

Elevated Retinal Zeaxanthin and Prevention of Light-Induced Photoreceptor Cell Death in Quail

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PURPOSE. Inferential evidence indicates that macular pigments (lutein and zeaxanthin) protect photoreceptors and/or retard age-related macular degeneration. These experiments tested the hypothesis that retinal zeaxanthin prevents light-induced photoreceptor cell death.

METHODS. Retinal damage was assessed in quail fed a carotenoid-deficient (C-) diet for 6 months. Groups of 16 birds (8 male, 8 female) were fed a C- diet supplemented with 35 mg 3R,3'R-zeaxanthin for 1, 3, or 7 days; one group was continued on C- diets. Half of each group was exposed to intermittent 3200-lux white light (10 1-hour intervals separated by 2 hours in dark). After 14 additional hours in the dark, one retina of each quail was collected for HPLC analysis, and the contralateral retina was embedded in paraffin for counts of apoptotic nuclei.

RESULTS. After 7 days' supplementation, concentrations of zeaxanthin in serum, liver, and fat had increased by factors of 50.8, 43.2, and 6.5, respectively (all $P < 0.001$). In contrast, retinal zeaxanthin fluctuated significantly upward on day 3, but there was no net change on day 7. The number of apoptotic rods and cones in light-damaged eyes correlated significantly and inversely with zeaxanthin concentration in the contralateral retina ($r = -0.61$; $P < 0.0001$ and $r = -0.54$; $P < 0.002$), but not with serum zeaxanthin. Similar correlations were observed with retinal lutein, which correlated strongly with retinal zeaxanthin ($r = 0.95$; $P < 0.0001$).

CONCLUSIONS. Retinal zeaxanthin dose dependently reduced light-induced photoreceptor apoptosis; elevated serum levels did not. These data provide the first experimental evidence that xanthophyll carotenoids protect photoreceptors in vivo. (*Invest Ophthalmol Vis Sci.* 2002;43:3538-3549)

Although it has been known for more than 50 years that the dark yellow color of the primate fovea and surrounding macula is due to the accumulation of the dietary xanthophyll carotenoids—zeaxanthin, lutein, and trace amounts of α -cryptoxanthin¹—little is known about the functional significance of these carotenoids. Five lines of indirect evidence suggest macular pigments protect photoreceptors from photo-oxidative damage and/or events associated with progression of age-related macular degeneration (AMD):

1. Epidemiologic and case-control studies suggest risk for age-related maculopathy,² and advanced AMD may³⁻⁵ (or may not⁶) be inversely related to xanthophyll concentrations in the diet or plasma (and presumably in macular pigments). Other factors associated with increased risk for AMD, including smoking, female gender, and lighter eye color,⁷⁻¹⁰ are also associated with reduced serum carotenoid levels and lower macular pigment density in normal human subjects.¹¹⁻¹³
2. Donor eyes with AMD had less macular pigment than control eyes¹⁴ and eyes at high risk for AMD had less macular pigment than selected control eyes.¹⁵
3. The spared central region in advanced bull's-eye geographic atrophy matches the region of greatest concentration of macular pigments.¹⁶ Early photoreceptor atrophy in AMD appears later and progresses more slowly in the fovea.^{17,18} However, foveal sparing is also the expected consequence of selective loss of rods in AMD.¹⁹
4. Greater age-related loss of sensitivity to blue light in retinal regions with lower macular pigment density and in older subjects with lower macular pigment density has been interpreted as evidence of protection.^{20,21} However, loss of sensitivity could also be due to decreased lens transmission of blue light in persons with low dietary carotenoids,²²⁻²⁵ or to local gain changes as a result of differential filtering of light.²⁶
5. Macular pigment absorbs blue light, which is associated both with increased risk for exudative AMD²⁷ and with production of reactive oxygen species by components of lipofuscin.²⁸⁻³⁰ Foveal sparing may derive from the combination of maximum macular pigment density,³¹ reduced levels of lipofuscin,^{16,32,33} and consequent sharp reduction in production of reactive oxygen species in the RPE. Accumulation of oxidatively damaged proteins, especially calcium-regulatory proteins, has been implicated in age-related degenerative changes (loss of cell function, impaired ability to withstand and repair physiological stress, and senescence).³⁴⁻³⁶

Collectively, these observations have led to the compelling hypothesis that macular pigment prevents light damage to the photoreceptors and/or delays events leading to photoreceptor atrophy.^{37,38} However, to date, case-control and clinical studies have linked risk for AMD only with dietary and plasma lutein and zeaxanthin levels.

To compare the possible protective action of retinal and plasma xanthophylls, we examined light-induced photorecep-

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TABLE 1. Composition of Experimental Diets

Diet	Zeaxanthin*		Lutein*		Total Xanthophylls†		Total Tocopherols‡	
	µg/kg	% T	µg/kg	% T	µg/kg	% T	µg/kg	% T
C-	0.2	20	0.34	11.9	0.54	13.5	61.6	115
Z+	35.7	3570	0.24	8.4	36.8	902	65.5	122
T	1.0	100	2.84	100	4.0	100	53.5	100

Data are expressed as micrograms per kilogram food and percentage of amount in the commercial turkey diet.

* *Cis*-isomers included.

† Sum of zeaxanthin, lutein, α -cryptoxanthin and β -cryptoxanthin.

‡ Sum of α -, δ -, and γ -tocopherols.

tor death in quail whose dietary carotenoids had been experimentally manipulated. A quail model for light damage was developed because the quail retina selectively accumulates zeaxanthin and lutein,³⁹⁻⁴¹ whereas the more thoroughly characterized rodent retinas do not accumulate carotenoids. The structure and function of the highly colored oil droplets in avian cones have been described.⁴²⁻⁴⁸ We have reported that quail fed for 6 months on zeaxanthin-supplemented diets had approximately 5-fold elevations in retinal zeaxanthin and more than 45-fold increases in zeaxanthin concentration in serum, liver, and fat.⁴¹ A further advantage of a quail model is that they age rapidly and their retina exhibits age-related loss of photoreceptors,⁴⁹ decline in contrast sensitivity,⁵⁰ and accumulation of RPE lipofuscin.⁵¹ Moreover, Bruch's membrane deposits found in quail of advanced age have ultrastructural features of soft drusen in humans.⁵² The effects of dietary manipulation and gender introduced a nearly 300-fold variation in serum zeaxanthin and approximately a 3-fold variation in retinal zeaxanthin. We report here that the number of apoptotic photoreceptors in light-damaged retinas was significantly and inversely correlated with the concentration of xanthophyll carotenoids (zeaxanthin and lutein) in retina, but not in serum.

METHODS

All animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and under protocols approved by the Animal Care and Use Committees of the Schepens Eye Research Institute and the University of British Columbia.

