RENEWAL OF GLYCEROL IN THE VISUAL CELLS 
AND PIGMENT EPITHELIUM OF THE FROG RETINA

CAROL BIBB and RICHARD W. YOUNG

From the Department of Anatomy and the Jules Stein Eye Institute, University of California at Los Angeles School of Medicine, Los Angeles, California 90024

ABSTRACT

The renewal of glycerol in the visual cells and pigment epithelium of the frog retina was studied by autoradiographic analysis of animals injected with [2-11]glycerol. Assay of chloroform:methanol extracts showed that the labeled precursor was used mainly in lipid synthesis, although there was also some utilization in the formation of protein. Radioactive glycerol was initially concentrated in the myoid portion of rods and cones, indicating that this is the site of phospholipid synthesis in visual cells. The glycogen bodies (paraboloids) of accessory cones were also heavily labeled, suggesting the diversion of some glycerol into glycogenic pathways. In the pigment epithelium, only the oil droplets became significantly radioactive. The outer plexiform layer (which contains the visual cell synaptic bodies) and the cone oil droplets gradually accumulated considerable amounts of labeled material. Within 1-4 h, labeled molecules began to appear in the visual cell outer segments, evidently having been transported there from the myoid portion of the inner segment. Most of these were phospholipid molecules which became distributed throughout the outer segments, presumably replacing comparable constituents in existing membranes. In rods only, there was also an aggregation of labeled material at the base of the outer segment due to membrane biogenesis. These highly radioactive membranes, containing labeled molecules of lipid and protein, were subsequently displaced along the rod outer segments due to repeated membrane assembly at the base. The distribution of radioactivity supported the conclusion that membrane renewal by molecular replacement is more rapid for lipid than it is for protein.

INTRODUCTION

Renewal of the stack of disk-shaped, light-sensitive membranes which comprises the outer segment of rod and cone visual cells has been studied extensively by autoradiography using radioactive amino acids as protein precursors (5, 9, 13, 29, 30, 32, 33, 37, 39). Within minutes after injection into vertebrate animals, labeled amino acids become concentrated in the myoid portion of the inner segment of the cell (Fig. 1). This cell compartment, which contains free and membrane-bound ribosomes, the Golgi complex, and essentially all of the cell's cytoplasmic RNA (3, 31) is the major site of protein synthesis in both rods and cones. Protein destined for the renewal of the outer segment membranes is transported past the mitochondria massed in the ellipsoid region, and then through
FIGURE 1 Diagram of rod and cone visual cells and their relationship to the pigment epithelium in the frog retina. In the pigment epithelium, the oil droplets are storage sites for vitamin A. Phagosomes contain groups of phagocytized membranes shed from the tip of the rod outer segments. In the visual cells, the outer segment consists of a photosensitive stack of disk-shaped double membranes, derived from the outer cell membrane by infolding. The ellipsoid is filled with mitochondria, whereas the myoid contains a granular endoplasmic reticulum, free ribosomes, and the Golgi complex. Together, the ellipsoid and myoid make up the inner segment of the cell. A double cone is shown. The principal member contains an oil droplet (of unknown function); the accessory member contains a glycogen mass (paraboloid). A few rods with short outer segments and some single cones bearing oil droplets are also present in small numbers in the retina.

Adjacent disks by a thin film of cytoplasm. When the proteins in new disks are labeled as a result of the injection of radioactive amino acids, the fate of these labeled disks may be followed by autoradiography. Repeated formation of membranous disks at the base of the rod outer segment displaces previously formed disks away from the base and towards the pigment epithelium. To maintain the length of the stack of outer segment membranes, continual membrane biogenesis at the base is balanced by the intermittent shedding of groups of old membranes from the apex. These discarded membranes are phagocytized and destroyed by the pigment epithelium (34, 37). This mechanism of renewal by membrane replacement apparently occurs only in rods (32, 35).

In rod outer segments, protein which is not used in the assembly of new membranes becomes diffusely distributed in or among the previously formed disks. In cones, all of the new protein delivered to the outer segment becomes diffusely distributed in this manner (5, 32). This represents a different type of renewal process—renewal by molecular replacement—in which new molecules replace old ones in or among the membranes of the existing membrane system (36).

