Distribution of Lutein and Zeaxanthin Stereoisomers in the Human Retina

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The distribution of macular pigment stereoisomers in the human retina has been mapped and a pathway to account for the presence of the non-dietary carotenoid, meso-zeaxanthin, is proposed. Adult neural retinas were cut into three concentric areas centered on the fovea, and the extracted carotenoids were analysed and purified by high-performance liquid chromatography. The dicarbamate or dibenzoate derivates of the collected zeaxanthin fractions for each tissue sample were further analysed by HPLC to determine their stereoisomer composition. Whole retinas from infant eyes were similarly analysed. The results show that, relative to zeaxanthin, the concentration of lutein in the adult neural retina increases with radial distance from the fovea whereas that of meso-zeaxanthin decreases. Infant retinas were found to have more lutein and less meso-zeaxanthin, relative to zeaxanthin, than adult retinas. Small quantities of (3S, 3’S)-zeaxanthin were also found in the adult retina, particularly in the macula. It is proposed that lutein and zeaxanthin are transported into an individual’s retina in the same proportions found in his or her blood serum. Some of the lutein is then converted into meso-zeaxanthin, primarily in the macula, by a mechanism which is less developed in infants than adults. © 1997 Academic Press Limited

Key words: macular pigment; carotenoids; zeaxanthin; lutein; stereoisomers.

1. Introduction

The macular pigment, consisting principally of the carotenoids zeaxanthin and lutein (Bone, Landrum and Tarsis, 1985), has an interesting and unresolved distribution in the human retina. In the inner macula, the concentration of zeaxanthin is about twice that of lutein. As eccentricity from the fovea increases, the ratio of concentrations continuously changes with lutein becoming the dominant component in the peripheral retina. At distances exceeding 6 mm from the fovea, the lutein:zeaxanthin ratio is between 2:1 and 3:1 (Bone et al., 1988; Handelman et al., 1988). Noting that this ratio increased from the fovea outwards in rough proportion to the increasing rod:cone ratio, Bone et al. (1988) suggested that this might reflect an association of lutein and zeaxanthin primarily with rods and cones respectively. Snodderly, Handelman and Adler (1991) on the other hand proposed that particular lutein:zeaxanthin ratios might be associated with specific cone types whose relative abundance varied across the retina.

After the age of about 2 years, no age-related changes appear to occur in either the concentration or composition (lutein:zeaxanthin) of the pigment in the maculas of normal individuals, though wide variation in the former has been observed (Bone and Sparrock, 1971; Bone et al., 1988; Hammond et al., 1996; Pease, Adams and Nuccio 1987; Werner, Donnelly and Klugl, 1987). Also prenatal whole retinas and postnatal retinas up to the age of about 2 years differ from older age groups, having a higher lutein:zeaxanthin ratio (Bone et al., 1988).

Zeaxanthin extracted from the macula has been shown to consist of similar amounts of the (3R, 3’R) and (3R, 3’S) stereoisomers, and possibly a much smaller amount of the (3S, 3’S) stereoisomer (Bone et al., 1993). [Lutein is present as the single stereoisomer, (3R, 3’R, 6’R). The structure of this and the zeaxanthin stereoisomers may be found in Straub (1987).] Of the zeaxanthin stereoisomers, only (3R, 3’R)-zeaxanthin has been detected in human blood serum (Bone et al., 1993). The other two stereoisomers may be present in the serum, but if so, they are below current levels of detection. This suggests that their presence in the eye is the result of carotenoid transformations occurring in the eye. In particular, it has been postulated that the presence of (3R, 3’S)-zeaxanthin might be due to isomerization of lutein within the retina (Bone et al., 1993).

Current interest in the macular pigment has been sparked by the intriguing possibility that it may play a protective role against some forms of age-related macular degeneration (AMD). Protection could be provided by the pigment in two ways: through its ability to quench free radicals and singlet oxygen, and by absorbing blue light before it reaches the sensitizers which initiate photochemical damage. AMD is the leading cause of vision loss among the elderly (Hyman, 1992) and is without cure. Assessing risk factors for its development is therefore of considerable importance.
Preliminary data have shown that AMD donor eyes have, on average, a somewhat lower concentration of zeaxanthin and lutein throughout the retina than do non-AMD controls (Landrum et al., 1995; Landrum, Bone and Kilburn, 1996a). In the same vein, Seddon et al., 1994 found that among dietary carotenoids, a higher intake of lutein and zeaxanthin was associated with a reduced risk of neovascular AMD. The Eye Disease Case-Control Study Group (1993) found the same to be true when examining serum levels of carotenoids, including lutein and zeaxanthin. However, this result is inconclusive at present. Another study (Mares-Perlman et al., 1995) has revealed no such association.