Nutritional and Light History of the Birds

All birds used in this study were first-generation chicks from selected adult Japanese quail (*Coturnix japonica*) raised in the Department of Animal Science in Vancouver. Parental stock in cyclic light (12 hours on, 12 hours off) were fed a carotenoid-deficient (C-) diet custom synthesized (Basal Diet 5636C-1; Purina Mills, Richmond, IN) to match the commercial turkey (T) diet in calorie and vitamin content, but having only 13.5% of the carotenoid content (Table 1). The xanthophyll levels in the yolks of eggs laid by these birds were scored daily according to a yolk color fan (Roche Diagnostics, Indianapolis, IN).⁵³ When the fan score was 1 for 4 consecutive days (indicating carotenoid depletion) eggs were collected and incubated. All eggs hatched within the same 48-hour period, and the newly hatched birds were placed on a starter mash with a matching carotenoid profile (basal starter mash: 5636C-2; Purina Mills). To encourage eating, the newly hatched birds were kept for 1 week in brooders, with environmental light inside ranging from 85 to 340 lux. From 7 days to 42 days, they were maintained on a C- grower diet in 5 to 20 lux cyclic light (12 hours on, 12 hours off). After 42 days of age, the light in the cages was maintained at 5 lux.

At 23 weeks, three groups of 16 birds raised on C- diets were given a zeaxanthin-supplemented (Z+) diet for 7 days, 3 days, or 1 day immediately before initiation of the light-damage experiments. The Z+ diet was prepared by supplementing the C- diet with 3*R*,3*R'*-zeaxanthin (35 mg/kg C- diet, biosynthesized by *Sphingobacterium multivorum* (basonym *Flavobacterium multivorum*) at Applied Food Biotechnology, Inc. (O'Fallon, MO), added as an emulsion in olive oil containing α -tocopherol as an antioxidant. Another group of birds was given 50 mg β -carotene/kg food for the 7 days preceding light damage.

Light Damage

A protocol for light damage of quail retinas was established before the full experiments at 6 months. At 3 months, the protocol was tested in 10 birds fed a C- diet and 3 fed the commercial turkey diet. They were given eye drops of atropine sulfate and phenylephrine hydrochloride (Atropine Care and AK-Dilate; Akorn, Inc., Abita Springs, LA) to dilate their pupils. (These drugs were not the optimum choice for birds and were only partially effective, in that some pupil contraction was observed upon exposure to light.) Birds were placed in the dark for 24 hours, given eye drops again, and placed immediately into a light-exposure chamber (45 cm high, 60 cm deep, and 43 cm wide). Daylight fluorescent tubes (20 W, 36 in., GE Daylight tubes; General Electric, Fairfield, CT) mounted on all sides of the chamber produced 3200 lux diffuse cool white light at the perimeter of the cage (where the quail stood facing the light for most of the exposure period). The spectrum of the exposure light is provided in Figure 1. Quail were exposed to intermittent light⁵⁴ (1 hour on, 2 hours off) for a total light period of 10 hours. The air temperature inside the cage increased by 2°C to 3°C during light exposure. The birds were given small quantities of C- food throughout the day and were provided water ad libitum from transparent glass gravity feeders. After light exposure, the birds were placed in the dark with access to food and water for 14, 24, and

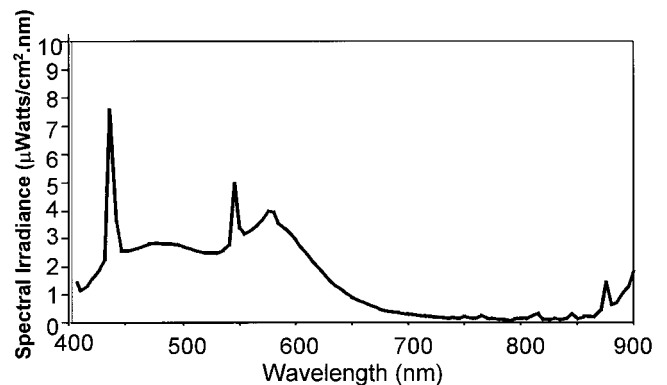


FIGURE 1. Spectral irradiance measured at the periphery of the light exposure chamber.

48 hours before death by decapitation. The eyes were enucleated and immediately dissected and processed for light microscopy (described later).

At 6 months, 4 males and 4 females from each of the 4 dietary groups (C-, and zeaxanthin supplemented for 1, 3, and 7 days (Z+1D, Z+3D, and Z+7D, respectively) were exposed to intermittent light as described earlier and killed after 14 additional hours in the dark. An equal number of birds from each group remained in dim light and served as unexposed controls.

After the light exposure, the birds were placed in the dark with access to food and water. After 14 additional hours in the dark, birds were killed by decapitation. The eyes were immediately enucleated, and samples of serum, right lobe of the liver, and fat from the breast collected and frozen in liquid nitrogen. All samples were stored at -70°C before analysis. The average time from sacrifice to freezing or fixation was less than four minutes per eye. All tissues were collected within one 5-day period to eliminate any differences due to age.

HPLC Analysis

Rapidly enucleated eyes were placed on ice within seconds after death, and dissection proceeded immediately. The anterior segment was removed, and the thick vitreous cut circumferentially with iris scissors close to the surface of the retina and removed. The retina was peeled from the eyecup, areas of major RPE contamination were removed, and the retina was frozen in liquid nitrogen. Dissection was completed before the next animal was killed.

Carotenoid and tocopherol content of retinas and sera of each animal were determined by high performance liquid chromatography by standard methods at Craft Technologies, Inc. (Wilson, NC), as previously described in detail.^{41,55} (Retinoids are not reported, because exposure to light during dissection resulted in variable loss.) Briefly, the samples were homogenized in phosphate-buffered saline, and precipitated with ethanol. After a portion was removed for protein analysis, they were ultrasonicated during 15 minutes of saponification in KOH containing pyrogallol, extracted with 90% hexane-10% ethyl acetate and washed with water, and the organic extract was evaporated under nitrogen. The residue was dissolved in ethyl acetate, diluted with mobile phase, and vortexed and sonicated before injection onto the separation column (Sphersorb ODS2; Phase Separations, Norwalk, CT). The separation was performed isocratically using a mobile phase of 80% acetonitrile, 15% dioxane, and 5% methanol/isopropyl alcohol containing 150 mM ammonium acetate and 0.1% triethylamine. A programmable UV/visible detector measured carotenoids at 450 nm, and a programmable fluorescence detector measured retinol at 326 nm excitation and 460 nm emission. Tocol and tocopherols were measured at 296 nm excitation and 340 nm emission.

