

RENEWAL OF FATTY ACIDS IN THE MEMBRANES OF VISUAL CELL OUTER SEGMENTS

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ABSTRACT

The renewal of fatty acids in the visual cells and pigment epithelium of the frog retina was studied by autoradiographic analysis of animals injected with tritiated palmitic, stearic, or arachidonic acids. Most of the radioactive material could be extracted from the retina with chloroform-methanol, indicating that the fatty acids had been esterified in lipids. Analysis of the extracts, after injection of [^3H]palmitic acid, revealed that the radioactivity was predominantly in phospholipid. Palmitic acid was initially concentrated in the pigment epithelium, particularly in oil droplets which are storage sites for vitamin A esterified with fatty acid. The cytoplasm, but not the nucleus of these cells, was also heavily labeled. Radioactive fatty acid was bound immediately to the visual cell outer segment membranes, including detached rod membranes which had been phagocytized by the pigment epithelium. This is believed to be due to fatty acid exchange in phospholipid molecules already situated in the membranes. Gradually, the concentration of radioactive material in the visual cell outer segment membranes increased, apparently as a result of the addition of new phospholipid molecules, possibly augmented by the transfer from the pigment epithelium of esterified vitamin A. Injected fatty acid became particularly concentrated in new membranes which are continually assembled at the base of rod outer segments. This localized concentration was short-lived, apparently due to the rapid renewal of fatty acid. The results support the conclusion that rods renew the lipids of their outer segments by membrane replacement, whereas both rods and cones renew the membrane lipids by molecular replacement, including fatty acid exchange and replacement of phospholipid molecules in existing membranes.

INTRODUCTION

How are cell membranes formed and renewed? That query represents one of the basic unanswered questions in the study of living cells. The visual cells of the vertebrate retina—the rods and cones—are useful for the investigation of these processes because the photoreceptive organelle, called the outer segment, is a site of continuous membrane biogenesis and renewal.

Vision is initiated when light is absorbed by

pigment molecules situated in the outer segment membranes (Fig. 1). The membranes occur as a tall stack of double-layered, circular disks. The thickness of these disks is approximately 150 Å, with each of the two membranes being about 67 Å thick (28). In rods, the distance between the disks is 150 Å; in cones, it is slightly larger.

Like other cell membranes, the membranes of the visual cell outer segments consist predomi-

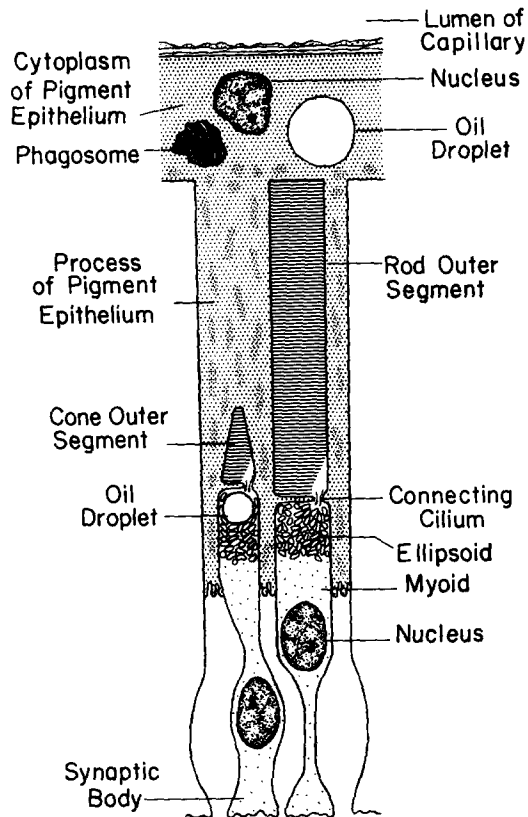


FIGURE 1 Diagram showing the relationship of the pigment epithelium (shaded, above) with the cone (left) and rod (right) visual cells in the frog retina. The outer segment of each visual cell consists of a stack of closely packed membranous disks, each disk being constructed of a double layer of cell membrane. Visual pigment molecules, which are sensitive to light, are situated in the outer segment membranes. The inner segment of the cell is subdivided into the ellipsoid which is filled with mitochondria, and the myoid, which contains the granular endoplasmic reticulum and Golgi apparatus.

nantly of protein combined with phospholipid. About 60% of the dry weight of the membranes is due to protein, and approximately 40% is lipid, most of which is phospholipid. The protein content is unusually simple, at least in rods, where as much as 80% of the membrane protein appears to be opsin (23, 24, 39). Opsin, when combined with vitamin A aldehyde (retinal), forms the visual pigment, rhodopsin.

The major phospholipids associated with rhodopsin in the membranes are phosphatidyl choline and phosphatidyl ethanolamine, which together comprise some 80–85% of total phospho-

lipids. Lesser amounts of phosphatidyl serine and very small amounts of phosphatidyl inositol and sphingomyelin are present. Glycolipids and traces of cholesterol have also been identified (3, 12, 19, 34). Attached to the glycerol backbone of each of the phospholipids (excluding sphingomyelin) are two fatty acids. Approximately 50% of the fatty acids are polyunsaturated and are located predominantly on the 2-position of the glycerol. The 1-position is occupied almost entirely by the saturated fatty acids, palmitic and stearic (3, 4, 12, 34, 37).

The presence of high proportions of unsaturated fatty acids coupled with the low content of cholesterol is associated with a fluidity of the membrane, the viscosity of the lipid phase being comparable to that of a light oil (14). The visual pigment molecules apparently float, partially immersed in this planar liquid, within which they may diffuse laterally (15, 49). They rotate freely, with retinal maintaining an orientation parallel to the plane of the membrane (13, 14).

When rhodopsin absorbs light, retinal may be released, then reduced to vitamin A in the visual cell outer segment, and esterified with a fatty acid—at least this seems to be the case if the retinal is not immediately reattached to an “empty” opsin molecule. The esterification does not take place in the outer segment membranes (5). Retinal microsomes are able to esterify vitamin A (5), but it appears likely that the major site of esterification is the pigment epithelium (29), for it is in these cells that vitamin A is stored in ester form (16, 25, 59). Palmitic and stearic acids are used preferentially in the esterification of vitamin A (5, 20, 29). These esters are then released from the pigment epithelium to the visual cells when needed (16, 26). The retina alone among the eye tissues is said to hydrolyze vitamin A ester readily (29), so that the fatty acids are probably detached from the vitamin A in the outer segments.

Fatty acids clearly play at least two important roles in the metabolism of the visual cell outer segment membranes: they are constituents of the membrane phospholipids, and they are esterified to vitamin A when that molecule is released from the membrane or returned to it from the pigment epithelium. Palmitic acid and stearic acid are major participants in both processes.

Renewal of the protein component of the membranes has been rather extensively studied. New protein molecules are synthesized predominantly

in the myoid portion of the visual cell inner segments (18, 50). A considerable part of the protein then moves through the Golgi complex and past the mitochondria massed in the ellipsoid, to reach the connecting cilium (60), through which it passes into the outer segment (51). In rods, most of the new protein which reaches the outer segment is concentrated at its base, where it is used in the assembly of new disk membranes (50). This occurs by a process which involves growth by infolding of the outer cell membrane, rather than *de novo* membrane assembly. A smaller proportion of the protein is used for molecular replacement, and becomes diffusely scattered among the membranes of the existing disks (11, 53). Opsin comprises the major part of the protein delivered to the rod outer segments (7, 22, 23, 31, 35), but once inserted in a new membrane, it does not appear to undergo renewal (23).

