

PARTICIPATION OF THE RETINAL PIGMENT EPITHELIUM IN THE ROD OUTER SEGMENT RENEWAL PROCESS

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ABSTRACT

The disposal phase of the retinal rod outer segment renewal process has been studied by radioautography in adult frogs injected with tritiated amino acids. Shortly after injection, newly formed radioactive protein is incorporated into disc membranes which are assembled at the base of the rod outer segment. During the following 2 months, these labeled discs are progressively displaced along the outer segment owing to the repeated formation of newer discs. When the labeled membranes reach the end of the outer segment, they are detached from it. They subsequently may be identified in inclusion bodies within the pigment epithelium by virtue of their content of radioactivity. These inclusions have been termed *phagosomes*. Disc membrane formation is a continuous process, but the detachment of groups of discs occurs intermittently. The detached outer segment fragments become deformed, compacted, undergo chemical changes, and are displaced within the pigment epithelium. Ultimately, the material contained in the phagosomes is eliminated from the cell. In this manner the pigment epithelium participates actively in the disposal phase of the rod outer segment renewal process.

INTRODUCTION

The light-sensitive outer segment of the retinal rod visual cell consists of a stack of many hundreds of densely packed discs, each of which represents a double layer of infolded plasma membrane. Recently, it has been established that the outer segments of mature rods are continually renewed (6). Protein constituents are synthesized in the inner segment of the cell, then transferred through the modified cilium of the connecting structure (7) to the base of the outer segment, where they are incorporated into new membranous discs which are assembled there (6, 8). At least part of this protein consists of the visual pigment, rhodopsin (1, 2, 4). Repeated formation of new discs at the base of the outer segment progressively displaces

older discs away from the site of assembly, and towards the apical end of the cell. This process does not take place in cones, in which the production of outer segment membranes is not continued in the mature cell (9).

The renewal of rod outer segments was revealed by radioautographic experiments which traced the fate of protein, labeled during synthesis in the presence of radioactive amino acids. Newly formed membranous discs, made radioactive by incorporation of the labeled protein during assembly, could be followed as they were gradually displaced along the outer segment (Fig. 1). In rats and mice, about 10 days were required for migration of the labeled discs from the base to the apex

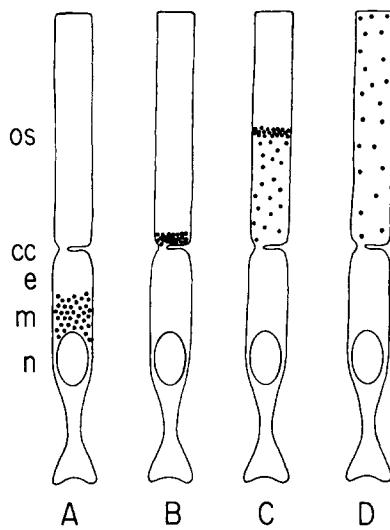


FIGURE 1 Diagrammatic summary of the renewal of rod outer segments as revealed by radioautography after injection of radioactive amino acids. The location of radioactive protein is indicated by black dots (see references 6-8 for details). *os*, outer segment; *cc*, connecting cilium; *e*, ellipsoid; *m*, myoid; *n*, nucleus. *A*, Minutes after injection, radioactive protein is concentrated in the myoid portion of the inner segment, the major site of protein synthesis. *B*, In the hours immediately following, newly formed protein aggregates at the base of the outer segment, the site of membrane assembly. *C*, The heavily labeled membranous discs are gradually displaced by the repeated formation of newer discs at the base of the outer segment. These new discs are weakly labeled, due to the continued presence of low levels of labeled precursor in the tissue fluids. *D*, The moving band of intense radioactivity disappears when it reaches the apex of the cell.

of the outer segment. In frogs raised at room temperature, the interval was about 6 wk (6). In all of the experiments, the radioactive protein disappeared from the outer segment when it reached the apical end of the cell.

Continued addition of new membranous discs at the base of a mature photoreceptor cell outer segment, the length of which remains constant, requires the synchronized removal of disc material at some other site. The radioautographic experiments indicate that the apex of the cell, which lies embedded in the cytoplasm of the pigment epithelium, is the site of disc disposal (6).

On the basis of indirect evidence, the concept was developed that the pigment epithelium par-

ticipates actively in this process (6, 9, 10). According to this idea, the pigment epithelium continually removes and destroys the older membrane material at the apex of the rod outer segment, thereby balancing the continued apposition of new discs at the base. However, there is as yet no direct evidence to implicate the pigment epithelium in the disposal phase of rod outer segment renewal.

The present report presents such direct evidence. It will be shown that radioactive protein which initially has been a constituent of the rod outer segment, and which subsequently has disappeared from the apex of that structure, may be identified in the pigment epithelial cell cytoplasm.

MATERIALS AND METHODS

The animal used was the leopard frog, *Rana pipiens*, raised under conditions of ordinary laboratory illumination.