Microscopic Analysis

The fresh eye was bisected to remove the anterior segment, and fixed in ice-cold, freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer for 12 hours and rinsed in buffered saline. The eye cups were cut parallel to the horizontal meridian across the area centralis, the superior tip of the pecten, and the area dorsalis.⁵⁶ A second parallel cut was made superior to the first, and this specimen was embedded in paraffin, and sectioned (8 μm thick) in a plane parallel to the horizontal meridian, along the first cut. The entire region from nasal periphery to temporal periphery in every bird was examined by light microscopy. Adjacent sections were stained with contrast blue (a nuclear stain; Kirkegarrd & Perry, Gaithersburg, MD) or immunostained with QH1, an antibody developed by Dieterlen-Lievre et al.⁵⁷ and obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa (Iowa City, IA) under contract NO1-HD-2-3144 from the National Institute of Child Health and Disease. We and others have demonstrated that photoreceptor cell death induces activation and migration of microglia from the inner plexiform layer into the photoreceptor layer in quail⁴⁰ and rats.⁵⁸ QH1

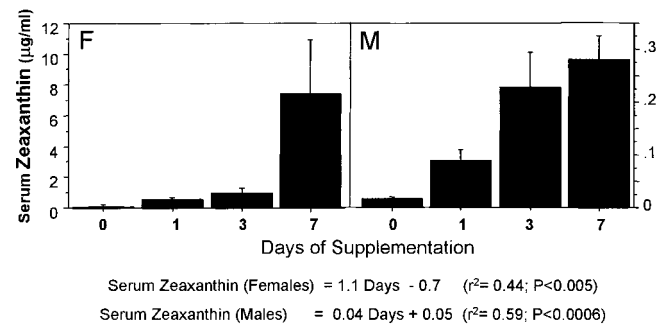


FIGURE 2. The mean (\pm SE) serum zeaxanthin concentration in 6-month-old female (F, left) and male (M, right) quail given supplemental zeaxanthin for 1, 3, or 7 days. Note difference in scales on ordinates. Regressions relate serum zeaxanthin to days of supplementation.

recognizes both microglia and endothelial cells in brain,⁵⁹ but only microglia in the avascular quail retina.⁶⁰

Photoreceptor cell death was examined by detecting nuclear condensation (i.e., pyknotic nuclei) and DNA fragmentation in 6- μm paraffin-embedded sections examined by light microscope (Axiovert; Zeiss, Thornwood, NY). DNA strand breaks were labeled with fluorescein by the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) reaction⁶¹ and detected with a fluorescein filter set. Damage was assayed by counting pyknotic nuclei in the central 1400- μm segment extending between the nasal and temporal peripheries. The highly organized outer nuclear layer permitted identification of rods nuclei distributed like beads on a string along the inner surface of the outer nuclear layer and the deeper layers of cone nuclei. QH1-positive microglia were counted only if cell bodies or thick processes were in the outer nuclear layer or among the inner or outer segments.

Statistical Procedures

A commercial statistics package (Statview; Abacus Concepts, Berkeley, CA) was used for analysis of data. Correlation analyses were performed to determine relationships between the death of rod and cone photoreceptors and the retinal or serum concentrations of zeaxanthin, lutein, or α -tocopherol. Effects of diet on specific variables were analyzed by ANOVA, using conservative Bonferroni/Dunn post hoc analyses.

RESULTS

Response to Dietary Zeaxanthin Supplements

As a result of the dietary manipulation and gender differences, the 6-month-old birds had mean serum zeaxanthin concentrations that were distributed over a 300-fold range, with maximum difference between basal males and zeaxanthin-supplemented females. Mean retinal concentrations varied by only threefold. Serum zeaxanthin concentrations in serum increased significantly with duration of supplementation and were higher in females than in males ($P < 0.0006$; Fig. 2). After 7 days of supplementation, the mean serum concentrations in supplemented females and males were 54.8 and 15 times higher, respectively, than in those maintained on the C- diet ($P < 0.01$ and $P < 0.0005$, respectively; Table 2). Zeaxanthin concentrations in females increased 43-fold in liver ($P < 0.01$) and 6.5-fold in fat ($P < 0.001$; Table 2). Males had smaller increases in zeaxanthin: 7.2-fold in liver ($P < 0.0001$) and 1.5-fold in fat (not significant; Table 2).

Zeaxanthin supplementation produced a rapid enrichment in the zeaxanthin fraction of total serum xanthophylls (Fig. 3A) and a slow increase in the zeaxanthin fraction of retinal xanthophylls (Fig. 3B). The lutein-zeaxanthin (L-Z) ratio in the

TABLE 2. Multiples of Change in Tissue Concentrations of Lutein and Zeaxanthin after 7-Day Dietary Supplementation with Zeaxanthin

Tissue	Multiples of Change During Supplementation			P-Values From ANOVA	
	Xanthophyll	Females	Males	Gender	Light Exp.
Serum	Zeaxanthin	54.8 ± 17.0†	15.1 ± 1.6¶	0.01	NS
	Lutein	1.76 ± 0.32*	0.60 ± 0.06‡	0.004	NS
Liver	Zeaxanthin	43.2 ± 13.0†	7.2 ± 1.1¶	0.01	T
	Lutein	1.86 ± 0.36*	0.27 ± 0.04§	0.004	NS
Fat	Zeaxanthin	6.5 ± 1.2§	1.5 ± 0.4	0.0006	T
	Lutein	1.29 ± 0.12	1.04 ± 0.11	NS	NS

The table summarizes changes in zeaxanthin and lutein concentrations induced by feeding C- birds zeaxanthin supplemented diet for 7 days. $n = 32$, 6-month-old quail raised on C- diet; 16 given supplementation; half of each group exposed to damaging light (Light Exp.). Symbols within the table indicate Bonferroni/Dunn comparisons with the unsupplemented birds: T, Trend (P between 0.1 and 0.2); NS, not significant.

* $P < 0.05$.

† $P < 0.01$.

‡ $P < 0.005$.

§ $P < 0.001$.

¶ $P < 0.0005$.

retina correlated inversely and significantly with the total zeaxanthin consumed ($r = -0.73$; $P < 0.0001$; Fig. 3C).

Despite their deficient diets, the retinas of C- birds had bright red, yellow, orange, and pale green oil droplets (Fig. 4). With supplementation, the concentrations of zeaxanthin and lutein in the retina appeared to fluctuate (Fig. 5). On day 3, the mean concentrations were significantly higher than on day 1 ($P < 0.005$ and $P < 0.0001$ for zeaxanthin and lutein, respectively), and there was substantial overlap in the range of values observed. Levels of retinal zeaxanthin on day 7 were not significantly different from those on day 1. Moreover, lutein and zeaxanthin concentrations were the same in birds given 7 days' supplemental β -carotene as in those supplemented with zeaxanthin (Fig. 5). These fluctuations occurred in animals that were and were not exposed to light. The steady enrichment in the retinal zeaxanthin content (Fig. 3) confirms that the fluctuations did not reflect a failure to deliver zeaxanthin to the retina. Females on all diets had higher retinal zeaxanthin concentrations than males ($P < 0.0001$; Fig. 5; Analysis of variance with Bonferroni-Dunn post hoc analysis).

The effect of zeaxanthin supplementation on lutein was strongly dependent on gender (Table 2). After 7 days, lutein concentrations had risen in serum ($P < 0.05$) and liver ($P < 0.05$) of females and declined in serum ($P < 0.005$) and liver ($P < 0.0001$) of males. Lutein concentrations in fat were unchanged.

