Lutein and Zeaxanthin Concentrations in Rod Outer Segment Membranes from Perifoveal and Peripheral Human Retina

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**Purpose.** In addition to acting as an optical filter, macular (carotenoid) pigment has been hypothesized to function as an antioxidant in the human retina by inhibiting the peroxidation of long-chain polyunsaturated fatty acids. However, at its location of highest density in the inner (prereceptoral) layers of the foveal retina, a specific requirement for antioxidant protection would not be predicted. The purpose of this study was to determine whether lutein and zeaxanthin, the major carotenoids comprising the macular pigment, are present in rod outer segment (ROS) membranes where the concentration of long-chain polyunsaturated fatty acids, and susceptibility to oxidation, is highest.

**Methods.** Retinas from human donor eyes were dissected to obtain two regions: an annular ring of 1.5- to 4-mm eccentricity representing the area centralis excluding the fovea (perifoveal retina) and the remaining retina outside this region (peripheral retina). ROS and residual (ROS-depleted) retinal membranes were isolated from these regions by differential centrifugation and their purity checked by polyacrylamide gel electrophoresis and fatty acid analysis. Lutein and zeaxanthin were analyzed by high-performance liquid chromatography and their concentrations expressed relative to membrane protein. Preparation of membranes and analysis of carotenoids were performed in parallel on bovine retinas for comparison to a nonprimate species. Carotenoid concentrations were also determined for retinal pigment epithelium harvested from human eyes.

**Results.** ROS membranes prepared from perifoveal and peripheral regions of human retina were found to be of high purity as indicated by the presence of a dense opsin band on protein gels. Fatty acid analysis of human ROS membranes showed a characteristic enrichment of docosahexaenoic acid relative to residual membranes. Membranes prepared from bovine retinas had protein profiles and fatty acid composition similar to those from human retinas. Carotenoid analysis showed that lutein and zeaxanthin were present in ROS and residual human retinal membranes. The combined concentration of lutein plus zeaxanthin was 70% higher in human ROS than in residual membranes. Lutein plus zeaxanthin in human ROS membranes was 2.7 times more concentrated in the perifoveal than the peripheral retinal region. Lutein and zeaxanthin were consistently detected in human retinal pigment epithelium at relatively low concentrations.

**Conclusions.** The presence of lutein and zeaxanthin in human ROS membranes raises the possibility that they function as antioxidants in this cell compartment. The finding of a higher concentration of these carotenoids in ROS of the perifoveal retina lends support to their proposed protective role in age-related macular degeneration. (Invest Ophthalmol Vis Sci. 2000;41:1200-1209)

Among the various carotenoids detected in human blood, only the dihydroxycarotenoids lutein and zeaxanthin (and minor isomeric forms) comprise the macular pigment of the human retina. Although macular pigment is present throughout the retina, it is most highly concentrated in the macula lutea (“yellow spot”), a slightly oval region of approximately 2-mm horizontal diameter that is centered on the fovea. Snodderly et al. examined the primate fovea by microdensitometry and found that the highest density of macular pigment occurs in the photoreceptor axons (outer plexiform layer) of the foveola and in the inner and outer plexiform layers of the immediate adjacent area. At these prereceptoral locations, macular pigment is thought to function as an optical filter that absorbs short-wavelength visible light, thereby reducing chromatic aberration and scatter in the foveal image. Filtration of short-wavelength light by macular pigment may also help to prevent photochemical damage to cones and retinal pigment epithelium (RPE) in the fovea.

In addition to its role as an optical filter, macular pigment has been widely hypothesized to have an antioxidant function in the human retina. In vitro studies have shown that lutein and zeaxanthin are highly effective antioxidants capable...
of scavenging peroxyl radicals and quenching reactive oxygen species.

However, a specific requirement for antioxidant protection would not be predicted for the plexiform layers of the fovea where macular pigment density is highest. On the other hand, photoreceptor outer segments are the primary target of oxidative insult in the retina due to their unusually high content of long-chain polysaturated fatty acids and the relatively high oxygen tension in which they exist. To function as lipophilic antioxidants in vivo, lutein and zeaxanthin would be expected to be located within or in close proximity to the membranes they are protecting. For these reasons, the photoreceptor outer segment membrane is the most likely site for an antioxidant function of lutein and zeaxanthin, if it occurs in the retina.