Six frogs, averaging 30 g body weight, were injected in the dorsal lymph sac with L-methionine-³H (sp act 600 mc/mmol) at a dosage of 10 μ c/g. The animals were maintained at 25°C, and were sacrificed at postinjection intervals of 30, 33, 35, 37, 42, and 44 days. At sacrifice, the eyes were removed and preserved by freeze-substitution (6). Paraffin sections were cut at 5 μ . The pigment granules of the pigment epithelium were then bleached so that the grains of reduced silver in the photographic emulsion could be more easily visualized over these cells. Deparaffinized

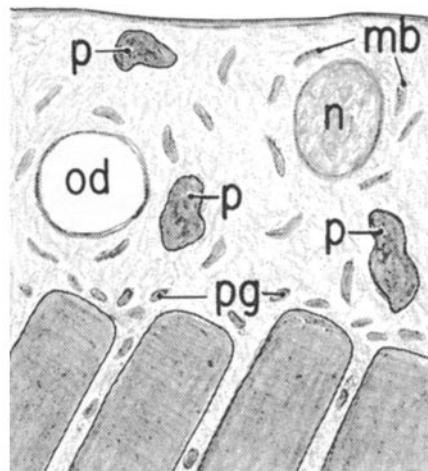


FIGURE 2 Diagram of the major structures visible within the retinal pigment epithelium of the frog. Abbreviations: *p*, phagosomes (inclusion bodies); *mb*, myeloid bodies; *od*, oil droplet; *pg*, pigment granules. The ends of the rod outer segments are shown at the bottom.

sections were bleached in a 1:1 mixture of 0.3% potassium permanganate and 0.3% sulfuric acid for 2 min, followed by incubation in 1% oxalic acid until the brown color disappeared. The sections were stained by the periodic acid-Schiff (PAS) technique, and prepared for radioautography by dipping in Kodak NTB2 emulsion maintained at 40°C. The preparations were exposed for 24 wk, then developed in Kodak Dektol for 2 min at 17°C, fixed in Kodak Acid Fixer (Eastman Kodak Co., Rochester, N.Y.), washed, and stained with hematoxylin.

In a second experiment, six frogs, averaging 11 g body weight, were injected with 0.3 mc/g of a mixture of L-leucine-³H (29.1 c/mmol) and L-phenylalanine-³H (5.0 c/mmol), containing equal amounts

of radioactivity due to each. These animals were maintained at 23°C, and were sacrificed at postinjection intervals of 47, 50, 54, 59, 61, and 68 days. The eyes were fixed for 1 hr at 4°C in a solution of 4% methanol-free formaldehyde, phosphate-buffered to pH 7.1, or a mixture of formaldehyde (2%) and glutaraldehyde (1%) in the same buffer. The tissues were next cut into smaller pieces, segments from the central and peripheral retina being kept separate. After a rinse in buffer, the specimens were postfixed in 1% osmium tetroxide in the same buffer, dehydrated, and embedded in Araldite. The retinal fragments, reoriented so that longitudinal sections of the photoreceptor cells could be obtained, were cut at $\frac{1}{2}\mu$. (The pigment granules do not interfere with

FIGURES 3-13 Junction of the rod outer segments with the pigment epithelium (See Fig. 2). In Figs. 3-10, the intensely labeled membranous discs are approaching the ends of the rod outer segments. Neither the oldest discs at the tip of the outer segments nor the inclusion bodies (*phagosomes*) within the pigment epithelium are reactive at this time. In Figs. 10-18, the heavily labeled discs have been detached from the ends of some of the rod outer segments. Only after this occurs can labeled phagosomes be observed. Figs. 3-6 are 47 days; Figs. 7-9, 50 days; Figs. 10-12, 54 days; and Fig. 13, 59 days postinjection. Radioautograms, $\frac{1}{2}\mu$ Araldite sections; toluidine blue. $\times 1,150$.

FIGURE 3 A row of small phagosomes is indicated by the arrow. Others are visible. None is labeled.

FIGURE 4 The phagosomes are rectangular in shape, heavily stained, and unreactive.

FIGURE 5 The darkly stained phagosome has an irregular shape and is unlabeled. The oil droplets remain free of radioactivity; the pigment epithelial cytoplasm is diffusely reactive.

FIGURE 6 Two nonradioactive phagosomes are shown.

FIGURE 7 Four phagosomes of variable shape are visible. None is labeled.

FIGURE 8 Two unlabeled phagosomes can be seen.