Serum zeaxanthin correlated positively and significantly with zeaxanthin concentrations in liver, fat, and retina (Table 3). Zeaxanthin in retina correlated positively with zeaxanthin in liver and fat. Serum lutein correlated positively and significantly with lutein in retina, fat, and liver (Table 3). Lutein in retina was not significantly related to liver lutein but correlated negatively with lutein in fat in females. The concentrations of zeaxanthin and lutein correlated strongly in every tissue of Z+7D birds (Table 3). This correlation reflects the constant relationship between zeaxanthin and lutein in the diet of these birds.

Light-Induced Photoreceptor Death

The hypothesis that photoreceptor apoptosis would be induced by exposure to intermittent light was tested in 3-month-old birds. The total number of apoptotic rods and cones were high 14 hours after light exposure, and declined rapidly, with few detected at 48 hours (Fig. 6). The 14-hour time point was

selected for subsequent light-exposure experiments in 6-month-old retinas.

All light-exposed retinas exhibited numerous, deeply stained, condensed, pyknotic nuclei along the inner aspect of the outer nuclear layer (compare Figs. 7A and 7B). Apoptosis was confirmed by a positive TUNEL reaction (Fig. 7C). Large activated microglial cells were frequently found among the dying photoreceptors in light-exposed retinas (Fig. 7B), but not in control retinas. The number of apoptotic photoreceptors and activated microglia increased from central to peripheral retina.

The number of apoptotic rods and/or cones in 6-month-old retinas correlated inversely and significantly with the concentration of retinal zeaxanthin ($r = -0.62$; $P < 0.0001$; Fig. 8A; Table 4). The r^2 of 0.37 indicates that approximately 37% of the variation in the number of apoptotic photoreceptors was related to retinal xanthophylls. Analysis of variance revealed that males were more vulnerable to light damage than females ($P < 0.0002$; Bonferroni/Dunn post hoc analysis). As has been described, males also had lower concentrations of retinal zeaxanthin ($P < 0.0001$).

Dying rod photoreceptors outnumbered cone photoreceptors by more than 3:1 in every retina and by more than 4:1 in 28% of retinas. The number of dying rods strongly correlated with the number of dying cones ($r = 0.86$; $P < 0.0001$), and every significant correlation with rods was therefore also a significant correlation with cones (Table 4).

The number of activated microglia that invaded the damaged photoreceptor layer also correlated negatively with retinal zeaxanthin concentration (Fig. 8B; Table 4). Photoreceptor apoptosis and microglial invasion both also correlated significantly and negatively with the concentration of retinal lutein and with total retinal xanthophylls (Table 4) but not with α -tocopherol concentrations (Table 4).

Photoreceptor apoptosis was not significantly related to serum concentrations of zeaxanthin or α -tocopherol (Fig. 8C; Table 4). A trend toward a negative correlation with serum lutein was found to derive from gender differences. Because zeaxanthin and lutein concentrations in the retina correlated strongly (Fig. 9) and because the sample size was limited, it was impossible to separate statistically how much of the relationship with apoptotic photoreceptors was due to zeaxanthin or lutein or to assess which variable had the stronger correlation.

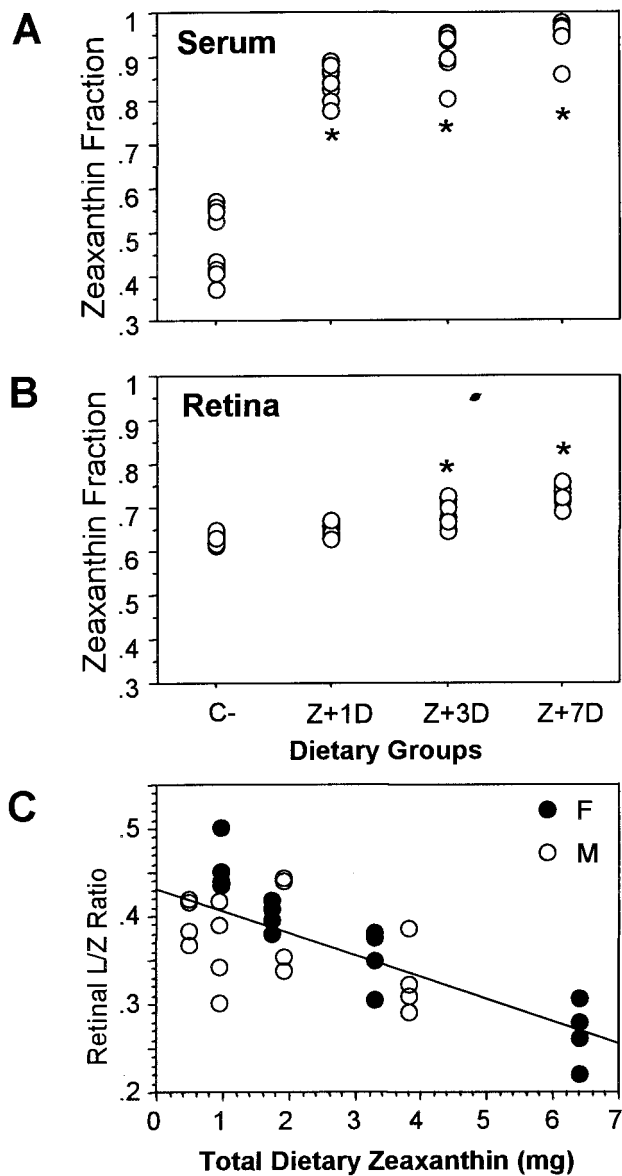


FIGURE 3. The ratio of zeaxanthin to total xanthophylls in serum (A) and in retina (B) increased after 1, 3, or 7 days of zeaxanthin supplementation in 6-month-old quail ($n = 8$ in each dietary group). (C) Inverse correlation between the total zeaxanthin consumed and the L/Z ratio (i.e., lutein concentration/zeaxanthin concentration) in the retina ($n = 8$ in each dietary group). *Significantly different from C- birds ($P < 0.0001$).

In effect, any significant association between zeaxanthin and any other variable would almost certainly also be true of lutein.

Retinal zeaxanthin was nonsignificantly reduced in birds fed supplemental β -carotene for 7 days (Fig. 3). A multiple regression analysis indicated that apoptosis was reasonably predicted from retinal zeaxanthin concentration; neither liver β -carotene nor liver retinol concentration (both significantly elevated in birds given supplemental β -carotene) was a significant variable in the regression model, indicating that supplementation with β -carotene did not influence photoreceptor cell death.

