

Transformations of Selected Carotenoids in Plasma, Liver, and Ocular Tissues of Humans and in Nonprimate Animal Models

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PURPOSE. To determine the stereochemistry of carotenoids in human ocular tissues in comparison with plasma and liver and to elucidate the possible transformations of dietary (3*R*,3'*R*,6'*R*)-lutein and (3*R*,3'*R*)-zeaxanthin in the eye. Similarly, to characterize the carotenoid profiles in the eye tissues, plasma, and liver of quails and frogs to determine whether these can serve as appropriate nonprimate animal models for metabolic studies.

METHODS. Configurational isomers of carotenoids and their nondietary by-products from pooled human plasma, liver, retinal pigment epithelium (RPE-choroid), ciliary body, iris, and lens were characterized and quantified by high-performance liquid chromatography (HPLC) on a chiral column. Carotenoids and their nondietary by-products in pooled extracts from quail and frog plasma, liver, retina, RPE-choroid, iris, and lens were similarly characterized and quantified.

RESULTS. (3*R*,3'*R*,6'*R*)-lutein, (3*R*,3'*R*)-zeaxanthin, (3*R*,3'*S*; *meso*)-zeaxanthin, (3*R*,3'*S*,6'*R*)-lutein (3'-epilutein), 3-hydroxy- β , ϵ -carotene-3'-one, and 5*Z*- and *all-E*-lycopene were detected in nearly all human ocular tissues examined. (3*R*,3'*S*; *meso*)-zeaxanthin was not detected in the human plasma and liver but was present in human macula, retina, and RPE-choroid. (3*S*,3'*S*)-zeaxanthin was detected in human macula in minute quantities. The carotenoid profiles in quail and frog ocular tissues were somewhat similar to those in humans, with the exception that lycopene was absent. Frog retina, plasma, and liver revealed the presence of (3*S*,3'*S*)-zeaxanthin.

CONCLUSIONS. The most likely transformations of carotenoids in the human eye involve a series of oxidation-reduction and double-bond isomerization reactions. Quail and frog appear to possess the appropriate enzymes for conversion of dietary (3*R*,3'*R*,6'*R*)-lutein and (3*R*,3'*R*)-zeaxanthin to the same nondietary by-products observed in humans and thus may serve as excellent nonprimate animal models for metabolic studies. (*Invest Ophthalmol Vis Sci.* 2002;43:3383-3392)

Although 25 dietary carotenoids and 9 of their nondietary by-products have been identified in human serum, milk, and tissues,¹⁻³ the major carotenoids in human ocular tissues are (3*R*,3'*R*,6'*R*)-lutein, (3*R*,3'*R*)-zeaxanthin, lycopene (*all-E* + 5*Z*), and their oxidative by-products.⁴⁻⁶ Findings in several epidemiologic studies conducted in the early 1990s suggest a beneficial role for carotenoids and antioxidant vitamins in the prevention of neovascular age-related macular degeneration (AMD).⁷⁻⁹ Meanwhile, in a nested case-control study consisting of subjects with retinal pigment abnormalities with the presence of drusen ($n = 127$) or with late AMD (geographic atrophy [$n = 9$] or exudative AMD [$n = 31$]) and an equal number of control subjects ($n = 167$), the serum concentration of lutein and zeaxanthin were found to be unrelated to the risk of incidence of AMD.¹⁰ This study also revealed that persons with the levels of serum lycopene (red carotenoid in tomato) in the lowest quintile were twice as likely to have AMD. More recently, the Age-Related Eye Disease Study (AREDS) research group found a beneficial effect for supplementation with a combination of zinc, vitamin C, vitamin E, and β -carotene in individuals at high risk for disease progression to advanced AMD.¹¹ For a review of the evidence for protection against AMD by carotenoids and antioxidant vitamins, see the publications by Snodderly¹² and Schalch et al.¹³

The possible role of lutein and zeaxanthin in protection from AMD has been investigated by Landrum et al.¹⁴ who have shown the loss of these macular pigments across all areas of the macula in AMD donor eyes in comparison with control eyes. Recently, Bernstein et al.¹⁵ demonstrated that eyes with AMD have 32% lower levels of macular carotenoids than do age-matched control eyes when measured in vivo by resonance Raman spectroscopy. Macular carotenoids are thought to function as an optical filter by absorbing short-wavelength visible light and reducing chromatic aberration.¹⁵ This may also prevent photochemical damage to cones and retinal pigment epithelium (RPE) in the fovea.^{15,16,17} Another mechanism by which macular carotenoids may provide protection against AMD may involve their antioxidant function.^{13,14,18-22} In 1997, we provided preliminary evidence for the possible photo-protective role of lutein and zeaxanthin in the retina as antioxidants in the prevention of AMD. This was accomplished by identification and quantification of lutein, zeaxanthin, and their oxidation products in human and monkey retinas.⁵ Based on these findings, we postulated that dietary lutein and zeaxanthin function as antioxidants that undergo a series of transformations to protect the macula against AMD. However, ca-

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rotenoids are highly concentrated in the plexiform layers of the fovea where an antioxidant mechanism of action would not be expected. Rapp et al.²³ have provided the "missing link" in this theory by detecting lutein and zeaxanthin in the photoreceptor outer segment (rod outer segment; ROS) membranes of the human retina—the region of the retina with the highest concentration of long-chain polyunsaturated fatty acids that exist in high oxygen tension and therefore the most susceptible to oxidative insult.^{24,25} More recently, we identified lutein, zeaxanthin, lycopene, and their nondietary by-products in nearly all structures of the human eye, including the peripheral retina, which contains ROS and many other cells of neural origin.⁶ These findings provide further support for the critical role of lutein and zeaxanthin in protecting the eye from light-induced oxidative damage. For example, although current evidence suggests that carotenoids in the eye may undergo photo-induced and/or enzymatic transformation to their by-products, the nature of the reactions and the enzymes that may be involved are not known. This is primarily due to the lack of an appropriate nonprimate animal model that can metabolize carotenoids in ocular tissues in a manner similar to humans.

