Visualization of $[^3\text{H}]$Docosahexaenoic Acid Trafficking Through Photoreceptors and Retinal Pigment Epithelium by Electron Microscopic Autoradiography

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Purpose. $[^3\text{H}]$docosahexaenoic (DHA) acid was followed through the retinal pigment epithelial cells and photoreceptors for up to 5 days after injection to specifically determine which membrane systems of the retinal pigment epithelial cells are used in the handling of $[^3\text{H}]$DHA after shedding and phagocytosis of rod tips.

Methods. Frogs (Rana pipiens) were injected with $[^3\text{H}]$DHA in the dorsal lymph sacs, and maintained for up to 5 days. Retinas were processed for electron microscopic autoradiography, stored for various periods of time, and then analyzed by transmission electron microscopy.

Results. After 1 day, $[^3\text{H}]$DHA had accumulated within photoreceptor ellipsoids, and had begun to appear as dense label in newly formed discs. By day 5, the basal region of dense label had expanded apically. Newly shed rod outer segment tips were diffusely labeled; but occasionally after several hours, they acquired additional label as they moved near Bruch’s membrane. Retinal pigment epithelial cytoplasm maintained a constant level of label, with myeloid bodies sometimes slightly labeled. Oil droplets of the retinal pigment epithelium accumulated dense label throughout this study.

Conclusions. When $[^3\text{H}]$DHA enters the retinal pigment epithelium, some is retained within oil droplets, whereas the rest is passed on to the photoreceptors. $[^3\text{H}]$DHA is initially taken up by inner segments and then dispersed to photoreceptor synaptic terminals as well as to ellipsoids where discs are assembled. Phagosomal labeling exactly matches rod outer segment tips, but occasionally increases as degradation occurs near Bruch’s membrane. Normally, density of label remains constant throughout the degradation process. Invest Ophthalmol Vis Sci. 1993;34:2402-2411.
orders of photoreceptors such as Usher’s Syndrome exhibit lowered blood plasma levels of DHA.\[^{6,7}\] Also, it has been shown that long-term maternal dietary deprivation of n3 fatty acids results in alterations in the ERG,\[^{8-10}\] as well as in visual acuity impairments,\[^{11}\] suggesting that DHA is extremely important to excitable membrane function, especially within photoreceptor outer segments.

The objectives of this study were to autoradiographically observe [\(^{3}H\)DHA at 5 days postinjection as it is added to disc membranes, and to describe the distribution of [\(^{3}H\)DHA within the retinal pigment epithelial (RPE) cells after shedding and phagocytosis. We selected frogs in these studies because they have large photoreceptors, making light- and electron microscopic-level autoradiography easier to analyze; they have a light-driven shedding response that is well characterized and easy to manipulate; they are poikilothermic, disc movement through the outer segment is 2–3 times slower in these poikilotherms than that in mammals, making it easier to select critical time points; and similarities in retinal lipid biochemistry of the frog and human make the frogs an excellent experimental model. Electron microscopic (EM) autoradiography was especially useful in determining the distribution of [\(^{3}H\)DHA within photoreceptors and RPE cells, and has enabled us to suggest a pathway for retinal DHA uptake, distribution, and subsequent recycling.

MATERIALS AND METHODS

Animals

Frogs (\textit{Rana pipiens}, Northern variety, 2–6 g) were obtained from J.M. Hazen Frog Co. (Alberg, VT) and maintained in incubators under a 24-hr diurnal photoregime of 14 hr of light: 10 hr of dark at 24°C for at least 1 mo before use. A light intensity of 15–20 µE/m² sec (1.2 × 10⁵ quanta/cm² sec) was produced from a door-mounted fluorescent source (40 W, cool white). Light readings were obtained with a LI-COR quantum/radiometer/photometer LI-185B and an LI-190SD quantum sensor (LI-COR, Lincoln, NE), that measured 400–700 nm. Animals were fed crickets (Fluker’s Cricket Farm, Baton Rouge, LA) once weekly.

Throughout this study research was carried out in accordance with statements about the proper care and use of animals as approved by the American Physiological Association, the Society for Neuroscience, and the Association for Research in Vision and Ophthalmology.

