Pure Appl. Chem., Vol. 74, No. 8, pp. 1419–1425, 2002.

New insights into the role of the macular carotenoids in age-related macular degeneration. Resonance Raman studies*

Paul S. Bernstein‡

Moran Eye Center, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, UT 84132, USA

Abstract: The human macula uniquely concentrates extraordinarily high levels of two xanthophyll carotenoids, lutein and zeaxanthin. The function, metabolism, and physiology of these yellow pigments are incompletely understood, but they are likely to prevent age-related damage to the foveal region by virtue of their ability to act as free-radical quenching antioxidants and to absorb phototoxic blue light with high efficiency. A wealth of circumstantial evidence suggests that high macular levels of these two carotenoids may protect against age-related macular degeneration (AMD), but definitive prospective clinical studies still remain to be conducted. It is imperative to gain a greater knowledge of the basic biochemical and physiological mechanisms underlying the specific uptake and metabolism of lutein and zeaxanthin in the macula and to develop improved methods of quantifying macular carotenoid levels noninvasively in order to facilitate the rational design of successful interventions against the leading cause of irreversible blindness in the elderly in the developed world. The development of resonance Raman spectroscopic methods for the objective measurement of macular carotenoid levels in living humans with and without AMD will be reviewed.

Age-related macular degeneration (AMD), the leading cause of irreversible blindness in the developed world, is a disorder that results from the complex interaction of numerous inherited and acquired risk factors including increasing age, smoking, family history, race, light exposure, and nutrition. It destroys the central vision of one or both eyes through damage to the macula, the cone-rich region of the retina responsible for high-acuity vision necessary for reading, driving, and recognizing faces. The damage may be either atrophic (dry AMD) or due to complications of choroidal neovascularization (wet AMD).

While many new treatments have been introduced for patients with advanced cases of the exudative form of AMD, the vast majority of these individuals will still progress to legal blindness. Thus, there is considerable interest among both patients and clinicians to intervene at much earlier stages of the disease so as to slow its progression at a time before substantial visual damage has occurred. Nutritional interventions with antioxidant supplements have been widely touted in the United States for many years to individuals at risk for AMD, but high-quality scientific data to support the marketing claims have been notably lacking. The recent publication of the results of the Age-Related Eye Disease Study (AREDS) finally puts some of these nutritional claims on solid scientific footing [1]. This very large long-term prospective placebo-controlled study demonstrated that oral supplementation with antioxidant vitamins and minerals (zinc, vitamin C, vitamin E, and beta-carotene) could slow the rate

*Lecture presented at the 13th International Symposium on Carotenoids, Honolulu, Hawaii, USA, 6–11 January 2002. Other presentations are presented in this issue, pp. 1369–1477.

‡Contact information: Tel: (801) 581-6078; Fax: (801) 581-3357; E-mail: paul.bernstein@hsc.utah.edu

of progression to advanced stages of AMD with severe visual loss in patients with intermediate stages of AMD or with advanced AMD in the contralateral eye.

While AREDS is an extremely important study, it is just the beginning of our understanding of the role of nutrition and antioxidants in AMD. The AREDS authors readily admit that their formulation is not optimized and that further studies are required. They specifically mention that the macular pigment carotenoids lutein and zeaxanthin have a very high potential to be protective against AMD and that they should be explored further [1,2]. It has been hypothesized that high levels of these two carotenoids in the macula may protect against age-related eye disease through antioxidant and light-screening mechanisms [3,4]. Two reports by the Eye Disease Case-Control Study Group demonstrated that high blood levels and high dietary intakes of these carotenoids were associated with a significantly lower risk of advanced AMD [5,6]. An autopsy study likewise has shown that eyes donated from patients with a clinical history of AMD had significantly lower macular and peripheral retinal levels of lutein and zeaxanthin relative to donor eyes from subjects without a known history of AMD [7].

