# Iron-Induced Fluorescence in the Retina: Dependence on Vitamin A

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***Purpose.*** Intravitreal iron injection induces fluorophore formation in the photoreceptor outer segments, followed by an accumulation of inclusions with lipofuscin-like fluorescence in the retinal pigment epithelium (RPE). The accumulation of RPE lipofuscin during aging is dependent on vitamin A availability. Experiments were conducted to determine whether iron-induced fluorophore formation in the outer segments and in RPE is also dependent on vitamin A, and thus whether oxidation promotes the participation of vitamin A in lipofuscin formation.

***Methods.*** For 23 weeks, beginning at weaning, albino Fischer rats were fed diets containing vitamin A either in the form of retinyl palmitate ($+A$), which can be metabolically converted into the retinoids involved in vision, or retinoic acid ($-A$), which does not support visual function. After 23 weeks, when rhodopsin levels had decreased more than 90% in the $-A$ rats, some animals in this group were given an intramuscular injection of all-*trans* retinol and were allowed to recover from retinoid deficiency for 7 days ($-A+A$). Animals in all three treatment groups were then given an intravitreal injection of ferrous sulfate. Both 1 day and 7 days after the iron injections, the retinas and RPEs were examined for fluorophores with excitation and emission properties similar to those of RPE lipofuscin fluorophores.

***Results.*** In retina sections examined with fluorescence microscopy 24 hours after the ferrous sulfate treatment, the photoreceptor outer segments of rats in all of the treatment groups displayed a fluorescence with a blue emission maximum. This outer-segment fluorescence was not present in untreated eyes. The in situ outer-segment fluorescence was correlated with the appearance of blue-emitting fluorophores in organic solvent extracts of the retinas. One week after the iron injections, the RPE cells of the $+A$ animals became filled with inclusions that displayed a golden-yellow fluorescence emission when excited by blue light. Very little of this lipofuscin-like fluorescence was observed in the RPE of the $-A$ rats 1 week after iron treatment. However, in the $-A$ rats that had been repleted with vitamin A, the ability of iron to induce the RPE fluorescence was restored. Several orange-emitting fluorophores were present in organic solvent extracts of the RPE-choroids of the $+A$ rats. The amounts of these fluorophores were not appreciably affected by the iron treatment. These orange-emitting compounds were not observed in extracts of any eyes in the $-A$ or $-A+A$ groups.

***Conclusions.*** The results of this study suggest that oxidation of the photoreceptor outer-segment lipids generates blue-emitting fluorophores that are not directly involved in RPE lipofuscin fluorophore formation. The findings also indicate that retinoids are direct precursors of RPE lipofuscin fluorophores, and that oxidative stress to the retina promotes participation of vitamin A in the formation of some of the compounds responsible for RPE lipofuscin fluorescence. Invest Ophthalmol Vis Sci. 1994;35:3613–3624.

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Lysosome-related inclusions with characteristic fluorescence properties accumulate in numerous postmitotic cell types during senescence. These inclusions have been designated lipofuscin, or age pigment. In the retina, age-related lipofuscin accumulation occurs almost exclusively in the retinal pigment epithelium (RPE).[1-3] The precise mechanisms involved in RPE lipofuscin fluorophore formation remain to be deter-

mined, but it appears that the contents of RPE lipofuscin granules are derived primarily from phagocytosed photoreceptor outer segments.[4-11] Of the molecular constituents of the outer segments, retinoids are most likely to be directly involved in the generation of RPE lipofuscin fluorophores. Animals fed diets deficient in the retinoids involved in the visual process show substantially less RPE accumulation of lipofuscin than do animals fed adequate amounts of retinoids.[10,12-16] In addition, recent evidence suggests that one of the fluorophores in human RPE lipofuscin is a reaction product of retinaldehyde and ethanolamine.[17]

Oxidative stress to the retina promotes the buildup of lipofuscin-like autofluorescent pigment granules in the RPE. For example, in animals fed diets deficient in antioxidant nutrients, the RPE becomes engorged with these inclusion bodies relatively quickly.[12,18-22] However, the effect of oxidative stress on RPE lipofuscin-like pigment accumulation is substantially reduced in animals depleted of visual cycle retinoids.[12,13] The latter observation suggests that oxidative stress to the photoreceptors promotes the formation of RPE lipofuscin constituents from outer-segment retinoids. Experiments were conducted to evaluate this possibility.

## MATERIALS AND METHODS

### Animals and Diets

Male Fischer 344 albino rats were obtained at 3 weeks of age from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed three to a cage in one room under dim 12-hour light/12-hour dark cyclic illumination, as described previously.[23] On receipt, the rats were divided into two dietary groups. One group was fed a synthetic diet that contained adequate levels of all nutrients known to be required by the rat.[24] This diet ($+A$) contained vitamin A in the form of retinyl palmitate, which can be metabolized to all of the retinoids involved in the visual process, as well as the retinoids used by other tissues. The second group was fed the same diet, except that retinoic acid was substituted for retinyl palmitate ($-A$).[24] Retinoic acid can satisfy the metabolic requirements of most tissues for vitamin A but cannot be metabolically converted into the retinoids involved in vision.[25] All investigations were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Vitamin A Repletion

Some animals fed the $-A$ diet for 23 weeks were given a single intramuscular injection of 75 $\mu$g of all-*trans* retinol.[26] This group, designated $-A+A$, was maintained on the $-A$ diet for an additional week before further treatment or analysis. Previous measurements have shown that in animals maintained on the $-A$ diet for 23 weeks, retinal rhodopsin levels are reduced to less than 10% of those in the $+A$ rats.[26] Mean outer-segment size is reduced by more than 50%, and visual sensitivity is reduced substantially.[26] However, no photoreceptor cell death occurred after 23 weeks.[26] One week after the retinol injection, retinal rhodopsin levels returned to more than 70% of normal, and both outer-segment size and visual sensitivity also showed significant recovery.[26]

### Iron Treatments

Animals in each of the vitamin A treatment groups described above were given a single intravitreal injection of ferrous sulfate in one eye and either an injection of saline vehicle or no injection in the other eye. Ferrous iron can catalyze oxidative decomposition of polyunsaturated fatty acids through Fenton-type reactions.[27] The rats were anesthetized with intramuscular injections of a combination of ketamine (80 mg/kg body weight) and xylazine (12 mg/kg body weight). One eye of each animal was then injected with 3 $\mu$l of freshly prepared 10-mM $FeSO_4$ in 150-mM NaCl. In some rats, the other eye was injected with the same volume of 150-mM NaCl. All injections were made into the vitreous behind the lens.