[\(^{3}H\)Docosahexaenoic Acid Labeling and Experimental Design

[\(^{3}H\)DHA (4, 7, 10, 13, 16, 19 [4, 5-\(^{3}H\) (N)]-22:6), obtained from New England Nuclear-DuPont (Wilmington, DE) (17.9 Ci/mmol), was dried under N\(_2\) and resuspended in ethanol. Approximately 200 µCi/g body weight was injected (final volume 10 µl) into the dorsal lymph sacs of frogs (4 g; electron microscopy, n = 7; light microscopy, n = 9). The animals were then individually maintained in 4-inch finger bowls in the light-cycled incubator, the water of which was changed daily. This is described in detail elsewhere.\[^{12}\]

After 4 days, animals from the 14L:10D photoregime were placed in constant light for 24 hr. Lights were turned off 1 hr for dark priming, and then turned on to trigger photoreceptor shedding\[^{13,14}\] Some animals were prepared for light-level autoradiography in a similar manner each day throughout this 5-day period.

Tissue Preparation and Electron Microscopy

Animals were killed by decapitation, then pithed. Eyes were rapidly removed, the corneas slit, and the tissue placed in cold fixative for 2 hr. The fixative contained 2% glutaraldehyde (Polysciences, Warrington, PA) and 2% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in 0.1 M sodium cacodylate buffer (Sigma, St. Louis, MO), pH 7.3. After all adhering tissue was removed from the eyeballs, the fronts dissected away, and the lens gently removed, they were trimmed into small pieces. Each eyecup was cut horizontally and the edges removed from each half, leaving a dorsal and a ventral square that was returned to cold fixative overnight. These pieces were rinsed in three changes (15-min each) of cold 0.1 M sodium cacodylate buffer, postfixed in cold, 1% osmium tetroxide, and again rinsed in three changes of buffer. Dehydration occurred in ethanol by 20% increments, followed by three rinses in dry acetone; all steps were of 15-min duration. Tissue was infiltrated overnight with a plastic mixture\[^{15}\] of Polybed 812 and Araldite (Polysciences, Warrington, PA), and embedded in flat molds.

Sections of 1µm thickness were taken for the purpose of orientation, and stained with 1% toluidine blue in 1% boric acid. Silver sections were cut with a diamond knife (Diatome U.S., Fort Washington, PA) on an MT6000-XL ultramicrotome (RMC, Tucson, AZ), expanded with chloroform vapors, and placed on hexagonal 200-mesh nickel grids. After staining with lead citrate and uranyl acetate, sections were viewed and photographed with a Zeiss C10 electron microscope (Zeiss, Jena, Germany).

Electron Microscope Autoradiography

Silver sections were placed on parlodion-coated 200-mesh nickel grids and stained with lead citrate and uranyl acetate. After 5 nm of carbon was evaporated onto them, they were stuck onto small corks over holes. Ilford L-4 emulsion (Polysciences, Warrington, PA) was prepared under dim red light, and the sec-
tions were coated by the loop technique described by Williams. After drying, the cork tips were placed in plastic tube holders in dry dark-boxes, wrapped in foil, and stored at 4°C. Exposures ranged from 1 to 13 months. Development occurred in Microdol-X (Kodak, Rochester, NY) at 20°C for 3 min, and produced characteristic "worm trails," easily distinguished from various sources of contamination.

Light-level autoradiography was also performed on this tissue. A detailed description of techniques employed was published previously.

### Analysis of Autoradiograms

Electron micrographs were taken from sections prepared from seven animals, and generally included the RPE and photoreceptor regions of the retina. Nine other animals were used to obtain the light-level autoradiograms. Electron microscopic autoradiograms of several different exposure times were observed. However, when different regions of the retina were compared, only autoradiograms of the same exposure time were used. A total of 58 micrographs were analyzed, 11 of which were photomontages of large retinal areas. When nuclear tracks were counted, tracings of specific regions were made onto clear acetate overlays. Generally, whole structures were not counted. Instead, counts were made only within circles of identical size placed over the micrographs. This ensured that all comparisons were in terms of label density, not total label. Only areas on micrographs of the same exposure times and magnifications were compared.