The spatial distribution of lutein and zeaxanthin in the extrafoveal regions of the human retina has been carefully mapped by Bone et al. using high-performance liquid chromatography (HPLC). The amount of macular pigment declines rapidly with distance from the foveal center and reaches a plateau in the periphery. Nonetheless, a substantial amount of the total retinal macular pigment is present within the perifoveal macula and peripheral retina because of the greater retinal expanse represented by these regions compared with the fovea. Zeaxanthin is the predominant pigment in the fovea, but its level declines more rapidly than lutein as a function of eccentricity. For this reason, the mass ratio of lutein to zeaxanthin ranges from approximately 0.4:1 at the foveal center to over 2:1 in the periphery.

Information remains limited on the subcellular localization of macular pigment in different regions of the retina. Bernstein et al. showed that carotenoids bind tubulin in retinal homogenates and proposed that tubulin was the site for macular pigment binding in the plexiform layers of the fovea. However, macular pigment has also been detected in the photoreceptor outer segment layer in the central fovea, even though the microtubules (which contain tubulin) do not reach this layer. This implies that macular pigment may be an integral component of the outer segment membranes or that it may be bound to membrane-associated proteins other than tubulin. Concerning retinal regions outside of the fovea, there is scant information about the cellular and subcellular distribution of lutein and zeaxanthin. Recently, Sommerburg and colleagues were the first to show that lutein and zeaxanthin are associated with rod outer segments (ROS) isolated from whole human retinas. However, lutein and zeaxanthin in ROS were expressed as their total amounts in the retina, and their concentrations in ROS membranes were not determined. Furthermore, no attempt was made to examine lutein and zeaxanthin concentrations in ROS from specific regions of the retina or in other membrane fractions.

A growing body of evidence from epidemiologic and experimental studies has implicated a role for macular pigment in protection against age-related macular degeneration (AMD). The most direct evidence comes from the Eye Disease Case-Control Study, which found a markedly reduced risk for neovascular AMD in persons with high serum levels of carotenoids and lutein/zeaxanthin specifically. High dietary consumption of foods containing lutein/zeaxanthin was also associated with a significant reduction in AMD risk. In considering the role of macular pigment in AMD, it should be recognized that AMD is a heterogeneous disease thought to be caused by a complex interaction of age-related, genetic, and extrinsic factors. Among other contributing factors, the likely involvement of oxidative stress in the etiology of AMD has received much attention in recent years. Although the mechanisms of AMD remain poorly understood, there is sufficiently compelling evidence to warrant a more complete understanding of the purported antioxidant function and protective value of macular pigment in the human retina.

The present study sought to determine whether lutein and zeaxanthin are components of ROS membranes in the human retina. Experiments were performed to evaluate the concentrations of lutein and zeaxanthin relative to membrane protein in ROS compared with residual (i.e., ROS-depleted) membrane fractions, and in the perifoveal compared with peripheral regions of the retina. We observed that outside of the fovea lutein and zeaxanthin occurred at their highest concentration in ROS membranes in the perifoveal region of the retina. These findings place lutein and zeaxanthin at sites in the human retina where they would most likely be required to function as antioxidants.

METHODS

Dissection of Retinal Tissue

Human retinal tissue was obtained from 18 eyes (nine pairs) from donors ranging in age from 46 to 73 years, with a mean age of 61 years. The eyes were provided by the Lions Eye Bank (Houston, TX) as either whole globes or posterior segments with the corneas removed. The procurement and use of human tissue in this study complied with the tenets of the Declaration of Helsinki. Causes of death included myocardial infarction, brain cancer, metastatic lung cancer, congestive heart failure, and trauma. The maximum interval between time of death and initiation of retinal dissection was 11.5 hours, with an average of 7.9 hours. The methods for retinal dissection were essentially the same as described by Handelman et al. with minor variations. During dissection, exposure of the retina to light was kept to a minimum, and the buffers used for dissection were purged with argon and maintained at 4°C. The globes were transected at the equator, the vitreous was extruded, and the eyecup was filled with phosphate-buffered saline containing 1 mM EDTA (PBS-EDTA). After severing its connection to the optic nerve, the retina was transferred to a dish containing PBS-EDTA. The remaining eyecup was filled with buffer and temporarily stored on ice in the dark. Using procedures similar to those described by Bone et al., a 3-mm-diameter retinal area (central disc) centered on the yellow spot (and therefore including the fovea) was obtained by trephination under the view of a dissecting microscope. A concentric annulus with inner and outer edges of 1.5- to 4-mm eccentricity was then obtained by centering an 8-mm-diameter trephine over the previously removed area. Typically, the outer border of this area corresponded superiorly and inferiorly to the retinal arcade vessels, and nasally to the edge of the optic nerve, which roughly defines the area centralis of the human retina. The remaining peripheral retina was then collected. In this study, the 1.5- to 4-mm annular region constituted the “perifoveal” retinal sample, and the remaining region outside of 4 mm, the “peripheral.”