The current study was designed to identify and quantify dietary carotenoids, their configurational isomers, and their by-products in human donor eye tissues (retina, macula, RPE-choroid, ciliary body, iris, and lens) in comparison with plasma and liver. It was anticipated that these comparative profiles would differentiate between those carotenoid by-products that may be formed locally in the eye by independent processes from those that may originate from the circulating blood. We used this strategy to demonstrate that Japanese quails (*Coturnix japonica*) and frogs (*Rana pipiens*) can serve as excellent nonprimate animal models for investigating the metabolism of dietary (3*R*,3'*R*,6'*R*)-lutein and (3*R*,3'*R*)-zeaxanthin. These studies draw parallels between the possible pathways of carotenoids in the eye tissues of the animal models and those of humans.

MATERIALS AND METHODS

Source and Sample Preparation

Human donor human eyes were obtained from the Utah Lions Eye Bank within 24 hours after death. Human liver samples were obtained from the Surgical Pathology Unit of Johns Hopkins Medical Institution (Baltimore, MD). Tissue procurement and distribution complied with the tenets of the Declaration of Helsinki. The use of quails and frogs was approved by the University of Utah's Animal Care and Use Committee, and the investigations reported in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes with any discernible ocular disease except for pseudophakia were excluded. All ocular tissues (retina, RPE-choroid, ciliary body, iris, and lens) were dissected at the University of Utah, frozen at -70°C , and shipped on dry ice to the University of Maryland. Dissection of the globes was performed under dim light and on ice to prevent degradation and photo-oxidation of carotenoids. Ciliary body was defined as the area of the uvula between the root of the iris and the ora serrata. Dissected ciliary bodies were separated from the lens and its zonules and gently rinsed in 10 mL of ice-cold isotonic phosphate-buffered saline to remove blood and vitreous. Maculae and submacular RPE-choroid were excised with a 5-mm circular trephine centered on the fovea, after which the retinal tissues were peeled from the underlying RPE-choroid and carefully separated from adhering vitreous. Frozen (-70°C) human plasma was procured from the American Red Cross (Diagnostic Manufacturing Division, Gaithersburg, MD).

Adult Japanese quails (*Coturnix japonica*) were purchased from Stromberg's Chicks & Gamebirds Unlimited (Pine River, MN), and wild-caught adult frogs (*Rana pipiens*) were purchased from Sullivan's Amphibian Supply (Nashville, TN). Dark-adapted quails were anesthe-

tized with carbon dioxide, and frogs were cooled on ice in the dark. Animals were killed by decapitation, and blood was collected by exsanguination supplemented by cardiac puncture. Eyes were enucleated, and ocular tissues were dissected with the aid of a dissecting microscope. Ocular tissues were stored in separate tubes at -70°C . Livers were removed and stored at the same temperature. Blood and animal tissues were shipped to the University of Maryland on dry ice. Plasma samples were prepared by centrifuging the blood in the presence of EDTA, disodium salt, dihydrate (2 mg/mL of blood) at approximately 12,000 g for 15 minutes and stored at -70°C .

Extraction of Carotenoids

Human and animal tissues were pooled, weighed, and extracted three times with tetrahydrofuran (THF, 3×10 mL) containing 0.1% butylated hydroxytoluene (BHT) in the presence of anhydrous sodium sulfate (20% by weight of tissue) by sonication at 5°C to 10°C for 30 minutes each time. The combined extracts were evaporated to dryness on a rotary evaporator under reduced pressure at below 40°C . The residue was dissolved in dichloromethane (4 mL) and filtered through a 0.45- μm disposable polyvinylidene fluoride filter assembly (Acrodisc; VWR Scientific Products, Bridgeport, NJ) into a 5-mL graduated micro-sample vial. The solvent was evaporated under nitrogen, and the extracts were redissolved in the appropriate HPLC injection solvent (mobile phase). The vials were centrifuged at approximately 2000g to remove the minor amounts of insoluble solid particles; 50- μL samples were injected onto the HPLC system. All animal tissues were examined under three different HPLC conditions, before and after saponification.

Saponification of Human Liver Extract

A tissue extract from human liver (4.4 g) in 20 mL THF was treated with 5 mL 10% potassium hydroxide in methanol and the mixture was stirred at room temperature for 2 hours. The solution was evaporated to dryness under reduced pressure, and the residue was partitioned between hexane (20 mL) and 5% sodium chloride (NaCl) solution (20 mL). The aqueous layer was removed, and the organic layer was washed twice (2×20 mL) with NaCl solution and dried over sodium sulfate. After evaporation of the solvent, the residue was redissolved in 5 mL hexane and passed through a solid-phase extraction cartridge containing 900 mg *n*-silica gel (Burdick & Jackson, Inc., Muskegon, MI). The cartridge was first washed with 30 mL hexane to remove the lipids and then with 30 mL of a 3:1 mixture of hexane-acetone to remove the carotenoids. The carotenoid fraction was evaporated to dryness and redissolved in 0.3 mL of the appropriate HPLC injection solvent for analysis.