### Fluorescence Microscopy

Either 1 or 7 days after the intravitreal injections, rats were killed with $CO_2$, and their eyes were enucleated. Eyes that were to be examined in cross-section were fixed as described previously.[23] The eyecups were then bisected in a plane parallel to the superior-inferior meridian, approximately 1 mm to one side of the optic nerve head. The larger portion of each tissue was washed for 10 minutes in 0.17-M sodium cacodylate, pH 7.4, and then for 10 minutes in 1:1 cacodylate buffer:Tissue-Tek embedding medium (Miles Laboratories, Naperville, IL), and finally was embedded in Tissue-Tek embedding medium and frozen. Sections of the frozen tissues were cut with a Bright cryostat (Bright Instrument, Huntington, UK) along the superior-inferior meridian at a thickness of 10 $\mu$m. The sections were mounted on glass slides in a solution consisting of 33% glycerol in 0.17-M sodium cacodylate, pH 7.4. The sections were examined and photographed with a Zeiss Photomicroscope I (Zeiss, Oberkochen, Germany) equipped for epi-illumination. Fluorescent emissions were stimulated by light from a 100-W high-pressure mercury vapor source. The light employed for excitation was passed through Zeiss filter set 487705, which consisted of a 400-nm to 440-nm bandpass excitation filter, an FT 460 dichromatic beam splitter and an LP 470 barrier. A 515-nm barrier filter was also placed in the emission path. Samples

were photographed with Kodak EL 135 400 film (Eastman Kodak, Rochester, NY).

Flat preparations of the RPE were made from the eyes of some animals 1 week after the intravitreal injections. The rats were killed with $CO_2$ and the eyes were enucleated. The corneas, irises, lenses, and retinas were removed, leaving the eyecups lined by the RPE.[24] Series of radial cuts were made in the eyecups to enable them to lie flat. The eyecups were mounted, RPE-side up, on glass slides in a 0.17-M sodium cacodylate buffer, pH 7.4. These preparations were then examined and photographed as described above for the sectioned tissues.

## Fluorophore Extraction and Chromatography

Both 1 and 7 days after iron treatment, analyses were performed to characterize the fluorophores that could be extracted from the retinas and the RPEs of treated and control eyes. Rats were killed with $CO_2$, and their eyes were enucleated immediately. The corneas, irises, and lenses were removed and discarded. The neural retinas were then dissected from the remainders of the eyecups, which included the RPE, choroid, and sclera. Thereafter, the neural retina and eyecup samples were treated identically.

The retinas from two eyes were pooled in a glass homogenization sleeve containing 400 $\mu$l of ice-cold buffer consisting of 10 mM HEPES, 150 mM NaCl, 1 mM $Na_2EDTA$, pH 7.4. The RPE–eyecups from the same eyes were pooled in another homogenization sleeve containing the same amount of buffer. The tissues were homogenized with approximately 10 passes of a motor-driven Teflon pestle (Wheaton, Millville, NJ) run at moderate speed. We have determined that this method dissociates only the RPE and choroid, leaving the sclera relatively intact.[24] The homogenates were then each transferred to an 8-ml vial with a Teflon-lined cap. Soluble nonpolar components of the homogenates were extracted with dichloromethane as described previously.[23] The fluorophore contents of both the organic and aqueous phases of each extract were analyzed with thin-layer chromatography (TLC). Some samples were extracted using the chloroform–methanol procedure described by Folch and colleagues.[28] No differences in the fluorophore compositions of the extracts were observed between the two extraction procedures. Therefore, all analyses conducted for this study were performed using the dichloromethane extraction technique.

The dichloromethane fraction of each extract was dried under a vacuum, and the residue was redissolved in 50 $\mu$l of 1:1 dichloromethane:methanol. The entire sample was spotted on a channeled silica gel G TLC plate with a preabsorbent zone (Analtech, Newark, DE). The TLC plates were developed for 20 minutes in a mobile phase consisting of dichloromethane:methanol:water:acetic acid (5:1:0.1:0.03). After development, the plates were dried and photographed under illumination from an ultraviolet source with a maximum output of 366 nm and a spectral bandwidth of approximately 50 nm at half-maximum.

It was recently reported that one of the orange-emitting fluorophores from human RPE lipofuscin is a reaction product of retinaldehyde and ethanolamine.[17] This compound was synthesized as described previously and purified using TLC.[17] A sample of the synthetic compound was chromatographed along with extracts of the RPE–eyecups of young (10-week) and old (20-month) rats that had been fed the $+A$ diet. Chromatography was performed as described in the previous paragraph.

The aqueous phase of each extract was lyophilized, and the residue was resuspended in 50 $\mu$l of butanol:acetic acid:water (4:1:1). A 20-$\mu$l aliquot of each sample was spotted on a silica gel G TLC plate with a preabsorbent zone (Analtech). The chromatograms were developed in butanol:acetic acid:water (4:1:1) for 100 minutes. After development, the plates were dried and photographed as described above.

## Morphologic Analyses

The effects of the $FeSO_4$ treatments on retinal structure were examined 1 and 7 days after iron administration. Rats were killed with $CO_2$, and their eyes were enucleated, fixed, dissected, and embedded as described previously.[2,23] Sections of the embedded tissue were cut at a thickness of 600 nm, stained with toluidine blue, and examined with Zeiss Axiophot microscope. Ultrathin sections were cut from the same blocks and were stained with uranyl acetate and lead citrate. The ultrathin sections were examined and photographed with a JEOL 1200 EX electron microscope (JEOL, Tokyo, Japan).

# RESULTS

## In Situ Iron-Induced Fluorescence in the Retina

Iron injection (regardless of vitamin A status) led to a rapid, transient appearance of autofluorescence in the photoreceptor outer segments. In animals fed both the $+A$ diet and the $-A$ diet, and in those fed the $-A$ diet and then repleted with retinol, a pronounced fluorescence was present in the photoreceptor outer segments 1 day after the $FeSO_4$ injection (Fig. 1). The outer-segment fluorescence appeared green-yellow when the samples were examined in the fluorescence microscope using filters that were optimum for visualization of lipofuscin fluorescence (400-nm to 440-nm bandpass excitation filter, 515-nm high-pass barrier emission filter) (Fig. 1). The outer-segment fluores-

