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Carotenoids in the human retina*

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Abstract: Lutein and zeaxanthin are the predominant carotenoids found in the human retina. The concentration of these carotenoids is greatest in the macula where they form the macular pigment. Within this region, *R*,*S*-zeaxanthin is found in nearly equal abundance to the more commonly occurring, dietary form, *R*,*R*-zeaxanthin. The distribution and metabolism of carotenoids in the tissues of the eye are described. Lutein and zeaxanthin concentration in the blood serum has been found to be positively correlated with a reduced risk of exudative age-related macular degeneration (AMD). The concentration of these carotenoids in the blood serum is also positively correlated with macular pigment density. Taken together, these findings suggest a protective function for the macular pigment against AMD. Results from a comparison of carotenoid levels in AMD and control donor eyes show lower average values in the former group. Recent studies have shown that dietary supplements of lutein or zeaxanthin at around 30 mg/day can significantly increase both the blood serum concentration of these carotenoids and the macular pigment optical density. This raises the possibility of dietary supplementation as a strategy for delaying or preventing the onset of AMD.

INTRODUCTION

The macula lutea [1,2] of the human retina was first recognized to be a carotenoid by George Wald [3] in 1945, nearly 200 years following its discovery [4]. In the early 1960s, Brown and Wald [5] attributed the identity to '. . . lutein and its *cis*-isomers'. As late as 1983, Britton [6] stated the '*The probable function of [the macula lutea] is to absorb . . . blue light around 450 nm . . . from its absorption spectrum the human macular pigment appears to be a carotenoid but it has not been characterized.*' A modern characterization of the components of the macula lutea, hereafter macular pigment, awaited the advent and ready availability of high performance liquid chromatography (HPLC), well suited to the detection and separation of the nanogram quantities of the carotenoids present.

Snodderly and coworkers [7–9], utilizing microdensitometry on thin sections of retinas from primates, have demonstrated that the macular pigment is localized primarily in the Henle fiber layer of the fovea. The thickness of the pigmented tissue was found to be near 50 μm (0.005 cm). The range of optical densities for the pigmented layer, obtained using heterochromatic flicker photometry, is typically 0.2–0.8. One can therefore calculate the concentration of carotenoids to be between 0.3 and 1.3 mM. This is arguably the highest carotenoid concentration found anywhere in the human body. The concentration in liver [10] and serum is three orders of magnitude lower. Despite the remarkable concentration of carotenoid present in the macula, the dimensions of this tissue are such that only nanogram amounts of carotenoids are present.

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CHARACTERIZATION OF THE MACULAR CAROTENOIDS

Our initial characterization of the macular pigment components utilized pooled extracts from several retinas in order to obtain accurate and reliable data [11]. Chromatography of these extracts was carried out on three separate stationary phases: normal silica, a calcium carbonate–calcium oxide–magnesium oxide mixture, and C-18 reversed phase. The order of elution and retention times of the two carotenoid components were shown to be identical to authentic samples of lutein and zeaxanthin by co-injection experiments. Separation and collection of the two components of the macular pigment provided samples of adequate concentration to obtain high quality UV/visible spectra, which again were compared and found to be identical to authentic lutein and zeaxanthin under identical conditions. Further characterization included chromatographic comparison of chemical derivatives of the macular pigment components with those of lutein and zeaxanthin. In later work [12], we were able to obtain convincing mass spectra of the purified macular pigment components and show them to be consistent with those of lutein and zeaxanthin obtained under identical conditions.

In subsequent investigations, we were able to characterize the stereochemical structure of the macular lutein and zeaxanthin utilizing a chiral chromatographic stationary phase after the methods of Maoka *et al.* [13]. The dibenzoate derivative of macular lutein was shown to elute as a single peak which co-eluted with an authentic standard of the 3*R*,3′*R*,6′*R*-stereoisomer of lutein. The dibenzoate derivative of macular zeaxanthin, however, was found to elute from the chiral column as two components of near equal abundance. Using authentic 3*R*,3′*R*-zeaxanthin and a racemic mixture of zeaxanthin stereomers produced by reduction of rhodoxanthin, the two macular zeaxanthin stereomers were shown to be indistinguishable from all-*E* 3*R*,3′*R*-zeaxanthin and all-*E* 3*R*,3′*S*-(*meso*)-zeaxanthin. Thus a final characterization of the macular pigment shows that it is composed of three carotenoids: lutein (3*R*,3′*R*,6′*R*-β,ϵ-caroten-3,3′-diol); zeaxanthin (3*R*,3′*R*-β,β-caroten-3,3′-diol); and *meso*-zeaxanthin (3*R*,3′*S*-β,β-caroten-3,3′-diol). Our identification of the non-dietary *meso*-zeaxanthin as a component of the macular pigment dramatically emphasizes the lack of understanding of xanthophyll metabolism by humans.