The ability to increase the amount of macular pigment by dietary supplementation with lutein has been demonstrated (Landrum et al., 1996a; Landrum et al., 1996b). Such a strategy may become recognized as an effective means of reducing the risk, and/or progression, of AMD in some individuals. Understanding the mechanism of pigment accumulation and possible transformation in the macula is vitally important to the development of such a therapy. If, for example, lutein is partially isomerized into meso-zeaxanthin, the retina may gain an advantage owing to the slightly greater quenching efficiency of zeaxanthin over lutein (Foote, Chang and Denny, 1970). In this study we have measured the distributions of lutein and the zeaxanthin stereoisomers across the retina using pooled samples from many eyes, as well as individual eyes. The results support the isomerization hypothesis and provide a straightforward explanation of the carotenoid distributions.

2. Methods

Sample Preparation

Human donor eyes were obtained fresh from the Florida Lions Eye Bank. These were stored in the dark at −20°C prior to analysis for periods not exceeding two weeks. Additional eyes, fixed in formaldehyde within approximately 6 hr of death, were provided by the National Disease Research Interchange (NDRI). These eyes were stored in the dark at 4°C for one to four weeks prior to analysis. All eyes were either from adults over 55, or from infants 0 to 7 months old. Some of the eyes in the older group, obtained exclusively from the NDRI, were from AMD patients. (They were also used in a parallel study on AMD whose results are not reported here.) These were diagnosed prior to death but the extent of the disease was unknown. Normal eyes were those for which no diagnosis of AMD or other eye disease had been made and which showed no visible abnormalities of the retina upon dissection. Procurement methods for tissues used in this study were humane, including proper consent and approval, and complied with the tenets of the Declaration of Helsinki. In order to obtain whole, unorn, neural retinas, each eye was immersed in 0.9% saline solution during dissection. Care was taken to minimize exposure of the tissues to bright light. In the case of adults, the intact retina was allowed to settle on a 1” Lucite sphere which was raised from the solution and placed in a device, previously described (Bone et al., 1988), which permitted cutting the retina into pieces of tissue concentric with the fovea. For the present study, the device included trephines of 3, 11, and 21 mm diameter, resulting in a central disk of tissue of area 7.1 mm² containing the yellow spot, and two concentric annuli of areas 93 and 343 mm². For the 0 to 7 month old age group, the entire retina was used.

In the earlier phase of the study, corresponding disks and annuli of tissue from 10 eyes were pooled for carotenoid analysis. A total of 60 eyes were analysed in this way. An alternative procedure was developed in the later phase to permit the analysis of individual tissue samples. Thirty seven eyes from 24 donors were thus analysed. To extract the carotenoids, the pooled or individual tissue samples were homogenized in a glass tissue grinder with 2 ml of ethanol/water (1:1). For the individually analysed eyes, 0.5 ml of an ethanol solution of lutein monohexyl ether were added as an internal standard. The solution was routinely calibrated spectrophotometrically, 0.5 ml containing ~12 ng of the standard. The homogenate was transferred to a large culture tube and the tissue grinder rinsed with three 2 ml aliquots of ethanol/water and two 5 ml aliquots of hexane, the rinses being added to the culture tube. After vortexing and centrifuging, the hexane layer was transferred to a pear-shaped flask and dried under a stream of N₂. The final preparation step was to concentrate the samples in 30 μl of the HPLC mobile phase (see below).

Reversed-phase HPLC

In order to separate and quantify the zeaxanthin and lutein components in each sample, a reversed-phase HPLC system was employed. This included a 250 × 2 mm C-18 column packed with 3 μm Ultracarb ODS (Phenomenex, Torrance, CA, U.S.A.). The mobile phase was 90% acetonitrile and 10% methanol, with 0.1% (v/v) of triethyl amine added to inhibit degradation of carotenoids during elution. The flow rate was 0.2 ml min⁻¹ and detection was at 452 nm. (The advantage of a 2 mm column over the standard 4.6 mm column used previously (Bone et al., 1988) cannot be over-emphasized. Peak height was increased by a factor of ~ 5 and solvent consumption reduced by the same factor.)