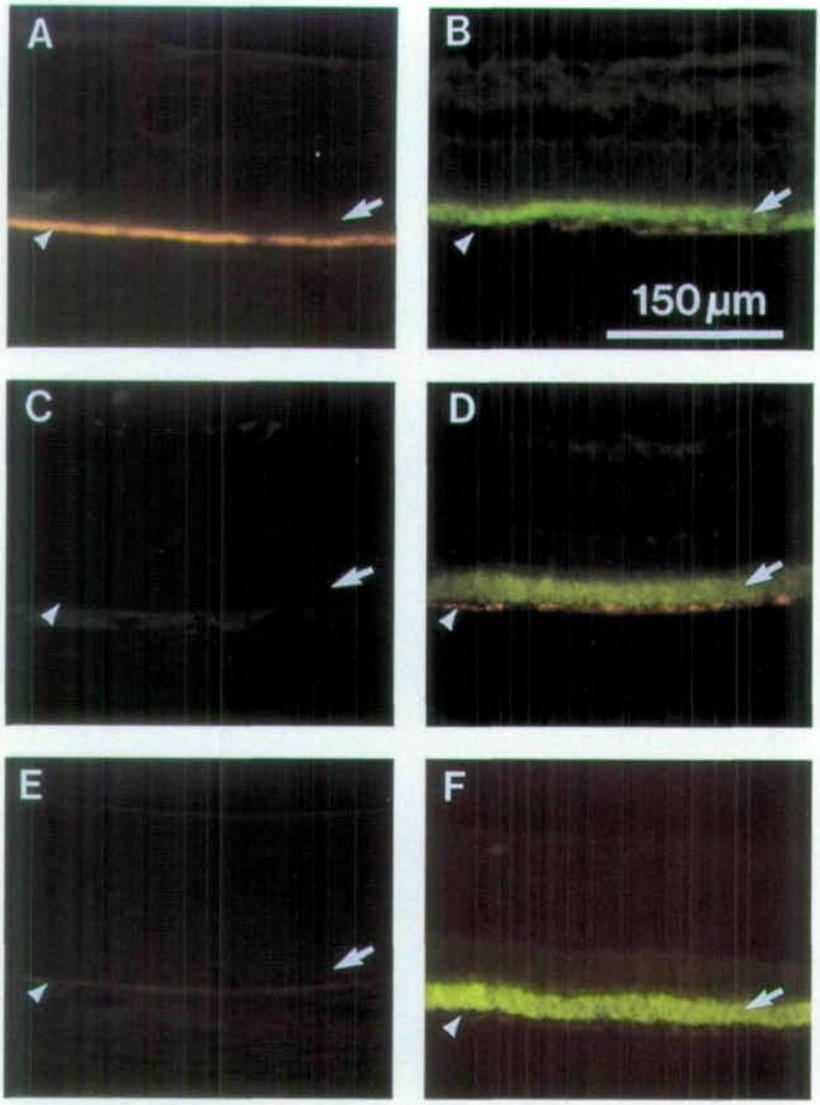


FIGURE 1. Fluorescence micrographs of cryostat sections of untreated retinas from $+A$ (**A**), $-A$ (**C**), and $-A+A$ (**E**) rats, and from retinas 1 day after iron treatment from $+A$ (**B**), $-A$ (**D**), and $-A+A$ (**F**) rats. The yellow band in (**A**) is the RPE with its typical lipofuscin-specific fluorescence. The photoreceptor outer segments are indicated by arrows, and the RPEs by arrowheads. Bar in (**B**) indicates the magnification of all six micrographs.

cence emission became more intense when examined with a combination of a narrow-bandpass excitation filter with peak transmission at 365 nm and a 395-nm high-pass barrier emission filter. With the latter filter combination, the outer-segment fluorescence emission appeared blue. With the dose given, the iron-induced fluorescence was restricted to the outer segments and did not occur in other parts of the retina. By 1 week after injection, the outer-segment fluorescence had largely disappeared from the retina in all three vitamin A treatment groups. Autofluorescence was not observed in the outer segments of any animals that did not receive the iron injection (Fig. 1).

Sustained dietary vitamin A led to an accumulation of golden-yellow-emitting fluorophores in the RPE, which was enhanced by iron injection. A similar golden-yellow autofluorescence appeared in the RPE after iron treatment in animals provided with vitamin A for only a short period. The RPE cells of the $+A$ rats not treated with $FeSO_4$ had amounts of autofluorescent age pigment typical of 6-month-old animals (Figs. 1, 2). The autofluorescent pigment was distributed uniformly throughout the RPE in this treatment group. One week after the iron treatment, many areas of the RPE in the $+A$ animals displayed a golden-yellow, lipofuscin-like fluorescence that was much more intense than that observed in rats not given the $FeSO_4$ injections (Fig. 2). In animals fed the $-A$ diet, the RPE age-pigment fluorescence was substantially less than that in rats given the $+A$ diet (Figs. 1, 2). Repleting the $-A$ animals with retinol for 1 week did not increase the RPE lipofuscin fluorescence (Fig. 2).