When labeled fatty acids are used as precursors of the lipid components of the membranes in frogs (2), a different autoradiographic pattern is observed. Instead of initially being concentrated in the myoid part of the cell, in the manner of amino acids, the fatty acids immediately appear throughout the cell, including the outer segments. Outer segment membranes contain abundant fatty acids as components of phospholipid molecules, but lack the enzymes to produce phospholipids (27). We therefore concluded that the direct incorporation of fatty acids in the outer segments is due to fatty acid exchange in phospholipid molecules already situated in the membranes (2). Gradually, the concentration of radioactive material in the visual cell outer segment membranes increases, apparently as a result of the addition of new phospholipid molecules containing labeled fatty acids, perhaps supplemented by the transfer to the outer segments from the pigment epithelium of vitamin A esterified with fatty acid. Within a few days, labeled fatty acid becomes intensely localized in the new membranes which have formed at the base of the rod outer segments since the injection. But this discrete concentration of radioactivity in new membrane is short-lived, apparently due to the rapid rate of fatty acid renewal. In contrast, when
proteins of the disk membranes are labeled, there is no significant decline in their content of radioactivity as they are displaced along the outer segment (13, 29).

These studies demonstrated that the lipids of the visual cell outer segment membranes are continually renewed, provided further evidence of both membrane and molecular replacement, and revealed some differences in the mechanisms by which the lipid and protein constituents of the membranes undergo turnover. However, the complexity of fatty acid metabolism in these cells, involving processes of exchange, vitamin A esterification, and phospholipid synthesis—all of which are superimposed in the autoradiographic image—did not enable us to answer the question of where phospholipid synthesis takes place.

Glycerol should serve as a useful lipid precursor in the study of phospholipid synthesis, as well as membrane biogenesis and renewal. About 40% of the dry weight of the outer segment membranes is lipid, most of which is phospholipid (1, 6, 11, 20). Glycerol is the structural backbone of the phospholipid molecule, does not undergo exchange once it has been incorporated into phospholipid, and is not involved in vitamin A esterification. Accordingly, we have used tritiated glycerol in the autoradiographic analysis of lipid renewal in the visual cells and pigment epithelium of the frog, with particular emphasis on the membranes of the rod and cone outer segments.

MATERIALS AND METHODS

 Autoradiography

Nine frogs (Rana pipiens), weighing between 2.4 and 15.0 g (average, 8.0 g), were injected with [2-3H]glycerol in aqueous solution at a concentration of 100 mCi/ml. The labeled compound was obtained from New England Nuclear Corp., Boston, Mass., at a specific activity of 7.85 to 8.81 Ci/mmol. The animals were killed at 15 min, 1 h (2 frogs), 4 h, 15 h, 1 day, 1 wk, 5 wk, and 8 wk after injection. The dosage was 1 mCi/g body weight, except that the frog killed at 15 min received 10 mCi/g. All injections were made intravenously, under anesthesia, except for the animals killed at 1 wk or later, which were injected in the dorsal lymph sac without anesthesia. At sacrifice, the eyes were fixed in phosphate-buffered 4% formaldehyde, pH 7.2, and left in that solution over night. The posterior half of the eye was then cut into smaller pieces, which were rinsed in buffer. Half of these were then fixed in 1% osmium tetroxide and embedded in Araldite (2). The remaining specimens were subjected to prolonged extraction with chloroform: methanol (2:1, vol/vol) to remove lipids. They were then fixed in osmium tetroxide and embedded in Araldite (2).

The autoradiograms were developed in Kodak Dektol (Eastman Kodak Co., Rochester, N. Y.) for 2 min at 17°C after an average exposure period (at 4°C under low humidity) of 1-2 mo.