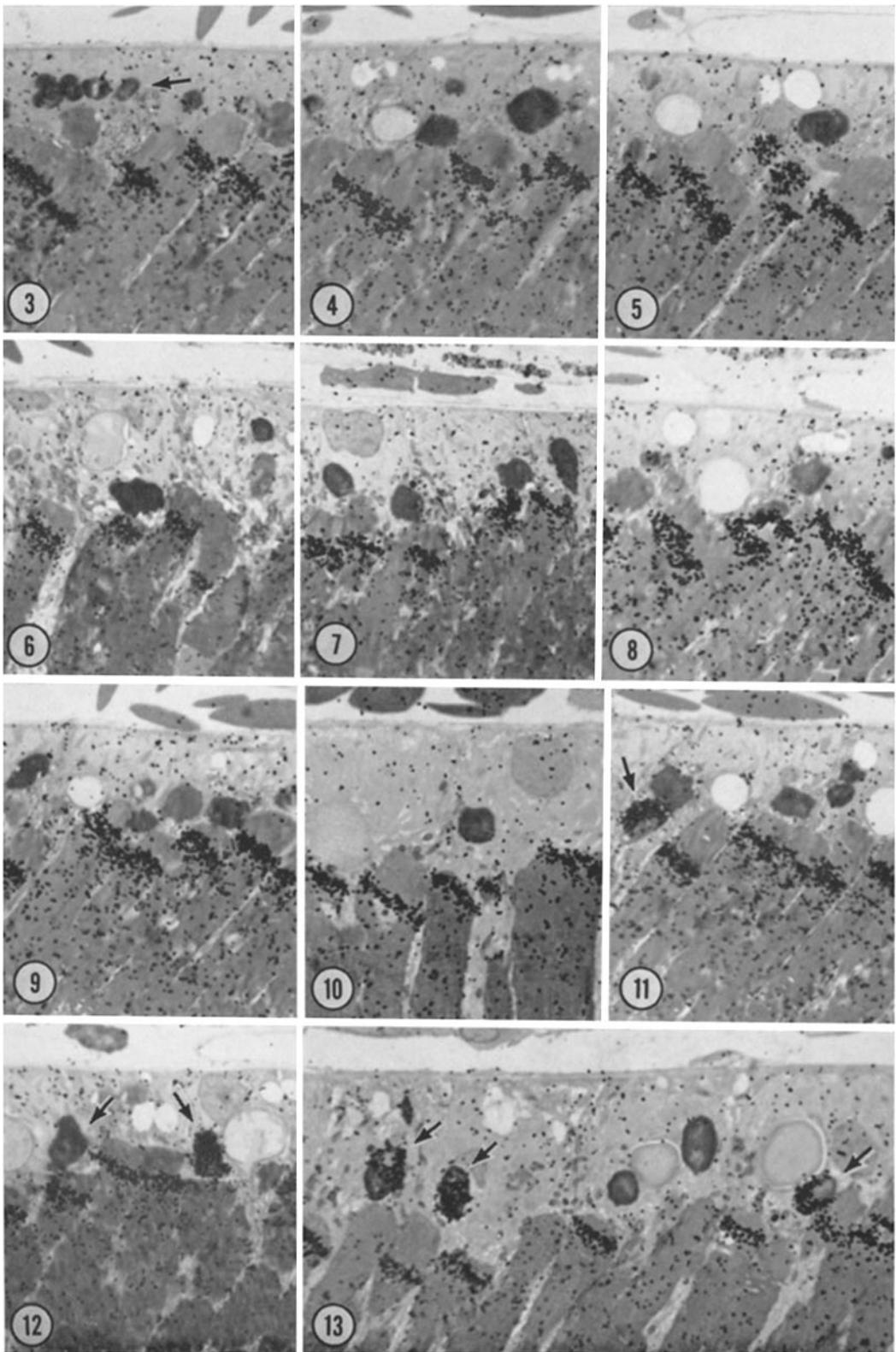
FIGURE 9 Several phagosomes, free of radioactivity, are present.

FIGURE 10 The heavily labeled discs have been displaced to the end of most of the rods in this field. The phagosome is unlabeled.

FIGURE 11 Four darkly stained phagosomes are visible. They are unreactive above background. A fifth, indicated by the arrow, contains a zone of intense radioactivity comparable to that in the adjacent rod outer segments, except that it has been tilted at a sharp angle.

FIGURE 12 Two phagosomes are indicated by arrows. One (left) is unlabeled. The other is intensely reactive.

FIGURE 13 The highly radioactive membranous discs are being shed from the apex of the rod outer segments. Two intensely labeled phagosomes on the left (arrows) bear witness to the fate of the labeled discs. Two other phagosomes (center) are unlabeled. On the right (arrow), a phagosome contains radioactive discs which have been deformed after detachment into a curved or "U" shape.



radioautographic analysis in sections of this thickness). The plastic embedment was removed by incubation in $\frac{1}{6}$ saturated NaOH in absolute ethanol. After being rinsed and dried, the sections were dipped in NTB2 emulsion, diluted 1:1 with distilled water, and maintained at 40°C. The preparations were exposed for 4 days at 4°C, then developed in Dektol for 2 min at 17°C. After photographic fixation, followed by being washed in water, the sections were stained through the emulsion with 1% toluidine blue in 1% sodium borate.

A few electron microscope radioautograms were prepared from retinal specimens taken from the frog sacrificed at 61 days. The procedures described in a previous report (8) were followed, except that development was carried out for 1 min at 18°C in Phenidon developer (3).