### RESULTS

#### Tissue Overview

Frog photoreceptor cell nomenclature used throughout this study is based on excellent descriptions of their ultrastructure and wavelength absorption characteristics from the literature. The in vivo distribution of [3H]DHA in photoreceptors and RPE of the frog, 5 days postinjection, displays a heterogenous pattern. Although DHA is sometimes found in the thin cytoplasmic regions of cells within the choriocapillaris, the low labeling of this richly vascularized region indicates that once [3H]DHA arrives, it rapidly crosses the microvasculature and enters the RPE. Little label accumulates within the cytoplasm of these cells, but RPE oil droplets become heavily labeled. The neural retina labels diffusely, with higher concentrations of [3H]DHA visible within the plexiform layers, especially after prolonged exposure times.

Photoreceptors, however, accumulate the majority of DHA label (Fig. 1). Rod inner segments are well labeled, showing slightly higher amounts distally in the photoreceptor cell ellipsoids. Outer segments have diffusely, evenly distributed label and dense [3H]DHA accumulation at the base. Cone photoreceptor labeling, however, does not follow this profile. Inner segments are diffusely labeled, with the ellipsoids demonstrating slightly less labeling than in rods, but oil droplets (found only in red-sensitive, 575-cones) show significant [3H]DHA uptake. Conversely, cones have only diffusely labeled outer segments; no basal accumulations occur (Fig. 2). Photoreceptor synaptic terminals also become well labeled, appearing as a single.

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**Figure 1.** Light microscopic autoradiograms showing accumulation of [3H]DHA in frog photoreceptor cells labeled in vitro. These unstained sections demonstrate the diffuse and the dense forms of the label within rod photoreceptor outer segments. (A) 1 day after injection; (B) 2 days after injection; (C) 5 days after injection; (D) diagram of B illustrating the relative positions of rod and cone outer segments. The small arrows point to cone oil droplets. In A-C, arrows delineate the dense basal region of labeling, whereas the more apical portions are diffusely labeled; in A, arrow indicates the base of the rod outer segments. Note in A that label has accumulated below in the ellipsoid region, as well as in the basal disc. Open arrows indicate the well-labeled synaptic regions of the photoreceptors. Scale bar, 20 μm.
DHA Distribution in Retina

FIGURE 2. Low magnification electron microscopic autoradiogram of frog photoreceptor cells showing general distribution of [3H]DHA at 5 days after injection. The dark, strongly labeled outer segments are at the top; the inner segments, rich in mitochondria and endoplasmic reticulum, are near the bottom. Label is concentrated within disc membranes at the base of rod outer segments (R) and in oil droplets (D) of cone inner segments. Though [3H]DHA is also localized around the mitochondria in ellipsoids (E), little label is found associated with membrane components of the myoid regions (black asterisks). Cone outer segments (C) label only sparsely, whereas their glycogen-rich paraboloids (P) have no label. [3H]DHA is not found in photoreceptor nuclei (N). The cone photoreceptor in the upper right, containing the oil droplet, is the primary component (red-sensitive, 575-cone) of a double cone. The secondary component (green-sensitive, 502-cone) lies immediately below, but because the section is slightly oblique, only the paraboloid region is apparent. Another secondary 502-cone appears near the middle (C), showing only sparse label in both the outer and inner segments. The primary component (575-cone) of this double cone is not in the plane of the section, with only a small portion of its inner segment cytoplasm visible (open arrow). The black arrow indicates a portion of the outer limiting membrane located near the apical region of the Müller cells (M), and between the nuclei of two red rods (green-sensitive, 502-rods). Scale bar, 5 μm.

Photoreceptor Cells

Though most [3H]DHA was taken up by photoreceptors, there is considerable variation between receptor types. Cone cells label sparingly, with inner segments approximately equivalent to outer segments (Fig. 3). Numerous vesicles occur within the inner segments and peripherally around the ellipsoids, but no label appears to be associated with them. Slightly more activity occurs within the cone ellipsoids, but mitochondria are very tightly packed, so that very little free cytoplasmic space, and no intermitochondrial vesicles, are seen. Near the distal end of red-sensitive cone (575-cone) inner segments is a large 3–5 μm diameter oil droplet (Figs. 2, 3), enclosed by a unit membrane.