Each dissected region was immediately placed in a sealed tube previously filled with argon in which the corresponding regions from the left and right eyes of each donor were com-
bined and then frozen at −70°C. RPE was then recovered as small sheets, which usually had begun to detach from the eyecups after remaining in cold buffer in the dark for approximately 1 to 2 hours. The detachment was aided by gentle trituration or by gently scraping with the blunt end of a scalpel blade. Samples of the RPE isolated in this manner were examined by phase contrast microscopy, which confirmed that the preparation consisted almost exclusively of RPE. However, the possibility that some ROS tips adhered to the RPE cells could not be excluded. RPE from the entire eyecup was collected with buffer into a tube and washed twice with low-speed centrifugation, and the pellet was covered with argon and frozen at −70°C. Subsequent analysis of frozen samples usually took place within a few weeks.

Dark-adapted bovine retinas were obtained from the W. L. Lawson Co. (Lincoln, NE). They were obtained only during the months November through March, because retinas harvested during other times of the year could be adversely affected by higher ambient temperatures. They were shipped on dry ice to the laboratory by overnight mail in packages of 50 retinas. The frozen retinas were stored at −70°C before analysis.

**Preparation of ROS Membranes**

ROS membranes were isolated according to the procedures described by Papertmaster and Dreyer, with minor modifications to accommodate the smaller amounts of tissue in this study. Human retinal tissue to be used for ROS preparations consisted of either the perifoveal or the peripheral regions from a particular donor (tissue from corresponding regions of the left and right eyes was pooled as described above). Portions of frozen bovine retina were thawed in the dark and approximately 1.0 to 1.2 g wet weight was used for ROS membrane preparation. Samples were suspended by vigorous vortexing for 2 minutes in 2 ml of a homogenizing medium of 34% sucrose (density of 1.15 g/ml) containing 65 mM NaCl, 2 mM MgCl₂, and 5 mM Tris buffer (pH 7.4). This step sheared off most of the ROS into the homogenizing medium, and the remaining retina was pelleted by centrifugation at 5000 rpm for 5 minutes. After collecting the supernatant (crude ROS), the pellet was resuspended with 2 ml of fresh sucrose homogenizing medium, and the steps to obtain crude ROS were repeated to maximize their yield. The supernatants of crude ROS were then combined and centrifuged at 15,000 rpm for 30 minutes with twice the volume of 10 mM Tris buffer (pH 7.4). This pellet was suspended in 2 ml of sucrose, density of 1.100 g/ml, containing 1 mM MgCl₂ and 10 mM Tris buffer, pH 7.4, and layered on top of a discontinuous sucrose gradient consisting of incremental 2 ml steps of 1.150, 1.130, and 1.110 g/ml, buffered with 10 mM Tris buffer (pH 7.4) and 1 mM MgCl₂. The resultant gradient was centrifuged at 25,000 rpm for 1 hour, and the band containing purified ROS, which appeared at the 1.110/1.130 g/ml interface, was collected. All the remaining bands were combined in a manner similar to that described by Fliesser et al. and were in turn combined with the membrane pellet from the earlier crude ROS isolation steps. This represented the “residual retina” membrane fraction. The results of other studies (see discussion in Ref. 28) suggest that cone outer segment membranes are enriched at the 1.130/1.15 g/ml interface rather than at 1.110/1.130 g/ml (containing the ROS membranes) and would therefore represent a small fraction of the residual membranes. The ROS and residual retina membrane fractions were then washed by adding approximately 10 ml of 10 mM Tris buffer (pH 7.4) and centrifuging at 15,000 rpm for 30 minutes. The resulting pellets were reconstituted in 0.5 to 2 ml of 10 mM Tris buffer (pH 7.4) and frozen at −70°C for subsequent analysis. Tissue processed in this manner yielded approximately 540 to 660 μg of ROS membrane protein, and approximately 5.8 to 7.6 mg of residual, per pair of human retinas. For the samples from the perifoveal retinal region, corresponding yields were approximately 32 to 53 μg of ROS, and approximately 420 to 610 μg of residual retinal membrane protein. The purity of membrane preparations was evaluated routinely by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli, and protein yields were determined by the BCA protein assay (Pierce Chemical, Rockford, IL).