Stereoisomer Analysis

Zeaxanthin stereoisomers were readily separable from each other by either of two methods. For the pooled samples, the zeaxanthin collected during
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(A) Inner

(B) Medial

(C) Outer

2 Mins

Fig. 1. HPLC chromatograms, obtained with a reversed-phase column, of macular pigment extracts from three different regions of a single human retina. (A) ‘Inner’ – disk centered on fovea obtained with 3 mm trephine, area 7.1 mm². (B) ‘Medial’ – annulus obtained with 3 and 11 mm trephines, area 93 mm². (C) ‘Outer’ – annulus obtained with 11 and 21 mm trephines, area 343 mm². L = lutein, Z̃T = combined zeaxanthin stereoisomers. The chromatograms have been truncated and do not show the internal standard.

reversed-phase HPLC was converted to the dibenzoate derivative and analysed on a chiral column as described elsewhere (Bone et al., 1993). The reaction, however, is highly inefficient and did not permit the analysis of carotenoids in single retinas or portions thereof. For these the following procedure, described by Rüttimann, Schiedt and Vecci (1983), was adopted.

Zeaxanthin collected during sample elution on the reversed-phase column was thoroughly dried under a stream of N₂ in a siliconized microcentrifuge tube. The tube was transferred to a glove box containing a dry N₂ atmosphere in order to carry out the derivatization procedure. The zeaxanthin, which had been concentrated into the bottom of the tube, was dissolved in 20 µl of anhydrous pyridine/benzene (50:50 v/v). To this was added 1 µl of (S)-(+)—1-(1-Naphthyl) ethyl isocyanate, and the reaction allowed to proceed at room temperature for ~ 48 hr.

The dicarbamate derivatives thus produced were analysed by HPLC, using a 250 x 2 mm normal-phase column packed with 5 µm Prodigy silica (Phenomenex, Torrance, CA, U.S.A.). The mobile phase was 88% hexane and 12% isopropyl acetate at a flow rate of 0.2 ml min⁻¹. Detection was at 451 nm. No internal standard was necessary; the total quantity of zeaxanthin stereoisomers was obtainable from the reversed-phase chromatography and the normal-phase separation permitted measurement of their relative proportions. The same was true for the dibenzoate derivatives analysed on the chiral column. It should be stressed that in a significant number of cases the normal-phase system was operating at, or close to, its signal-to-noise threshold (peak height ≈ twice noise amplitude). As a result, a number of samples did not yield useful data and were therefore excluded from further analysis.

For comparison purposes, standards of the three zeaxanthin stereoisomers were prepared from rhodohaxanthin as previously described (Maoka et al., 1986), and converted to the dibenzoate and dicarbamate derivatives.

3. Results

Retinal Distribution

Reversed-phase chromatograms of extracts from the central disk (‘inner’) and two concentric annuli of tissue (‘medial’ and ‘outer’) consistently displayed the trend, previously observed (Bone et al., 1988; Handelman et al., 1988), of an increasing lutein (L):zeaxanthin ratio with increasing distance from the fovea. This is apparent from the sample chromatograms, obtained from a single eye, shown in Fig. 1. The zeaxanthin collected during reversed-phase chromatography from the inner, medial and outer regions for the pooled samples subsequently yielded chiral column chromatograms such as those shown in Fig. 2. Figure 3 depicts a similar set obtained for a single eye, the collected zeaxanthin having been converted to the dicarbamate derivative and analysed on a normal-phase column. Note that the order of elution of the stereoisomers is different for the two chromatographic systems.

The chromatograms of Figures 2 and 3 were generally representative of all adult donor eyes analysed, exhibiting a decreasing ratio of meso-zeaxanthin (MZ) to zeaxanthin (Z) with increasing eccentricity from the fovea. For the inner, medial and outer regions, the average MZ:Z ratios ± s.d. obtained using individuals eyes were 0.83 ± 0.15, 0.39 ± 0.12, and 0.24 ± 0.16 respectively. In computing such
Fig. 2. HPLC chromatograms, obtained with a chiral column, of dibenzoate esters of zeaxanthin stereoisomers. These were obtained from the three different regions defined in Fig. 1 and represent the pooled extracts from 10 retinas. In order of elution, the stereoisomers are meso-zeaxanthin (MZ), zeaxanthin (Z) and (3S, 3’S)-zeaxanthin (SZ).

Fig. 3. HPLC chromatograms, obtained with a normal-phase column, of dicarbamate esters of zeaxanthin stereoisomers. These were obtained from the three different regions, defined in Fig. 1, of a single human retina. In order of elution, the stereoisomers are (3S, 3’S)-zeaxanthin (SZ), meso-zeaxanthin (MZ) and zeaxanthin (Z).