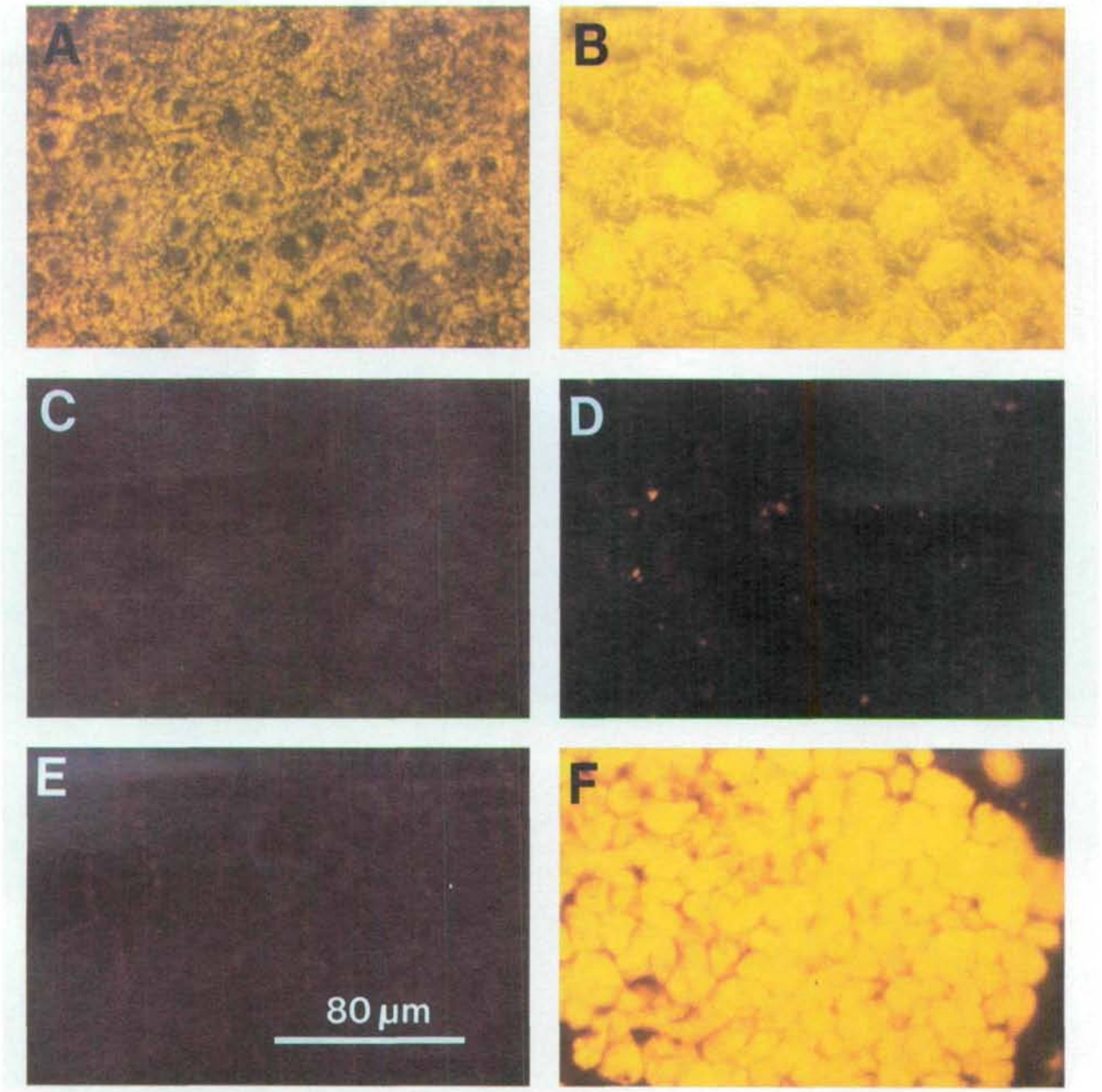


FIGURE 2. Fluorescence micrographs of whole-mount flat preparations of the RPE from untreated eyes from $+A$ (**A**), $-A$ (**C**), and $-A+A$ (**E**) rats, and from eyes 1 week after iron treatment from $+A$ (**B**), $-A$ (**D**), and $-A+A$ (**F**) rats. Bar in (**E**) indicates the magnification of all six micrographs.

Unlike the $+A$ rats, 1 week after the iron treatment the $-A$ animals did not display a detectable increase in autofluorescence anywhere in the RPE (Fig. 2). However, in $-A$ rats that had been repleted with vitamin A 1 week before treatment with $FeSO_4$, an iron-induced, lipofuscin-like fluorescence was observed in many regions of the RPE 1 week after injection that was as intense as the RPE fluorescence produced by iron treatment of the $+A$ rats (Fig. 1). In both the $+A$ and the repleted $-A$ rats, the RPE-associated fluorescence was not uniformly distributed over the choroid but was interspersed with nonfluorescent regions.

## Soluble Fluorophores

Analyses were conducted to evaluate the chromatographic and spectral properties of the fluorophores that could be extracted from the retina and RPE into dichloromethane. Regardless of vitamin A availability, the injection of iron led to formation of organic solvent-extractable, blue-emitting fluorophores in the neural retina. One day after injection, several of these fluorophores were present on thin-layer chromatograms of dichloromethane extracts of the neural retinas from the treated eyes of rats in all three vitamin A treatment groups (Fig. 3). The blue-emitting fluor-

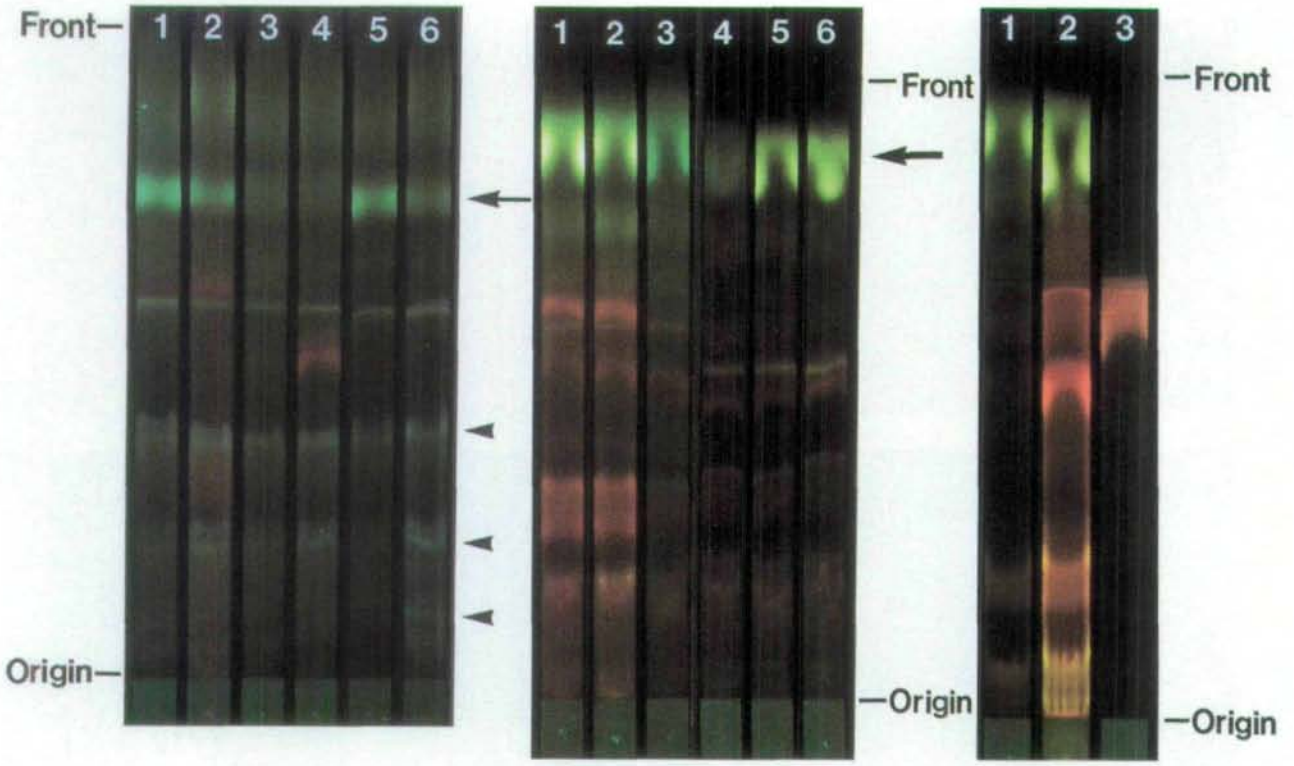


FIGURE 3. (*left*) Thin-layer chromatogram of dichloromethane extracts of neural retinas. The chromatogram was photographed under ultraviolet illumination. The extracts were from retinas of animals in the following treatment groups: lane 1, $+A$ control; lane 2, $+A$ iron-injected; lane 3, $-A$ control; lane 4, $-A$ iron-injected; lane 5, $-A+A$ control; lane 6, $-A+A$ iron-injected. All injected eyes were enucleated 1 day after iron treatment. Arrow indicates retinol fluorescence. Arrowheads indicate iron-induced fluorophores.