RESULTS

The larger structures observed within the cytoplasm of the pigment epithelium are depicted in Fig. 2. Nucleus, pigment granules, lipid droplets, myeloid bodies, and inclusion bodies may be distinguished. The inclusion bodies are of irregular shape and size. They may be elongate, oval, rectangular, rounded, or indented on one side. The outer surface is generally smooth, although irregularities and protuberances are occasionally present. The diameter of the inclusions may be as great as that of the rod outer segments, but is almost always smaller. The smaller inclusion bodies tend to be located nearer the base of the pigment epithelial cell. The inclusions and the rod outer

FIGURE 14-23 Junction of the rod outer segments with the pigment epithelium. This plate depicts various aspects of the process by which the intensely labeled membranous discs are detached from the ends of the rods and engulfed by the pigment epithelium. Figs. 14 and 15 are 59 days; Figs. 16-23 are 61 days postinjection. Radioautograms; $\frac{1}{2}\text{-}\mu$ Araldite sections; toluidine blue. $\times 1,150$.

FIGURE 14 Three phagosomes are indicated by arrows. The one on the left is free of radioactivity. The others are heavily labeled.

FIGURE 15 The detached tip of a rod outer segment, containing the heavily labeled discs (arrow), has been tilted at an angle compared to the orientation of the intact outer segments.

FIGURE 16 A small, unlabeled phagosome is visible near the base of the pigment epithelium (upper left). Two highly radioactive phagosomes are indicated by arrows. The oil droplet is not reactive.

FIGURE 17 Four labeled phagosomes are shown, two of them with distinctly banded labeling patterns (arrows).

FIGURE 18 Both of the phagosomes show evidence of localized labeling.

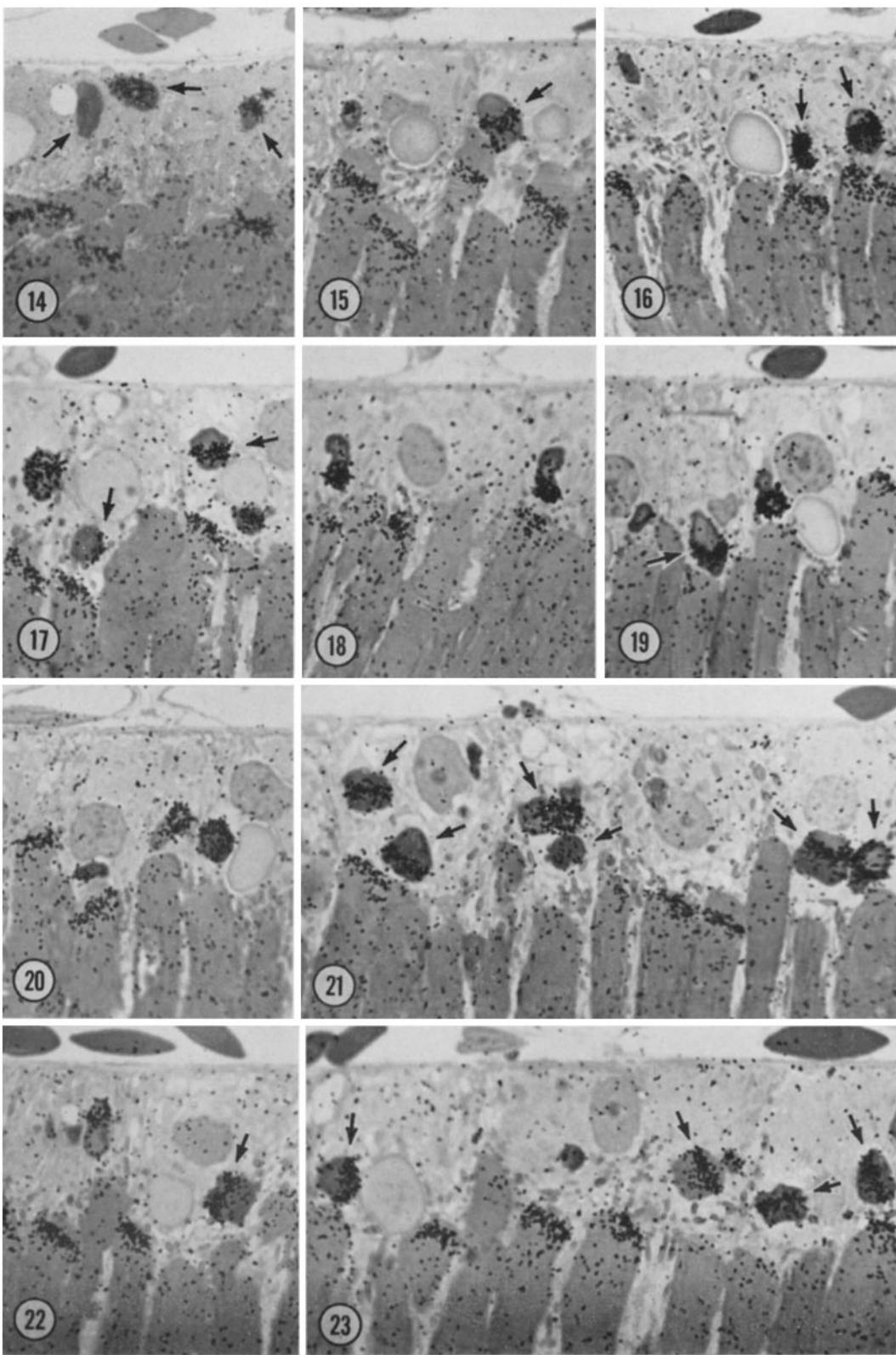
FIGURE 19 Note the curved shape of the reaction band in the labeled phagosome (arrow), indicating a deformation of the constituent discs (See Fig. 35).