Developed silver grains overlaying components of the visual cells and adjoining pigment epithelium were counted at several intervals in order to obtain a quantitative estimate of the distribution of radioactivity. Autoradiograms of frogs killed at 1 h, 15 h, 1 wk, and 5 wk were analyzed, using preparations in which lipids had been preserved. The lipid-extracted specimens from the 5-wk animal were similarly assessed. Counts were made using a grid mounted in the ocular lens of the microscope, and were expressed as grains/grid square. Each square had an area of 25 μm² at the magnification used. The cytoplasm, oil droplets, nuclei, and phagosomes of the pigment epithelium were analyzed, as were the outer segments (rods only), ellipsoids, myoids, nuclei, and synaptic bodies of the visual cells. Grains over the small cone outer segments were not counted. However, the labeling of the oil droplets and paraboloids (Fig. 1) in cone visual cells was analyzed.

Radiobiochemistry

45 frogs, with an average weight of 34.7 g, were injected intravenously with 1.7 μCi/g of [2-3H]glycerol (7.85 Ci/mmol) and were then raised under normal laboratory lighting conditions. The frogs, divided into three groups of 15, and dark-adapted 2 h before sacrifice, were killed at 1 day, 7 days, and 14 days after injection. Rod outer segments were prepared by sucrose floatation and repeated pH 7.1 phosphate buffer washes (15). Two-thirds of the outer segment preparation was extracted with chloroform:methanol (2:1, vol/vol), washed with 0.2 vol of 0.1 M NaCl solution, and the upper, water-methanol layer was discarded. The lower layer was taken to dryness, redissolved in chloroform:methanol, and the content of radioactivity determined by liquid scintillation spectrometry. The remaining material was solubilized in 10% hexadecytrimethylammonium bromide (CTAB) in 0.067 M phosphate buffer, pH 7.1, then fractionated on a column of agarose (13) which separates visual pigment from phospholipid. The visual pigment fraction was identified by measurement of absorption at 280 nm and 500 nm, and the content of radioactivity was determined by liquid scintillation spectrometry. These analyses were carried out in the laboratory of Dr. M. O. Hall, Jules Stein Eye Institute.

RESULTS

 Autoradiography

Despite the very high levels of radioactive glycerol administered to the animal killed 15 min after injection, very little radioactivity had accu-
mulated in the retina during that interval. With prolonged autoradiographic exposure it could be determined that the most heavily labeled retinal structures were the paraboloids of the accessory cones (Fig. 2). Otherwise, the most intense concentrations of radioactivity occurred in the myoid portion of the rod and cone inner segments. A weak, scattered labeling was discernible in the outer nuclear layer, containing the rod and cone nuclei, and in the outer plexiform layer, in which are situated the synaptic endings of the visual cells and adjoining neurons. Labeling of the ellipsoids was negligible, and the outer segments were not reactive. A few grains were detected over the cytoplasm of the pigment epithelium, but the nuclei and phagosomes were not labeled. Occasionally an oil droplet was overlaid by 1 to 3 silver grains.

By 1 h (Figs. 3–5, Table I) the intensity of the autoradiographic reaction had increased. In the pigment epithelium, radioactive material now had accumulated in significant amounts in the oil droplets, which were labeled to a considerably higher degree than the surrounding, weakly reactive cytoplasm. Even fewer grains were associated with the nuclei, and the phagosomes remained unlabeled. In the visual cells, the accessory cone paraboloids were highly radioactive. The myoid portion of the inner segment contained the next highest concentrations of radioactive material, although the synaptic bodies were now nearly as heavily labeled, due to the marked increase in labeling which had taken place in the outer plexiform layer. A few grains lay over the ellipsoids, often near the periphery. At the base of a few rod outer segments the suggestion of beginning accumulation of radioactive material was visible. Otherwise, the rod and cone outer segments were devoid of radioactivity.

4 h after injection (Figs. 6–8), labeling formerly confined to the visual cell myoids appeared to be spreading through the ellipsoids towards the outer segments. Although the concentration of labeling in the ellipsoid rose only slightly, the preferential aggregation of silver grains at the base of each rod outer segment was now apparent (Fig. 8). Otherwise, there was only a weakly scattered labeling of the outer segments in both rods and cones. In cones with oil droplets, a few grains were associated with the perimeter of these structures, although the lipid within the droplets showed no evidence of radioactivity. The paraboloids of accessory cones continued to contain the greatest concentration of radioactivity in the visual cells, but the accumulation of labeled material in the outer plexiform layer had continued, so that the