FIGURE 4. (*middle*) Thin-layer chromatogram of dichloromethane extracts of RPEs. The chromatogram was photographed under ultraviolet illumination. The extracts were from RPEs of animals in the following treatment groups: lane 1, $+A$ control; lane 2,, $+A$ iron-injected; lane 3, $-A$ control; lane 4, $-A$ iron-injected; lane 5, $-A+A$ control; lane 6, $-A+A$ iron-injected. All injected eyes were enucleated 1 week after iron treatment. Arrow indicates retinyl ester fluorescence.

FIGURE 5. (*right*) Thin-layer chromatogram of dichloromethane extracts of RPEs and retinal-ethanolamine reaction product. The chromatogram was photographed under ultraviolet illumination. The extracts were from RPEs of untreated $+A$ animals 10 weeks (lane 1) and 87 weeks (lane 2) of age. Lane 3 contains the synthetic reaction product of all-*trans* retinal and ethanolamine.

ophores were either not detectable or were much less abundant in the extracts from retinas that had not been exposed to $FeSO_4$ (Fig. 3). One week after the iron injections, when the in situ autofluorescence in the neural retinas had disappeared, the blue-emitting fluorophores were no longer observed. Thin-layer chromatograms of extracts from the retinas of $+A$ and $-A+A$ rats not treated with iron contained a relatively nonpolar yellow-green emitting component (Fig. 3) that has previously been identified as retinol.[29] The retinol band was weak or absent in extracts of retinas from $-A$ animals. The retinol bands were slightly less intense 24 hours after $FeSO_4$ treatment in both the $+A$ and the $-A+A$ rats (Fig. 3).

Sustained dietary vitamin A intake led to accumulation of organic solvent-extractable, orange-emitting fluorophores in the RPE. Dichloromethane extracts of the RPE–eyecups of eyes from $+A$ rats not injected with iron contained a number of orange-emitting constituents that were separated from one another on TLC (Fig. 4). These fluorophores appear to be components of RPE lipofuscin,[29] and they clearly accumulate in the RPE during senescence (Fig. 5). One of the orange-emitting fluorophores had the same chromatographic mobility as that of a stable reaction product of all-*trans* retinal and ethanolamine (Fig. 5).[17] The RPE extracts also contained a yellow–green-emitting component that migrated near the solvent front

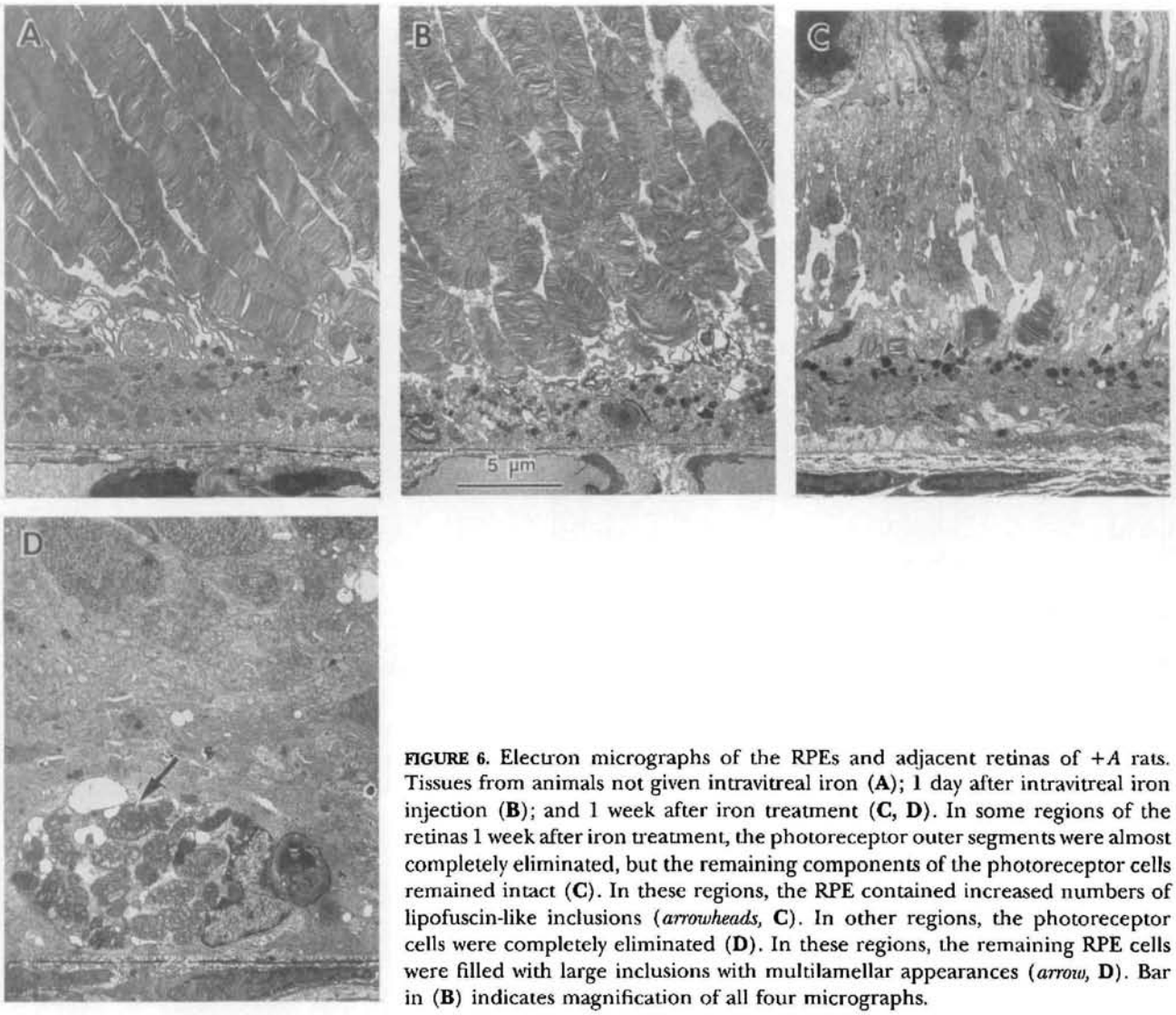


FIGURE 6. Electron micrographs of the RPEs and adjacent retinas of $+A$ rats. Tissues from animals not given intravitreal iron (**A**); 1 day after intravitreal iron injection (**B**); and 1 week after iron treatment (**C, D**). In some regions of the retinas 1 week after iron treatment, the photoreceptor outer segments were almost completely eliminated, but the remaining components of the photoreceptor cells remained intact (**C**). In these regions, the RPE contained increased numbers of lipofuscin-like inclusions (*arrowheads,* **C**). In other regions, the photoreceptor cells were completely eliminated (**D**). In these regions, the remaining RPE cells were filled with large inclusions with multilamellar appearances (*arrow,* **D**). Bar in (**B**) indicates magnification of all four micrographs.