As new membranes are continually formed at the base of the rod outer segments, they displace previously formed membranes away from the base. To compensate for the repeated assembly of new disk membranes, groups of old disks are intermittently shed from the tips of the rod outer segments. The detached disks are immediately phagocytized and destroyed by the pigment epithelium (27, 55, 58).

The renewal of protein by molecular replacement is very prominent in cone outer segments (11), but this class of visual cells appears not to form new membranes and shed old ones in the manner observed in rods (52, 54).

In summary, *biogenesis* of outer segment membranes takes place when individual molecules (or membrane subunits) produced at one or more synthetic sites are transported to the outer segment, where they are used in membrane assembly by a process involving growth of existing membrane. *Renewal* of the outer segment membrane systems occurs by two different mechanisms. In rods, renewal by *membrane replacement* takes place by assembly of new membranes coupled with shedding of old ones. In both rods and cones, new proteins also become distributed among the existing membranes. This constitutes renewal by *molecular replacement*. (Further support for these conclusions is presented in a recent review: 56.)

The process by which lipid molecules are produced and supplied for the assembly of new outer segment membranes is completely unknown, nor is there any published evidence bearing on the question of whether the lipids, once assembled

into the membranes, are subsequently renewed. The role of the pigment epithelium in such processes is also unexplored. We have therefore embarked upon the analysis of lipid renewal in the visual cells and pigment epithelium, using the frog as an experimental animal, and employing autoradiographic and radiobiochemical analyses of the distribution of radioactive material in these cells after the administration of tritiated fatty acids.

MATERIALS AND METHODS

Palmitic Acid

Seven frogs (*Rana pipiens*), weighing between 5.5 and 8.5 g, were injected with 1 mCi/g of [9,10-³H] palmitic acid (sp act, 50 Ci/mmol; Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N.Y.) dissolved in 70% isopropyl alcohol at a concentration of 100 mCi/ml. The animals were killed at 30 min; 1, 4, and 24 h; and 1, 4, and 8 wk after injection. An additional frog, weighing 4.2 g, received 10 mCi, and was killed 5 min after injection. All of the frogs killed before 1 wk after radioisotope administration were injected intravenously under anesthesia. The frogs killed at 1, 4, and 8 wk were injected in the dorsal lymph sac without anesthesia.

Stearic Acid

Three frogs, weighing 10–15.5 g, were injected in the dorsal lymph sac with 1 mCi/g [9,10-³H] stearic acid (sp act, 75 Ci/mmol; Schwarz/Mann) dissolved in 70% isopropyl alcohol at a concentration of 133 mCi/ml. The frogs were killed 1 day, 4 days, and 1 wk after injection.

Arachidonic Acid

One frog, weighing 1.8 g, was injected in the dorsal lymph sac with 1 mCi [5, 6, 8, 9, 11, 12, 14, 15-³H] arachidonic acid (sp act, 10 Ci/mmol; New England Nuclear, Boston, Mass.) dissolved in 70% isopropyl alcohol at a concentration of 100 mCi/ml. The frog was killed 1 h later.

All experiments were carried out at room temperature (22–24°C) under conditions of normal laboratory lighting.

Preparation of Tissues

The eyes were enucleated, punctured through the cornea with a needle, then placed in ice-cold, phosphate-buffered 4% formaldehyde, pH 7.2. The fixative was allowed to come to room temperature, and the eyes were left in this solution overnight. The posterior half of each eye was then cut into small

pieces which were rinsed in buffer, then divided into two groups. These were processed separately in order to retain lipids or, alternatively, to extract them from the tissues. For *lipid preservation*, the specimens were postfixed for 2 h in phosphate-buffered 1% osmium tetroxide (pH 7.2), rinsed in buffer, and then dehydrated rapidly in 70% and 95% ethanol (twice for 10 min in each) at 4°C, followed by transfer through three changes of pure Araldite, 1 h each, at 4°C. Next, the specimens were placed in complete Araldite mixture (containing catalyst) and were stored overnight in the refrigerator. They were then transferred to fresh Araldite mixture for 1 h at 37°C, then overnight at room temperature, followed by polymerization at 60°C for 3 days. This procedure is based upon that of Stein and Stein (43). For *lipid extraction*, the specimens were placed in three changes, 1 h each, of chloroform:methanol (2:1, vol/vol) at room temperature, using gentle agitation on a rotating platform. The tissues were then left in the solution at room temperature overnight, without agitation. Next, they were placed in 4% osmium tetroxide dissolved in carbon tetrachloride (two changes, 10 min each). After rinsing in CCl₄, the specimens were immersed in absolute ethanol (two changes, 10 min each), then propylene oxide for 30 min, followed by 1 h in a 1:1 mixture of propylene oxide and complete Araldite mixture, after which they were embedded in the Araldite mixture, left overnight at room temperature, and then polymerized at 60°C for 3 days.

Tissues from the arachidonic acid-injected frog were prepared by the lipid preservation sequence only.

Analysis of Lipid Extracts

The chloroform:methanol extracts from the palmitic acid experiments were pooled, washed with 0.2 vol of 0.1 M NaCl solution, and the upper, water-methanol layer (containing approximately 3% of the total radioactivity in the sample) was discarded. The lower layer was then taken to dryness in a stream of nitrogen, redissolved in a small volume of chloroform-methanol (2:1), and then analyzed by two-dimensional thin-layer chromatography in a system designed to separate phospholipids (2). The components of the mixture, detected by charring, were individually scraped into vials, and their content of radioactivity determined by liquid scintillation spectrometry. These analyses were carried out in the laboratory of Dr. M. O. Hall, Jules Stein Eye Institute.

Preparation of Autoradiograms

The specimens were oriented so that longitudinal sections through the visual cells could be obtained. For light microscope examination, sections were cut

at a thickness of 0.5 μm , collected on glass microscope slides, and then coated with emulsion by dipping in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, N.Y.) which had been diluted 1:1 with distilled water and was maintained at 40°C. In some cases, the Araldite embedment was removed before dipping by incubation in $\frac{1}{6}$ saturated NaOH in absolute ethanol. The preparations were exposed for 10–30 days in the dark at 4°C in the presence of a desiccant, and then developed in Kodak Dektol (Eastman Kodak Co.) for 2 min at 17°C. Next, they were fixed in Kodak Acid Fixer (Eastman Kodak Co.), washed in water, stained with 1% toluidine blue in 1% sodium borate, and protected by cover slips.

Labeling of the retina in frogs killed at 1, 4, and 8 wk after injection was intense enough to permit analysis by electron microscope autoradiography. These autoradiograms were prepared according to methods published previously (58) using Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) and Phenidon developer (30).

Analysis of Autoradiograms

Developed silver grains overlying components of the pigment epithelial cells and visual cells were counted in the light microscope in order to obtain a quantitative estimate of the distribution of radioactivity. For these purposes, the cytoplasm, oil droplets, nuclei, and phagosomes (ingested fragments of rod outer segments) of the pigment epithelium, and the ellipsoids, myoids, nuclei, and synaptic bodies of the visual cells were analyzed. Labeling of the rod outer segments was assessed, but grains over the much smaller cone outer segments were not counted. However, labeling of cone oil droplets was analyzed. Counts were made using a grid mounted in the ocular lens of the microscope, and were expressed as grains per grid square. Each square had an area of 25 μm^2 at the magnification used.

RESULTS

Palmitic Acid: Autoradiography

The following description of the distribution of tissue radioactivity is based upon material in which neither lipids nor plastic embedment were removed before coating the sections with emulsion.