Lutein and zeaxanthin are dihydroxycarotenoids that are derived exclusively from dietary sources such as dark green leafy vegetables and orange and yellow fruits and vegetables. They are xanthophylls by virtue of the presence of at least one oxygen atom on the basic $C_{40}H_{56}$ carotene structure, and lutein and zeaxanthin differ only in the position of one double bond. Metabolism of carotenoids in the mammal is quite limited—hydrocarbon carotenoids such as β-carotene cannot be converted to xanthophylls, and xanthophylls cannot be metabolized to hydrocarbon carotenes. Some carotenoids, such as beta-carotene, can be cleaved at the 15-15′ bond to generate vitamin A aldehyde (retinal), but most xanthophylls are not substrates for the cleavage enzyme, and even if they were, they would generate 3-hydroxy-retinoids, which possess no vitamin A activity [8,9].

The macula of the human eye contains by far the highest concentrations of these two carotenoids relative to any other tissue in the body, and their uptake into neural retinal tissues is extraordinarily selective. Over 15 different dietary carotenoids are detectable in human serum, yet only lutein and zeaxanthin and their metabolites are found to any substantial extent in the retina [10]. By contrast, other adjacent ocular tissues, such as the RPE/choroid and the ciliary body contain a much wider spectrum of carotenoids, including lycopene, beta-carotene, and beta-cryptoxanthin (Fig. 1) [11]. In the foveal region of the retina, lutein and zeaxanthin concentrations are at their highest, and they are spatially localized to the Henle fiber layer and to the inner plexiform region [12]. The concentration of the mac-

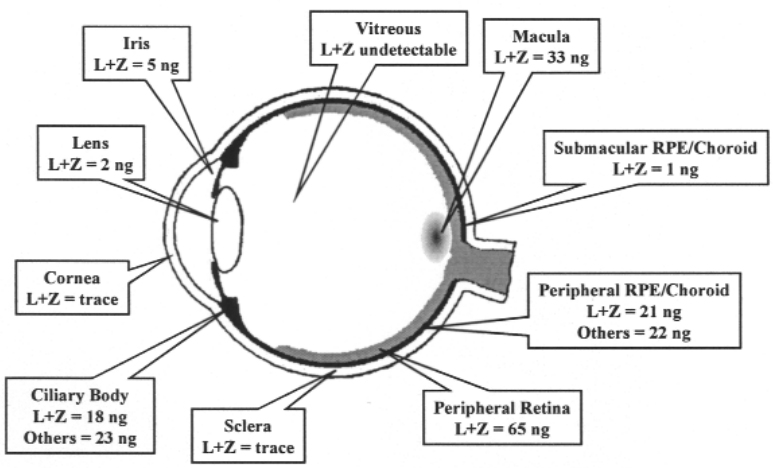

Fig. 1 Typical lutein (L) and zeaxanthin (Z) levels in the human eye [11].

ular carotenoids falls precipitously outside of the foveal region—the concentration of lutein and zeaxanthin in the peripheral retina per unit area is just 1 % of the concentration at the fovea [13,14], and a considerable portion of the extrafoveal carotenoids is associated with the rod outer segments [15,16].

Whenever a tissue exhibits highly specific uptake and deposition of a biological molecule, it is likely that appropriate binding proteins are involved. These proteins may act as cell surface receptors, as transmembrane transport proteins, as intracellular mediators of the biological actions of the ligand, or as sites for the deposition and stabilization of the ligand. The structure, function, and molecular biology of retinoid binding proteins in the retina have been explored in exquisite detail, but much less is known about specific carotenoid binding proteins in the vertebrate eye. In fact, while a number of carotenoid binding proteins have been described in plants [17], microorganisms [18], and invertebrates [19–21], there was only one report of a specific carotenoid binding protein in any mammalian system before 2001, a beta-carotene binding protein prepared from ferret liver [22]. Recently, a membrane-associated xanthophyll binding protein (XBP) from human retina that binds lutein and zeaxanthin with high affinity and specificity has been partially purified and characterized [23].

In order to explore the role of lutein and zeaxanthin in modifying the risk of AMD, it is important to be able to quantify their levels noninvasively with high reliability. While the subjective psychophysical test known as heterochromatic flicker photometry has been used most frequently [24], we and others have found the test to be quite unreliable in elderly subjects, especially if they have significant ocular pathology [25]. As an alternative to flicker photometry, we have developed and patented an objective laser optical method based on resonance Raman spectroscopy [26–28].