**Lutein and Zeaxanthin Analysis by Normal-Phase HPLC**

The methods for HPLC analysis of lutein and zeaxanthin and the synthesis of 3'-ethoxylutein to be used as the internal standard were adapted from those reported by Khachik et al. Photo-isomerization and degradation of the carotenoids were minimized by conducting all procedures under yellow light (Kodak OO safelight filter) or occasionally under very low levels of indirect room light. Aliquots of known protein amounts of retinal membranes or ROS, or the 3-mm retinal area corresponding to the macula lutea plus immediate surrounding area, were homogenized in 0.5 ml of 10 mM Tris buffer. The homogenate was then combined with 0.5 ml ethanol containing 2.5 ng of internal standard and 0.1% BHT. Two milliliters of hexane was then added, followed by vortexing for 180 seconds. Phase separation was accomplished by centrifuging at 1900 rpm, and the upper hexane layer was transferred to a clean vial. Two milliliters of hexane was added to the remaining sample, and the extraction steps were repeated. The extracts were combined, dried under argon, and resuspended in 50 μl of mobile phase. Separations were carried out on a silica-based, nitrile-bonded (normal-phase) column (5 μm particle size, 250 × 4.6 mm ID; Rainin, Woburn, MA). The mobile phase consisted of an isocratic mixture of 80% hexane/19.4% dichloromethane/0.5% methanol/0.1% N,N-diisopropylethylamine, and column flow rate was set at 0.7 ml/min. The monitoring wavelength of the eluent was 450 nm. Using these methods, the internal standard eluted at approximately 7 minutes and therefore did not interfere with any of the carotenoid peaks, the first of which appeared approximately 8 minutes later. Lutein and zeaxanthin were quantified as previously described, by determining their peak areas relative to that of the internal standard and converting to nanograms, using standard curves derived from external standards of lutein (Sigma) and zeaxanthin (generously donated by Hoffmann-LaRoche, Nutley, NJ) that were injected daily on the column. Analytic recovery of lutein and zeaxanthin determined by adding known amounts of external standard to rat ROS (which we established have no endogenous lutein or zeaxanthin) was greater than 98%. Sensitivity of the assay was estimated at 0.1 ng for both carotenoids.

**Fatty Acid Composition**

Samples of frozen ROS and residual retinal membranes were shipped on dry ice by overnight mail to the University of
Oklahoma Health Sciences Center in Oklahoma City, OK, where gas-liquid chromatographic analysis (GLC) of fatty acids was performed by Maureen Maude (laboratory of R. E. Anderson) according to previously reported methods.\(^4\) In brief, lipids from thawed samples were extracted in 2:1 chloroform/methanol, and a portion of the extract was applied to a silica gel plate and developed in hexane/ethyl ether/glacial acetic acid. The region of the plate containing the total phospholipids was scraped into a tube and methyl esters prepared with BF\(_3\)-methanol. Their masses were quantitated by GLC using 17:0, 19:0, and 21:0 as internal standards.

**Statistical Analysis**

A three-way ANOVA was applied to the data for lutein and zeaxanthin concentrations (nanograms per milligram of protein) in human retinal membranes, examining factors of carotenoid (lutein versus zeaxanthin), region (perifoveal versus peripheral), and membrane fraction (ROS versus residual). A two-way ANOVA was applied to the data obtained on human ROS membranes, specifically examining factors of carotenoid (lutein versus zeaxanthin) and region (perifoveal versus peripheral). A two-way ANOVA was applied to the data for the lutein-to-zeaxanthin concentration ratio, examining factors of region (perifoveal versus peripheral) and membrane fraction (ROS versus residual). The level of significance for all tests was 0.05.

**RESULTS**

**Evaluation of ROS Membrane Purity by SDS–PAGE and Fatty Acid Analysis**

Figure 1 shows SDS–PAGE patterns of proteins present in ROS and residual membranes that were prepared from bovine retina and peripheral human retinal samples. In the ROS membranes, the major band corresponded to opsin (36–38 kDa), indicating that the ROS preparation was of high purity.\(^3\) Absence of the opsin band in the residual membrane fraction indicated that they were relatively free of ROS contamination. Gel patterns were similar for bovine (Fig. 1A) and human (Fig. 1B) retinal membranes. Retinal membranes from the perifoveal human retina were similarly analyzed, and the banding patterns for ROS and residual membranes were essentially the same as those shown from the peripheral human retina shown in Figure 1B.

