discs which have not yet pinched-off from the outer membrane. But it is significant that we have never observed partially formed discs except at the very base of cones — perhaps indicating that the extreme base of the outer segment is the most active in assembling new membrane. In comparison, our data suggest that rods possess a restricted region at the outer segment base where several discs are formed simultaneously — an observation consistent with the band of radioactive protein seen in autoradiograms (47).

What are the controlling factors which determine the presence or absence of disc shedding from cone outer segments? The emerging evidence suggests that two factors may be important: First, the process may be species dependent. The only published evidence of disc shedding from cone outer segments has come from mammals, specifically from humans and squirrels. Disc shedding apparently does not occur in the cone outer segments of either the frog (Rana pipiens) or the salamander (Ambystoma tigrinum) (44), nor have phagosomes been identified in the pigment epithelium of several diurnal lizards which possess all-cone retinas (5). Ishikawa and Yamada (17) failed to find phagosomes in their preliminary examination of the pigment epithelium in snake, chick, or turtle retinas — a rather puzzling observation, however, since both the turtle and chicken retinas contain substantial numbers of rods (23, 26, 28). Secondly the shape of the cone outer segment may prove to be a significant factor in the shedding process. Squirrel cone outer segments are cylindrical, and human extrafoveal cone outer segments may be described as slightly tapered. Young (44) has already pointed out that the significant tapering of cone outer segments in such species as the frog and salamander is not compatible

with a disc shedding process. Before any further generalizations can be made, a variety of mammalian and nonmammalian species should be examined for evidence of cone disc shedding and/or assembly.

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