These oil droplets rapidly label and maintain their [3H]DHA for long periods of time, and, with the exception of rod outer segments, are the most highly

FIGURE 3. Electron microscopic autoradiogram showing the inner segment regions of a rod and a cone photoreceptor cell 5 days after injection. A 575-cone (middle) has a well-labeled oil droplet situated within the mitochondrial mass of the ellipsoid (C). The cone outer segment is only diffusely labeled. The inner segment of a 502-rod lies to the left with slight ellipsoid (R) labeling. The outer segment is just visible at the upper left. Numerous dark melanin granules within processes of the retinal pigment epithelial cells are present. Scale bar, 5 μm.
labeled structures within the retina proper. (RPE cell oil droplets, however, have a much higher labeling density than those of cone oil droplets.) Green-sensitive cones (502-cones) generally label like the 575-cones (Fig. 2), although they do not possess oil droplets. Just proximal to the ellipsoid is the paraboloid, a granular glycogen-rich area found in 502-cones. No $[^3H]$DHA is observed there. Both 502- and 575-cone outer segments label diffusely; silver grains appear randomly scattered over the entire outer segment, but are not concentrated within specific areas (Figs. 2, 3).

The inner segments of rod and cone photoreceptors had accumulated label to the same extent by 5 days after injection (Figs. 2, 3). $[^3H]$DHA is generally dispersed across the cytoplasm, but does not appear to be associated with vesicles. The ellipsoid contains the highest labeling of all rod inner segment regions. Some ellipsoidal vesicles are present, usually peripherally or in the distal portions of rod inner segments, but at that time of sampling, no label appeared nearby. Minimal differences in labeling between red rods (green-sensitive, 502-rods) and green rods (blue-sensitive, 435-rods) were observed after 5 days of labeling, even though there were marked structural differences.

Rod outer segments show two well-defined patterns of labeled lipids (Fig. 4). Generally, the entire outer segment labels evenly, in a random manner not unlike that found within cone outer segments. In addition, a densely labeled region occurs at the base of the outer segment, extending distally to a height equivalent to 5 days of synthesized discs. This label is three times as dense as the rest of the outer segment, and is evenly distributed within this densely labeled area (Figs. 2, 4).

Synaptic terminals are also labeled in these 5-day autoradiograms (Fig. 5). Rod spherules accumulate slightly more $[^3H]$DHA than cone pedicles, and approximately ten times more than regions immediately adjacent to them, with label usually concentrated in the more densely contrasted, vesicle-laden cytoplasm. Areas near synaptic ribbons are not highly labeled, but some concentration of $[^3H]$DHA can be observed in processes of the outer plexiform layer that appear to be associated with photoreceptor terminals (Fig. 5).

RPE Cells

RPE cells accumulate $[^3H]$DHA diffusely, with slightly more DHA appearing in the basal regions than apical regions (Fig. 6). Slight labeling occurs along Bruch's membrane, but no tritium is associated with RPE intercellular spaces, located either distal or proximal to the tight junctions. The basal half of the RPE cells contain most of the smooth endoplasmic reticulum and associated stacks of membranes that form the lens-shaped myeloid bodies. DHA is sometimes associated with these structures, but not in a uniform or consistent manner (Fig. 6), and not usually in signifi-
significant quantities. Similarly, DHA is found occasionally associated with mitochondria along the basal edge of the RPE cells. The apical half of the cytoplasm contains numerous pigment granules and some myeloid bodies, but label is sparse. Autoradiographic grain counts of RPE cell cytoplasm show that approximately 16% of total [3H]DHA is dispersed throughout the basal half. The villar processes extending inward between rod outer segments from the apical surface of the RPE cells, like the apical cytoplasm, demonstrate very little DHA accumulation (Fig. 2). Large oil droplets accumulate as much as 65% of total [3H]DHA in the RPE cells, whereas another 13% appears within phagosomes. No nuclear labeling is seen. Three large area photomontages were analyzed to obtain information about RPE cytoplasmic distribution of [3H]DHA.

Newer phagosomes occur closer to the rod outer segment tips, but gradually become repositioned near the basal surface as degradation progresses. Phagosomes within the RPE cells demonstrate labeling equal to that of rod outer segment tips (Fig. 6). Occasionally, when older partially degraded phagosomes become lodged against Bruch's membrane, they can accumulate up to 7 times more [3H]DHA than younger phagosomes. Compare, for example, the labeling density of the two phagosomes in Figure 6. The phagosome at the top, near Bruch's membrane, has a labeling density of > 5 times that of the centrally located phagosome.