Saponification of Pooled Extracts from Tissues of Quails and Frogs

Pooled extracts from liver and ocular tissues of quails and frogs were dissolved in dichloromethane (2 mL) and were saponified with 1 mL of 1% potassium hydroxide in methanol (wt/vol) for 30 minutes at room temperature. The mixture was transferred into a separatory funnel and washed with water (4×5 mL) until the pH of the aqueous layer was 7, and then the mixture was dried over anhydrous sodium sulfate. The solution was evaporated to dryness on a rotary evaporator under reduced pressure at below 40°C . The residue was dissolved in dichloromethane (4 mL), microfiltered through a 0.45- μm disposable filter assembly, reconstituted in the appropriate HPLC solvents, and centrifuged as above before analysis.

Extraction of Plasma from Human, Quails, and Frogs

Human plasma (12 mL) was treated with ethanol (12 mL) and ethyl ether (12 mL) containing 0.1% BHT, and centrifuged to remove the proteins. The proteins were re-extracted with ethyl ether (3×12 mL), and the combined extract was evaporated to dryness on a rotary evaporator under reduced pressure at below 40°C . The residue was

microfiltered through a 0.45- μm disposable filter assembly with dichloromethane. After evaporation of the solvent, the residue was reconstituted in the appropriate HPLC solvents and centrifuged at 2000g before analysis. Pooled blood samples from quails (40 mL) and frogs (5 mL) resulted in 18 and 2 mL plasma from these animals, respectively. These plasma samples were similarly extracted with ethanol and ethyl ether, and prepared for HPLC, according to the procedures described earlier.

Chromatographic System and HPLC Analysis

The analyses were performed on an HPLC system (model 1100; Agilent Technology, Palo Alto, CA) equipped with a quaternary solvent delivery system autosampler, thermostat-controlled column compartment, and diode array detector. The data were stored and processed by computer (Kayak XM600 with Windows NT, with HP Chem-Station software, a 19-in. color display monitor, and a Laserjet 4050 printer, all from Hewlett Packard, Palo Alto, CA). Plasma and tissue extracts from human, quail, and frog were analyzed by HPLC with a reversed-phase, normal-phase, and chiral columns as follows.

Separation of Carotenoids by Reversed-Phase HPLC.

Before saponification, carotenol fatty acid esters in the pooled extracts from tissues of quails and frogs were first analyzed by reversed-phase HPLC, according to our published procedures.^{1,2,26} These methods separate all the common carotenol fatty acid esters of lutein, zeaxanthin, and other hydroxycarotenoids.

Separation of Carotenoids by Normal-Phase HPLC with Eluent A. Separations were performed on a silica-based nitrile bonded (25 cm length \times 4.6 mm internal diameter [ID]; 5- μm spherical particle) column (Regis Chemical, Morton Grove, IL). The column was protected with a Brownlee nitrile-bonded guard cartridge (3-cm length \times 4.6 mm ID; 5- μm particle size), according to our published methods.^{1,2} Eluent A consisted of an isocratic mixture of hexanes (75%), dichloromethane (25%), methanol (0.3%), and *N,N*-diisopropylethylamine (DIPEA, 0.1%). The column flow rate was 0.7 mL/min. The column temperature was maintained at 20°C, and the monitoring wavelength was 446 nm. Eluent A was also used as the injection solvent.

Separation of Carotenoids by Chiral HPLC with Eluent B. Chiral separations with eluent B were conducted on a chiral column (Chiralpak AD, 25 cm length \times 4.6 mm ID; purchased from Chiral Technologies, Exton, PA). The column packing consists of amylose tris-(3,5-dimethylphenyl)carbamate coated on 10- μm silica gel substrate. The column was protected with a silica gel guard cartridge (3 cm length \times 4.6 mm ID; 5 μm particle). The eluent consisted of a three-step gradient, beginning with an isocratic mixture of hexanes (94.5%) and 2-propanol (5.5%) for the first 40 minutes. Between 40 and 50 minutes, the composition of 2-propanol was linearly increased from 5.5% to 15%, whereas that of hexane was reduced from 94.5% to 85%. At 50 minutes, the composition of the two solvents was changed to 50% each during 5 minutes and maintained at this ratio for 90 minutes. Although all carotenoids were eluted after the first 70 minutes, considerable amounts of noncarotenoid pigments in tissue extracts were detected as broad HPLC peaks after this time. Therefore, the HPLC runs were extended to 90 minutes to clean up the column. At the end of each run, the column was re-equilibrated under the original isocratic conditions for 25 minutes. The flow rate with eluent B was 0.7 mL/min and the HPLC runs were monitored at $\lambda = 450$ and 470 nm. A mixture of hexanes (94.5%) and 2-propanol (5.5%) was used as the injection solvent.

Source of Carotenoid Standards

(3*R*,3'*R*,6'*R*)-lutein (85% pure) was isolated from a saponified extract of marigold flowers (Kemin Foods, LC, Des Moines, IA) and was purified to greater than 98% by two consecutive crystallizations, according to our published procedure.²⁷ (3*R*,3'*R*)-zeaxanthin, (3*S*,3'*S*)-zeaxanthin, and (3*R*,3'*S*)-*meso*-zeaxanthin—as well as a racemic mixture of these carotenoids, (3*S*,3'*RS*)-zeaxanthin—were synthesized by

Hoffmann-La Roche (Basel, Switzerland), according to published procedures.^{28–30} (3*R*,3'*S*,6'*R*)-lutein (3'-epilutein) and (3*R*,6'*R*)-3-hydroxy- β , ϵ -carotene-3'-one were synthesized from (3*R*,3'*R*,6'*R*)-lutein according to published procedures.^{1,31} *all-E*-lycopene and 5*Z*-lycopene were synthetic samples from Hoffmann-La Roche.³² 2,6-Cyclolycopene-1,5-diol was synthesized from lycopene according to our published procedure.³³ BHT and *N,N*-diisopropylethylamine (DIPEA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). THF, ethyl ether, ethanol, and the HPLC-grade solvents, hexanes, dichloromethane, methanol, and 2-propanol (VWR Scientific Products), were used without further purification.