| Table I |

Concentration of Radioactivity* in Components of the Pigment Epithelium and Visual Cells at Different Intervals after Injection of [1H]Glycerol

| Components                  | Time after injection |
|-----------------------------|----------------------|
|                             | 1 h       | 15 h      | 1 wk       | 5 wk       |
| Pigment epithelium          |           |           |            |            |
| Cytoplasm                   | 0.5       | 2.8       | 3.1        | 1.3        |
| Oil droplets                | 2.7       | 10.0      | 8.9        | 2.2        |
| Nucleus                     | 0.4       | 1.8       | 1.3        | 0.4        |
| Phagosomes                  | 0.1       | 0.5       | 1.9        | 1.2        |
| Visual cells                |           |           |            |            |
| Outer segment (rods)†       | 0.2       | 0.5       | 1.5        | 1.3        |
| Band                        | —         | —         | 22.3       | 19.2       |
| Ellipsoid                   | 1.4       | 1.3       | 1.4        | 0.9        |
| Myoid                       | 5.5       | 6.5       | 6.1        | 1.2        |
| Nucleus§                    | 1.0       | 1.9       | 2.0        | 0.8        |
| Synaptic body‖              | 4.6       | 24.7      | 11.6       | 5.3        |
| Paraboloid (cones)          | 28.6      | 30.1      | 25.0       | 4.6        |
| Oil droplet (cones)         | 0.4       | 1.6       | 7.5        | 2.0        |

* Silver grains per unit area in autoradiograms; 1 mCi/g dose; 2-mo exposure.
† Region scleral to the band of intensely labeled disks.
§ Outer nuclear layer.
‖ Outer plexiform layer.
synaptic bodies of both rods and cones were now very heavily reactive. In contrast, the nuclei were practically devoid of labeling. What radioactive material was present in the outer nuclear layer appeared to reside predominantly between the nuclei, rather than in them (Fig. 8). In the pigment epithelium, there had been no increase of labeling in the cytoplasm or nuclei, but the amount of reactive material in the oil droplets continued to rise (Fig. 6). Phagosomes remained unreactive.

At 15–24 h after injection (Figs. 9, 10, Table I) it was apparent that the gradual rise in labeling had continued. The greatest relative increase had occurred in the synaptic bodies, which were now as heavily labeled as the paraboloids of accessory cones. The aggregation of radioactive material at the base of the rod outer segments was more pronounced, and a weak, diffuse reaction was now more clearly visible over the rod and cone outer segments. Most of the cone oil droplets showed weak labeling. The grains formerly situated at their perimeters were no longer present. The myoids in both rods and cones were still prominently labeled, but the nuclear reaction was very weak, and largely restricted to the sparse, perinuclear cytoplasm. Labeling of the pigment epithelium had also increased slightly. Highest concentrations occurred in the oil droplets. A few grains were now observed over some of the phagosomes.

In the frog killed 1 wk after injection of [H]glycerol (Fig. 11, Table I), the synaptic bodies of the visual cells and the paraboloids of accessory cones continued to be the most heavily labeled retinal structures, although both had declined in

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**FIGURES 2-8** Pigment epithelium and visual cells from frogs injected intravenously with [2-1H]glycerol and killed between 15 min and 4 h later. Autoradiograms; 1/2 µm Araldite sections; lipid-preserved specimens; toluidine blue staining.

**FIGURE 2** 15 min after injection. Despite a tenfold increase in dosage, and prolonged (2-mo) autoradiographic exposure, there was only a weak reaction in autoradiograms prepared at this interval. The greatest accumulation of radioactivity had taken place in the accessory cone paraboloids (arrow). Next most heavily labeled were the myoids of the rods and cones (m). x 800.

**FIGURE 3** 1 h after injection. The intense concentration of labeled material in the myoid portion of the rod and cone inner segments is readily apparent. The darkly stained, round structures (arrow) are cone oil droplets. Note the relative absence of significant labeling in the cytoplasm (c) of the pigment epithelium. x 800.