DISCUSSION

Electron microscopic autoradiography shows dense [3H]DHA labeling within the basal region of rod outer segments that corresponds to disc membranes assembled during the 5 days after [3H]DHA injection. This has been demonstrated previously at the light level by comparing the distribution of [3H]leucine (to label newly synthesized opsin within outer segment discs) with [3H]DHA.12 This dense area contrasts with a diffuse, low-density label that appears to be evenly distributed throughout the entire rod outer segment. These electron microscopic observations strongly support biochemical and light microscopic autoradiographic studies that demonstrate avid uptake by photoreceptors, and preferential usage of [3H]DHA-phospholipids for disc membrane synthesis.2,15,17,21 Additionally, high performance liquid chromatographic evaluations of retinal lipids through 46 days after [3H]DHA labeling, demonstrated that all radioactivity was recovered within the DHA peak, and more than 84% remained after 67 days.12 Finally, short-term [3H]DHA uptake studies have demonstrated very rapid esterification of DHA into glycerolipids, with only 5% remaining as free DHA at 6 hr.17 Of the total esterified [3H]DHA, 92% appears as phospholipids with 80% distributed among phosphatidylcholine, phosphatidyleth-
There are differences in $[^3H]$DHA distribution in rod and cone inner segments. Light-level autoradiography of short-term in vitro labeled retinas demonstrates much less labeling in cone inner segments than in rods, whereas long-term in vivo labeling, such as this 5-day postinjection study, shows equal rod and cone inner segment labeling. The in vitro preparations involve removal of retinas from the RPE cells and the eyecups, thereby eliminating any regulation by the RPE. This labeling difference could imply a significant functional role for the RPE cells in normal DHA handling, possibly demonstrating that during in vivo conditions the RPE cells carefully limit the amount of DHA released to the photoreceptors. Although this in vitro/in vivo comparison is also a short-term/long-term comparison, it is probably valid because, even after 25 days, exogenous (anywhere from outside the photoreceptor cell) sources of DHA replace the inner segment DHA that has been moved to new outer segment discs and the synaptic terminal. It may be, however, that the initial, short-term differences in photoreceptor labeling are overcome as cone inner segments slowly accumulate DHA. In either case, there appear to be differences in the way these two cell types take up DHA.

$[^3H]$DHA within the inner segments is not evenly distributed. In fact, labeling of the myoid region is sparse, and no accumulations occur over the Golgi complex, endoplasmic reticulum, or vesicles. Heavier labeling is found in ellipsoid regions in both rods and cones, but it is not possible from this study to determine if the $[^3H]$DHA is intramitochondrial or intermitochondrial. There is only minimal space between these organelles, and no vesicles occur there, even though substantial labeling exists. This is particularly interesting because photoreceptor disc membranes are assembled at the base of the outer segments from components arriving from the myoid. It has been shown that specific vesicles transport the protein opsin from the Golgi apparatus to the apical surface of the inner segment, where they fuse with the plasma membrane. In addition, it is known that vitamin A does not form a complex with opsin until the opsin has been added to assembled disc membranes in the outer segments. Monensin and brefeldin A inhibit some lipids from being added to outer segments, whereas many others continue to accumulate in the newly forming disc membranes, suggesting an alternative route for the trafficking of some lipids, different from that followed by proteins. High $[^3H]$DHA labeling of ellipsoids devoid of labeled vesicles can be interpreted in one of two ways. Either mitochondria are taking up and retaining DHA from the myoid or DHA is not significantly transported within the membranes of vesicles. Also, it is possible that opsin-laden vesicles peak at some time other than when these retinal samples were analyzed. Alternatively, $[^3H]$DHA-phospholipids could be transported by protein carriers within the cytosol and not become associated with transport vesicles. There is evidence for the existence of phospholipid transfer proteins, as well as free fatty acid carriers, such as fatty acid binding proteins. In either case, some $[^3H]$DHA could have become associated with mitochondria.