When illuminated with monochromatic light, all compounds scatter the light. The vast majority of this light is scattered elastically at the same wavelength, a phenomenon known as Rayleigh scattering. A small proportion is scattered inelastically with wavelength shifts dependent on vibrational modes determined by the compound's molecular structure, a phenomenon known as Raman scattering. Carotenoids' Raman spectra result primarily from their long rigid polyene backbones with characteristic peaks at 1008 cm^{-1} ($C–CH_3$ bend), 1159 cm^{-1} (C–C stretch), and 1525 cm^{-1} (C=C stretch), and signal heights scale linearly with concentration [29]. Ordinarily, Raman scattering from a molecule is very weak, and high-powered lasers are required along with high-sensitivity detectors, and Raman spectra from biological materials can be quite difficult to interpret due to their complexity. Under certain circumstances in which the illuminating laser light overlaps with a major absorption band of the compound, the generated Raman signals can be resonantly enhanced by many orders of magnitude [29]. A resonant enhancement of up to 10^5 can be achieved when 488 nm argon laser light is used to illuminate carotenoids, greatly facilitating detection of characteristic carotenoid Raman spectra even at very low concentrations in complex biological systems.

All carotenoids measured in vivo or in vitro have very similar Raman spectra because they share a common isoprenoid polyene backbone (Fig. 2). We would not expect a major shift of the Raman peaks of the carotenoid ligand on XBP unless there is major distortion of the molecule in a rigid binding pocket, a very unlikely possibility in light of the relatively small bathochromic shift in the visible spectrum [30]. Indeed, we found the resonance Raman spectrum of XBP carrying a lutein or zeaxanthin ligand to be indistinguishable from the carotenoids in organic solution (data not shown). While this was disappointing, we realized that resonance Raman spectroscopy could be a valuable method to quantify macular carotenoid pigment levels noninvasively. Our initial work employed a bulky, expensive laboratory-grade Raman spectrometer and liquid nitrogen-cooled detector along with a high-power argon laser. Using human cadaver maculae mounted between glass slides, we were able to measure macular carotenoid Raman signals at approximately 100-μm spatial resolution, and we were able to show that the Raman signal scales linearly with macular carotenoid content as determined by high-performance liquid chromatography (HPLC) (Fig. 3) [27]. Next, using this same apparatus, we were able to record carotenoid Raman spectra from intact frog eyes [27].

Encouraged by these initial results, we constructed a resonance Raman device more suitable for use in a clinical setting (Fig. 4) [31]. Laser power was lowered, illumination time was decreased, and

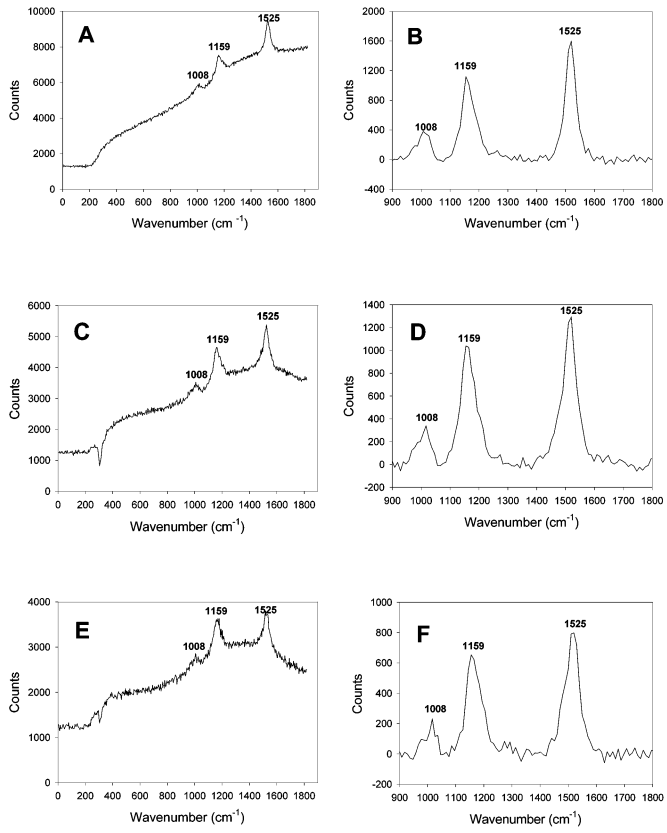

Fig. 2 Resonance Raman spectra of living human macula (A,B), zeaxanthin (C,D), and lutein (E,F) before (A,C,E) and after (B,D,F) subtraction of background fluorescence [32].