FIGURE 20 Two intensely labeled phagosomes are apparent immediately adjacent to two rod outer segments from which the groups of highly radioactive discs have been detached.

FIGURE 21 Six labeled phagosomes of various shapes are visible in this field (arrows). In four of these a localized band of intense radioactivity can be detected.

FIGURE 22 Two radioactive phagosomes are present. Note that in one of them (arrow) there is a discrete band of radioactivity which has been turned at an angle of 90° with respect to the comparable bands in the rod outer segments.

FIGURE 23 Four of the phagosomes visible in this field contain intensely labeled groups of outer segment discs (arrows). These have been tilted at various angles, demonstrating that the detached fragments are readily displaced within the pigment epithelium.



segments stain similarly with PAS, but the inclusions stain more intensely with toluidine blue.

In the first experiment, in which the paraffin technique was used, the heavily labeled discs had not yet reached the apex of the rod outer segments 30 days after injection of methionine-³H. The pigment epithelial cell inclusion bodies were not labeled. However, at 33 and 37 days, radioactivity began to appear in the inclusion bodies situated near the retinal periphery. This corresponded to the disappearance of the moving band of radioactivity from many of the rod outer segments in this

region. By 44 days, the reaction band had also vanished from many of the rods situated near the center of the retina. At the same time, the incidence of radioactive inclusion bodies in the central retina had risen markedly. In the periphery, no heavily labeled outer segment discs remained, but the intense labeling of many of the inclusion bodies was strikingly apparent.

In the second series of animals, in which the frogs were injected with a mixture of radioactive leucine and phenylalanine, the fate of the moving band of outer segment protein could be followed

FIGURES 24-34 Junction of the rod outer segments with the pigment epithelium. The final stages in the disposal of the intensely radioactive membranous discs are depicted in this plate. 68 days postinjection. Radioautograms; $\frac{1}{2}\mu$ Araldite sections; toluidine blue. $\times 1.150$,

FIGURE 24 The heavily labeled outer segment discs have been shed from all but one of the rods visible in this field. A phagosome (arrow) contains a group of these highly radioactive membranes which have been bent into a curved arrangement (See Fig. 35).

FIGURE 25 A single rod outer segment still retains a group of intensely reactive discs. Radioactive material derived from such discs is present in the phagosome.

FIGURE 26 Several radioactive phagosomes can be seen. They contain intensely labeled membranous discs which have been detached from the adjacent rod outer segments.

FIGURE 27 The band of intense radioactivity has disappeared from all of the rods (See Fig. 1, D). A residual mass of radioactive material persists in the phagosome located near the base of the pigment epithelium (arrow). A second phagosome (center) is weakly radioactive, as are the outer segments.

FIGURE 28 Weakly labeled and intensely labeled phagosomes are both visible.

FIGURE 29 A phagosome, well removed from the rod outer segments, and containing material derived from the heavily labeled outer segment membranes, is visible at the upper right, above the oil droplet.

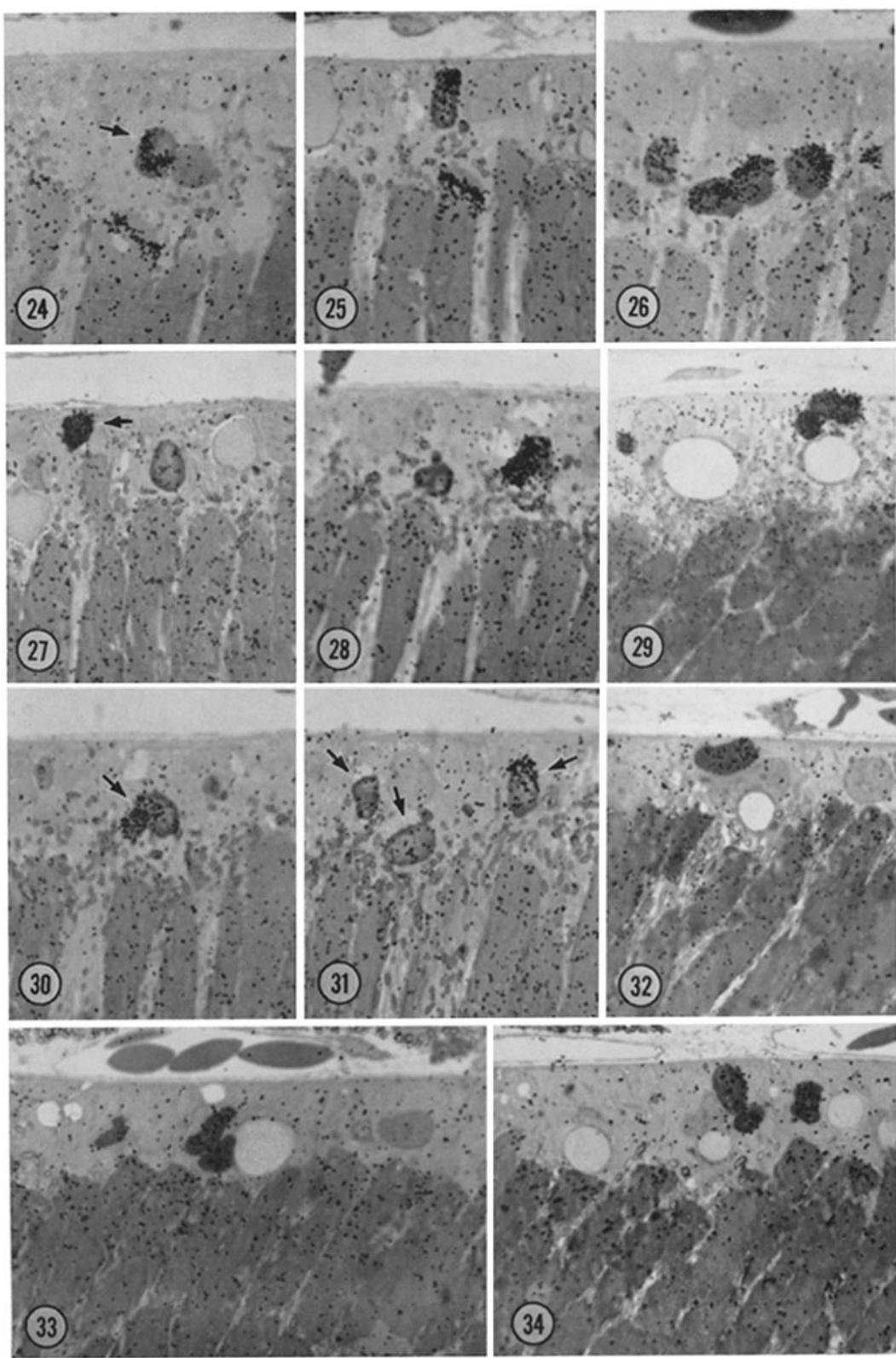
FIGURE 30 The heavily labeled phagosomes which persist 68 days after injection (arrow) are the most intensely radioactive structures in the retina at this interval.