DISTRIBUTION WITHIN THE RETINA

The distribution of these carotenoids within the retina is not uniform [14–16]. HPLC of extracts of tissue taken from the central fovea shows that the zeaxanthin isomers dominate over lutein and a zeaxanthin to lutein ratio of 2.4 : 1 is observed. This ratio varies rapidly as a function of eccentricity, shifting to a 1 : 1.9 ratio over a 3 mm spacing and eventually reaches the limiting ratio of 1 : 2.2 at an eccentricity of 6 mm distance from the center of the fovea. The ratio of zeaxanthin to lutein in the serum is between 1 : 3 and 1 : 4, somewhat different from the peripheral retina, but consistent with the dominance of lutein in the diet. An active retinal metabolism of the xanthophylls is implicated by this variability in the distribution. An early suggestion for this was that there might be a preferential uptake by foveal cones for zeaxanthin in preference to lutein. We believe recent data suggest a more likely cause.

The *meso*-zeaxanthin isomer described earlier can be mapped as a function of eccentricity in a manner similar to that used to characterize the relative abundances of lutein and zeaxanthin [17,18]. The *meso*-zeaxanthin isomer is found to parallel the relative abundance of zeaxanthin as a component in the macular pigment. Therefore the percentage of lutein in the macula reaches its lowest value in the same place (the central fovea) where *meso*-zeaxanthin is at its greatest.

A MECHANISM FOR *meso*-ZEAXANTHIN FORMATION

These data suggest a relationship between the two isomeric carotenoids, possibly a conversion of lutein into *meso*-zeaxanthin. This hypothesis would apparently be supported by a comparison of the absolute stereochemical configurations of lutein and *meso*-zeaxanthin. The 3′ C absolute configuration is identical in these two compounds, consistent with a conversion hypothesis. More careful investigation of this idea shows that, while lutein to zeaxanthin conversion may be occurring, conservation of the stereochemical configuration at the 3′ C may be chemically naive.

Khachik *et al.* [19] recently published results from a study of human and primate retinas in which

minor carotenoid components were studied. A significant number of carotenoids were detected, which were characterized on the basis of retention times, UV/visible and mass spectra. Their structures are readily explainable as metabolites of lutein and zeaxanthin via one or more oxidation–reduction steps. It is suggested that *meso*-zeaxanthin is the result of oxidation at the 3′ C of lutein (or zeaxanthin), producing 3-hydroxy-β,ε-caroten-3′-one, followed by reduction. If reduction is accompanied by concomitant migration of the double bond, both *R*,*R*-zeaxanthin and *R*,*S*-*meso*-zeaxanthin result. If the reduction process occurs without double bond migration, *epi*-lutein results. Khachik *et al*. report both *epi*-lutein and 3-hydroxy-β,ε-caroten-3′-one to be present in the retina. An oxidation–reduction pathway is a reasonable pathway between lutein and *meso*-zeaxanthin which does not involve a conservation of the absolute configuration of the stereochemical structure at the 3′ C. The reason zeaxanthin accumulates over lutein may be a result of the more readily oxidized allylic hydroxyl group in lutein and a slight preference in the thermodynamic stability of zeaxanthin as a product relative to lutein.

The study of canthaxanthin accumulation within the primate retina [20,21] and its metabolism is consistent with the oxidation–reduction conversion hypothesis. Canthaxanthin is dramatically accumulated by primate and human retinas. Canthaxanthin, or 'gold dust', retinopathy is a well-documented result of high canthaxanthin consumption and is characterized by the presence of high levels of canthaxanthin, including crystalline inclusions observable in the peripheral retina. In addition to canthaxanthin, the two compounds 4-hydroxy-echinenone and isozeaxanthin (4-hydroxy-β,β-caroten-4′-one and β,β-caroten-4,4′-diol) are present. These compounds are the sequential products formed by reduction of the carbonyls to the corresponding alcohols, apparently within the retina. Significantly, the greatest relative amounts of these two compounds were detected in the macula. The peripheral retina also had detectable quantities, but in lower percentages, reminiscent of the distribution of *meso*-zeaxanthin in the human retina. This is strong circumstantial evidence that keto-carotenoids can be readily reduced within the retina and that the greatest activity exists in the macula.