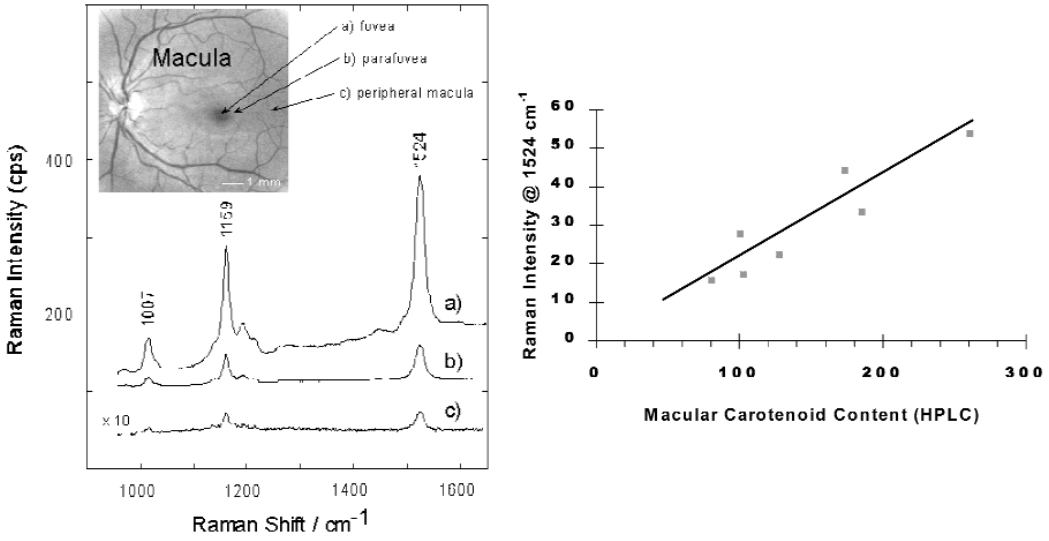

Fig. 3 Spatial dependence of macular carotenoid pigment distribution measured by Raman method on a human donor retina (left panel) and linear relation of macular carotenoid pigment distribution measured on the human macula samples by HPLC or by the resonance Raman method (right panel) [27].

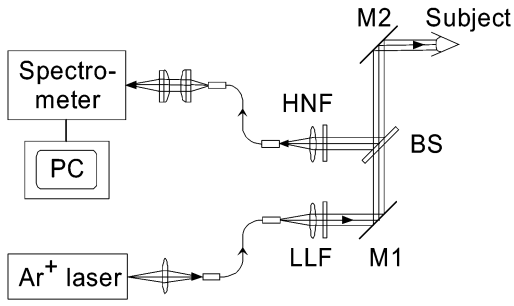

Fig. 4 Schematic diagram of macular resonance Raman spectrometer designed for human studies. LLF: laser line filter; HNF: holographic notch filter; BS: beam splitter; M1: mirror 1; M2: mirror 2; PC: personal computer. Typical settings are 0.5 mW of 488 nm light for 0.5 s with a 1-mm spot size on the macula [31,32].

spot size was enlarged in order to bring the illumination levels of the macula within ANSI standards. Fiber optics replaced the laboratory optical bench to make it more stable and less prone to misalignment. The spectrometer was changed to a high-throughput lower-resolution spectrometer coupled with a low-noise, Peltier-cooled charge-coupled device (CCD) detector to increase the signal-to-noise ratio. Windows-based software was developed to allow for data display and analysis in less than 1 s. The device could be readily calibrated against known concentrations of lutein and zeaxanthin in a 1-mm path length quartz cuvette at the focal point of an optically correct model eye [32]. We initially interfaced the device with a fundus video camera and tested it on living Macaque monkeys, and we were able to confirm spatial resolution and linearity of detector response with actual carotenoid content as determined by HPLC [31].