**FIGURE 4** 1 h after injection. The paraboloids of accessory cones (arrows) are highly radioactive, presumably due to the incorporation of the labeled precursor into glycogen. Heavy labeling of the myoid (m) is believed to be a result of the utilization of [H]glycerol in the synthesis of protein and phospholipid. e, ellipsoid. Radioactive material has begun to aggregate in the outer plexiform layer (oPL), which contains the synaptic bodies of the visual cells. x 1,250.

**FIGURE 5** 1 h after injection. Although there is relatively little incorporation of [H]glycerol into the cytoplasm (c) and nuclei (n) of the pigment epithelium, there is appreciable deposition of the precursor in the oil droplets (arrows). x 1,250.

**FIGURE 6** 4 h after injection. In the pigment epithelium the oil droplets (arrow) continue to contain radioactive material, but the phagosomes (p) are not labeled. x 1,250.

**FIGURE 7** 4 h after injection. There has been a relative and absolute increase in labeling of the outer plexiform layer (oPL). The myoids (m) of the visual cells remain heavily reactive. Between these two layers, the nuclei of the rods and cones are practically devoid of radioactivity, although some exposed silver grains are associated with the nuclear peripheries or internuclear material. Labeled molecules have begun to accumulate at the base of the rod outer segments (arrow). (See Figs. 8). Otherwise, there is only a weak scattering of silver grains over the outer segments. In the pigment epithelium, only the oil droplets (oD) are well labeled. x 800.

**FIGURE 8** 4 h after injection. Note the heavy labeling of the visual cell myoids (m) and the outer plexiform layer (oPL). The intervening nuclei have not used much of the injected [H]glycerol. Labeled material, evidently including both protein and phospholipid, is aggregating at the base of each rod outer segment (arrow), presumably having been transported from the myoid region. This aggregation is due to the assembly of new membranes. x 1,250.
labeling intensity. The decrease in radioactivity was most pronounced in the region of the synaptic bodies, which now contained less than half of the labeled material which had been present at 15–24 h. In contrast, labeling of the cone oil droplets had noticeably increased. The diffuse labeling in rod and cone outer segments and in phagosomes within the pigment epithelium also had increased. In rods, the discrete, intense band of radioactive material, which had been located near the base of each outer segment at 1 day, was now slightly displaced from the base.

5 wk after injection, this band of labeled material was located near the apical ends of the rod outer segments (Fig. 12). There was a weak, diffuse labeling on the outer (scleral) side of the band and a slightly heavier labeling on the inner (vitreal) side, within the outer segment. Radioactive material had been lost from all other constituents of the visual cells and pigment epithelium to such a considerable extent that this group of preferentially radioactive disk membranes was now by far the most intensely reactive component in the retina. Labeling of the synaptic bodies, myoids, and cone paraboloids, as well as that of the oil droplets in the cones and the pigment epithelium, had declined to about 20% of its peak intensity.

By 8 wk, the intense reaction band, formerly present in the rod outer segments, had disappeared. Only a weak, random labeling could be discerned in association with the visual cells and pigment epithelium, except that a few phagosomes showed evidence of significant residual content of radioactivity.

Extraction of the tissue specimens with chloroform-methanol solution before fixation with osmium tetroxide removed practically all of the radioactivity from the oil droplets in cones and in the pigment epithelium. Grain counts in the case of

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**Figures 9-14** Pigment epithelium and visual cells from frogs injected with [2-3H]glycerol and killed between 15 h and 5 wk later. Lipid-preserved specimens, except for Fig. 14, which is lipid-extracted. Autoradiograms; ½ µm Araldite sections; toluidine blue staining.

**Figure 9** 15 h after injection. The accumulation of radioactivity at the base of each rod outer segment and in the outer plexiform layer has increased. × 1,250.

**Figure 10** 15 h after injection. In the pigment epithelium, the nuclei (n) contain very little radioactivity, the cytoplasm is diffusely and rather lightly labeled, but the oil droplets (od) are intensely reactive. A weak labeling of the rod outer segments (ros) and phagosomes (arrows) is now apparent. × 1,250.