FIGURE 31 Three phagosomes are shown (arrows). One contains remnants of the highly labeled outer segment discs.

FIGURE 32 The moderately labeled phagosome is separated from the rod outer segments by a nucleus and oil droplet in the pigment epithelium.

FIGURE 33 Three oddly shaped and darkly stained phagosomes are shown. They are weakly and diffusely labeled, as are the adjacent rod outer segments.

FIGURE 34 The phagosomes now contain outer segment material formed after the specific activity of the injected radioactivity has dropped to relatively low levels. Both unlabeled and intensely labeled phagosomes have been eliminated from the pigment epithelium.



with greater clarity, owing to the improved fixation obtained with aldehyde-osmium tetroxide, and the increased resolution resulting from the use of thinner sections. The time at which the moving group of heavily labeled discs reached the apical end of the rod outer segments was somewhat later in this series of frogs, which had been maintained at a slightly lower ambient temperature (6). The more rapid disappearance of the reaction band from the peripheral retina was confirmed. The following description refers to the central retina.

By 47 days after injection, the intensely labeled group of rod outer segment discs had been considerably displaced, but had not yet reached the extremity of the cell. In front of the moving reaction band the outer segment was unlabeled. Behind the band, the outer segment was weakly and diffusely reactive all the way to its base. In the pigment epithelium, a weak, unlocalized labeling of cytoplasm and nucleus was evident. However, the lipid droplets and inclusion bodies were uniformly free of radioactivity (Figs. 3-6).

At 50 days, the band of heavy radioactivity was now closer to the end of the outer segment. A few exceptional cases were observed in which the reaction band had disappeared from a rod. In these regions, intensely labeled inclusion bodies were observed in the adjacent pigment epithelium. Apart from these rare examples, the inclusion bodies were unlabeled (Figs. 7-9).

Between 54 and 59 days, the groups of heavily labeled discs completed their migration to the ends of the outer segments. The reaction bands were now present either at or near the apical extremity of the outer segments, or else they had disappeared. Some of the inclusion bodies remained unlabeled. However, by 59 days the majority of the inclusion bodies were intensely reactive (Figs. 10-15).

A localization of labeling could be observed in many of the inclusion bodies. Frequently, this consisted of a distinct banding, comparable to that observed in the rod outer segments (Figs. 11, 15-17, 21-23). Sometimes the reaction band in the inclusions was curved or bent into a "U" shape, something which never occurred in the rod outer segments (Figs. 13, 19, 24). In other instances, the band was straight, but tilted at an angle to the outer segment bands, which are characteristically oriented in the same plane (Figs. 11, 15, 22, 23).

At the latest intervals studied (61 and 68 days),

outer segment reaction bands were extremely rare. When present, they occurred at the apical extremity of the cell (Figs. 16-25). The entire rod outer segment was now weakly and diffusely reactive. In the pigment epithelium, there was a comparable diffuse cytoplasmic and nuclear labeling. The lipid droplets remained unlabeled, but all the inclusion bodies were now radioactive. A few were intensely reactive, but the majority showed a diffuse, relatively weak labeling, comparable to that of the rod outer segments (Figs. 26-34).