We have measured macular carotenoid pigment levels in hundreds of individuals with and without significant macular pathology, and we have been able to demonstrate conclusively that macular carotenoid levels decline with age (Fig. 5) and that levels are significantly lower in unsupplemented AMD eyes relative to age-matched controls (Fig. 6) [32]. If AMD patients have begun taking high-dose lutein supplements, their levels are in the "normal" range. These findings are consistent with the hypothesis that low levels of macular carotenoid pigment are associated with an increased risk of developing AMD. The Raman instrument is well accepted by patients, and it appears to be a substantial

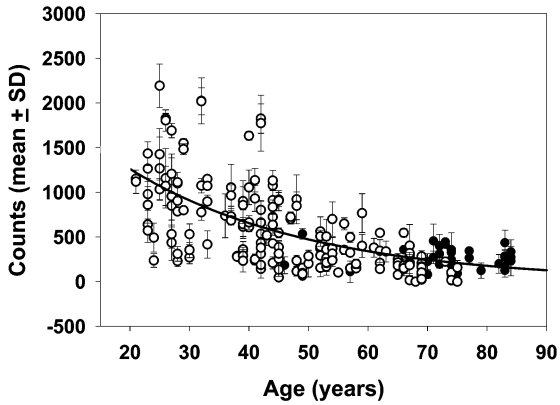

Fig. 5 Decline of macular carotenoid pigment with age in normal subjects as measured by resonance Raman spectroscopy. Open circles are eyes with natural crystalline lenses. Filled circles are eyes with prosthetic intraocular lenses placed after cataract surgery [31].

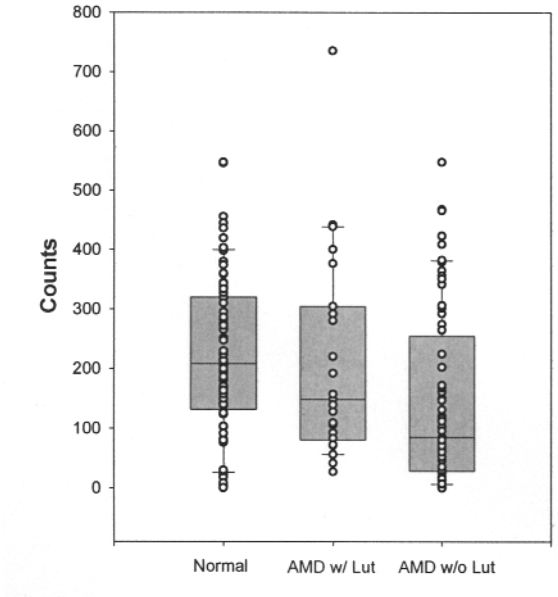

Fig. 6 Box plot of macular carotenoid levels in normal elderly individuals and in age-matched AMD patients with and without regular high dose (≥4 mg/day) lutein (Lut) supplementation [32].

improvement over the psychophysical test commonly used in the past to measure macular pigment levels—heterochromatic flicker photometry. As a psychophysical test, flicker photometry is by definition subjective, and it has never been proven reliable in subjects with significant macular pathology such as AMD [25]. Raman measurement of macular pigment levels, on the other hand, is an objective optical technique that is quite reliably performed as long as the subject has central fixation—typically an acuity of 20/80 or better. Currently, we are comparing the Raman device to flicker photometry in a prospective placebo-controlled lutein supplementation trial in normal subjects. We and others plan to use the Raman device in prospective AMD trials in the near future. Eventually, this instrument might be used as a screening tool to detect young individuals with abnormally low levels of macular pigment who may be at increased risk of developing AMD later in life. Dietary interventions could be instituted, and response to treatment could be monitored with the device.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Eye Institute (EY-11600 and EY-12324), Kemin Foods, Des Moines, IA, and Research to Prevent Blindness, New York, NY.

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