FUNCTION

Our knowledge of the function of the macular pigment [22] has improved over Britton's 1983 evaluation [6]. The macular pigment indeed may have as its primary function the absorption of blue light, but now we are able to point to possible consequences for the absence or presence of only low levels of macular pigment in human retinas. We now know that the carotenoids can function as antioxidants, singlet oxygen quenchers, and/or as triplet excited state deactivators [23]. If such a function occurs within the retina, it would mitigate the potentially harmful effects of radicals, generated by the presence of light and oxygen, on the high levels of polyunsaturated fatty acids found in the retinal membranes.

MACULAR PIGMENT AND AGE-RELATED MACULAR DEGENERATION

Oxidative insult has been recognized as an important factor in the etiology of age-related macular degeneration (AMD), thereby raising the possibility of a protective function for the macular pigment against this disease. Support for this hypothesis is provided by the Eye Disease Case-Control Study Group. Seddon *et al.* [24] examined carotenoid intake in relation to the incidence of exudative (wet) AMD. Those subjects in the highest quintile of lutein and zeaxanthin intake had a 57% lower risk for AMD compared with those in the lowest quintile. An even greater reduction in risk was observed for those in the highest quintile of lutein and zeaxanthin concentration in the serum compared with those in the lowest quintile [25]. Further support for the protection hypothesis is provided by the observation that certain risk factors for AMD are correlated with relatively low concentrations of macular pigment. Thus subjects of female gender, those having light colored irises, or those who smoke were found to have lower concentrations than males, those with dark irises, or non-smokers, respectively [26–28].

In order to investigate the protection hypothesis, we have conducted a comparative study of carotenoid concentrations in human donor retinas both with and without AMD. The study, to date, has involved 56 AMD donors and 45 control donors, reasonably matched in terms of both age and sex (see Table 1). The retinas were removed from each eye and cut into three concentric regions using a device previously described [14]: a disk centered on the fovea covering the range of visual angles 0–5 ° ('inner') and two

Table 1 Human donor characteristics

	AMD	Control
Number	56	45
Age	81.6 ± 7.7	77.2 ± 7.5
(Range)	(65–98)	(58–95)
Sex	21M, 34F	17M, 22F, 5?

annuli covering the ranges 5 °–19 ° and 19–38 ° ('medial' and 'outer'). After adding an internal standard (lutein monopropyl ether), the carotenoids were extracted using standard procedures [18] and analyzed by reversed-phase HPLC (Ultracarb 3 μm ODS, 250 × 2 mm column; acetonitrile–methanol (90 : 10) at 0.2 ml/min). From the resulting chromatograms, the combined quantities of lutein and zeaxanthin per unit area of retinal tissue were determined. For most donors, data were obtained from both eyes and averaged. In a few instances, data were available from one eye only.

The results are summarized in Fig. 1. A direct comparison of the total carotenoid per unit area in the inner disk and medial and outer annuli showed that the AMD group had, on average, 61%, 72% and 80%, respectively, of the amounts found for the control group. These differences are significant at the $P < 0.001$, < 0.005, and < 0.05 levels, respectively.

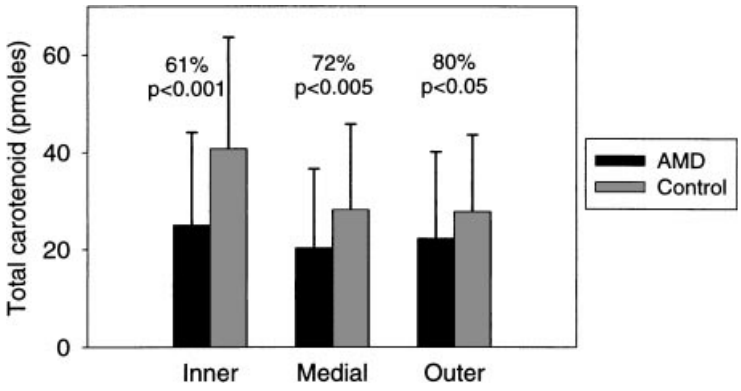

Fig. 1 Comparison of carotenoid concentrations in different regions of the retinas of AMD and control donors.

A limitation of this study is the sparsity of available donor information. In most cases, the only information on AMD donors was that they had, at some stage, been diagnosed with the disease, but the duration or severity was not recorded. In a few cases, the diagnosis included the type of AMD, wet or dry. It is also conceivable that some of the donors had AMD but this was not diagnosed at the time of death. The net result would be to blur any genuine differences in macular pigmentation that might exist between AMD cases and controls. Thus the differences in Fig. 1 may significantly underrepresent the true state of affairs.