Although the frog killed 5 min after injection received more radioactive palmitic acid than the frog killed at 30 min, the labeling intensity at the earlier interval was significantly less. The distribution of radioactivity was similar, however, so that these two intervals will be described together.

There was a scattering of silver grains over the entire retina, but only the pigment epithelium and visual cells showed regionally localized concentrations of radioactivity. Most heavily labeled were the oil droplets in the pigment epithelium. The surrounding cytoplasm was also very heavily labeled (Figs. 2, 3, Table I). Slightly less reactive were the cytoplasmic processes which extend between the rod and cone outer segments. The nuclei contained very little radioactivity. Labeling of phagosomes was rather variable. Some were reactive only to a slight degree; others were heavily labeled. On the average, however, the intensity of labeling was about the same as the cytoplasm in which they were embedded, and much higher than that of the adjacent rod outer segments from which they had been derived.

In the visual cells, the greatest amount of radioactive material occurred in the outer segments (Fig. 3). At 30 min, labeling in some of the cone outer segments was higher than that in the rods. There was no evident localization of radioactivity within the outer segments; the silver grains were randomly scattered over those structures. Nuclear labeling was very low and many of the grains in the outer nuclear layer appeared to lie over the thin rim of perinuclear cytoplasm. Scattered grains were recorded over the inner segment (ellipsoid and myoid) and the synaptic body, but these were less concentrated than those over the outer segments (Table I). Labeling of cone oil droplets was negligible.

Between 30 min and 4 h, the amount of radioactive material in visual cells and pigment epithelium continued to increase. In the pigment epithelium, radioactivity was present in highest concentration in the oil droplets (Figs. 3-5, Table I). The phagosomes and cytoplasm were also heavily labeled, although to a lesser degree. Less reactive were the cytoplasmic processes. The nuclei contained very little radioactive material.

Rod and cone outer segments were both characterized by diffuse labeling which appeared to be higher in cones (Figs. 4, 6), but cone oil droplets were practically free of radioactivity. Visual cell nuclei contained little significant labeling, but were rimmed by silver grains which were apparently exposed by a source in the perinuclear cytoplasm. The inner segments continued to be less heavily labeled than the outer segments in both rods and cones, and there was no localization within either the ellipsoid or myoid.

A further increment in labeling was recorded

between 4 and 24 h in some structures (Fig. 7, Table I). The greatest change was observed in the cone oil droplets, practically all of which were now noticeably radioactive. Some of the cone outer segments continued to be more heavily labeled than those of rods.

1 wk after injection, labeling in the pigment epithelium continued to exceed that in the visual cells. As before, the oil droplets were the most intensely labeled structures (Figs. 8, 9, Table I). There was a marked increase in the intensity of labeling in cone oil droplets. Except for the oil droplets in the pigment epithelium, these were now the most heavily labeled structures in the retina. Rod and cone outer segments were diffusely labeled at about the same level; no longer did some of the cones appear more reactive. Furthermore, there was evidence of a concentration of radioactive material at or near the base of rod outer segments (Fig. 10).

At 4 wk, the oil droplets in the pigment epithelium and in the cones were the two most heavily labeled structures in the retina, with those in the pigment epithelium containing a slightly higher concentration of radioactive material (Fig. 11, Table I). The pigment epithelial cytoplasm and processes, phagosomes, and rod outer segments were all diffusely labeled at about the same level. No localized labeling was evident in the rod outer segments. The cone outer segments continued to show a diffuse labeling, but it now appeared, on the average, to be slightly less than that of the rods. Nuclear labeling was minimal, and the visual cell inner segments remained less radioactive than the outer segments.

At 8 wk, the intensity of labeling in all structures had declined, with the reduction being most pronounced in the oil droplets and cytoplasm of the pigment epithelium (Fig. 15, Table I). Radioactivity in the rod outer segments was now higher in concentration than that in the phagosomes and cytoplasm of the pigment epithelium. The rod outer segments were still more heavily labeled than the inner segments, and appeared to be more heavily labeled than cone outer segments as well.

Extraction of Lipids and Removal of Plastic Embedment

Table II and Figs. 11-14 present the results of extraction with chloroform-methanol solution, removal of the plastic embedment, or a combina-

tion of the two procedures. From 66 to 100% of the radioactivity (average, 90%) was extracted in the lipid solvent, leaving little doubt that most of the tissue radioactivity was due to incorporation of [³H]palmitic acid into lipids. Removal of Araldite by immersion of sections in an alkaline solution of ethanol also removed a considerable proportion of the tissue radioactivity, probably due in part to the saponification of fatty acid esters. A combination of lipid extraction and plastic removal depleted the tissue of essentially all of its radioactive substances (93–100%; average, 98%). Neither of these procedures, alone or in combination, removes radioactivity from the retina after injection of protein precursors, such as [³H]leucine.

Analysis of the Chloroform-Methanol Extract

Most of the radioactivity was recovered in phosphatidyl choline (59.9%), whereas only 3%

occurred in phosphatidyl ethanolamine (Table III). These are the two major phosphorus-containing lipids in the retina. Recovery of most of the radioactivity in only one of these two dominant phospholipids agrees well with fatty acid analyses (Table III, last column) which have shown that nearly half of the fatty acids associated with phosphatidyl choline are palmitic acid, whereas less than 6% of the fatty acid constituents of phosphatidyl ethanolamine are accounted for by palmitic acid. Approximately 5% of the radioactivity in the lipid extract was recovered with sphingomyelin, which is a minor retinal phospholipid, but one with an appreciable content of palmitic acid (23% of total fatty acids). Another minor phospholipid component, phosphatidyl inositol, with a significant content of palmitic acid (12.8%) contained 2.9% of the radioactivity in the extract. Less than 1% was recovered in phosphatidyl serine. Therefore, the major part of

FIGURES 2-7 Pigment epithelium (above) and visual cells from frogs injected intravenously with [9, 10-³H]palmitic acid and killed between 5 min and 1 day later. Autoradiograms, 1/2- μ m Araldite sections; lipid-preserved specimens, toluidine blue staining.

FIGURE 2 5 min after injection. The cytoplasm (*c*) and oil droplets (*od*) of the pigment epithelium are heavily labeled, in contrast to the nuclei (upper arrows), which are practically free of radioactivity. In the visual cells, the heaviest autoradiographic reaction occurs over the outer segments of the rods (*ros*) and cones (arrow, lower right). $\times 800$.

FIGURE 3 30 min after injection. Although more radioactive palmitic acid has been incorporated into the cells, the distribution of labeling is similar to that at 5 min. The oil droplets and cytoplasm of the pigment epithelium have concentrated the tritiated fatty acid. The outer segments are the most heavily labeled portion of the rod visual cells. In contrast, nuclei of the rods and cones, located in the layer delineated by the two horizontal lines, are relatively free of labeling. Many of the silver grains are located at the perimeter of the nuclei or between them. $\times 800$.

FIGURE 4 1 h after injection. The oil droplets of the pigment epithelium continue to show the greatest concentrations of radioactivity, whereas the nuclei (upper arrows) have not taken up the labeled fatty acid. Phagosomes (*p*), representing rod outer segment membranes phagocytized by the pigment epithelium, are labeled at about the same level as the surrounding cytoplasm. The visual cell outer segments retain higher concentrations of [³H]palmitic acid than do other parts of these cells. Some cone outer segments (arrow, lower right) are more heavily labeled than the rod outer segments. $\times 800$.