Fig. 4. UV-visible absorption spectrum of the SZ peak in Figs 2 and 3. Solvent: hexane/isopropyl acetate (88:12). The spectrum is identical to that of zeaxanthin and meso-zeaxanthin.

averages, here and elsewhere, left and right eye data for a donor, where available, were first averaged. This conservative approach was adopted even though
left/right differences in macular pigment density have been found (Bone et al., 1988; Landrum, Bone and Kilburn, 1996a; Landrum et al., 1996b). A repeated measures, one way analysis of variance indicated a significant difference among the three regions [F(2, 46) = 150.07, P < 0.001]. A Fisher’s least significant difference post hoc test performed at a 5% significance level indicated that all pairs of means differed from one another. These MZ:Z ratios, obtained from individually analysed eyes, were generally consistent with those obtained from the pooled samples where the average ratios were 0.79 ± 0.06, 0.51 ± 0.06, and 0.30 ± 0.08 respectively. (An independent samples, two-tailed t-test returned P values of > 0.5, > 0.02, and > 0.2 for the differences between the pooled and individual inner, medial and outer regions respectively.) From these results, the combined averages for all donor eyes were 0.82 ± 0.12, 0.41 ± 0.10, and 0.25 ± 0.13 respectively.

The small leading peak in Fig. 3(A) has been
tentatively identified as (3S, 3’S)-zeaxanthin (SZ). The same component appears as the small trailing shoulder in Fig. 2(A). Accumulated material from many experiments yielded the UV-visible absorbance spectrum shown in Fig. 4. The spectrum was indistinguishable from that of the other two zeaxanthin stereoisomers. (The different spatial orientations of the hydroxyl groups of the three stereoisomers do not affect their spectra.) Furthermore, coinjection with the mixture of derivatized stereoisomer standards (Z, MZ and SZ) on the normal-phase HPLC column produced enhancement of the SZ peak.

For each eye that was analysed individually, the mass of total zeaxanthin stereoisomers, \( \text{Z}_{\text{T}} \), in an inner, medial or outer tissue sample was determined from the reversed-phase chromatogram as the product of the ratio of the \( \text{Z}_{\text{T}} \) peak area to that of the internal standard, the mass of the standard, and a weighting factor which accounted for the different extinction coefficients of \( \text{Z}_{\text{T}} \) and the standard at the detection wavelength. A similar calculation gave the mass of L.

<table>
<thead>
<tr>
<th>Donor number†</th>
<th>Inner (7-1 sq. mm)</th>
<th>Medial (93 sq. mm)</th>
<th>Outer (343 sq. mm)</th>
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<tr>
<td></td>
<td>L (µg)</td>
<td>Z (µg)</td>
<td>MZ (µg)</td>
</tr>
<tr>
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<tr>
<td>29A†</td>
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</tr>
<tr>
<td>30A†</td>
<td>0.189</td>
<td>0.320</td>
<td>0.285</td>
</tr>
</tbody>
</table>

* Units are pmole of carotenoid per sq. mm of tissue.

** Undetectable.

† A and B refer to the 2 eyes of a donor. Where only one eye was fully analysed, only A is used.

Table I
Concentrations* of lutein (L), zeaxanthin (Z), meso-zeaxanthin (MZ) and (3S, 3’S)-zeaxanthin (SZ) in the inner, medial and outer regions of 37 individual retinas from 24 donors.
normal-phase chromatograms, it was then possible to apportion the mass of Z₁ among the zeaxanthin stereoisomers represented. The numbers of pmoles mm⁻² of the different carotenoids present in the inner, medial, and outer regions of 37 retinas (24 donors) are shown in Table I, together with the averages. Data were included in this study only when both reversed- and normal-phase HPLC analyses were successfully accomplished for all three regions.

**Age Effects**

The average L:Z mass ratio in the whole adult retina was calculated to be 1.86 ± 0.63. This was based on the amounts of L and Z in the three combined regions of the individually analysed retinas. [The effect on this L:Z ratio of the very small amounts of carotenoids which we have measured in the retina beyond the outer annulus (data not shown) is negligible.] By comparison, the same ratio determined for five donors comprising the 0 to 7 month old age group was 3.3 ± 1.8. The difference is significant at P < 0.005 in a two-tailed, independent samples t test. Also from Table I, the average MZ:Z ratio in the whole adult retina was 0.50 ± 0.13, whereas for the 0 to 7 month old group, it was 0.29 ± 0.28. This difference is significant at P < 0.02.