on TLC. This component has previously been identified as a mixture of the retinyl esters that are stored in the RPE.[24,29] The orange-emitting fluorophores were not observed in the RPE–choroid extracts from either the $-A$ or the $-A+A$ groups, demonstrating that these fluorophores are clearly related to vitamin A (Fig. 4). Their presence depended on long-term intake of retinyl palmitate. Iron treatment did not result in appreciable changes in the amounts of any of the age-related yellow-emitting or orange-emitting fluorophores extracted from the RPE of any of the rats (Fig. 4). Thus, the iron-induced fluorophores of the RPE were not extractable. The blue-emitting fluorophores observed in extracts of iron-treated neural retinas after 1 day were not detected in extracts of the RPE–choroid either 1 day or 1 week after iron injection.

TLC analysis was also performed to characterize any soluble polar fluorophores that may have been present in the aqueous phases of the extracts from iron-treated and control eyes. None of the aqueous phases of extracts from the neural retinas obtained 1 day after iron injection had any detectable fluorophores. The chromatograms of the aqueous phases of the RPE–eyecup extracts from both control eyes and eyes collected 1 week after iron treatment each had a single blue-emitting fluorescent component with an $R_f$ of approximately 0.5. Neither vitamin A nor iron treatment affected the amount of this component that was extracted.

## Effect of Iron on Retinal Structure

Iron treatment caused major disruptions in outer-segment structure. These disruptions were more pronounced when vitamin A was available in the retina. Significant iron-induced alterations in outer-segment structure were observed in the $+A$, $-A$, and $-A+A$ groups 24 hours after injection (Figs. 6, 7, 8). Iron treatment caused apparent outer-segment fragmentation, folding, and disruption of outer-segment orienta-

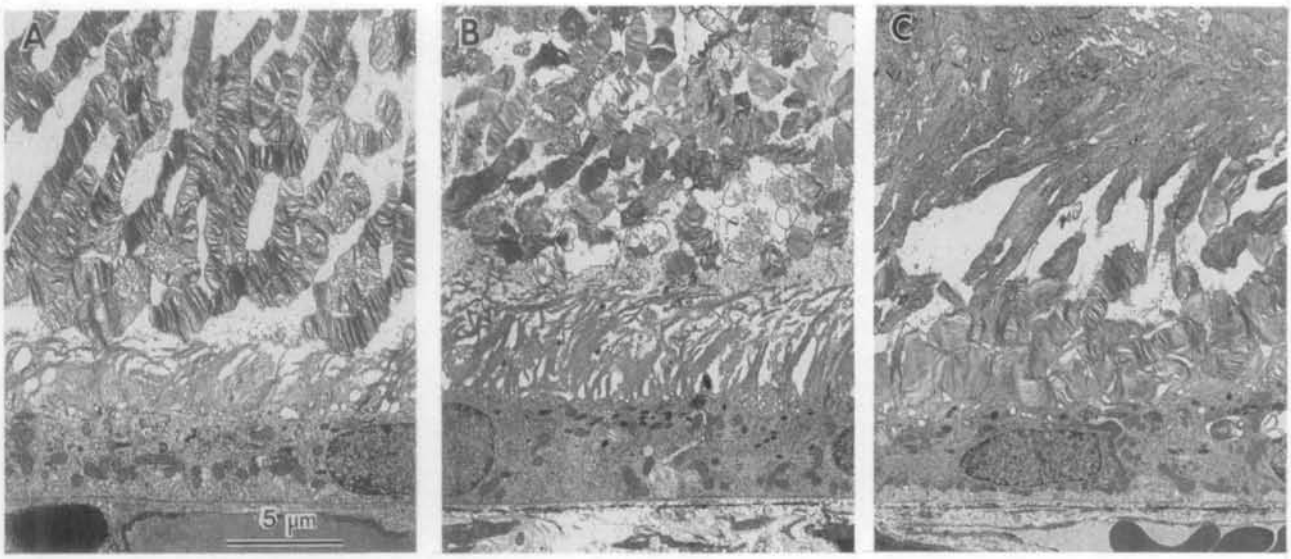


FIGURE 7. Electron micrographs of the RPEs and adjacent retinas of $-A$ rats. Tissues from animals not given intravitreal iron (**A**); 1 day after intravitreal iron injection (**B**); and 1 week after iron treatment (**C**). Vitamin A deficiency resulted in a vesiculation of many outer-segment disks (**A**). One day after iron treatment, the outer segments were severely fragmented (**B**). One week after the iron injection, the remaining outer segments were greatly shortened (**C**). The RPE had few lipofuscin-like inclusions either before or after iron treatment. Bar in (**A**) indicates magnification of all four micrographs.

tion. One week after the iron treatment, the outer segments either were greatly reduced in size or were completely absent in many regions of the retinas of animals in all three vitamin A treatment groups (Figs. 6, 7, 8). In some regions of the retinas from the $+A$ and $-A+A$ rats, the entire photoreceptor cell layer had disappeared by 1 week after the iron treatment (Figs. 6, 8). In these regions, the inner nuclear layer or outer plexiform layer was immediately adjacent to the RPE or Bruch's membrane. In the $-A$ animals, no regions were observed in which the entire photoreceptor cell layer had been eliminated.