FIGURE 5 4 h after injection. The amount of bound radioactive material has continued to increase. Note the intense labeling of the pigment epithelium oil droplets (arrows), and the persistent, heavy autoradiographic reaction over the rod outer segments. $\times 800$.

FIGURE 6 Between 30 min and 24 h, some of the cone outer segments (arrows) were more heavily labeled than the rod outer segments. Fig. 6 *a* is 1 h, 6 *b* 4 h after injection. The cone oil droplets (*od*) are still quite free of labeling at these intervals. Note that the cytoplasmic processes of the pigment epithelium (*z*) are labeled at about the same intensity as the rod outer segments. $\times 1,625$.

FIGURE 7 1 day after injection. The content of radioactivity in the oil droplets of the pigment epithelium has continued to increase. A few grains are now observed over many of the cone oil droplets (arrow). $\times 800$.

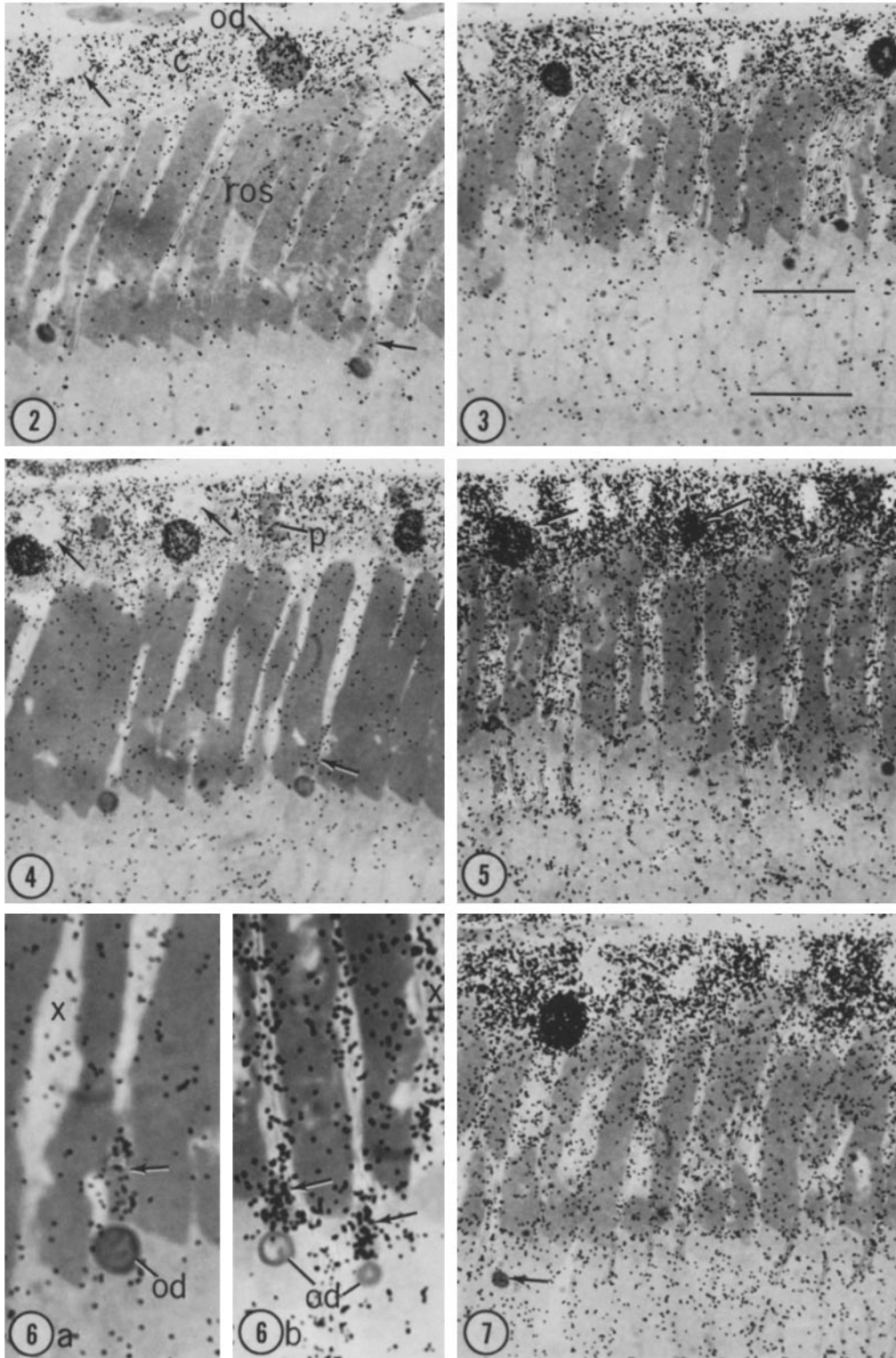


TABLE I
Concentration of Radioactivity* in Components of the Pigment Epithelium and Visual Cells at Different Intervals after Injection of [³H]Palmitic Acid

	Time after injection				
	30 min	4 h	1 day	1 wk	8 wk
Pigment epithelium					
Cytoplasm	14.5	25.5	25.6	29.4	7.3
Oil droplets	17.0	48.2	59.8	96.7	24.4
Nucleus	1.9	7.8	8.4	4.4	1.1
Phagosomes	13.1	26.2	27.6	28.0	8.2
Visual cells					
Outer segment (rods)	2.0	8.2	8.2	21.8	11.8
Ellipsoid	1.2	5.7	7.4	20.4	8.8
Myoid	1.5	5.2	7.0	14.5	6.4
Nucleus	0.6	4.7	4.1	10.1	3.2
Synaptic body	1.2	6.7	7.6	18.3	5.2
Oil droplet (cones)	0.9	1.1	7.4	53.6	23.6

* Silver grains per unit area in autoradiograms; 1 mCi/g dose; 1 wk exposure.

the radioactivity in the chloroform-methanol extract was recovered in phospholipids, more than half in phosphatidyl choline, with the distribution reflecting the normal partition of palmitic acid among those phospholipids. Some 27% of the radioactivity traveled with the solvent front. Triglycerides and fatty acid esters of vitamin A (predominantly esters of palmitic acid) run with the solvent front in this system, and are the most likely compounds to have contained the radioactivity.

Stearic Acid: Autoradiography

The overall pattern of labeling in the retina after the injection of tritiated stearic acid was similar to that after [³H]palmitic acid administration (Figs. 16, 17, Table IV). The heaviest labeling was in the pigment epithelium oil droplets, followed by the pigment epithelium cytoplasm and phagosomes, and the cone oil droplets, with the lowest concentration of radioactivity occurring in the cell nuclei. The level of diffuse labeling in the rod and cone outer segments appeared to be similar. At 4 days, there was a slightly greater concentration of silver grains near the base of the rod outer segments than elsewhere in those membranous organelles, but this regional localization was not clearly discernible 3 days later. Practically

all of the radioactivity was removed by extraction with chloroform-methanol solution.