Evaluation of purity by SDS–PAGE was performed on all membrane preparations to be used for HPLC analysis of carotenoids. In retinal tissue derived from two of the donor eyes, a detectable opsin band was seen in the residual membrane fraction, indicating some ROS contamination had occurred (not shown). In the corresponding ROS membranes, opsin appeared as a visible band in the gel, but relative to other proteins it was not as pronounced as in Figure 1. Using a conservative criterion, the central and peripheral membranes prepared from these two donor eyes were not used for quantitative assessment of lutein and zeaxanthin concentrations. However, tissues from both eyes were used as part of the samples for determination of lutein and zeaxanthin concentrations in the RPE and in the 3-mm-diameter retinal area centered over the macular lutea. Of the nine pairs of eyes used in this study, there were seven pairs in which the preparations of ROS and residual membranes from both the central and peripheral regions of the eye were determined to be highly pure by SDS–PAGE. The membrane preparations from these seven pairs of eyes were used to obtain data on lutein and zeaxanthin concentrations to which statistical analyses were applied.
As an additional test of ROS membrane purity, fatty acid composition was determined on ROS and residual membranes from bovine and human retinas. A distinguishing characteristic of ROS membranes is their enrichment in long-chain polyunsaturated fatty acids, and in particular docosahexaenoic acid (DHA; 22:6n3), relative to total retinal membranes or other neural tissue. In the bovine retinal membrane preparations in this study, DHA represented 29.1 mol% of the total ROS membrane fatty acids and only 14.5 mol% of the total residual membrane fatty acids. A similar relationship was seen in the human ROS and residual retinal membranes, where DHA represented 33.5 and 12.2 mol% of the total fatty acids, respectively (Table 1). This finding of enriched DHA in ROS preparations confirmed the purity of the membranes to be used for lutein and zeaxanthin analyses.

**HPLC Analysis of Lutein and Zeaxanthin**

Lutein and zeaxanthin were found by HPLC analysis to be present in the ROS and residual membranes of the perifoveal and peripheral regions of the human retina (Figs. 2A–2D, and Table 2). There was a considerable (five- to eightfold) variation in carotenoid concentrations among individual donor eyes for each membrane fraction and retinal region. Figure 3 illustrates that donors with relatively high levels of lutein in the perifoveal ROS membranes (represented by filled symbols) generally also had relatively high levels of both lutein and zeaxanthin in the other (peripheral and residual) retinal membranes. Three-way ANOVA indicated that lutein and zeaxanthin concentrations depended on the main factors of carotenoid type, retinal region, and membrane fraction. Lutein was the predominant carotenoid (outside the central 3-mm disk), being overall 1.7 times more concentrated than zeaxanthin in ROS and residual membranes of the perifoveal and peripheral retina (P < 0.02).

The concentration of lutein plus zeaxanthin (combined means) was 2.5 times greater in the perifoveal than in the peripheral region of the retina (P < 0.001), and 1.7 times higher in the ROS than the residual membranes (P < 0.01). No significant interactions were found between the main variables (P for all > 0.1). Because ROS membranes were of particular interest in this study, concentrations were computed and analyses performed to compare carotenoid concentrations in ROS membranes independent of residual membranes. Most notably, lutein plus zeaxanthin concentrations in ROS membranes were 2.7 times higher in the perifoveal than in the peripheral retinal region (P < 0.001).

The ratio of lutein-to-zeaxanthin concentrations in human retinal membranes was calculated individually for each donor, and mean values are shown in Table 2. Two-way ANOVA indicated that lutein-to-zeaxanthin concentration ratios were significantly higher in the peripheral than in the perifoveal retina (P < 0.001) and in ROS than in residual human membranes (P < 0.05). To examine this relationship for ROS membranes independent of residual membranes, a paired t-test was applied, which confirmed that the lutein-to-zeaxanthin ratio in ROS membranes specifically was higher in the peripheral retinal region than in the perifoveal region (P < 0.02; Table 2).