**4. Discussion**

Lutein and zeaxanthin are two of the main xanthophylls found in higher plants (Goodwin, 1992), with lutein generally predominating, and this is the principal source of these carotenoids in the human diet. Higher animals are unable to synthesize carotenoids de novo. Analysis of zeaxanthin in a number of higher plants including corn and pumpkin, and also in commercial chicken egg yolks, indicates that it has the (3R, 3'R, 6'R) configuration, i.e., it is Z (Maoka et al., 1986). It is perhaps not surprising therefore, considering the dietary origin of carotenoids in animals, that, among the zeaxanthin stereoisomers, only Z has been detected in human blood plasma (Bone et al., 1993).

Carotenoids ingested by animals may undergo metabolic transformations into other carotenoids, and this is presumably what is occurring in the human eye where MZ and SZ are present. The lack of detectable levels of these two stereoisomers in human blood argues against their formation elsewhere and subsequent transportation to the retina. Such a mechanism might exist but would require an efficiency of uptake of MZ by the macula greatly exceeding that of L and Z. Apart from minor components identified tentatively as carotenoid cis isomers (Bone and Landrum, 1992), zeaxanthin and lutein are the only known carotenoids deposited in the retinal tissues. As such, they are obvious candidates for precursors of MZ and SZ. The amount of MZ in the macula is considerably. From Table I, MZ represents on average approximately 25% of the total amount of carotenoids in the central disk of tissue. We previously put forward the hypothesis that this stereoisomer might be derived in the eye from L (Bone et al., 1993). The process would require only the migration of a double bond in lutein to the 5',6' position to form meso-zeaxanthin.
would not vary with eccentricity and, furthermore, would match the L:Z ratio in the blood. In fact the average values of (L+MZ):Z for the inner, medial and outer regions of the individually analysed adult retinas are 2.16±0.60, 2.32±0.77, and 2.91±1.21 respectively. (For the pooled samples, the corresponding values, which differ insignificantly (P > 0.5) from the above, are 2.22±0.33, 2.28±0.36, and 3.13±0.60.) These figures are consistent with various analyses of blood serum carotenoids which indicate L:Z ratios in the range of about 2:1 to 4:1 (Krinsky et al., 1990; Craft, 1992; Handelman, Shen and Krinsky, 1992). Analysis of variance of the (L+MZ):Z ratios indicated a significant difference among the three regions [F(2, 46) = 7.57, P < 0.003]. A Fisher’s LSD post hoc test at a 5% significance level indicated no difference between the inner and medial regions but a difference between these and the outer region. The higher (L+MZ):Z ratio here is compatible with an imperfect conversion mechanism which degrades a fraction of L during the postulated conversion process. In the central region, where the conversion rate is high, the loss of L would be correspondingly high and the (L+MZ):Z ratio would be lowered. The low rate of conversion in the outer region would mean a smaller loss of L and a relatively higher (L+MZ):Z ratio.

A logical candidate for the conversion mechanism would be an enzyme, specifically an isomerase, capable of catalysing the reaction of L into MZ. If this were found only in the cone cell axons, the density of which decreases with eccentricity, the corresponding decrease in conversion rate could be rationalized. We have also considered the possibility of a photochemical process. A [1,3]-sigmatropic shift of hydrogen from the 6’ to 4’ carbon of L’s e-ionene ring would convert L into MZ. This process is thermally forbidden but photochemically allowed. There are, however, a number of objections to this mechanism. (1) In vitro experiments with L show no similar conversion into MZ. (2) There is no reason to believe that the average retinal illuminance decreases rapidly with eccentricity from the fovea in a way that would account for the changing conversion rate of L into MZ. (3) A similar process occurring in the β-ionene ring of Z would be equally likely. This would produce [3R, 3’S, 6’R]-lutein, an isomer not found in the retina.

Further support for our hypothesis regarding the conversion of L into MZ comes from our 0 to 7 month old data. Here, a somewhat higher L:Z ratio in the whole retina, compared with adults, is once again associated with a low MZ:Z ratio, reminiscent of the outer region of the adult retina. To reconcile this observation with our model, the purported enzyme would be presumed to be absent or less effective in the newborn.

The origin in the retina of the small quantity of SZ, which is not found in the serum, could be due to a process involving loss of configuration at the 3 and 3’ carbons of Z. This could occur through the biological oxidation of the two hydroxyl groups and subsequent non-specific reduction to produce the three zeaxanthin stereoisomers.

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References