RESULTS

Nomenclature

The nondietary (3*R*,3'*R*)-*meso*-zeaxanthin, 3-hydroxy- β , ϵ -carotene-3'-one, and 3'-epilutein, which were identified in the serum, liver, and ocular tissues of human, quail, and frog are referred to as nondietary by-products of (3*R*,3'*R*,6'*R*)-lutein and/or (3*R*,3'*R*)-zeaxanthin throughout this article. The proposed transformations leading to the formation of these carotenoid by-products may or may not be light induced and/or enzymatically derived. Therefore, at present it is unclear whether these carotenoid by-products can be considered as metabolites of dietary lutein and/or zeaxanthin. The terms *all-E* and *Z* isomers of carotenoids refer to *all-trans* and *cis* isomers of carotenoids, respectively. For in-chain geometric isomers of carotenoids, the terms *all-trans* and *cis*, which have been used with the old nomenclature, are not appropriate. The *R* and *S* symbols refer to those carotenoids with known absolute configurations.

Separation and Identification of Carotenoids

We have extensively reported on the distribution and characterization of carotenoids and their nondietary by-products in the human plasma and ocular tissues.^{1–3,5,6} However, the absolute configuration of zeaxanthin and the *E/Z* geometry of lycopene were not investigated. Establishing the constitution of zeaxanthin is highly significant, because in addition to dietary (3*R*,3'*R*)-zeaxanthin, two other nondietary stereoisomers of this carotenoid are possible: (3*R*,3'*S*)-*meso*-zeaxanthin and (3*S*,3'*S*)-zeaxanthin. Consequently, the absolute configuration of zeaxanthin and the comparative profile of other carotenoids in human plasma, liver, and ocular tissues could provide an insight into the transformation of these pigments in the human eye.

The qualitative and quantitative distribution of carotenoids in plasma and tissues of humans and two animal models (quails and frogs) are shown in Tables 1 and 2, respectively. The chemical structures of the characterized carotenoids are shown in Figure 1. The strategy for separation and identification of these carotenoids was to analyze the various extracts by three different HPLC methods that involve photodiode array detection: reversed-phase HPLC, normal-phase HPLC on a silica-based nitrile bonded column, and chiral HPLC. Each of these methods allowed the separation of a specific group of carotenoids that could not be achieved by the other methods. For example, when the retinas of quail and frog were analyzed by reversed-phase chromatography according to our published procedures, it was revealed that all the lutein and zeaxanthin were esterified with fatty acids, such as palmitic and myristic acids.²⁶ We have shown that esterified carotenoids do not make their way into human blood, liver, and ocular tissues.^{1–3,5,6} However, the reversed-phase separation in this case is useful, because it allows the characterization of a wide range of dietary carotenoids.^{1,2} The normal-phase HPLC separation on a silica-based nitrile bonded column with eluent A separated

TABLE 1. Qualitative and Quantitative Distribution of Ocular Carotenoids and Their Nondietary By-products in Human Plasma and Pooled Ocular Tissues

Human Plasma and Pooled Ocular Tissues	Average Concentration of Ocular Carotenoids and Their By-products in Plasma (ng/mL), Liver (ng/g), and Pooled Ocular Tissues (ng/Wet Tissue)						
	(3R,3'R,6'R)-lutein*† (L)	(3R,3'R)-zeaxanthin*† (Z)	(3R,3'S; meso)-zeaxanthin‡ (M)	Z:M Ratio	All-E-lycopene* and 5Z-lycopene* All-E/5Z ratio	(3R,3'S,6'R)-lutein‡ (3'-epilutein)	3-hydroxy-β,ε-carotene-3'-one‡
Plasma (12 mL)	53.2	33.2	—§	—	All-E + 5Z: 189 All-E: 98.0 5Z: 91.0 E/Z: 1.1	12.8	12.2
Liver (4.4 g)¶	404	72.0	—§	—	All-E + 5Z: 352 All-E: 236 5Z: 116 E/Z: 2.0	9.5	—§
Retina (n = 4) (0.27 g/tissue)	38.6	16.0	2.9	5.5	—§	3.8	9.7
Macula (n = 5)¶ (0.021 g/tissue)	14.8	9.4	3.2	2.9	—§	—§	—§
RPE/choroid, (n = 20) (0.16 g/tissue)	10.3	2.0	0.7	2.9	All-E + 5Z: 12.3 All-E: 7.8 5Z: 4.5 E/Z: 1.7	3.7	1.3
Ciliary body, (n = 20) (0.28 g/tissue)	10.9	2.8	0.6	4.7	All-E + 5Z: 14.0 All-E: 8.1 5Z: 5.9 E/Z: 1.4	1.5	0.8
Iris, (n = 20) (0.02 g/tissue)	8.5	0.9	0.2	4.5	All-E + 5Z: 1.7 All-E: 1.6 5Z: 0.1 E/Z: 16.0	0.6	0.4
Lens, (n = 18) (0.21 g/tissue)	0.8	0.5	0.1	5.0	—§	0.4	0.2

* Dietary carotenoids.

† Concentrations of the E/Z geometrical isomers have been combined.

‡ Carotenoid by-products.

§ Not detected; detection limit for HPLC analysis of carotenoids was 0.1 ng/g of liver, 0.1 ng/wet ocular tissue, and 0.1 ng/mL plasma.

¶ Human liver contained substantial amounts of a presumed lycopene metabolite 2,6-cyclolycopene-1,5-diol (613 ng/g).

|| Human macula also contained minute quantities of (3S,3'S)-zeaxanthin (1.5 ng/wet tissue).