Because the RPE was isolated as intact cells and analyzed without any further isolation of its membranes, lutein and zeaxanthin concentrations were expressed on the basis of total protein (membrane plus soluble). Lutein and zeaxanthin were detected in all samples (n = 6 donors) of human RPE (Table 3). As in retinal membranes, lutein was the predominant carotenoid in the RPE. Three unidentified peaks consistently appeared in RPE chromatograms in addition to lutein and zeaxanthin (Fig. 2E). The unidentified peak eluting just before lutein was also seen in most of the retinal membrane preparations but was always more prominent in the RPE samples.

HPLC analyses were performed on the 3-mm-diameter central disks to provide a basis for comparing the carotenoid amounts in the donor eyes of our study to those in other investigations. The level of lutein plus zeaxanthin combined in the central disks was 4.36 pmol/mm² (Table 3), which compares favorably with the 4.87 pmol/mm² previously reported for exactly the same region (see Table 1 in Ref. 2; amounts of lutein plus zeaxanthin and its two stereoisomers combined for comparison to our value). Zeaxanthin was found to be the predominant carotenoid in this region, which is also consistent with previous findings.

The relationship between the carotenoid concentration in ROS membranes and the carotenoid level in the central disks was determined for donor eyes in which both measurements were made (Fig. 4). Among donors, the lutein plus zeaxanthin concentration of the perifoveal ROS was highly correlated with the carotenoid level in the central disks (P < 0.001; r = 0.983). A slightly weaker but still significant association was observed between the carotenoid concentration in the peripheral ROS and the level in the central disks (P < 0.05; r = 0.823).

Very low levels of lutein and zeaxanthin were found in bovine retinal membranes. In samples from two preparations, human retinal membrane samples were obtained from peripheral retinas. Values expressed as mol% of total fatty acid; Human retina values are means for 4 donors and bovine values represent the average of duplicate runs on two separate membrane preparations. ROS, rod outer segment.

**Table 1. Fatty Acid Composition of ROS and Residual Membranes from Bovine and Human Retinas**

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For values of lutein, zeaxanthin, and all other carotenoids, see Table 2. See Table 1 for abbreviations.
lutein and zeaxanthin in ROS and residual membranes were detected at concentrations of 0.2 to 0.4 ng/mg protein. There was no apparent predominance of lutein or zeaxanthin in bovine retinal membranes.

**DISCUSSION**

The data presented herein demonstrate that macular pigment is a component of ROS membranes in the human retina. In this study, HPLC carotenoid analysis was performed on ROS membranes that were isolated from human retinas and shown to be of high purity by their protein gel pattern and fatty acid composition. Expressing carotenoid amounts in ROS membranes relative to membrane protein allowed for direct comparisons

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<table>
<thead>
<tr>
<th>Membrane</th>
<th>Lutein (ng/mg protein)</th>
<th>Zeaxanthin (ng/mg protein)</th>
<th>Lutein/Zeaxanthin</th>
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<td>$15.25 \pm 12.22$</td>
<td>$5.83 \pm 5.01$</td>
<td>2.63 $\pm 0.36$</td>
</tr>
<tr>
<td>Peripheral Residual</td>
<td>$9.49 \pm 5.40$</td>
<td>$4.76 \pm 3.67$</td>
<td>2.00 $\pm 0.50$</td>
</tr>
</tbody>
</table>

Values are mean $\pm$ SD for a sample of seven donors. Lutein-to-zeaxanthin concentration ratios were individually calculated for each of the donors, and the mean values shown are the average of these values. Mean concentration ratios determined in this manner do not show exact correspondence to the ratios of lutein and zeaxanthin average concentrations.
of their concentration between ROS membranes in the perifoveal and peripheral regions of the human retina, and between ROS and residual membrane fractions. Lutein plus zeaxanthin was nearly three times more concentrated in membranes from perifoveal ROS membranes than in those from the periphery. Carotenoid concentration in ROS overall was 70% higher than in residual retinal membranes. However, the greater portion (total mass) of the carotenoids would appear to be in the residual membranes, because of the much greater proportion of the total retinal membranes represented by this fraction. Furthermore, residual membranes were derived from various cells and subcellular compartments. The carotenoid concentration in any specific residual retinal membrane could therefore be quite high.

Based on the protein yields of the membrane preparations and the concentrations of lutein and zeaxanthin reported herein, the total amounts of lutein plus zeaxanthin are estimated to be 145 ng in the entire retina and 14.9 ng in the ROS membranes. Considering the possibility of loss of some ROS during their isolation, lutein plus zeaxanthin amounts in ROS membranes would therefore represent approximately 10% to 15% of their amounts in the entire retina. In another recent study, approximately 25% of the total retinal carotenoids were in found in the ROS. However, a direct comparison between the two studies is not possible because different methodology was used for the isolation of ROS and the testing of their purity.