Preliminary examination of this material by electron microscope radioautography has revealed that the inclusion bodies in the pigment epithelium closely resemble rod outer segments. They are composed of stacked membranous elements, tightly and regularly packed together. They differ from the outer segments in that they are more dense, and appear to be more heavily stained by the uranium-lead procedure. The constituent membranes are often curved or bent (Fig. 35), and may form complex whorl-like arrangements. Occasionally, they are uniformly straight, as in the outer segments themselves. The derivation of these pigment epithelial inclusion bodies from the outer segments of the retinal rods is apparent from the radioautographic detection of a group of heavily labeled membranes in the inclusion bodies at the time when such membranous discs are being shed from the apex of the rod outer segments (Fig. 35).

DISCUSSION

Newly formed protein, made radioactive by the injection of labeled amino acids, is delivered from its site of synthesis in the rod inner segment to the base of the rod outer segment, where it is incorporated into membranous discs in process of assembly there. The labeled membranes are then progressively displaced along the outer segment, as a result of the repeated formation of new discs at the base of that structure (6-10).

That part of the outer segment in front of the moving band of highly radioactive discs is unlabeled during the period studied.¹ These older discs

¹ A relatively small amount of radioactive protein does infiltrate between or into the older discs, already assembled at the time of injection (8). This "diffuse" protein renewal component has a relatively short half-life (D. Bok, unpublished), and has essentially disappeared as a source of protein radioactivity at the intervals herein analyzed.