The difference in macular pigment density between AMDs and controls tends to decrease from the inner to medial to outer regions, raising the possibility that the disease itself may be partly responsible for a preferential loss of lutein and zeaxanthin in the macula. Therefore to test the hypothesis that low amounts of lutein and zeaxanthin in the macula represent a risk factor for AMD, it may not be appropriate to use the carotenoid concentration in the inner disk as a measure of the eye's ability to accumulate carotenoids. On the other hand, the carotenoid concentration in the outer annulus should be relatively unaffected by AMD when this is present in a donor's eye. Furthermore, the outer annulus provides a reasonable measure of the carotenoid concentration elsewhere in the normal retina, as can be judged from Fig. 2.

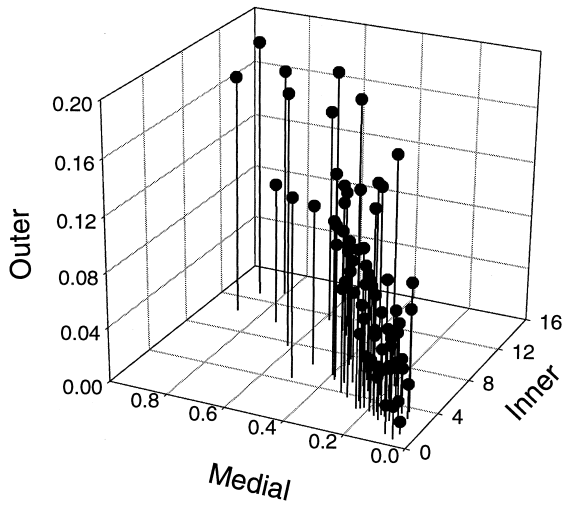

Fig. 2 Comparison of carotenoid concentrations (pmol/mm^2) in the 'inner', 'medial', and 'outer' regions of the retina. Only control eyes were used.

Following this line of reasoning, control subjects were divided into quintiles of carotenoid concentration in the outer annulus. The number of AMD donors in each quintile, and hence the odds ratios relative to the lowest quintile, together with the 95% confidence intervals (CI) were determined. These results are shown in Table 2, along with the median carotenoid concentrations for each quintile. The table clearly shows a trend of decreasing risk of AMD with increasing carotenoid concentration in the outer annulus. In particular, it was only 27% as likely for AMD donors to be in the highest compared to the lowest quintile than for controls. In other words, those in the lowest quintile had a 73% higher risk for AMD compared with those in the highest quintile.

Table 2 Odds ratios relative to the lowest quintile of carotenoid concentration in the outer annulus (L, lutein; Z, zeaxanthin)

Quintile	Median L + Z (pmol/mm^2)	AMD/control	Odds ratio	95% CI
1	0.025	22/9	1.0	
2	0.048	12/9	0.55	0.17–1.74
3	0.074	9/9	0.41	0.12–1.37
4	0.108	7/9	0.32	0.09–1.12
5	0.142	6/9	0.27	0.08–0.99

From these data, we conclude that very low carotenoid levels in the retina are associated with an increased incidence of AMD. However, this association may not be causal. It could be, for example, that processes which lead to AMD are also responsible for the retina's sluggishness in accumulating carotenoids.

For 24 of the donor eyes, a comparison was made between the combined amount of lutein and zeaxanthin in the outer annulus and the concentration of these carotenoids in the donor's blood serum. The latter was also determined by HPLC. As shown in Fig. 3, these quantities were reasonably linearly correlated, with an r of 0.58 that was highly significant ($P < 0.003$). Therefore, about one-third of the variability in the subjects' macular pigment density can be inferred from the serum concentration of lutein and zeaxanthin. The rather low correlation coefficient is perhaps not surprising. The concentration of lutein and zeaxanthin in the serum can change on a time-scale of days, while, in the retina, changes are much slower (see below) and the macular pigment density probably reflects a long-term average dietary intake.

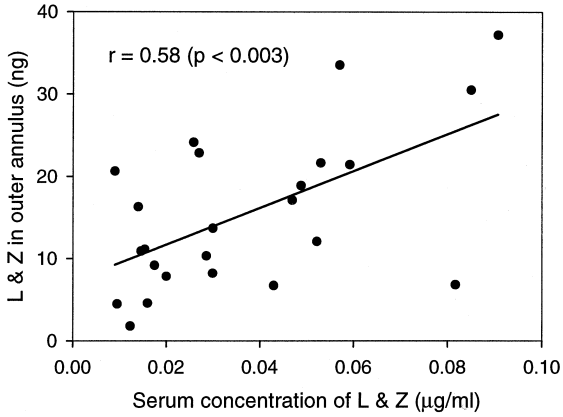

Fig. 3 Total lutein (L) and zeaxanthin (Z) in the outer annulus versus serum concentration of these carotenoids in 24 donors.