DISCUSSION

These data provide the first experimental evidence for the long-held hypothesis that photoreceptors are protected by

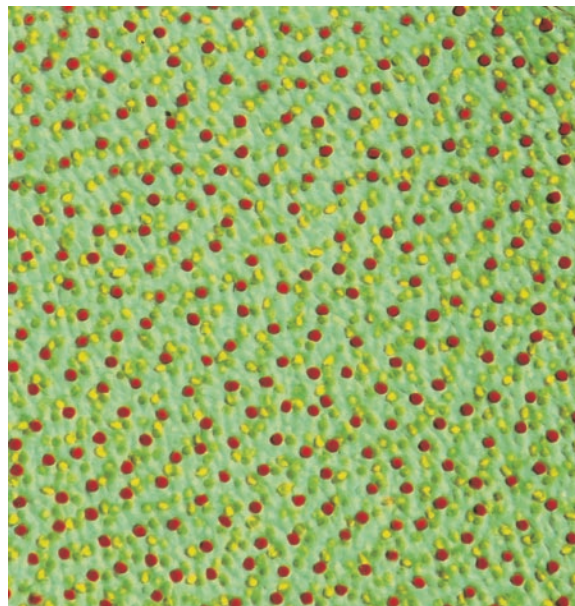


FIGURE 4. Prominent red and yellow oil droplets in flatmounted retina of female quail raised on C- diet demonstrate high content of zeaxanthin and lutein. Pale green droplets located in a deeper image plane were occasionally visible. Magnification, $\times 350$.

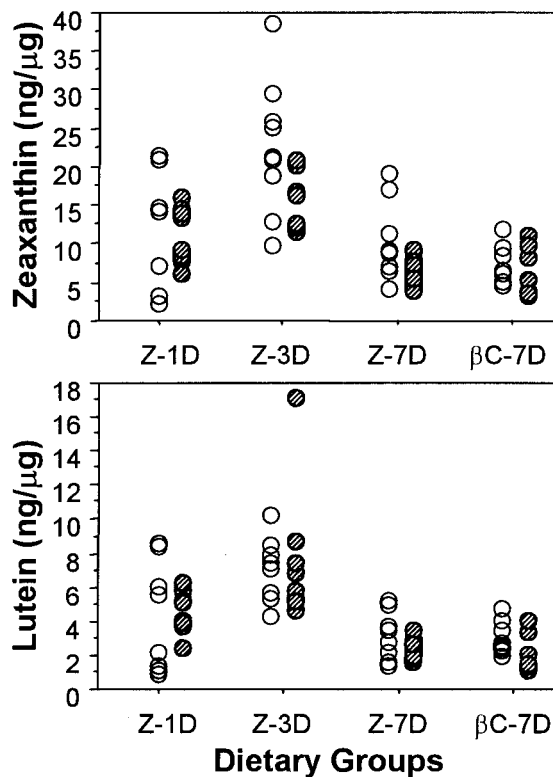


FIGURE 5. Parallel fluctuations in concentrations of retinal zeaxanthin and lutein in quail fed a C- diet for 6 months and then a diet supplemented with zeaxanthin (Z) for 1, 3, or 7 days (D) and with β -carotene (β C) for 7 days. Concentrations of both zeaxanthin and lutein increased on day 3 ($P < 0.005$ and $P < 0.0001$) and returned to day 1 levels by day 7. Quail that were (open circles) and were not (batched circles) exposed to damaging light had equivalent levels of zeaxanthin ($P > 0.7$) and lutein ($P > 0.7$).

TABLE 3. Correlations among Carotenoids in Different Tissues of Quail Fed Supplemental Zeaxanthin for 7 Days

Carotenoid	Tissue	Tissue	<i>r</i>	<i>P</i>	
Zeaxanthin	Serum	Liver	0.97	<0.0001	*
	Serum	Fat	0.80	0.0001	*
	Serum	Retina	0.80	0.0001	
	Liver	Fat	0.88	<0.0001	*
	Liver	Retina	0.72	0.001	
	Retina	Fat	0.47	0.08	Both genders
	Retina	Fat	0.63	0.10	Females
Lutein	Retina	Fat	-0.08	NS	Males
	Serum	Liver	0.72	0.002	*
	Serum	Fat	0.32	0.05	*
	Serum	Retina	0.50	0.06	
	Liver	Fat	0.57	0.02	*
	Retina	Liver	0.13	NS	
	Retina	Fat	-0.3	NS	Both genders
Zeaxanthin and lutein	Retina	Fat	-0.85	0.01	Females
	Retina	Fat	-0.33	NS	Males
	Serum		0.94	<0.0001	*
	Liver		0.73	0.001	*
	Fat		0.98	0.003	*
	Retina		0.94	<0.0001	*

n = 16.

* These relationships were also significant when birds from different dietary groups were analyzed together.

macular pigment—that is, by xanthophyll carotenoids in the retina, but not by those in serum. Three lines of evidence support this conclusion. First and foremost, the number of apoptotic photoreceptors in light-exposed retinas correlated inversely with the retinal concentration of zeaxanthin. In these experiments, and in those of Fite et al.,⁵¹ female quail sustained less light damage than males, and females had significantly higher retinal zeaxanthin. Finally, retinal zeaxanthin concentration correlated negatively with the number of activated microglia that migrated from the inner and outer plexiform layers into the photoreceptor layer (in response to photoreceptor damage^{40,58,62}).

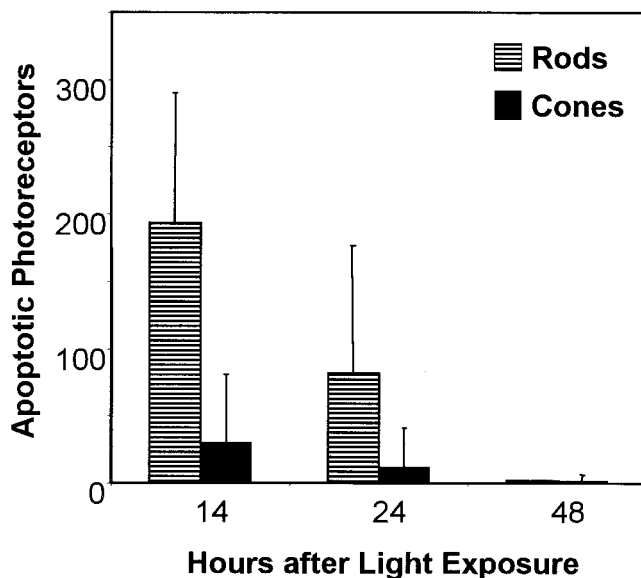


FIGURE 6. Number (mean ± SD) of apoptotic rods and cones in 3-month-old pigmented quail that were fed a C- diet for 3 months, exposed to intermittent 3200-lux white light for a total of 10 hours, and examined 14, 24, and 48 hours after the last interval of exposure to light.