TABLE 2. Average Concentration of (3R,3'R,6'R)-Lutein, (3R,3'R)-Zeaxanthin, and Their Nondietary By-products in Pooled Ocular Tissues, Plasma, and Liver of Quail and Frog

Pooled Plasma and Tissues	Average Ocular Tissue (ng/Wet Tissue), Plasma (ng/mL), and Liver (ng/Wet Tissue) Concentration					
	(3S,3'S)-zeaxanthin	(3R,3'S; meso)-zeaxanthin	(3R,3'R)-zeaxanthin	(3R,3'R,6'R)-lutein	(3R,3'S,6'R)-lutein (3'-epilutein)	3-hydroxy-β,ε-carotene-3'-one
Quail						
Retina (n = 28, 0.029 g/tissue)	0.3	42.5	361	41.9	173	—*
RPE/Choroid (n = 27)	—*	2.1	23.1	5.2	12.3	—*
Iris (n = 28)	—*	—*	0.3	0.3	—*	—*
Lens (n = 28)	—*	—*	—*	—*	—*	—*
Plasma (18 mL)	—*	—*	190	186	2.2	9.0
Liver (n = 14, 0.175 g/tissue)	—*	1.0	46.5	39.6	—*	—*
Frog						
Retina (n = 12, 0.037 g/tissue)	14.9	4.1	50.0	103	43.1	—*
RPE/choroid (n = 12)	9.7	1.4	46.1	138	40.2	—*
Lens (n = 12)	—*	—*	—*	0.1	—*	—*
Plasma (2 mL)	2.8	2.2	14.9	66.2	0.9	3.5
Liver (n = 6, 0.993 g/tissue)	859	324	3587	16800	575	—*

Carotenoids were quantitated by HPLC on silica-based nitrile bonded and chiral columns after saponification. Concentrations of the E/Z geometric isomers of lutein and zeaxanthin have been combined. Frog liver also contained α-cryptoxanthin (740 ng/tissue), β-cryptoxanthin (4200 ng/tissue), neurosporene (750 ng/tissue), γ-carotene, (2400 ng/tissue), α-carotene (4700 ng/tissue), and β-carotene (13300 ng/tissue). These carotenoids were not detected in quail liver.

* Not detected; detection limit for HPLC analysis of carotenoids was 0.1 ng/wet tissue and 0.1 ng/mL.

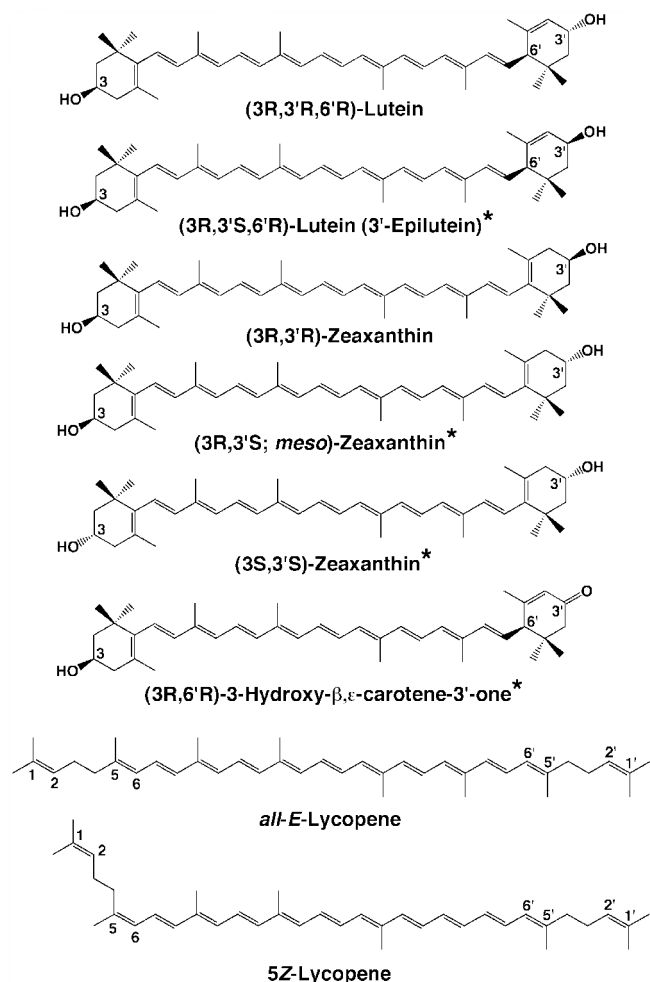


FIGURE 1. The chemical structures of carotenoids and their presumed metabolites identified in plasma, liver, and ocular tissues of human, quail, and frog. (*) Nondietary carotenoids.

dietary (3*R*,3'*R*,6'*R*)-lutein and (3*R*,3'*R*)-zeaxanthin as well as their geometric isomers.^{5,34} An example of this separation is the carotenoid HPLC profile of an extract from quail retina (saponified) in comparison with plasma (unsaponified) as depicted in Figures 2A and 2B, respectively. As shown in these chromatograms, this approach also allows the separation and identification of 3-hydroxy- β,ϵ -carotene-3'-one and (3*R*,3'*S*,6'*R*)-lutein (3'-epilutein), which are the presumed metabolites of dietary lutein and zeaxanthin identified in human plasma and eye tissues.^{1,4,5,6} In addition, this HPLC method can verify the presence or absence of 2,6-cyclolycopene-1,5-diol in various extracts. This compound is a proposed metabolite of dietary lycopene and has been previously identified in human plasma and retina.^{2,5,35}