A similar study was conducted using a group of 19 human volunteers. Its purpose was to examine correlations between blood serum concentrations of lutein and zeaxanthin, macular pigment density, and dietary intake of lutein and zeaxanthin. The latter was determined using food frequency questionnaires, and each subject's macular pigment density was determined by heterochromatic flicker photometry. With this technique, the subject views a stimulus that alternates between 460 nm (peak macular pigment absorbance) and 540 nm (zero absorbance). The subject adjusts the 460 nm intensity to obtain a flicker null, the setting depending on the relative pre-receptor transmittances and relative receptor sensitivities at these two wavelengths. By making measurements in both the fovea and extra-fovea, the macular pigment transmittance at 460 nm may be found from the ratio of intensity settings in these two regions.

A highly significant correlation was found to exist between the subjects' serum concentration of lutein and dietary intake of lutein and zeaxanthin ($r = 0.67$, $p = 0.002$) (see Fig. 4). Similarly, between the macular pigment density and serum concentration of lutein, the correlation was significant ($r = 0.52$, $p = 0.002$), a result comparable to that obtained with autopsy eyes (see Fig. 5). Once again the correlation coefficients are not expected to be high since the food frequency questionnaire reflects long-term eating habits, while the serum is influenced by recent intake. Nonetheless, we may conclude that, in general, serum concentrations of lutein and zeaxanthin are determined by the diet and themselves determine the density of macular pigment.

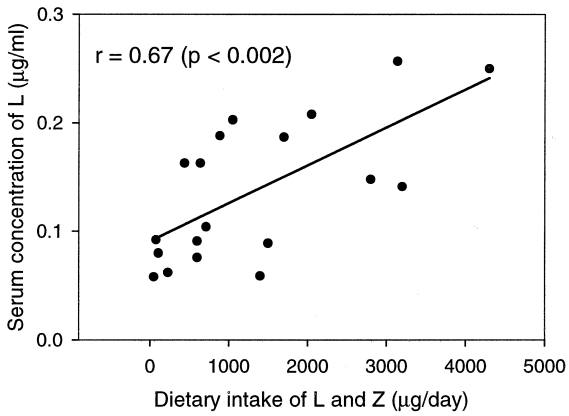

Fig. 4 Serum concentration of lutein versus dietary intake of lutein and zeaxanthin for 19 human subjects.

With this in mind, and the possible protection afforded by the macular pigment against AMD, it is of interest to know whether the macular pigment density can be altered significantly by modification of the

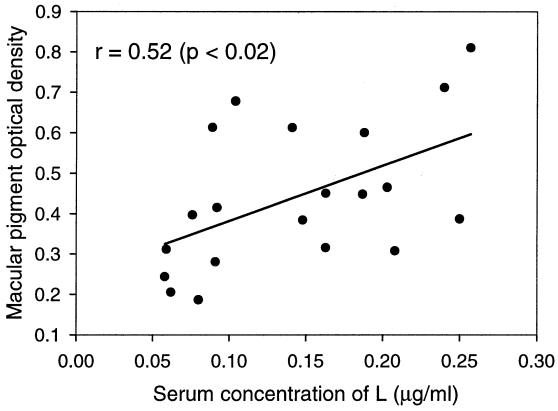

Fig. 5 Macular pigment optical density versus serum concentration of lutein for 20 human subjects.

diet. We have conducted supplementation studies using both lutein (as diester) and zeaxanthin. With dosages of 30 mg day for periods of 3–4 months, significant increases were achieved in both the subjects' serum concentration of the carotenoids and their macular pigment optical density [29,30]. In addition, studies have been conducted elsewhere on the effects of increased intake of foods rich in lutein and zeaxanthin. Subjects were fed spinach and corn, these providing 10.8 mg/day of lutein and 0.3 mg/day of zeaxanthin. Increases in macular pigment density were observed for most, but not all, of the subjects [31].

Thus, if future studies indicate that a low level of macular pigment in a patient constitutes a risk factor for AMD, there is a high probability that modification of the diet to increase the intake of lutein and/or zeaxanthin will be an effective way of minimizing that particular risk.

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