Because the proportions of dietary lutein and zeaxanthin were constant, lutein and zeaxanthin correlated highly in all quail tissues. Thus, any correlation with zeaxanthin was also true with lutein, making it impossible to distinguish whether protection was primarily due to zeaxanthin, lutein, or both. That zeaxanthin was preferentially taken up by the quail retina⁴¹ and that it accounted for the majority of the retinal xanthophylls strongly suggests that the observed protection against retinal light damage can be attributed to the zeaxanthin present in the retina. This possibility is further supported by evidence that zeaxanthin is more effective than lutein in preventing UV damage to lipid membranes.⁶³ It is worth noting that zeaxanthin is preferentially accumulated in human and monkey foveas⁶⁴⁻⁶⁶ and possibly in retinas of other species without a macula.⁶⁷

The presence in quail retinas of several cone types with different colored oil droplets⁴⁵ is consistent with the concept that both xanthophylls protect the photoreceptors. If replacement of lutein by zeaxanthin was sufficient to reduce photoreceptor damage, the number of apoptotic photoreceptors should decline with decreasing L-Z ratio in the retina. In fact, photoreceptor apoptosis was not significantly related to retinal L-Z ratio ($r = -0.09$; $P > 0.57$). These considerations imply that lutein and zeaxanthin may protect different classes of cones in the avian retina. By extension, rod photoreceptors—and cones without carotenoids in their oil droplets—may be more vulnerable to light damage. This may explain the selective loss of rods in light-damaged quail and suggests that there may have been selective loss of the cones with colorless (or carotenoid-deficient) oil droplets. In this context, it is interesting that Hodos et al.⁶⁸ have reported that double cones are selectively lost during aging in pigeons, for the smaller member of the double cone has a colorless oil droplet.⁶⁹

Possible Mechanisms for Carotenoid Protection of Photoreceptors

The dose-dependent protection of rod and cone photoreceptors by xanthophyll carotenoids may be related to their absorption of short-wavelength (blue) light, antioxidant capacity, and/or induction of antiapoptotic genes.

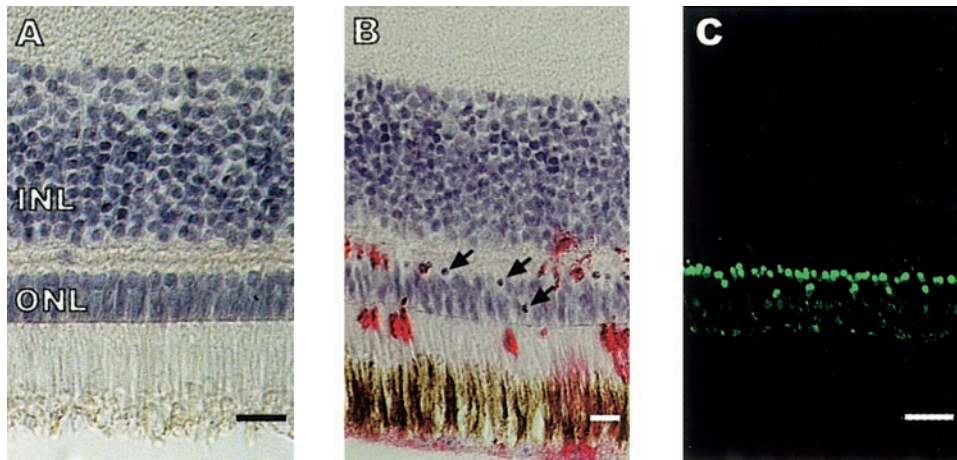


FIGURE 7. Photomicrographs of inner (INL) and outer (ONL) nuclear layers and outer segments of retinas of C- 6-month-old quail. Unexposed retina (A) shows regular array of rod photoreceptor nuclei along the inner aspect of the ONL. (B) Fourteen hours after light exposure, nuclear condensation identified dying rods (upper arrows) and cones (lower arrow). Red-stained (QH1 positive) microglial cells are seen among damaged photoreceptors. (C) Positive TUNEL reaction (green fluorescence) in apoptotic rods and a few cones. Scale bars, 50 μm .

Absorption of Short-Wavelength Light. Rhodopsin, the major photosensitizer in rod photoreceptors,^{70,71} has peak absorption in green light (not absorbed by carotenoids). However, Grimm et al.⁷² clearly demonstrated that light-mediated damage to rod photoreceptors in mice is exacerbated by blue-light-mediated photoregeneration of rhodopsin. The spectral characteristics of this photoregeneration and its relevance to normal environmental conditions remain to be determined. The observed carotenoid protection of rod photoreceptors

may relate to this process. Protection may be more efficient if the xanthophyll carotenoids are found in rod outer segments in quail, as observed in human.⁷³

Inverse spatial and quantitative relations between macular pigment concentration and sensitivity to blue light in aging human retinas^{20,21} led to the hypothesis that xanthophylls protect the short-wavelength cones. The quail data provide the first experimental evidence that retinal zeaxanthin and lutein dose dependently prevent light-induced cone apoptosis. Carot-

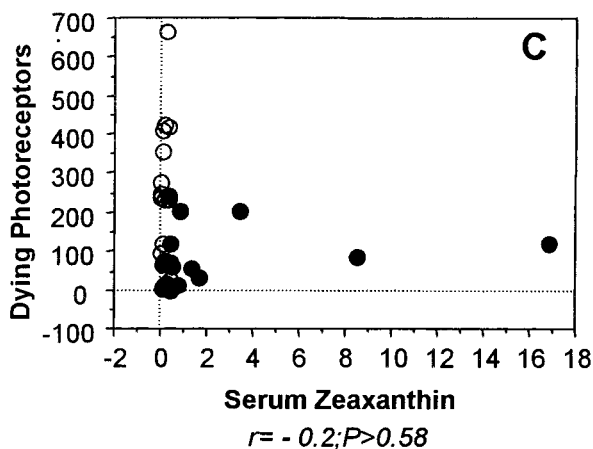
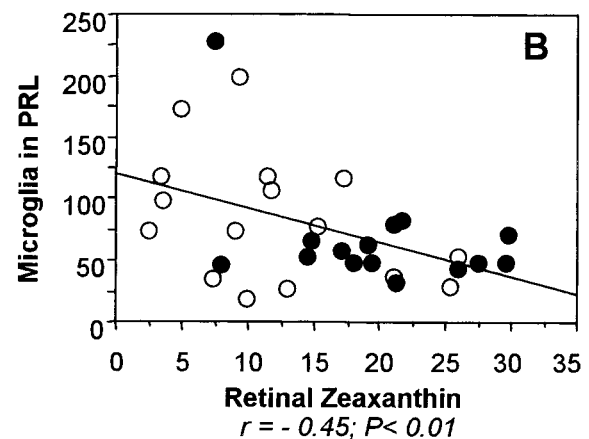
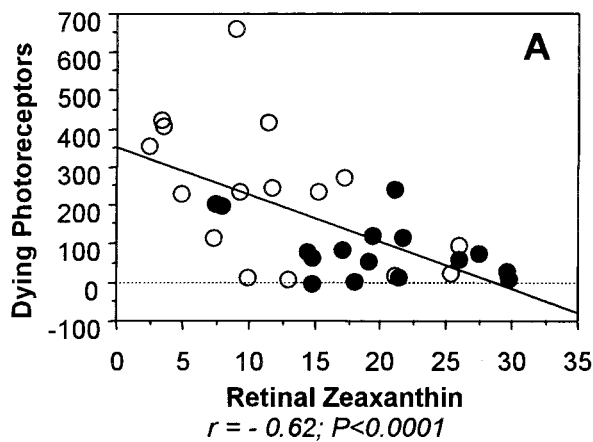


FIGURE 8. Both the number of apoptotic photoreceptors (A) and the number of activated microglia that invaded the photoreceptor layer (B) correlated negatively with the concentration of zeaxanthin (nanograms per milligram protein) in the contralateral retina of 32 light-damaged, 6-month-old quail (eight birds from each dietary group: C-, Z+1D, Z+3D, and Z+7D). Neither photoreceptor death (C) nor microglial invasion (not shown) correlated with serum zeaxanthin. Open circles: males; filled circles: females.