Iron-induced accumulations of lipofuscin-like inclusions in the RPE were observed that were dependent on vitamin A availability. The RPEs of the untreated $+A$ rats contained moderate numbers of inclusions with structural appearances typical of lipofuscin (Fig. 6A). The numbers of these inclusions were significantly less in both the $-A$ and $-A+A$ animals not treated with intravitreal iron (Figs. 7A, 8A). In the $+A$ rats, the numbers of lipofuscin-like inclusions were greatly increased 1 week after iron treatment in regions of the retinas that showed only outer-segment loss or diminution (Fig. 6C). In regions where the photoreceptor cells had completely disappeared, the adjacent RPE either was absent in patches or was filled with large multilamellar inclusions (Figs. 6D, 8D). No accumulations of iron-induced inclusions were observed in the RPEs of the $-A$ rats 1 week after iron treatment (Fig. 7C). RPE structure was relatively well preserved in the iron-treated eyes of $-A$ rats, except that the photoreceptor outer segments were substantially shortened 1 week after the iron treatment. In the $-A+A$ animals 1 week after iron treatment, most regions of the RPEs appeared similar to those of the most severely affected regions in the $+A$ rat retinas (Fig. 8C). The RPE had disappeared in patches and in other areas was filled with the same type of large, striated inclusions observed in the $+A$ rat RPEs 1 week after iron treatment (Figs. 8C, 8D). The structural appearances of these inclusions in both the $+A$ and $-A+A$ animals suggested that they were derived from the photoreceptor outer segments (Fig. 8). Overall, the damage to the photoreceptors and RPE was more severe in the $+A$ and $-A+A$ animals than in the $-A$ rats.

## DISCUSSION

A substantial amount of evidence indicates that RPE lipofuscin is derived primarily from components of the photoreceptor outer segments.[4-7,20] Although several molecular constituents of the outer segments may participate in RPE lipofuscin formation, the evidence that outer-segment retinoids are directly involved in RPE lipofuscin fluorophore formation is the clearest.[12-17,30] Dietary deficiency in retinoids results in a dramatic reduction in the rate of lipofuscin-like autofluorescent pigment accumulation in the RPE.[12,13,15] Recently, evidence has been presented indicating that one of the fluorophores from human RPE lipofuscin is a reaction product of retinaldehyde and ethanol-

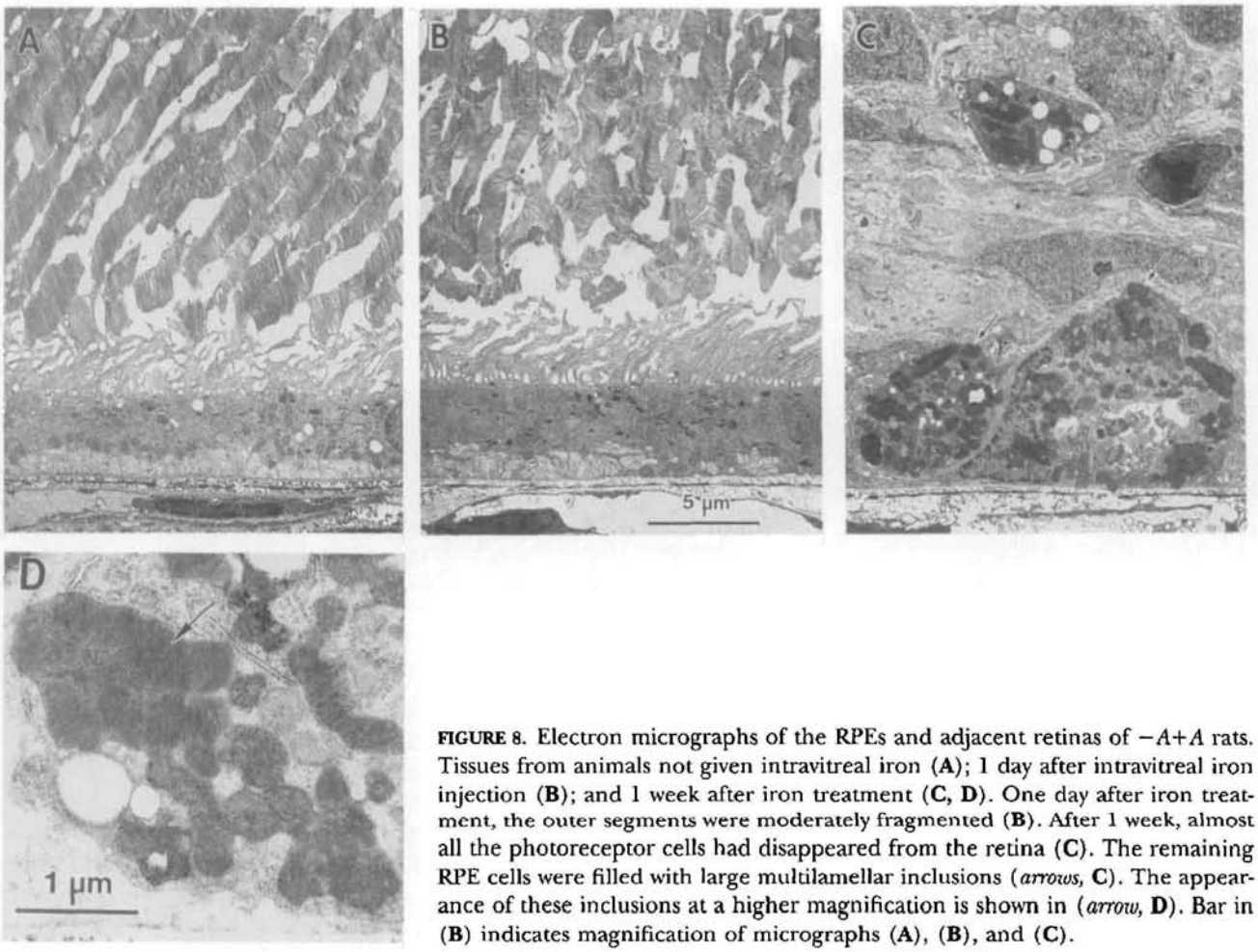


FIGURE 8. Electron micrographs of the RPEs and adjacent retinas of $-A+A$ rats. Tissues from animals not given intravitreal iron (**A**); 1 day after intravitreal iron injection (**B**); and 1 week after iron treatment (**C, D**). One day after iron treatment, the outer segments were moderately fragmented (**B**). After 1 week, almost all the photoreceptor cells had disappeared from the retina (**C**). The remaining RPE cells were filled with large multilamellar inclusions (*arrows*, **C**). The appearance of these inclusions at a higher magnification is shown in (*arrow*, **D**). Bar in (**B**) indicates magnification of micrographs (**A**), (**B**), and (**C**).

amine.[17] Thus, conditions that result in an elevation of the steady-state levels of retinal in the photoreceptor outer segments and in RPE would be expected to increase the rate of RPE lipofuscin fluorophore formation.