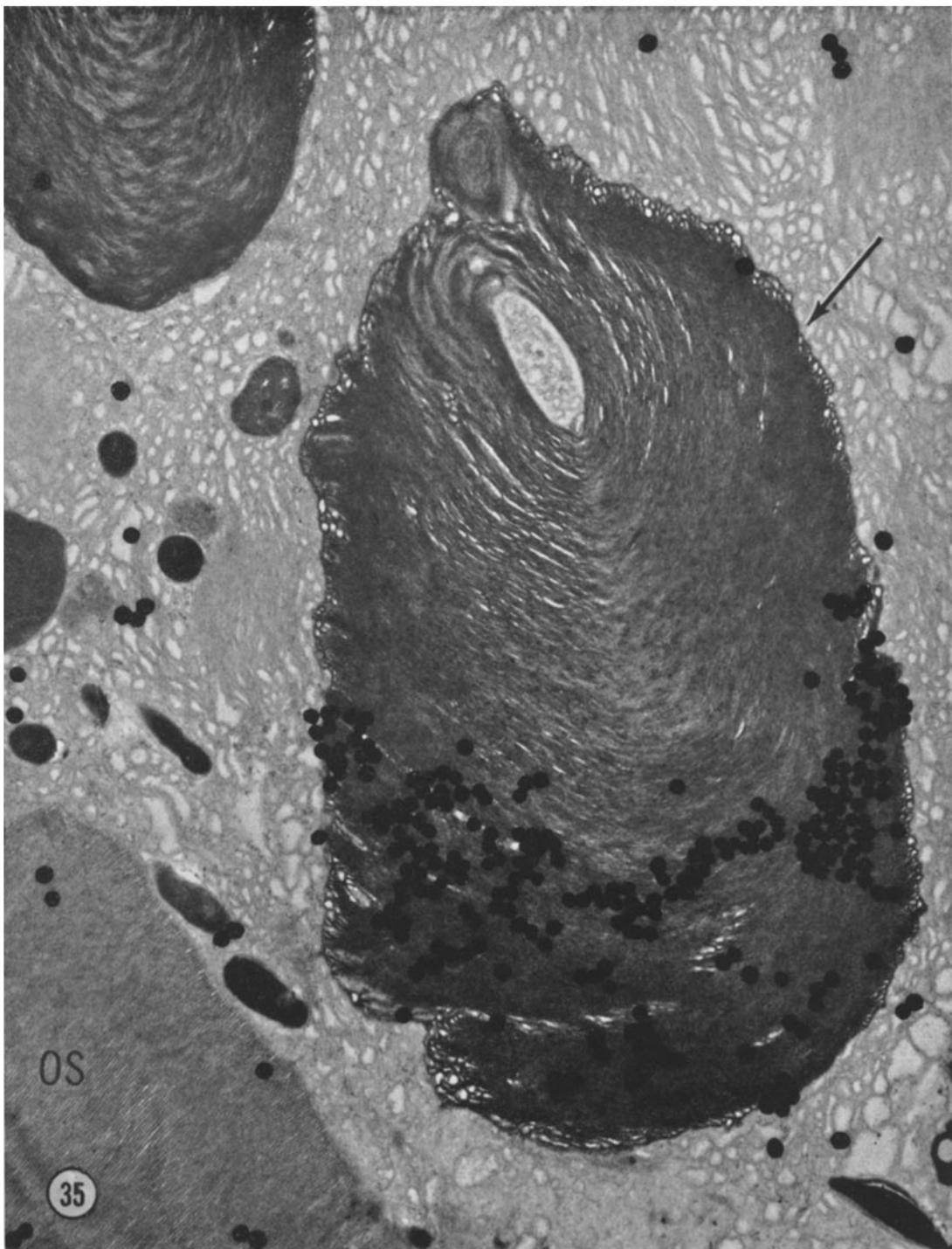


FIGURE 35 Electron microscope radioautogram of retinal rod outer segment fragment (arrow) within the pigment epithelium. Such inclusions have been termed *phagosomes* to clearly denote that they are foreign bodies engulfed by the pigment epithelium, and therefore different in origin from the cellular inclusions produced by synthesis within the pigment epithelium. A portion of an intact rod outer segment (*os*) is visible, lower left. Note that the membranous discs of the detached outer segment fragment have been compacted and deformed. The membranes in the lower part of the phagosome are intensely radioactive, whereas those comprising the upper part are unlabeled. This indicates that the membranous discs of the upper portion are older, and were assembled (at the base of the rod outer segment) before the animal was injected with radioactive amino acids. Both phagosomes are surrounded by smooth endoplasmic reticulum in the cytoplasm of the pigment epithelium. 68 days postinjection. $\times 12,000$.

were synthesized and assembled before the radioactive amino acids were injected. In contrast, the discs formed during the first few hours after injection are the most intensely labeled of all, because they were formed during the period when the concentration of radioactive precursor molecules was highest. The outer segment behind the moving band of heavily labeled discs is weakly labeled, owing to the availability of progressively lower levels of circulating precursor in the tissue fluids for an extended period after injection (6).

None of the pigment epithelial inclusion bodies show any radioactivity for several weeks after injection, despite the availability of labeled precursors. Thus, these structures do not appear to be undergoing synthesis from amino acid precursors in the pigment epithelium. The inclusions remain free of radioactivity as long as the heavily labeled membranes are retained within the adjacent rod outer segments.

Ultimately, the moving group of intensely radioactive discs arrives at the apical extremity of the outer segment, which is embedded in the cytoplasm of the pigment epithelium. In the frog, this arrival occurs slightly earlier in green rods than in red rods, is influenced to a minor degree by retinal illumination, and is accelerated considerably by an increase in ambient temperature (6). The present work demonstrates that it also occurs slightly earlier at the periphery than in the center of the retina.

As soon as the intense radioactivity leaves the end of the outer segment, a comparable level of radioactivity appears in the adjacent pigment epithelial cell inclusion bodies. The source of this radioactivity must be the rod outer segments. In fact, every available indication is perfectly consistent with the conclusion that *the pigment epithelial cell inclusion bodies are fragments of rod outer segments*—groups of membranous discs detached from the ends of the adjacent rods as a component of the normal renewal process. We shall hereafter refer to these bodies as *phagosomes*, to more clearly denote that they are engulfed foreign bodies, different in origin from the cellular inclusions (pigment granules, oil droplets, myeloid bodies) produced by the pigment epithelial cells themselves. The phagosomes unquestionably correspond to the “laminated lipid bodies” previously described in the frog pigment epithelium (5).

During the first few weeks after injection, the phagosomes contain no detectable amounts of

radioactivity, because they consist of detached groups of outer segment discs formed before the injection of the radioactive amino acids. When the heavily labeled discs reach the outer segment apex and are detached from it, they are readily identified among the phagosomes because of their high content of radioactivity. Unlabeled, intensely labeled, and weakly labeled portions of phagosomes are observed at this time. These represent groups of rod outer segment discs formed before, during, or after the period when radioactive amino acids first became available. The unlabeled phagosomes disappear from the pigment epithelium after the last of the unlabeled discs have been shed from the ends of the rod outer segments. Subsequently, the intensely labeled phagosomes also disappear, and are replaced by phagosomes containing a low level of radioactivity, comparable to that of the rod outer segments from which they are directly derived.

Previous work indicates that the intensely labeled disc membranes are formed during a period of 4–6 hr, and that about 36 discs per day are assembled in frog red rods at room temperature (8). The large size of many of the phagosomes indicates that they represent the product of several days of repeated disc formation. Available evidence strongly suggests that there is a continual synthesis of rod outer segment discs (which continues even in total darkness [6]). In contrast, the removal of old disc material appears to be an intermittent process.

As soon as a group of discs has been detached from the rod outer segment, the membranous material begins to undergo chemical changes, revealed by changed staining properties. Within the pigment epithelial cytoplasm the phagosomes may be turned or rotated, and the detached discs may become warped, bent, or otherwise deformed. Phagosomes occur near the base of the pigment epithelium, well removed from the ends of the outer segments, indicating that the engulfed cell fragments tend to be shifted towards the surface of the cell which faces the capillary network of the choroid. The phagosomes in this zone tend to be smaller, suggesting that a reduction in volume may occur during this displacement.

Ultimately, the membranous material is eliminated from the pigment epithelium. This is evident (a) from the fact that there is no progressive increase in the number of phagosomes, (b) from the

disappearance of unlabeled phagosomes following the detachment of radioactive discs from the extremities of the rod outer segments, and (c) from the subsequent disappearance of intensely labeled phagosomes.

The intracellular mechanism by which the material contained in the phagosomes is destroyed remains to be explored. We are continuing the

study of this problem with the use of electron microscope radioautography.

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