TABLE 4. Correlations between Photoreceptor Apoptosis and Components of Retina or Serum

	Zeaxanthin		Lutein		Total Xanthophylls		α -Tocopherol	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Retina								
Rods	-0.62	0.0001	-0.62	0.0001	-0.63	0.0001	-0.13	NS
Cones	-0.54	0.002	-0.50	0.004	-0.53	0.002	-0.02	NS
Total PR	-0.62	0.0001	-0.61	0.0002	-0.61	0.0001	-0.1	NS
Microglia	-0.45	0.01	-0.46	0.01	-0.46	0.01	-0.1	NS
Serum								
Rods	-0.1	NS	-0.3	T	-0.1	NS	-0.3	T
Cones	-0.1	NS	-0.3	T	-0.1	NS	-0.3	T
Total PR	-0.1	NS	-0.3	T	-0.1	NS	-0.3	T
Microglia	-0.05	NS	-0.1	NS	-0.05	NS	-0.2	NS

Correlations between number of apoptotic photoreceptors (PR) and concentration of xanthophyll carotenoids and α -tocopherol in contralateral eyes or in serum of 32 quail raised for 6 months on C-diets and supplemented or not with zeaxanthin during the 1, 3, or 7 days before light exposure. Light exposure had no significant effect on the retinal concentration of zeaxanthin ($P > 0.6$), lutein ($P > 0.6$), or α -tocopherol ($P > 0.35$). NS, not Significant; T, trend ($0.08 < P < 0.1$), lost when males and females analyzed separately. $n = 32$.

enoid accumulation in oil droplets would dose dependently reduce blue light in outer segments of blue cones. If blue light also regenerates bleached cone opsins,⁷² carotenoids in oil droplets would offer significant protection from light damage.

It is unlikely that the protection offered by xanthophylls in quails was mediated by reduced short-wavelength damage to

mitochondrial cytochrome oxidase,⁷⁴ because the mitochondria are situated vitread to the cone oil droplets.^{43,46} Similarly, paucity of RPE lipofuscin in the quail suggests that carotenoid protection was not related to reduced production of damaging species of oxygen by lipofuscin and/or one of its components.^{28,30}

Modulation of Apoptosis. An early increase in intracellular calcium during photoreceptor death induces production of superoxide and activation of neuronal nitric oxide⁷⁵ and probably mitochondrial nitric oxide synthase.⁷⁶ The resultant peroxynitrite causes nitration and/or release of mitochondrial cytochrome *c*, a potent proapoptotic signal.⁷⁶⁻⁷⁸ By reacting with peroxynitrite, zeaxanthin and lutein could interrupt this apoptotic pathway.^{79,80} The possibility that xanthophylls influence photoreceptor expression of genes regulating apoptosis cannot be excluded. Zeaxanthin, lutein, and a number of carotenoids induce expression of a gap junction protein in liver epithelial cells, and lutein influences the balance in Bcl-xL-Bax proteins in normal mammary epithelial cells.^{81,82}

Modulating Oxidation and/or Microglial Activity. Zeaxanthin may reduce production of radicals by microglia among the photoreceptors, as has been shown in macrophages.⁸³ Both zeaxanthin and lutein inhibit lipoperoxidation in vitro and in vivo.^{84,85} Zeaxanthin prevents oxidative damage to tissues by quenching peroxy radicals,⁸⁵ and is better than lutein in preventing UV-induced oxidation of lipid membranes during prolonged exposure.⁸⁶ However, it is important to note that peroxy radicals formed in outer segments could only be quenched by zeaxanthin located in outer segments, not in the more distant oil droplets in quail retinas or in plexiform layers in primate retinas.

Response of Retinal Zeaxanthin to Short-Term Supplementation

As previously reported in human subjects and primates,^{66,87-89} zeaxanthin in quail serum increased rapidly in response to dietary supplementation, whereas zeaxanthin in retina fluctuated but had not increased at the end of 7 days of supplementation. The discordant responses of serum and retina to dietary supplementation are not unprecedented. Serum lutein increased rapidly, but macular pigment density increased only after several weeks of supplementation in human subjects.⁸⁷⁻⁸⁹ Although we do not know when retinal zeaxanthin would increase in quails, birds supplemented for 6 months had

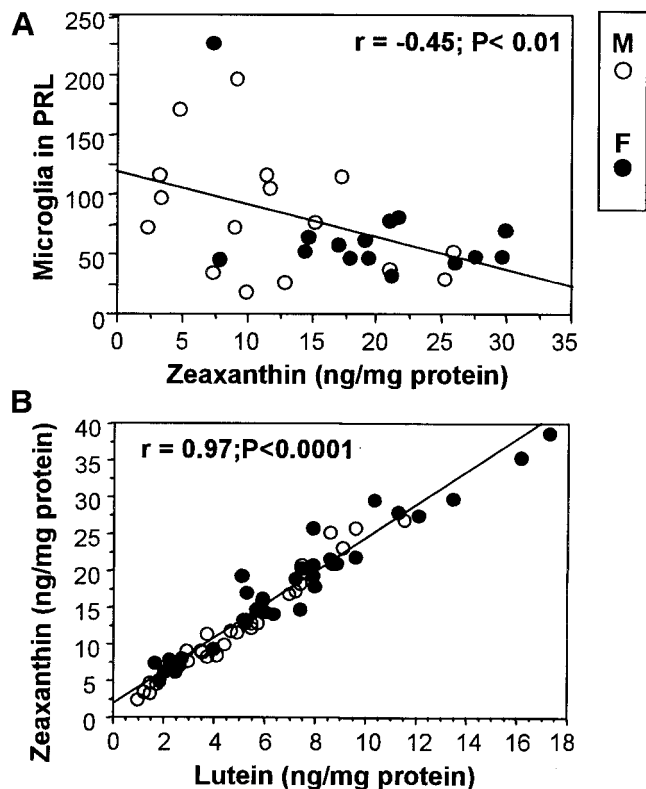


FIGURE 9. Retinal concentrations of zeaxanthin were significantly correlated with the number of microglia in the photoreceptor layer (PRL) (A; $n = 32$), and also with retinal lutein (B; 32 light-exposed and 32 control retinas, with equal numbers of females (F) and males (M) in both groups). Among the light-exposed retinas the correlation was $r = 0.95$; $P < 0.0001$. Note that retinas had 2.4 times as much zeaxanthin as lutein.