When the visual pigments of mammalian retinas are bleached, the vitamin A chromophore is released from the visual pigment protein as all-*trans* retinal. Under normal conditions, the retinal is rapidly reduced to retinol through the action of a photoreceptor-specific retinol dehydrogenase.[31,32] Conditions that impede this reduction could result in an elevation of the steady-state levels of retinal. Retinal spontaneously reacts with primary amines, including phosphatidylethanolamine and protein lysine residues.[33,34] Reaction of retinal with the former constituent of the outer segments apparently results in the formation of at least one of the fluorescent components of RPE lipofuscin.[17]

Oxidative stress to the retina is expected to decrease the steady-state levels of NADPH used by retinol dehydrogenase to reduce retinal to retinol, because much of the reducing potential in the outer segments would be drained to counter the effects of direct oxidative damage to outer-segment constituents.[20,35–37] Oxidative damage to the outer-segment lipids generates lipid hydroperoxides. These hydroperoxides are reduced through the action of glutathione peroxidase in a reaction that oxidizes glutathione. Regeneration of reduced glutathione consumes NADPH through the action of glutathione reductase.

The direct oxidative damage to the retina is reflected in the structural changes in the outer segments and in the appearance of in situ outer-segment fluorescence and extractable blue-emitting fluorophores in the retina 1 day after iron treatment. The outer-segment fluorophores are not vitamin A dependent and probably represent oxidation products of disk membrane polyunsaturated fatty acids.[38] These blue-emitting fluorophores do not accumulate in the RPE but are apparently either degraded or eliminated from the retina and the RPE. A number of studies have shown that impairment of antioxidant protection of the retina results in the accumulation of large amounts of an autofluorescent, lipofuscin-like pigment in the RPE.[18–20,22] This accumulation is dependent on the availability of outer-segment retinoids.[12,13,16] In the present study, iron was used to pro-

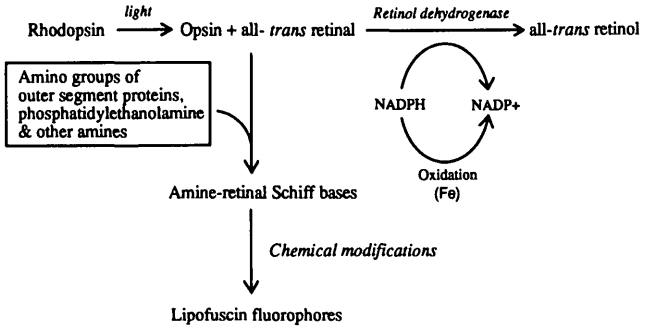


FIGURE 9. Proposed model to explain the effects of dietary vitamin A and iron treatment on RPE lipofuscin fluorophore formation.

mote oxidative damage to the outer segments.[23,38] When outer-segment retinoids were present (in the $+A$ and $-A+A$ rats), iron-induced oxidative damage to the outer segments was followed by the deposition of an autofluorescent, lipofuscin-like pigment in the RPE. However, in the absence of visual cycle retinoids in the retina ($-A$ rats), the lipofuscin-like fluorescence failed to develop. Thus, it appears that accelerated oxidative damage to the retina impairs reduction of retinal to retinol, and thus allows a larger-than-normal amount of retinal to react with outer-segment amines to generate fluorescent products that accumulate in the RPE. A proposed model to explain the interaction between oxidative damage and retinoids in the formation of RPE lipofuscin is shown in Figure 9.

The data from the present study are generally consistent with this model for RPE lipofuscin fluorophore formation. According to this model, the fluorescent products of outer-segment fatty acid oxidation are not directly involved in RPE lipofuscin fluorophore formation.[38] Indeed, the fluorescent compounds present in the neural retina 1 day after iron treatment were not observed in extracts of the RPE that were rich in iron-induced inclusions with lipofuscin-like fluorescence or in extracts of the RPE from old, untreated animals.

One aspect of the results is puzzling, however. Normal RPE lipofuscin contains both extractable and insoluble fluorophores.[29] Several of the extractable fluorophores were observed on thin-layer chromatograms of RPE extracts from $+A$ rats that were 26 weeks of age or older (Figs. 4, 5). All of these age-related fluorophores were clearly vitamin A dependent (Fig. 4). Iron treatment failed to increase the amounts of these extractable vitamin A-dependent fluorophores in either the $+A$ or the $-A+A$ animals, despite the fact that iron induced the accumulation of RPE inclusions with lipofuscin-like fluorescence in these vitamin A treatment groups. Thus, it appears that iron treatment induces the formation of only the insoluble fluorescent components of RPE lipofuscin. One can only speculate why this is the case. One difference between normal age pigment and the iron-induced RPE inclusions is their age. Age pigment is formed throughout life, so some of the pigment granules may be as old as the animals from which they are obtained. By contrast, the iron-induced inclusions in this study were all less than 1 week of age. Perhaps the orange-emitting and yellow-emitting fluorophores, formed initially during lipofuscinogenesis, are all insoluble reaction products between retinal and outer-segment proteins. As these compounds are acted on by lysosomal enzymes over a long period of time, they may produce soluble products, including those fluorophores extracted from normal age pigment. To determine whether this is the case, young $+A$ animals could be given intraocular iron injections, and after a period of perhaps 20 weeks or more, the RPE could be analyzed to determine whether the amounts of extractable fluorophores were enhanced. Such experiments may enable us to clarify further the precise pathway by which vitamin A is involved in RPE lipofuscin fluorophore formation.

Not only was the presence of vitamin A necessary for generation of autofluorescent, lipofuscin-like inclusions in the RPE, but the presence of visual cycle retinoids also potentiated iron-induced damage to the retina. There were many regions of the retinas in the $+A$ and $-A+A$ groups in which the photoreceptor cells and the RPE were completely eliminated within 1 week after the iron treatment. In the $-A$ animals, however, the effect of iron treatment was limited to a shortening of the photoreceptor outer segments. This effect of vitamin A on iron-induced cell loss in the retina can be explained as follows. When vitamin A was present in the retina, iron treatment induced a massive accumulation of outer segment-derived inclusions in the RPE. This accumulation resulted in either severe impairment of RPE cell function or RPE cell death. The adjacent photoreceptor cells then died as a consequence of the elimination of RPE cell support. In the absence of vitamin A, direct damage occurred to the photoreceptor outer segments, but no accumulation of inclusions in the RPE occurred. Thus, RPE function was preserved, and the RPE was capable of providing the support functions necessary for the survival of the moderately damaged photoreceptor cells in the $-A$ rats. This interpretation is consistent with the proposal that lipofuscin accumulation in the RPE is involved in the photoreceptor cell death that occurs during normal aging.[39]

*Key Words*

autoxidation, photoreceptors, retinoids, fluorophores, retinal pigment epithelium, lipofuscin

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