Because the silica-based nitrile bonded column did not distinguish between the configurational isomers of zeaxanthin, a chiral HPLC method was used to directly resolve (3*R*,3'*R*)-zeaxanthin, (3*R*,3'*S*; *meso*)-zeaxanthin, and (3*S*,3'*S*)-zeaxanthin. In the past, the method of choice for resolving these zeaxanthins was to derivatize their racemic mixture before HPLC analysis on a chiral column. For example, Rüttimann et al.³⁵ resolved a racemic mixture of zeaxanthin through the dicarbamates of (*S*)-(+)- α -(1-naphthyl) ethyl isocyanate. In another approach, Maoka et al.³⁶ used a different chiral HPLC column (Sumipax OA-2000; Sumisho Electronics, Tokyo, Japan) to resolve the dibenzoate derivatives of the three config-

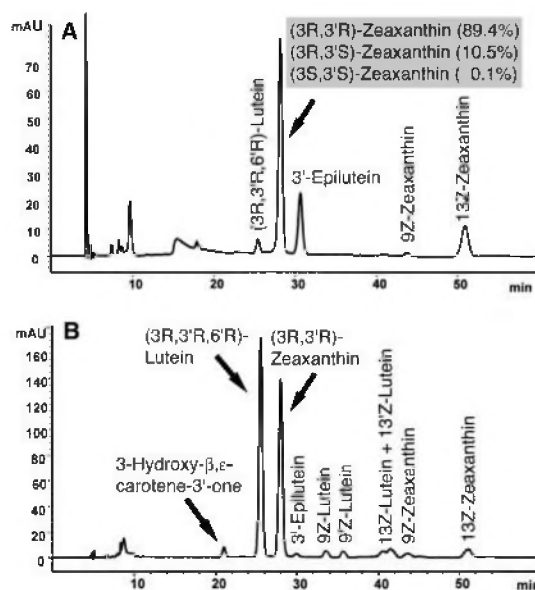


FIGURE 2. Comparison of the carotenoid HPLC profiles of a saponified extract from quail retina (A) with an unsaponified extract of quail plasma (B) on a silica-based nitrile bonded column with eluent A.

urational isomers of zeaxanthin. This method was later used by Bone et al.⁴ who resolved (3*R*,3'*S*; *meso*)-zeaxanthin from (3*R*,3'*R*)-zeaxanthin in human retina. We have also reported on a modification of the method of Maoka et al.³⁶ for the resolution of some of the optical isomers of zeaxanthin, lutein, and several keto- and hydroxycarotenoids in an extract from human plasma.³⁷ The main disadvantage with the methods of Rüttimann et al.³⁵ and Maoka et al.³⁶ is that the small-scale derivatization of hydroxycarotenoids in extracts from human ocular tissues is not consistently quantitative and is often accompanied by some losses. Therefore, our chiral chromatographic method which does not require derivatization of zeaxanthin and lutein provided an accurate qualitative and quantitative account of the optical isomers of these carotenoids in various extracts. A typical separation of carotenoid standards on the chiral amylose tris-(3,5-dimethylphenylcarbamate) HPLC column with eluent B is shown in Figure 3. In addition to resolving the configurational isomers of zeaxanthin, this column is also capable of separating *all-E*- and 5*Z*-lyco-

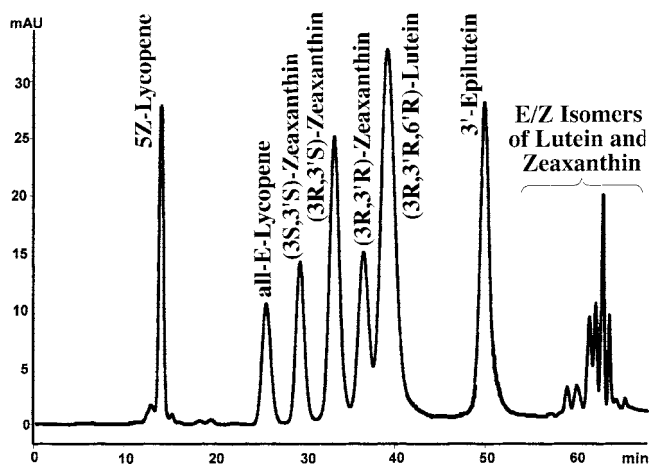


FIGURE 3. HPLC separation of a mixture of carotenoid standards on a chiral amylose tris-(3,5-dimethylphenylcarbamate) HPLC column with eluent B.