almost four times as much retinal zeaxanthin as the unsupplemented birds.⁴¹

We have no explanation for the fluctuation in retinal zeaxanthin and lutein. The increases in serum and liver zeaxanthin and the relative enrichment in retinal zeaxanthin (Fig. 3) verify that the dietary zeaxanthin was absorbed from the gut and delivered to the retina. The fluctuations cannot be attributed to light-induced loss of photoreceptors, because equivalent fluctuations in xanthophyll concentrations were observed in retinas of birds that were not exposed to light. The probability that the fluctuations were real is further supported by the fact that the changes in retinal zeaxanthin correlated very significantly and negatively with photoreceptor vulnerability and paralleled changes in retinal lutein. Fluctuations may also occur during supplementation in human subjects. Hammond et al.⁸⁸ reported that 2 of 13 subjects given supplemental dietary sources of lutein and zeaxanthin were retinal nonresponders—that is, they had significant increases in serum lutein, but a mean 11% decrease (not significant) in macular pigment density.

Physiological, diurnal rhythms may have contributed to the observed fluctuation in retinal xanthophyll concentration. Supplementation was initiated exactly 1, 3, or 7 days before light exposure, which for logistic reasons started in early morning or afternoon. It is feasible that the fluctuation in retinal carotenoids reflects physiological variation in the relative carotenoid content of LDL and HDL during the day and their relative rates of delivery to retina and liver. The production of cholesterol (and presumably secretion of LDL) and the expression of the LDL receptor in rats^{90,91} and the concentrations of LDL, HDL, and carotenoids in human serum follow a diurnal rhythm.^{92,93} Diurnal variation in delivery to the retina would reduce the accuracy of our estimates of retinal zeaxanthin present at the initiation of light damage, suggesting that the correlation between retinal zeaxanthin and photoreceptor loss was underestimated in these experiments. Although diurnal variation in retinal sensitivity to light damage is also well documented,⁹⁴ it would not have contributed to variation in light damage, because light exposure extended over a 27-hour period, throughout any diurnal cycle.

Fat and Liver Zeaxanthin

Rapid elevation in liver zeaxanthin in supplemented quails is consistent with changes in liver carotenoid concentrations in poultry, monkeys, and mice given dietary lutein or zeaxanthin supplements.^{66,85,95} Carotenoids and cholesterol absorbed from the diet are carried on chylomicrons from the gut directly to the liver,⁹⁶ where they are absorbed and resecreted on plasma lipoproteins, including VLDL, LDL, and HDL. HDL carry primarily lutein and zeaxanthin, whereas LDL carry both xanthophylls and hydrocarbon carotenoids (e.g., lycopene, β -carotene).^{97,98}

HDL and LDL Transport

Available evidence suggests that carotenoids may reach the central nervous system and retina on apolipoprotein E (ApoE) containing HDL. Brain capillary endothelial cells absorb α -tocopherol five times more efficiently from apoE-containing HDL than from LDL.⁹⁹ Receptors for ApoE are present on most central nervous system (CNS) neurons,¹⁰⁰ and ApoE containing HDL binds to membranes isolated from human brains.¹⁰¹ HDL may also transport carotenoids within the retina, for retinal Müller cells synthesize ApoE-containing lipoproteins.¹⁰² Tissues absorbing lutein and zeaxanthin from LDL may exhibit more rapid accumulation than those absorbing the xanthophylls from HDL, in that every LDL particle contains a carotenoid molecule, whereas only 2.5% of the HDL molecules carry carotenoid.⁹⁷ Thus, preferential uptake of HDL in the retina

could explain both the absence of hydrocarbon carotenoids in the retina and the slow response of the retina to elevated serum carotenoids.

Tissue Competition for Plasma Carotenoids

Positive correlation of retinal zeaxanthin with those in fat indicates that adipose and fat do not compete for zeaxanthin. In contrast, retinal lutein concentrations correlated negatively with those in fat in female quail but not in males. Similarly, in humans temporal changes in macular pigment density correlated negatively with lutein concentrations of adipose in women, but not in men.⁸⁹ The concept that fat and retina may compete with lutein is consistent with our previous observation that fat in quail captures serum lutein in preference to serum zeaxanthin by a margin of 4:1.⁴¹ Competition between retina and adipose for serum lutein (more prevalent in the diet) may help explain why macular pigment density is reduced in subjects with higher body mass index.¹⁰³ These observations suggest that macular pigment density may be more efficiently increased by supplementation with zeaxanthin than with lutein, especially in older subjects with higher body mass indices.

Relevance to AMD

The absence of a significant relationship between the number of damaged photoreceptors and serum zeaxanthin establishes that serum xanthophylls offer no protection to the photoreceptors. Thus, the observations that those with elevated dietary or serum lutein and zeaxanthin (xanthophyll carotenoids) had reduced risk for drusen in early age-related maculopathy, for pigmentary abnormalities in late age-related maculopathy, and for exudative AMD^{2,5} may substantially underestimate the protection offered by macular pigment. Higher serum levels of lutein and zeaxanthin tend to be associated with higher macular pigment density, but the correlation between these variables is weak,^{88,89,104,105} and may be influenced by interaction with other tissues (e.g., adipose) that accumulate these carotenoids.⁸⁹ Indirect evidence that the retina is protected by accumulation of lutein and zeaxanthin in macular pigment includes findings that macular pigment is significantly reduced in eyes at high risk for AMD,¹⁵ in central and peripheral regions of eyes with AMD,¹⁴ and in smokers¹³ (who have increased risk for AMD).^{3,106}

The role of "chronic" or life-long light exposure in the loss of photoreceptors in aging and in retinal degenerations remains unknown. Increased risk for late AMD was associated with increased exposure to blue and visible light in a case-control study of Chesapeake watermen^{107,108} and with increased exposure to sunlight in the Beaver Dam Eye study.¹⁰⁹ Reduction of blue light by macular pigment would reduce the vulnerability of the rod photoreceptors associated with photoregeneration of rod photopigments,⁷² and could therefore reduce the preferential loss of rods during aging and in AMD.^{19,110} Evidence for an inverse relationship between retinal zeaxanthin and rod apoptosis provides strong support for the hypothesis that elevating macular pigment with supplemental dietary zeaxanthin could be an effective means to reduce the incidence of, or delay the progression of, age-related macular degeneration.³⁷ Although most of the zeaxanthin in the human retina is accumulated in the fovea, the mechanism(s) leading to that distribution has not been identified, and there is little reason to assume a priori that supplemental zeaxanthin would increase macular pigment only in the fovea.

In summary, these data provide the first experimental evidence that xanthophyll carotenoids within the retina prevent light-induced photoreceptor death, whereas those in the serum

do not. Further clinical studies of the relationship between AMD and macular pigment should employ a noninvasive means for measuring macular pigment.¹¹¹⁻¹¹³

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