**Figure 11** 1 wk after injection. Labeling in the outer plexiform layer (opl) has declined. In contrast, the diffuse reaction over the rod outer segments (ros) has perceptibly increased. The highly radioactive disk membranes, assembled at the base of the rod outer segments during the first hours after the injection, have now been displaced from the base due to the repeated assembly of newer membranes. Note the grains overlying the cone oil droplets (arrows), which attain their peak labeling at about this time. × 800.

**Figure 12** 5 wk after injection. Labeling in all structures has declined, except for the intensely radioactive membranes in the rod outer segments (arrows), which are now the most heavily labeled components in the retina. Observe that they have been displaced more than half-way along the outer segments. × 800.

**Figure 13** 5 wk after injection. This figure, when compared with Fig. 14, demonstrates the loss of radioactive material which resulted from extraction of tissue specimens with chloroform-methanol solution. With maximal retention of lipid shown here, the outer segments are filled with radioactive material, which is particularly concentrated in a few disk membranes which are situated in the outer half of the outer segment. The radioactive molecules which are located in the outermost portion of the outer segments (above the most heavily labeled disks) have infiltrated membranes which had been assembled before the injection of [3H]glycerol in the process of renewal by molecular replacement. 3-mo exposure, × 1,250.

**Figure 14** 5 wk after injection. This autoradiogram was prepared from the same retina which yielded the specimen shown in Fig. 13, except that the retinal fragment was extracted with chloroform:methanol (2:1, vol/vol) before fixation in osmium tetroxide. Most of the radioactivity situated in the outermost portion of the rod outer segments has been extracted, demonstrating that it was in lipid. There has also been loss of material from the rest of the outer segment, with the least decrease occurring in the intensely labeled membranes. 3-mo exposure, × 1,250.
the animal killed 5 wk after injection (Figs. 13, 14) showed a decrease of 93–95% in oil droplet labeling. No other structure showed a greater loss. The least amount of radioactivity was removed from the discrete band of labeled material which moved along the outer segments of rods between 4 h and 5 wk after injection. At 5 wk, quantitative analysis showed a loss of only 25% of the labeling from this band as a result of extraction with chloroform-methanol solution. This extraction procedure removed about half of the radioactivity from other compartments of the visual cells and pigment epithelium (range, 36–67%). Labeling on the outer (scleral) side of the rod outer segment reaction band was reduced more than half (about 60%). On the inner (vitreal) side, it was reduced by about 54%.

**Radiobiology**

Part of the purified rod outer segment preparation was extracted with chloroform-methanol solution to determine the amount of radioactivity in lipid. The remainder was solubilized in CTAB solution, then fractionated to separate the visual pigment (which consists of the protein, opsin, combined with vitamin A aldehyde). The total dpm/eye in the chloroform-methanol extract expressed as a ratio of the total dpm/eye in purified visual pigment at 1, 7, and 14 days after injection was 3.6, 3.9, and 4.8, respectively. This indicates that approximately four times as much glycerol was incorporated into lipid as into visual pigment protein in the rod outer segment membranes.

**DISCUSSION**

Glycerol, a small, water-soluble molecule, was administered to frogs in radioactive form by intravenous or intralymphatic injection. The visual cells and pigment epithelium incorporated some of the tritiated glycerol which had passed from the blood stream into the retina and used it in the synthesis of products which were thereby rendered radioactive. Prolonged initial fixation in aqueous formaldehyde solution and subsequent buffer rinses assured the extraction of unbound precursor molecules, so that residual tissue radioactivity could be ascribed to the larger product molecules retained by the aldehyde-osmium fixation procedure and the abbreviated dehydration-embedding sequence which we used to assure maximal retention of lipids.

One of our primary goals was to determine where phospholipids are produced in visual cells. Glycerol constitutes the structural backbone of phospholipids (excluding sphingomyelin), which are the major lipid constituents of the visual cell outer segment membranes. That glycerol was actually used by the cells in producing outer segment phospholipids was shown by purifying rod outer segments from [3H]glycerol-injected frogs, extracting the lipids from them, and then assaying the content of radioactivity in the extract. The vast majority of the radioactivity in these membranes was indeed in the lipid fraction. We further demonstrated the utilization of the injected glycerol in lipid synthesis by extracting samples of tissue with chloroform:methanol (2:1) solution. This solvent, which extracts lipids, removed a significant part of the radioactivity from the retina. Having demonstrated that the glycerol was used in the synthesis of lipids (probably almost entirely phospholipids), it remained to determine in what part of the cell the labeled precursor molecules were initially concentrated, because the site at which labeled product molecules are first detected may be presumed to be the site at which they are synthesized. 15 min after injection, only one region of all the rod and cone visual cells showed an accumulation of radioactive material—the myoid portion of the inner segment. We may therefore tentatively conclude that it is in the myoid region that phospholipids are produced.