Arachidonic Acid: Autoradiography

Arachidonic acid is a polyunsaturated, "essential" fatty acid which cannot be synthesized in animals (at least in higher vertebrates). The distribution of radioactivity 1 h after injection of [³H]arachidonic acid was similar, but not identical to that of [³H]palmitic acid at the same interval. The most heavily labeled structures were the oil droplets in the pigment epithelium (Fig. 18), but these were relatively less heavily labeled with arachidonic acid than with palmitic acid. Slightly less reactive were the surrounding cytoplasm and the phagosomes embedded in it. Nuclei contained very little radioactivity. In the visual cells, scattered labeling was observed over all cell components, except that the nuclei and cone oil droplets were practically devoid of radioactivity. Diffuse labeling over the outer segments 1 h after injection of arachidonic acid was about the same as that

TABLE II
Percentage Loss of Radioactivity due to Chloroform: Methanol Extraction and Removal of Plastic Embedment*

	Lipid extraction		Plastic removal		Lipid extraction + plastic removal	
	1 wk †	4 wk †	1 wk	4 wk	1 wk	4 wk
	Pigment epithelium					
Cytoplasm	80	73	56	66	94	94
Oil droplets	100	97	30	27	100	100
Nucleus	100	98	70	88	100	99
Phagosomes	90	87	70	76	94	100
Visual cells						
Outer segment (rods)	90	66	80	67	98	95
Ellipsoid	90	85	80	76	98	96
Myoid	87	95	78	81	99	99
Nucleus	95	88	85	82	97	93
Synaptic body	94	85	85	82	99	95
Oil droplet (cones)	100	90	25	22	100	100

* Percentage loss of silver grains in autoradiograms compared to sections from which lipids were not extracted and the plastic embedment was not removed.

† Frogs killed 1 wk and 4 wk after injection of [³H]palmitic acid.

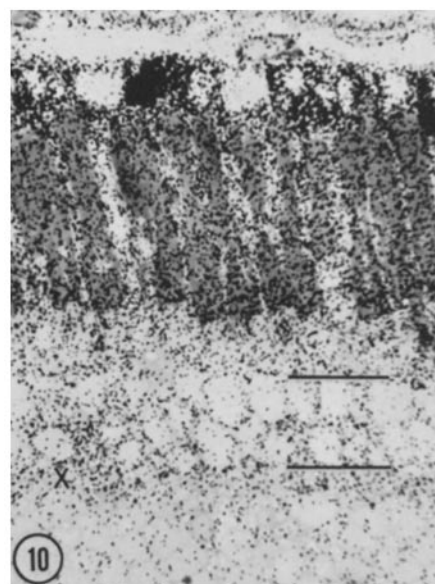
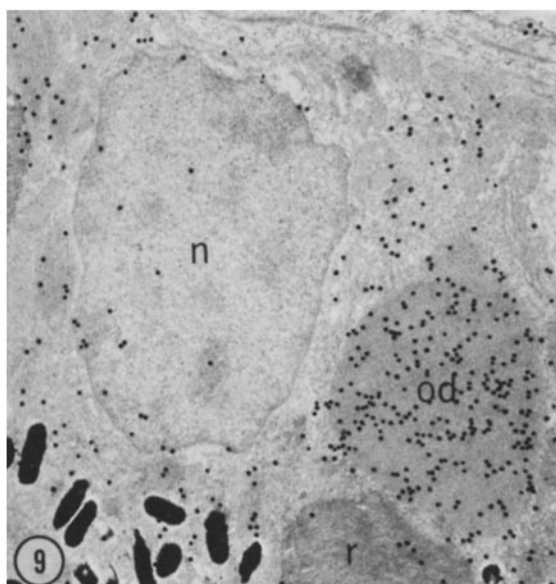
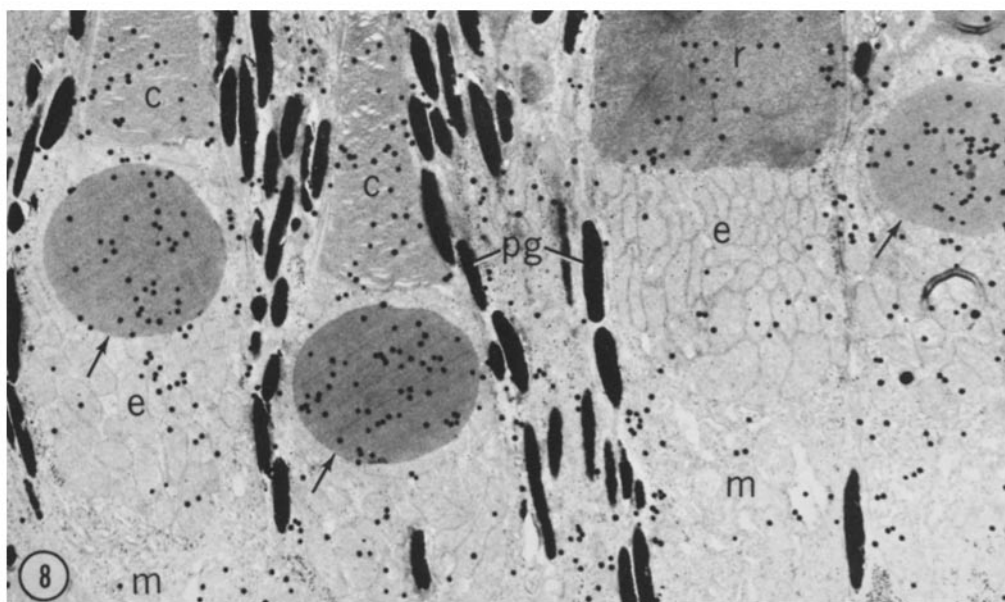


FIGURE 8 1 wk after injection of [^3H]palmitic acid. This electron microscope autoradiogram shows the concentration of labeled material in cone oil droplets (arrows) which are the most intensely labeled structures in the visual cells at this interval. Significantly labeled in a diffuse manner are the outer segments of cones (*c*) and rods (*r*). *e*, ellipsoid; *m*, myoid; *pg*, pigment granules in the cytoplasmic processes of the pigment epithelium (see Fig. 1). $\times 5,500$.

FIGURE 9 1 wk after injection. The prominent accumulation of radioactive palmitic acid in an oil droplet (*od*) within the pigment epithelium is shown in this electron microscope autoradiogram. The cytoplasm is also well labeled, but the nucleus (*n*) is practically free of radioactivity. The tip of a rod outer segment (*r*) is visible below. $\times 7,325$.

FIGURE 10 1 wk after injection. The most intensely radioactive structures are the oil droplets in the pigment epithelium. The nuclei appear as pale, unlabeled areas embedded in the heavily reactive cytoplasm. In the visual cells, the rod outer segments are also characterized by prominent labeling, which is perceptibly greater at the base, where the dark-stained outer segment joins the lighter-stained inner segment. This regional localization is due to the utilization of the labeled fatty acids for new membrane formation. The nuclei of the visual cells, located in the layer limited by the two horizontal lines, are quite free of labeling, but silver grains are present at the periphery of each nucleus. There is some accumulation of radioactive material in the region of the synaptic bodies (*x*). Autoradiogram, $\times 800$.

over the inner segments, whereas it was slightly higher over the outer segments at the same interval when palmitic acid was used as the precursor.

DISCUSSION

Within 5 min after intravenous injection of [^3H]palmitic acid in the frog, significant concentrations of radioactivity were detected in the pigment epithelium, and lesser amounts in the adjacent visual cells. The concentration of labeling continued to increase for at least a week, although the level of circulating [^3H]palmitic acid must have dropped markedly during that period. Apparently, the pigment epithelium avidly takes up the fatty acid, but does not readily relinquish it to the blood stream. This conclusion is consistent with the results of experimental fatty acid deficiency in which the retina (and brain), in contrast to other tissues, are relatively resistant to the effects of the dietary deficiency (21). (The retina is similarly resistant, probably for the same reason, to the effects of vitamin A deficiency.)