By 5 days postinjection, cone cell and RPE cell oil droplets have accumulated label, with their central portions labeled as well as their peripheries. RPE oil droplets have a much higher labeling density than those of cones from the same tissue (Figs. 2, 3, 5). It is difficult, however, to make assumptions on uptake and turnover rates within the oil droplets of these two cell types because the overall cytoplasmic labeling of RPE cells is higher than that of the 575-cone inner segments in these preparations. It has been shown that RPE oil droplets can be composed mostly of triglycerides (>95%) and vitamin A esters (<5%). Cone oil droplets become well labeled with tritiated fatty acids, as well as with $[^3H]$glycerol. Additionally, recent studies with propranolol, an inhibitor of phosphatidate phosphohydrolase, showed an overall 74% reduction of $[^3H]$DHA-labeled retinal triglycerides (but no decrease in diglycerides), and highly reduced cone oil droplet labeling, suggesting that triglycerides are important components of these oil droplets as well. It therefore is possible that a significant proportion of the $[^3H]$DHA may have become esterified into triglycerides. The autoradiographic labeling profile demonstrates that a portion of the incoming $[^3H]$DHA, taken up by the RPE, is accumulated in oil droplets where it is retained. Even though DHA levels in animals must normally fluctuate from variations in diet, photoreceptors continually assemble disc membranes, thus requiring a constant DHA input from the RPE, through the interphotoreceptor matrix. It may be that RPE oil droplets serve as a buffer, facilitating a sustained delivery of DHA to photoreceptors, even during relative dietary shortage.

The high labeling of oil droplets in RPE contrasts with the low labeling of cytoplasm and intracellular membranes. In fact, light-level autoradiograms of retinas 1–46 days postinjection have demonstrated constant but low levels of RPE label. Membrane organelles within the RPE cytoplasm, such as the myeloid bodies, have been considered as potential sites where lipids could be manipulated as they pass from the circulation to the interphotoreceptor matrix. Additionally, it has been shown in newts that myeloid bodies disappear when the neural sensory retina is removed, and only reappear after retinal regeneration is
complete and outer segments are again present and shedding normally, linking these structures with photomembrane turnover. A recent study with chick retinas describes the phospholipid content of myeloid bodies, showing them to be highly enriched in polyunsaturated fatty acids, and comparing their lipid profile to that of the photoreceptor outer segments. Based on these profiles, it has been suggested that chick myeloid bodies may be involved in the recycling of fatty acids from shed photoreceptor tips back to the sensory retina, implying that lipid molecules from phagosomes undergoing degradation will soon appear transiently within the myeloid bodies. At 5 days postinjection, when plasma displays the highest [3H]DHA labeling, no association of [3H]DHA with myeloid bodies can be demonstrated on EM autoradiograms, suggesting minimal or no participation in the trafficking of DHA as it enters the RPE cells from the circulatory system. This does not rule out a functional role for these membrane systems in fatty acid metabolism. Our electron microscopic study investigates the distribution of [3H]DHA 5 days after injection and several hours after normal shedding of diffusely labeled rod outer segments. It is questionable whether this low, diffuse label could be detected once it leaves the degraded phagosome, if it becomes dispersed among membranes of the myeloid bodies. It would be interesting to observe the distribution of [3H]DHA under identical conditions after shedding of densely labeled phagosomes. This would, however, require 30 days or more to ensure that shed discs are heavily labeled, and has not yet been studied at the ultrastructural level. Therefore, though myeloid bodies are not remarkably labeled in this study, it is not possible to determine if they are involved in the trafficking of phagosomal DHA through the RPE cells. In summary, [3H]DHA could have been rapidly moved through the myeloid bodies only after injection, or only at specific times of the day. The first possibility seems unlikely because DHA levels remain high throughout this time interval; the second alternative may occur because myeloid body disposition in the RPE cytoplasm changes throughout a 24-hr period. However, at the time of sampling, an acceleration of disc assembly has occurred, requiring more DHA. Also, it is possible that myeloid bodies do not handle incoming DHA-labeled lipids, but are involved with the specific task of recycling DHA-rich phospholipids immediately back to photoreceptors once they are removed from disc membranes of newly shed phagosomes. As newly shed phagosomes are degraded, they are moved away from the photoreceptor tips to a position near Bruch’s membrane. Electron microscopic autoradiography clearly shows that phagosomes sometimes become enriched in [3H]DHA. When this occurs, it is only in phagosomes adjacent to Bruch’s membrane; phagosomes located near the photoreceptor tips or centrally within the RPE cell have never been observed to have a labeling density higher than that of the underlying rod outer segment tips. A typical example is shown in Figure 6. Bibb and Young, observing similar labeling patterns with [3H]palmitic acid, suggested that fatty acids could become involved in an exchange reaction (turnover) with phospholipids that retain their positional integrity. Because our study demonstrates a similarity between phagosomal labeling (originating from diffusely labeled outer segment tips) with [3H]DHA and [3H]palmitic acid labeling over the same period of time, this form of labeling may be nonselective for DHA. Also, palmitic acid labeling of photoreceptor outer segments occurs rapidly and becomes evenly dispersed, similar to the diffuse label of DHA. However, palmitic acid does not form a dense, nondiffusing basal region associated with newly formed disc membranes, as does DHA. This further suggests that the diffuse outer segment labeling and additional uptake demonstrated by older phagosomes, once they reach Bruch’s membrane, for both fatty acids, may occur by a similar mechanism (ie, turnover of acyl groups), and is distinct from the dense, [3H]DHA labeling of new discs.