Further support for this conclusion is provided by biochemical studies on the retina (27) and on other cell systems, particularly rat liver, which have revealed that the enzymes involved in phospholipid synthesis are localized in the "microsome" fraction of cells (7, 10, 19, 28). This fraction contains the endoplasmic reticulum, ribosomes, and Golgi complex—all of which are situated in the myoid region of visual cells. Autoradiographic studies are also consistent with this conclusion. These have indicated that fatty acids are esterified with glycerol in the endoplasmic reticulum of mouse mammary gland (24), rat heart muscle (25), and rat liver cells (22, 23), and that the phospholipids, phosphatidyl choline and phosphatidyl ethanolamine, are synthesized mainly in the endoplasmic reticulum of rat liver cells (26).

In summary, available evidence indicates that the incorporation of [3H]glycerol into the myoid portion of visual cells reflects the synthesis of phospholipids. However, some of the labeled precursor was used in protein synthesis, which also is localized in the myoid portion of the cell (29, 39).
Most of the glycerol which came to reside in the rod outer segment membranes was recovered in lipids (which are predominantly phospholipids; 11), but a significant labeling of visual pigment protein was also recorded. Furthermore, on the average only half of the radioactivity was removed from most components of the visual cells and pigment epithelium by extraction with a lipid solvent before autoradiography, indicating a significant utilization of the [3H]glycerol in nonlipid synthetic pathways. Labeling of cone paraboloids, which are glycogen deposits, further implies that some of the glycerol was used in glucose formation. Because there is some glycogen in the myoid of frog visual cells (31), and because glucosamine is incorporated into the myoid region (4), a small portion of the myoid labeling may also be due to carbohydrate metabolism. It is evident that the myoid region is the visual cell's major synthetic center and appears capable of producing all of the major constituents of the outer segment membranes.

Excess carbohydrate is stored in the retina as glycogen, particularly in avascular retinas such as that of the frog (12, 17, 31). In frog visual cells, the most prominent storage site is the paraboloid of accessory cones. However, glycogen is also abundant in the outer plexiform layer, where much of it is apparently located in the rod and cone synaptic bodies (although some may be in Müller fibers). After injection of [3H]glycerol, the paraboloids were already heavily labeled within 15 min. Evidently the synthesis of glycogen occurs in situ; that is, in the region where it is stored. Much of the intense buildup of labeled material in the outer plexiform layer was probably due also to glycogen production.

The paraboloids had practically reached peak labeling within 1 h, but the greatest content of radioactivity in the outer plexiform layer was not attained until 15–24 h after injection. Furthermore, labeling in this region dropped off rapidly. By 1 wk the concentration of radioactivity in the outer plexiform layer had declined to less than half of that in the paraboloids, whereas at 15 h they were at a similar level. This suggests a different metabolic function for the labeled material in these two zones. This is also revealed by their different responses to temperature. A drop in temperature increases glycogen in the plexiform layers, but diminishes it in the cone paraboloids; with higher temperatures, the situation is reversed (18, 31).

The rise in labeling of cone oil droplets was even more gradual, first becoming significant at 15 h, and reaching a peak (among the intervals sampled) 1 wk after injection. These colorless lipid droplets, of unknown function, apparently do not contain protein (39), but gradually become heavily labeled after administration of fatty acids. Peak labeling with fatty acids, as in the case of glycerol, is reached about a week after injection (2).