The heavy incorporation of palmitic acid by the pigment epithelium is not due simply to the fact that these cells intervene between the adjacent

capillary network and the visual cells. Other membrane precursors (such as amino acids and glycerol) are incorporated at higher levels in the visual cells than in the pigment epithelium after similar intravenous injections (57). Thus, we conclude that the pigment epithelium is highly active in fatty acid metabolism. The predominant cytoplasmic organelle in this cell, the smooth-surfaced endoplasmic reticulum, may play a significant role in these metabolic pathways.

As early as 5 min after injection of [^3H]palmitic acid, the oil droplets in the cytoplasm of the pigment epithelium were heavily labeled. These droplets similarly take up, concentrate, and store vitamin A (59). Since vitamin A is stored primarily as the ester of palmitic or stearic acid, the labeling of oil droplets after injection of these fatty acids must be due in part to the esterification of vitamin A. Arachidonic acid apparently is not used to esterify vitamin A (5, 20), at least in calf eyes. Nevertheless, in these frogs, although labeling was less intense, the oil droplets were also radioactive within 1 h after the injection of this polyunsaturated fatty acid. The oil droplets of the pigment epithelium also become labeled after the injection of radioactive glycerol and choline

FIGURES 11-14 This plate shows the distribution of radioactivity in visual cells and pigment epithelium 4 wk after injection of [9,10- ^3H]palmitic acid, and demonstrates the loss of radioactive material associated with lipid extraction and removal of the plastic (Araldite) embedment. Autoradiograms, $\frac{1}{2}$ - μm Araldite sections, 10 days exposure, toluidine blue staining. $\times 800$.

FIGURE 11 With maximal retention of lipid, resulting from aldehyde-osmium fixation combined with an abbreviated dehydration-embedding sequence, the heavy labeling of the pigment epithelium and visual cells is illustrated. The dark bodies, such as those indicated by arrows, are oil droplets in the pigment epithelium (above) and cones (below), which are heavily radioactive at this interval (4 wk).

FIGURE 12 This autoradiogram was prepared from the same block of tissue depicted in Fig. 11, except that the plastic embedment was removed with an alkaline ethanol solution before autoradiography. This procedure also removed a considerable amount of radioactive material, with the loss of oil droplet labeling being less than that in other structures.

FIGURE 13 This autoradiogram was prepared from the same retina which yielded the specimens depicted in Figs. 11 and 12, except that the tissue specimen was extracted with chloroform:methanol (2:1, vol/vol) before fixation in osmium tetroxide. Most of the radioactivity, including essentially all of it in the oil droplets, has been removed, demonstrating that the precursor was used predominantly in the synthesis of lipid.

FIGURE 14 This autoradiogram was prepared from the same block of tissue depicted in Fig. 13, except that the plastic embedment was removed with an alkaline ethanol solution before autoradiography. Practically all of the labeled material is lost from the tissues due to the combined procedures of chloroform-methanol extraction and plastic removal. These procedures do not cause any significant loss of labeled protein.

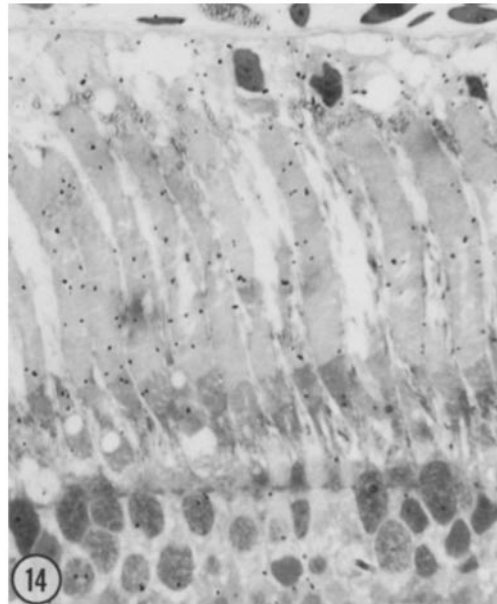
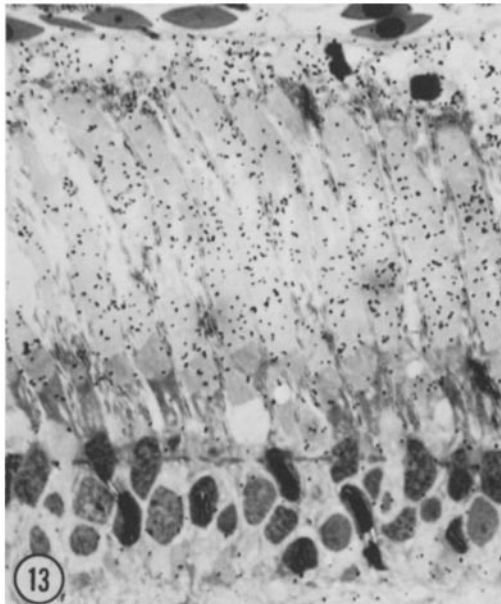
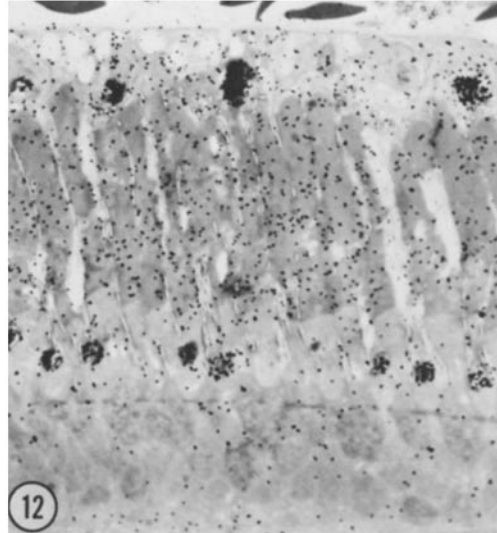
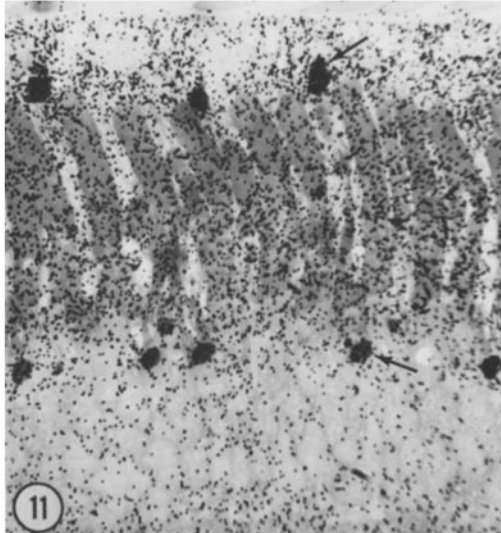


TABLE III
Distribution of Radioactivity in the Chloroform-Methanol Extract after Injection of [³H]Palmitic Acid

	Lipid extract percent radioactivity	Phospholipid percent composition*	Palmitic acid percent composition†
Phosphatidyl choline	59.9	46.0	42.5
Phosphatidyl ethanolamine	3.0	32.2	5.8
Phosphatidyl serine	0.5	8.7	6.2
Phosphatidyl inositol	2.9	4.9	12.8
Sphingomyelin	4.7	3.7	23.0
Other	1.3	4.5	—
Origin	0.7	—	—
Solvent front	27.0	—	—
Total	100.0	100.0	—

* Total retinal phospholipids; average of six mammalian species (2).

† Percentage of total fatty acids in retinal phospholipids accounted for by palmitic acid; average of six mammalian species (2).

(unpublished data), suggesting that some of the fatty acid in these structures may be associated with glycerides or phospholipids.