The lutein-to-zeaxanthin concentration ratios determined for human retinal membranes in the present study (Table 2) are consistent with the mass ratios determined by Bone et al. In making comparisons to the intact (i.e., not undergoing fractionation) retina samples examined in previous studies, it is important to note that ROS membranes represented approximately 8% to 9% of the total retinal membranes and should therefore be weighted less heavily in these comparisons. Thus, the closer correspondence of the residual rather than ROS retinal membranes to the ratios reported by Bone et al. supports this reasoning. Of greater significance, however, is the finding that lutein-to-zeaxanthin ratios in ROS membranes alone showed regional variation. Previous theories addressing the increase in the lutein-to-zeaxanthin ratio with linear distance from the fovea have proposed an association of lutein with rods and zeaxanthin with cones, or a preferential association of particular lutein–zeaxanthin ratios with different cone phenotypes. Our findings show that central (perifoveal) to peripheral differences in lutein-to-zeaxanthin ratio can also occur in ROS membranes independent of cones. Because the basis for this difference cannot involve different photoreceptor types, alternative possibilities include a spatial dependence of uptake of lutein and zeaxanthin into the ROS, or in carotenoid metabolism. Bone et al. proposed a lutein to meso–zeaxanthin conversion mechanism in the human retina that, if spatially distributed, could explain decreasing levels of meso–zeaxan-

**Table 3. Mean Lutein and Zeaxanthin Levels and Concentration Ratio in Human RPE and Central Disc**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lutein Level</th>
<th>Zeaxanthin Level</th>
<th>Lutein/ Zeaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPE</td>
<td>2.90 ± 1.89</td>
<td>1.39 ± 0.97</td>
<td>2.19 ± 0.17</td>
</tr>
<tr>
<td>Central disc</td>
<td>1.70 ± 1.16*</td>
<td>2.66 ± 1.68*</td>
<td>0.64 ± 0.11</td>
</tr>
</tbody>
</table>

Units are nanograms per milligram of total protein for RPE and picomoles per millimeter squared for central disc. Values are mean ± SD for sample sizes of 6 and 8 for RPE and central disc, respectively. Lutein-to-zeaxanthin concentration ratios were individually calculated for each of the donors, and the mean values shown are the average of these values. Mean concentration ratios determined in this manner do not show exact correspondence to the ratios of lutein and zeaxanthin average concentrations.

* The levels of lutein and zeaxanthin in the central disc compare favorably with Bone et al.² where values of 1.92 pmol/mm² for lutein and 2.95 pmol/mm² for zeaxanthin plus its two stereoisomers were reported for the same region.

**Figure 3.** Lutein and zeaxanthin concentrations in human ROS and residual retinal membranes. Superimposed on each bar are the individual data points (represented by a different symbol for each donor), illustrating the range of values for each group. The filled symbols represent the donors with the three highest concentrations of lutein in perifoveal ROS membranes (which, for the most part, also tended to be relatively high in the other regions and fractions for both carotenoids). Statistical analysis of these data (see text) indicated that the concentrations of lutein plus zeaxanthin (nanograms per milligram of protein) were on average higher in ROS than in residual membranes, and in the perifoveal versus peripheral retinal regions. Vertical bars represent SD of the mean.
thin and increasing levels of lutein with increasing distance from the fovea. With the HPLC methodology of the present study, zeaxanthin and meso–zeaxanthin co-eluted in a single peak. Therefore, even if such a conversion mechanism existed in human ROS membranes and varied with retinal region, it would not affect the zeaxanthin concentration values in our study. However, the possible involvement of this mechanism in lowering lutein levels in the perifoveal versus peripheral ROS cannot be excluded and is worthy of further evaluation.