Neither the nuclei nor the ellipsoids of the visual cells were prominently labeled at any interval examined. A possible role for the nuclear membrane in phospholipid synthesis (8) cannot be excluded, because the scattering of grains in the outer nuclear layer was heaviest between the closely packed nuclei—but whether this was due to labeling of the nuclear membrane, the thin rim of perinuclear cytoplasm, or the slender intervening extensions of the Müller cells, we could not determine. Nuclei in the pigment epithelium also failed to make significant use of the injected glycerol. Lack of glycerol uptake in nuclei has also been observed in liver cells (23). Similarly, nuclei fail to incorporate appreciable quantities of fatty acids (2, 23), indicating that these organelles do not participate directly in a major way in lipid metabolism.

The outer segments of rods and cones failed to take up [3H]glycerol from the tissue fluid, as did the detached rod membranes present in the cytoplasm of the pigment epithelium (phagosomes). This is direct evidence that these membranes are incapable of synthesizing their own lipid constituents, a conclusion supported by in vitro radiobiological studies (27). Nevertheless, after a delay of 1–4 h, radioactive material began to appear in the outer segments of both classes of visual cells. As noted earlier, the source of these molecules is evidently the myoid region of the cell inner segment. In rods, much of the labeled material aggregated at the base of the outer segments, a sign that new lipid (and protein) was being used in the assembly of outer segment membranes. The displacement of these new, heavily labeled membranes along the rod outer segments, due to repeated formation of newer disks (29), was observed at 1 and 5 wk after injection. By 8 wk these highly radioactive membranes were no longer present in the outer segments. They had been shed from the end of the cell and phagocytized by the pigment epithelium (37), within which a few well-labeled phagosomes remained as evidence of this scavenging process.

Our failure to detect a small group of preferentially labeled membranes in cone outer segments at
any interval after injection is consistent with all previous autoradiographic studies of this class of visual membranes. Cones do not appear to form new membranes and shed old ones in the same manner as rods (32, 35).

Radiobiochemical assay indicated that approximately four times as much glycerol was incorporated into rod outer segment lipid as into visual pigment protein (which constitutes at least 80% of total protein in rod outer segment; 13, 14, 21).

Autoradiograms revealed that approximately 60% of the diffuse outer segment labeling was extractable with a lipid solvent. The 60% loss is based upon comparison with the “lipid-preserved” tissues, in which there is actually some loss of lipids—probably on the order of 10–15% (23). These measurements indicate that most of the diffusely distributed rod outer segment labeling is due to lipid.

In contrast, only about 25% of the radioactivity was extracted (in autoradiograms) from the “reaction band,” representing the heavily labeled new membranes which were assembled in the first few hours after the injection of radioactive glycerol. In other words, more than half of the radioactivity diffusely distributed among membranes which had been assembled before the injection was in lipid, but only one-fourth of the label in disks assembled immediately after the injection was in lipid. This means that—compared to protein—a greater proportion of new lipid is used for molecular replacement, and a lesser proportion is used for membrane assembly. However, we presume that a full complement of lipid, as well as protein, must be supplied for the assembly of the new membranes. Therefore, renewal by molecular replacement is apparently more rapid for lipid than it is for protein, a conclusion we also derived from our studies of fatty acid renewal (2). In fact, there is evidence that visual pigment protein is not replaced at all, once it has been inserted into the membrane (5, 13). The rate of molecular replacement is even more rapid for fatty acids than for the intact phospholipid molecules, because they are replaced by fatty acid exchange as well as by molecular replacement of phospholipids (2).

In the pigment epithelium, there was initially very little incorporation of [3H]glycerol, although by 1 h it was apparent that radioactive material was gradually accumulating in the oil droplets. This was the only organelle in the pigment epithelium which attained significant labeling. The oil droplets also contain radioactive material after injection into frogs of tritiated galactose, vitamin A (38), and fatty acids (2). The presence of a glycerol derivative and fatty acids in the oil droplets (which are completely extracted with chloroform-methanol) suggests that they may contain triglycerides and phospholipids as well as vitamin A esterified with fatty acid.

The technical assistance of Ms. Mirdza Lasmanis and Ms. Zoja Trirogoff is gratefully acknowledged. Drs. Dean Bok and Michael Hall offered helpful suggestions concerning the manuscript.

This research was supported by United States Public Health Service Grants EY 00095 and EY 00444.

Received for publication 28 December 1973.

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