All parts of the visual cells and pigment epithelium showed renewal of fatty acids, but turnover in the nucleus was extremely low. A similar lack of significant fatty acid metabolism in the nucleus has been observed in other tissues (43–45). Cone oil droplets were initially devoid of radioactivity, but began to accumulate significant stores of labeled material 1 day after injection, and ultimately became the most intensely labeled structures in the visual cells. The function of these colorless lipid droplets in the frog is unknown. This is the first report that their constituents (possibly triglycerides) are renewed.

Fatty Acid Exchange

Phagosomes are the detached tips of rod outer segments which have been phagocytized by the pigment epithelium and are undergoing degradation (27, 55, 58). Therefore, it is highly unlikely that phagosomes are sites of lipid synthesis, although they unquestionably contain lipids. In

unpublished work we have observed that there is no direct incorporation of glycerol into phagosomes or rod outer segments. This is direct evidence that synthesis of phospholipids and glycerides does not take place in these structures. Biochemical studies also indicate that rod outer segments are devoid of the enzymes involved in phospholipid synthesis (46). Nevertheless, there was an immediate labeling of both phagosomes and rod outer segments within 5 min after injection of [³H]palmitic acid. We believe that the most likely reason for this is an exchange reaction. There is considerable evidence, obtained in studies of other cell systems, that fatty acids can exchange in phospholipids by mechanisms which maintain positional specificity of the saturated and unsaturated acids (6, 33, 36, 40–42). The rather variable labeling of phagosomes may be due to the presence of membranes in different states of degradation. In addition, the higher initial labeling of phagosomes, relative to that of the rod outer segments, could be related to the fact that the phagosomes are embedded in the intensely radioactive cytoplasm of the pigment epithelium, whereas the rod outer segments are surrounded by the less heavily labeled cytoplasmic processes of that cell.

Phospholipid Replacement

The progressive accumulation of diffusely distributed radioactivity in the rod and cone outer segments is probably due largely to incorporation of new phospholipid molecules into existing membranes. Labeling of the rod outer segments continued to increase for at least 1 wk after a single injection of [³H]palmitic acid, whereas phagosome labeling did not increase significantly after 4 h. If both were the result of an exchange reaction, they would be expected to increase or decrease at a similar rate. A more compelling argument that the gradual increase in outer segment labeling is due at least in part to phospholipid replacement is derived from our work with [³H]glycerol.¹ In these unpublished experiments we have observed an initial incorporation of radioactivity in the myoid portion of the visual cell inner segment, followed by a spreading of radioactive lipid throughout the outer segment. Glycerol uptake is

¹ Bibb, C., and R. W. Young 1973. Renewal of glycerol in the visual cells and pigment epithelium of the frog retina. Submitted to this journal.

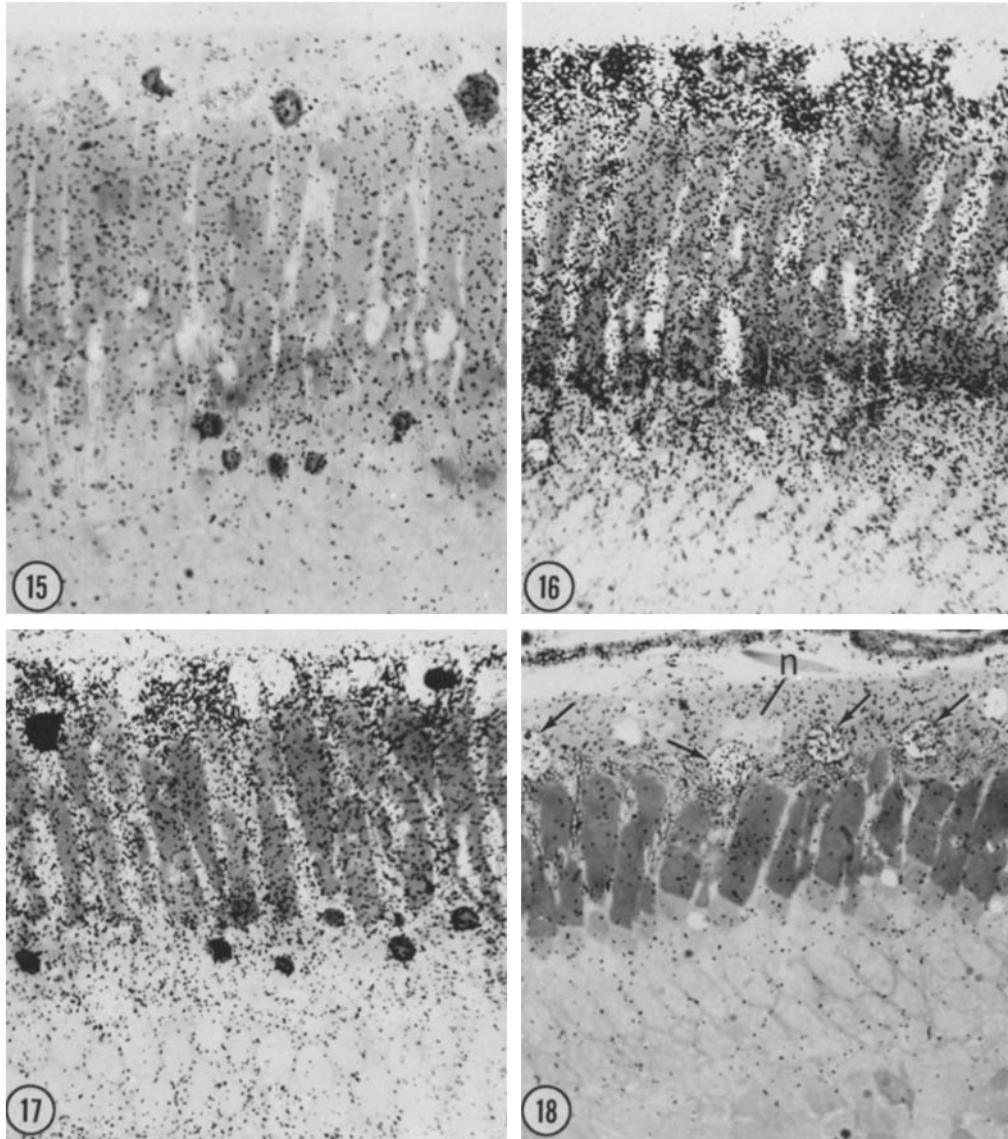


FIGURE 15 8 wk after injection of [^3H]palmitic acid. The intensity of labeling in all structures has decreased, particularly in the oil droplets and cytoplasm of the pigment epithelium. Although there has also been a loss of radioactive material from the highly reactive rod outer segments and cone oil droplets, the decline is proportionately less than that in the pigment epithelium. Autoradiogram, $\times 800$.

FIGURE 16 4 days after injection of [^3H]stearic acid. The distribution of radioactive material is similar to that observed after injection of [^3H]palmitic acid, with heavy labeling of the oil droplets and cytoplasm of the pigment epithelium, and weak labeling of the nuclei, which appear as pale areas free of overlying silver grains. The rod outer segments are heavily labeled, with the greatest intensity occurring at the base, indicative of new membrane assembly. Autoradiogram, $\times 800$.

FIGURE 17 1 wk after injection of [^3H]stearic acid. Note the very heavy labeling of the oil droplets in the pigment epithelium. The cone oil droplets are also labeled, although to a lesser degree. The preferential labeling at the base of the rod outer segments is no longer apparent. Note that the visual cell nuclei have used very little of the labeled fatty acid, but are rimmed with silver grains, as was also the case with [^3H]palmitic acid. Autoradiogram, $\times 800$.