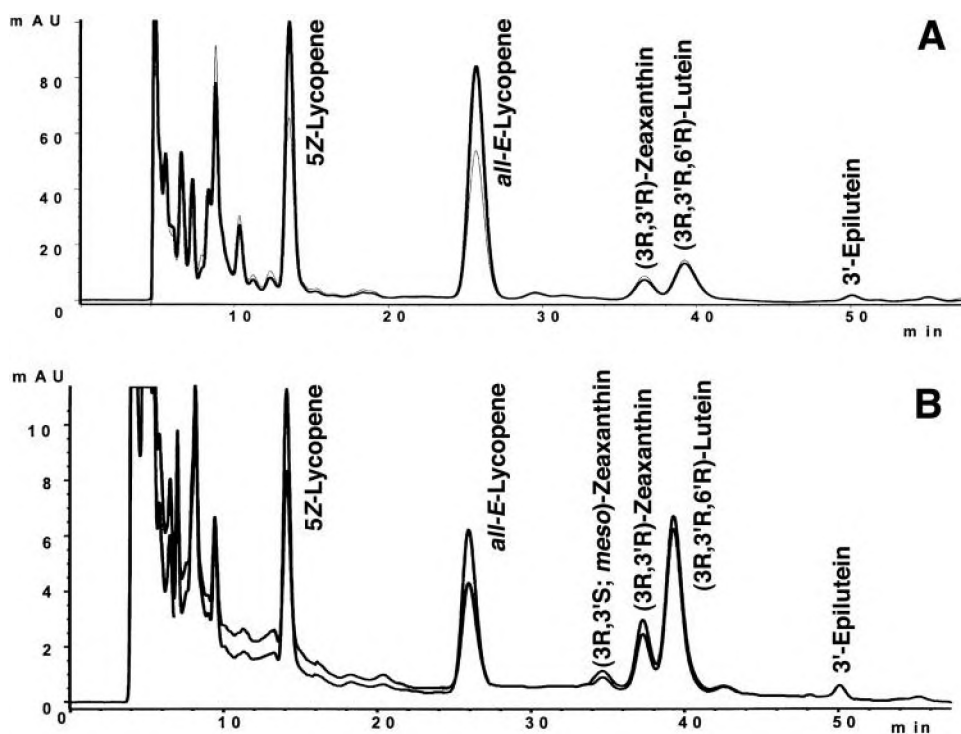


FIGURE 4. Comparison of the carotenoid HPLC profiles of extracts from human plasma (A) and RPE-choroid (B) on a chiral amylose tris-(3,5-dimethylphenylcarbamate) HPLC column with eluent B.

ene, (3*R*,3'*R*,6'*R*)-lutein, and 3'-epilutein. Therefore, carotenoids in plasma, liver, and ocular tissues of human, quail, and frog were separated by the aforementioned methods and identified by comparison of their HPLC retention times and UV-visible absorption spectra and by coinjection with synthetic or isolated standards. Details regarding the UV-visible absorption maxima of carotenoids can be found in our previous publications.^{1,2,5,6}

Distribution of Carotenoids in Human Plasma, Liver, and Eye Tissues

The analysis of human plasma and liver by reversed-phase HPLC according to our published methods revealed the presence of a wide range of dietary carotenoids (data not shown).¹⁻³ Herein, we have presented only the data relevant to lutein, zeaxanthin, lycopene, their stereoisomers, and their nondietary by-products. These data were obtained from the carotenoid HPLC analysis of extracts on the silica-based nitrile bonded and chiral columns with eluents A and B, respectively. Because the use of an internal standard could interfere with the presence of unknown carotenoids, no internal standard in the extraction of the various samples was used. To monitor the accuracy and reproducibility of the HPLC analysis of carotenoids, a solution containing known concentrations of (3*R*,3'*R*,6'*R*)-lutein, (3*RS*,3'*RS*)-zeaxanthin (racemic mixture), lycopene, and 3'-epilutein was routinely analyzed by HPLC with eluents A and B. The recovery and reproducibility of the HPLC analysis for carotenoids with these eluents was greater than 95%.

In nearly all cases, the extracts from human plasma, liver, and ocular tissues contained (3*R*,3'*R*,6'*R*)-lutein, (3*R*,3'*R*)-zeaxanthin, 3'-epilutein, and 3-hydroxy- β , ϵ -carotene-3'-one (Table 1). A comparison between the carotenoid HPLC profiles of extracts from human plasma and human RPE-choroid, obtained by chiral chromatography with eluent B, is shown in Figures 4A and 4B, respectively. These data clearly show the presence of (3*R*,3'*S*; *meso*)-zeaxanthin in human RPE-choroid, whereas this carotenoid is absent in human plasma. (3*R*,3'*S*; *meso*)-

zeaxanthin was also identified and quantified in other tissues of the human eye at varying concentrations but was not detected in human liver (Table 1). In an earlier investigation, we used the method of Maoka et al.³⁶ and reported that human plasma contains up to 2% (3*R*,3'*S*; *meso*)-zeaxanthin.⁵ However, this involved isolation of zeaxanthin from plasma by preparative HPLC followed by derivatization with benzoyl chloride, and purification of the resultant zeaxanthin dibenzoate by chromatography, before chiral HPLC analysis. Because of this extensive sample preparation and purification, it is quite likely that small quantities of (3*R*,3'*S*; *meso*)-zeaxanthin were generated as an artifact in our earlier investigation. The current methodology does not require isolation of zeaxanthin and derivatization with benzoyl chloride and therefore the extracts from human plasma can be directly subjected to chiral chromatography. The data presented here unequivocally demonstrate that (3*R*,3'*S*; *meso*)-zeaxanthin is absent in human plasma and liver. (3*S*,3'*S*)-zeaxanthin was also absent in human plasma, liver, and all ocular tissues examined except for trace amounts in the macula. *All-E*- and 5*Z*-lycopene were present in human plasma, liver, and ocular tissues, with the exception of neural retina, macula, and lens. Human liver contained a high concentration of 2,6-cyclolycopene-1,5-diol (613 ng/g) in comparison with plasma, in which the levels were approximately 2.7 ng/mL.