Finally, [3H]DHA also accumulates within the synaptic terminals of photoreceptors. Although little is known about the biochemistry of DHA in this region of the receptors, it does appear from autoradiograms that rod spherules may accumulate more label than do cone pedicles. The synaptic regions of these cells are densely packed with vesicles to the extent that photoreceptor terminals often appear denser than nearby cells in electron micrographs. Because DHA is a major constituent of photoreceptor membranes, it is likely that it is also being incorporated into the membranes of synaptic vesicles as they are formed. This suggests several interesting things. First, there could be specific handling mechanisms for newly acquired phospholipids that direct these molecules either toward the outer segment or to the synaptic region. Second, the phospholipid components of rod synaptic vesicle membranes could be different from those of other cell types, possibly suggesting differences in metabolic, transmitter, and/or vesicle fusion/release mechanisms. Third, if no difference in phospholipid makeup exists between rod and cone terminals, differential labeling might still be observed if the vesicle synthesis/depletion rates for these two cells are different.

Thus, [3H]DHA autoradiograms of frog retinas sampled at 5 days postinjection show the following: (A) Cone photoreceptors, especially outer segments, label differently than rod photoreceptors, taking up less label. (B) Although there are vesicles present within the inner segments of both rods and cones, no [3H]DHA appears to be associated with them. This includes small
vesicles found around the periphery of the ellipsoid’s mitochondrial mass. There is, however, significant [3H]DHA found within this region. (C) Oil droplets in both cone cells and RPE cells label much more than the surrounding cytoplasm. (D) Myeloid bodies within the RPE only accumulate label occasionally. (E) Some older phagosomes, when near Bruch’s membrane, increase their level of labeling considerably above the level found in rod outer segment tips. (F) [3H]DHA accumulates within the synaptic terminals of photoreceptor cells.

In summary, based on the ultrastructural observations in this study and earlier light-level autoradiographic and biochemical studies,10,17,21,22,34,41 we can now suggest a trafficking pathway for DHA through the retina, after its arrival to the eye. DHA lipids enter RPE cells from the choriocapillaris where they are altered for storage within oil droplets and/or are rapidly passed on to the photoreceptors. Myeloid bodies do not seem to be involved in the initial handling of DHA, and DHA-phospholipids are not retained within RPE cell membranes and cytoplasm. After uptake by the myoid of photoreceptors, some DHA-containing lipids are moved to synaptic terminals, whereas most others accumulate within ellipsoids. There is no evidence of vesicular transport to the apical portion of the inner segments, although it cannot yet be ruled out. In cone photoreceptors, DHA actively accumulates within oil droplets. Some DHA becomes associated with mitochondria, but most enters the outer segments. DHA rapidly labels outer segment disc membrane phospholipids in an even and diffuse pattern in both rods and cones. However, three times as much DHA is continually assembled basally into the disc membranes of rod outer segments. These DHA-rich phospholipids follow exactly the apical migration of [3H]leucine,12 as first described by Young.42 After shedding and phagocytosis of photoreceptor tips by the RPE cells, degradation of disc membranes begins. DHA-containing lipids are removed and recycled back to the photoreceptors.25 It is possible that RPE myeloid bodies may play a role in this recycling, but we have not yet demonstrated it autoradiographically. As phagosomes are degraded, they often collect near Bruch’s membrane where they may accumulate DHA entering from the circulation. This transiently bound DHA may be destined for temporary storage within oil droplets, or returned to the underlying photoreceptors, along with newly arriving DHA-phospholipids.

Key Words
frog, phagosomes, pigment epithelium, polyunsaturated fatty acids, retina, synaptic terminals

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