FIGURE 18 1 h after injection of [^3H]arachidonic acid. The distribution of this polyunsaturated fatty acid is similar to that of the saturated fatty acids, palmitic and stearic, except that labeling of the oil droplets of the pigment epithelium (arrows) is not quite as great, relative to that in the cytoplasm (cf. Fig. 4). There is little incorporation into nuclei (n). The outer segments are diffusely labeled at about the same level as the inner segments in the visual cells. Autoradiogram, $\times 800$.

TABLE IV
Concentration of Radioactivity* in Components of the Pigment Epithelium and Visual Cells at Different Intervals after Injection of [³H]Stearic Acid

	Time after injection			
	1 day		1 wk	
	Stearic acid	% Palmitic†	Stearic acid	% Palmitic acid
Pigment epithelium				
Cytoplasm	8.9	35	18.9	64
Oil droplets	31.8	70	39.4	41
Nucleus	.9	11	1.6	36
Phagosomes	3.7	13	9.1	23
Visual cells				
Outer segment (rods)	1.1	13	3.4	16
Ellipsoid	1.4	19	4.5	22
Myoid	1.5	21	3.3	23
Nucleus	1.0	24	2.3	23
Synaptic body	1.4	18	2.6	14
Oil droplets (cones)	2.2	30	6.0	11

* Silver grains per unit area in autoradiograms; 1 mCi/g dose; 1 wk exposure.

† Labeling with stearic acid expressed as a percent of labeling with palmitic acid under comparable conditions of dosage, time, and autoradiographic exposure.

considered to be indicative of the synthesis of new lipid molecules (6), since there is no evidence that glycerol undergoes exchange in existing glycerides or phospholipids. Radiobiochemical assay (carried out by Dr. M. O. Hall) shows that most of the glycerol is used in phospholipid synthesis. Studies of liver, erythrocytes, and other extraocular tissues have revealed that new phospholipid molecules may be inserted into existing membranes (1, 9, 32, 38, 47, 48).

Some cone outer segments were more heavily labeled than those of rods between 30 min and 1 day after injection of [³H]palmitic acid. This may be due to a faster molecular replacement or a higher content of the fatty acid in cone visual cells.

Vitamin A Transfer

The progressively decreasing amount of radioactivity in the cytoplasm and oil droplets of the pigment epithelium compared to that in the rod

outer segments suggests that labeled material may be transferred from the pigment epithelium to the outer segment membranes. The glycerol experiments just cited offer no indication that the pigment epithelium is a source of phospholipids for the membranes. However, there is good reason to believe that the pigment epithelium does supply vitamin A, esterified with fatty acid, to the outer segments (16, 25, 26, 29). In the membranes, the vitamin A (converted to 11-*cis* retinal) is bound to protein to form the visual pigment molecules in both rods and cones. The fate of the fatty acid is not known. Perhaps it too is incorporated into the membrane.

Thus, there is evidence that the lipids of the visual cell outer segment membranes are renewed by at least two types of *molecular replacement*: fatty acid exchange in phospholipids situated in the membranes, and the insertion of new phospholipids into the membranes. (In both cases we presume that there is a balanced loss of comparable molecules.) Furthermore, there may be a transfer of vitamin A, esterified with fatty acid, between the pigment epithelium and the membranes.

Membrane Replacement

In addition to these indications of renewal by *molecular replacement*, the autoradiograms provide further evidence for the process of renewal by *membrane replacement*, in which assembly of new membranes is balanced by shedding of old ones. A concentration of radioactivity was observed near the base of the rod outer segments in frogs killed 1 wk after injection of [³H]palmitic acid and 4 days after injection of [³H]stearic acid. This localization of labeled material results from the *biogenesis of new membranes*. It was not observed in cones, which do not form new membranes and shed old ones in this manner (52, 54). When the *proteins* of new disk membranes in frog rods are preferentially labeled, the disks can be traced for several weeks because their content of radioactivity is stable. Autoradiography reveals that they are displaced along the outer segment to its tip, from which they are shed (50, 58). In contrast, in these experiments we could no longer detect preferential labeling of any disks at intervals examined later than 1 wk after injection of radioactive palmitic and stearic acids. The disappearance of preferential labeling as the disks were displaced is believed to be a result of the rapid

renewal of membrane fatty acids. These observations therefore indicate that the fatty acids in the membranes are replaced more rapidly than the protein.

The source of the proteins used in the biogenesis and renewal of outer segment membranes is the myoid portion of the visual cell inner segment. After injection of radioactive amino acids, labeled protein can be traced as it moves from the myoid through the ellipsoid and connecting cilium to the outer segment. Glycerol, the backbone of the phospholipid molecule, is also concentrated in the myoid before being transported to the outer segment (57). However, no such sequence of events is observed after the injection of radioactive palmitic acid. Labeling in the myoid never exceeds that in the outer segment, and does not decline as outer segment labeling rises. If phospholipid destined for the outer segment membranes is produced in the myoid region of the inner segment (as the glycerol experiments suggest), why do the autoradiograms fail to show this shifting distribution of radioactive lipid? Several possible explanations may be considered.

The fatty acids might be broken down in the pigment epithelium, with the resulting distribution of radioactivity not being representative of fatty acids. Alternatively, the visual cells might synthesize phospholipids in the myoid region, but without using exogenous fatty acids. Both of these suggestions appear unlikely because of the evidence that the fatty acids *were* incorporated into phospholipids. Most of the tissue radioactivity occurring after injection of labeled palmitic and stearic acids was soluble in the lipid solvent, chloroform-methanol. Nearly 75% of the extracted palmitic acid radioactivity proved to be in phospholipids, with a distribution approximating the normal disposition of palmitic acid. The results do not exclude the possibility that the pigment epithelium, which concentrates fatty acids, might supply some phospholipids to the outer segment membranes. However, our unpublished experiments which show that the pigment epithelium incorporates relatively little glycerol, and in vitro studies in which visual cells, incubated without the pigment epithelium, apparently continue to assemble outer segment membranes (8, 35) indicate that this process, if it occurs at all, probably is of minor significance.

On the basis of available evidence, we think it is more likely that fatty acid exchange in outer segment membranes, coupled with the esterifica-

tion of vitamin A and its transfer between the pigment epithelium and these membranes, so dominate the autoradiographic pattern that any shift of newly synthesized, labeled phospholipid from the myoid to the outer segment may be obscured when [³H]fatty acids are used as the radioactive precursor—although it is clearly evident when [³H]glycerol is used as precursor.

The present study demonstrates unequivocally that the lipids of visual cells and pigment epithelium are continually renewed. In rods, the stack of disks is renewed by the process of *membrane replacement*, which entails the repeated assembly of new lipoprotein membranes at the base of the outer segment, in conjunction with the shedding of old membranes from the apex of the cell. In addition, in both rods and cones, *molecular replacement* contributes to lipid renewal. New phospholipid molecules are inserted into existing membranes, presumably accompanied by a removal of comparable molecules. Secondly, fatty acids appear to undergo exchange in phospholipid molecules already situated in the membranes. Furthermore, there is an indication that vitamin A, esterified with fatty acid, may be transferred between the pigment epithelium and the membranes. Fatty acids are replaced more rapidly than protein in the rod outer segment membranes. The pigment epithelium is significantly involved in retinal fatty acid metabolism, sequestering these molecules from the blood stream, storing them, using them to esterify vitamin A, and supplying them to the visual cells.

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