Low levels of macular pigment have been detected in human RPE (also Bernstein PA, personal communication, August 1999), but no data have been provided regarding its concentration in this tissue. Our findings showed that human RPE isolated from all regions had a mean lutein plus zeaxanthin concentration of 4.29 ng/mg total (membrane plus soluble) protein. Assuming membrane protein to represent roughly half the total in RPE, the concentration of lutein plus zeaxanthin relative to membrane protein would likely be no more than 8 to 10 ng/mg. For most regions of the retina, this represents less than half the concentration found in ROS membranes. The possibility exists that some of the lutein/zeaxanthin measured in RPE is attributable to outer segment phagosomes or tips of ROS that remained attached during dissection. However, an equally likely possibility is that the human RPE stores some macular pigment to replenish what would be lost in the ROS due to turnover. The peaks in human RPE chromatograms other than those of lutein and zeaxanthin could not be identified by the HPLC methodology of the present study. However, the mobile phase and normal-phase columns used in this study were essentially the same as those used by Khachik et al. and in that study, oxidation products or isomers of lutein and zeaxanthin were identified that may represent the unknown peaks in the present study. Further experiments are needed to evaluate the possible relevance of these minor carotenoids as metabolites or oxidation products of lutein and zeaxanthin.

The location of macular pigment in the retina provides important clues to its function and role in protecting against AMD. The physical properties of macular pigment (i.e., wavelengths it absorbs and orientation relative to the direction of incoming light), and its very high density in the pre-receptor fovea, indicate that macular pigment is an ideal filter for improving the visual image and for protecting against short-wavelength visible light damage. The involvement of light exposure as a risk factor in AMD is uncertain, because epidemiologic studies have provided equivocal evidence regarding this relationship. However, the annular pattern of certain macular retinal degenerations (including those resulting from photosensitizing drug toxicity and AMD) shows a close correspondence between the region where macular pigment absorbs most strongly and the region of central sparing in these degenerations.

The concentrations of lutein and zeaxanthin in the ROS of the perifoveal retina provides additional compelling support for an evolving theory pertaining to the role of oxidative stress and antioxidant protection in AMD. ROS membranes are uniquely susceptible to peroxidative insult because of their high content of long-chain polyunsaturated fatty acids and the high oxygen tension of their microenvironment. For unknown reasons, accumulation of diffuse lipid deposits, thought to be linked to the pathogenesis of AMD, occurs preferentially in the posterior pole of the eye. Recent studies have shown that lipids in these deposits are of cellular rather than blood origin, a likely source being debris derived from incompletely digested photoreceptor membranes. Most of the photoreceptor debris would originate from rods, being that they have a much higher density than cones (ranging from 4–30 times greater) in the perifoveal macula. Very recently, it has been shown that Bruch’s membrane isolates from the human macula contain peroxidized lipids that were derived in part from long-chain polyunsaturated fatty acids, typical of those found in outer segment membranes. Taken together, this evidence provides a direct link between peroxidation of human ROS in the perifoveal retina and the accumulation of debris that is associated with AMD. Furthermore, the location of relatively high concentrations of lutein and zeaxanthin in the perifoveal ROS corresponds to that which would be predicted to have the greatest requirement for antioxidant protection in the human retina.

Vitamin E is thought to be an important antioxidant in biological membranes and is found in high levels in mammalian ROS. If carotenoids function as antioxidants in human retinal membranes, they must do so in addition to, or in synergy with, vitamin E. In liver microsomes, the inhibition of lipid peroxidation for vitamin E and β-carotene combined was found to be greater than the sum of their individual inhibitions. The vitamin E concentrations determined for the human retina have varied among studies, but the midrange was approximately 1 nmol/mg protein. Converting our concentration values to nanomoles, there is approximately 0.06 to 0.1 nmol/mg protein of lutein plus zeaxanthin in the membranes of perifoveal retina. The possibility for antioxidant synergism
in membranes where vitamin E is in higher concentration than carotenoids has been proposed. Although the location of lutein and zeaxanthin in the ROS of the human retina suggests an antioxidant function, there has as yet been no direct demonstration of this role. Further studies are needed to determine whether or not the concentrations of macular pigment in human ROS membranes are capable of inhibiting lipid peroxidation at rates that might be expected to occur in the human retina. A protective role of lutein and zeaxanthin in AMD has been indicated by epidemiologic studies. These observations have prompted the inclusion of lutein in several commercially available nutritional and vitamin supplements. Lutein and zeaxanthin in animals are derived exclusively from dietary sources (there is no de novo synthesis), and macular pigment density was shown to increase in human subjects taking lutein supplements. However, until the physiological role of macular pigment in the retina is more fully understood and long-term safety established, caution should be exercised in taking such supplements, which may provide excessive doses. At this time, a sensible approach to optimizing visual health and preventing age-related retinal disease would be to consume a balanced diet that is rich in fruits and vegetables that contain these carotenoids.

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References