Distribution of Carotenoids in Plasma, Liver, and Eye Tissues of Quails and Frogs

Reversed-phase chromatography showed that hydroxycarotenoids in plasma of quail and frog are not esterified with fatty acids and as a result, the plasma extracts were directly analyzed by HPLC (eluents A and B) without saponification. Quail plasma contained a high concentration of (3*R*,3'*R*,6'*R*)-lutein, (3*R*,3'*R*)-zeaxanthin, and 3'-epilutein, whereas frog plasma showed the presence of (3*S*,3'*S*)- and (3*R*,3'*S*; *meso*)-zeaxanthin in addition to these carotenoids (Table 2). C_{18} -reversed-phase and normal-phase HPLC of the extracts from quail and frog livers also showed the presence of unesterified lutein and

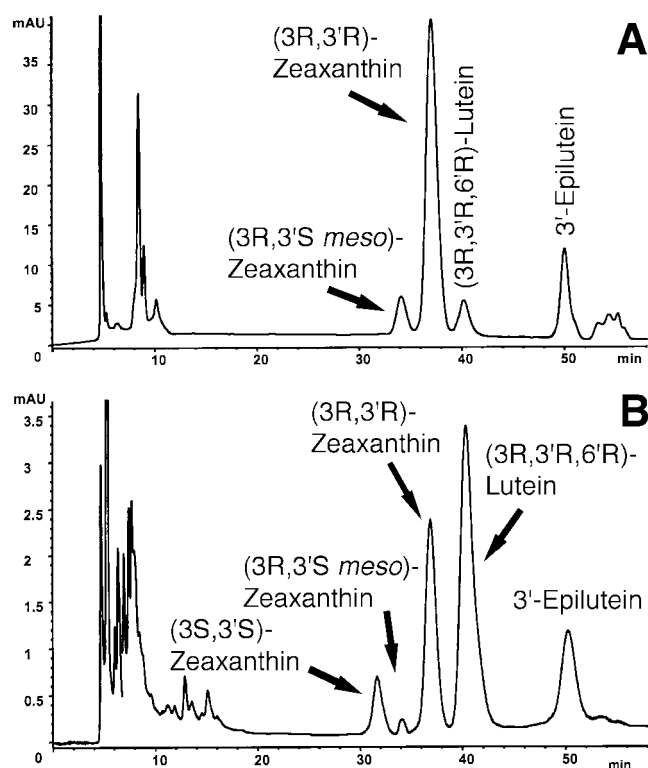


FIGURE 5. Comparison of the carotenoid HPLC profiles of saponified extracts from quail (A) and frog (B) RPE-choroid on a chiral amylose tris-(3,5-dimethylphenylcarbamate) HPLC column with eluent B.

zeaxanthin. Nonetheless, these samples were saponified to remove the lipids and fatty acids before HPLC analysis on the chiral column. Frog liver appeared to accumulate a much higher concentration of $(3R,3'R,6'R)$ -lutein and $(3R,3'R)$ -zeaxanthin relative to quail. In addition to the carotenoids listed in Table 2, frog liver showed the presence of other dietary carotenoids such as α -cryptoxanthin (740 ng/tissue), β -cryptoxanthin (4,200 ng/tissue), neurosporene (750 ng/tissue), γ -carotene (2,400 ng/tissue), α -carotene (4,700 ng/tissue), and β -carotene (13,300 ng/tissue). These carotenoids were identified and quantified by reversed-phase HPLC similar to our published procedures.^{1,2} Quail liver did not contain any significant level of the aforementioned carotenoids. $(3R,3'R,6'R)$ -lutein and $(3R,3'R)$ -zeaxanthin in quail and frog retinas and RPE-choroid were esterified, and as a result the tissue extracts from these animals were saponified before HPLC analysis with eluents A and B. A comparison between the carotenoid HPLC profiles (eluent B) of extracts from quail and frog RPE-choroid is shown in Figures 5A and 5B, respectively. The most notable difference between frog and quail was the presence of $(3S,3'S)$ -zeaxanthin in frog RPE-choroid but not in quail. Quail RPE-choroid appear to accumulate a higher concentration of $(3R,3'R)$ -zeaxanthin than $(3R,3'R,6'R)$ -lutein, whereas in frogs this trend was reversed. The RPE from both animals showed a relatively high concentration of 3'-epilutein. Retina of quail, as in human, had a much higher concentration of carotenoids than did the RPE-choroid whereas in frog, carotenoid levels were generally high in both RPE-choroid and retina. A low concentration of $(3R,3'R,6'R)$ -lutein and $(3R,3'R)$ -zeaxanthin was found in quail iris, but no detectable levels of carotenoids were present in lens. Frog lens contained a very low concentration of $(3R,3'R,6'R)$ -lutein. No detectable amount of lycopene was found in plasma, liver, and ocular tissues of quails and frogs.

DISCUSSION

Our detailed analysis of human plasma and serum has previously revealed that at least 13 dietary *all-E* carotenoids and 12 of their *Z* isomers are absorbed, metabolized, and used routinely by humans.^{1,2} In addition, we have characterized one *Z* and eight *all-E* carotenoids in human serum that are not of dietary origin and are presumably formed by metabolic transformation of dietary $(3R,3'R,6'R)$ -lutein, $(3R,3'R)$ -zeaxanthin, and lycopene.³⁷ In 1997, we demonstrated that in addition to dietary lutein and zeaxanthin, human and monkey retinas accumulate some of the by-products of these carotenoids, particularly, 3'-epilutein and 3-hydroxy- β , ϵ -carotene-3'-one.³ More recently, a complete spectrum of lutein, zeaxanthin, and the aforementioned by-products were identified and quantified in all tissues of the human eye, such as RPE-choroid, peripheral retina, ciliary body, iris, and lens.⁶ Although the function of dietary lutein and zeaxanthin in the human ocular tissues is not fully understood, it is hypothesized that macular carotenoids may act as an optical filter by absorbing short-wavelength visible light, reducing chromatic aberration, and preventing photochemical damage to cones and RPE in the fovea.^{13,16,17} Another proposed mechanism for the role of macular carotenoids may be their antioxidant function. In this regard, understanding the metabolism of dietary $(3R,3'R,6'R)$ -lutein and $(3R,3'R)$ -zeaxanthin in the human eye can provide valuable information about the function of these carotenoids in the prevention of retinal disorders such as AMD.

One of the major difficulties in elucidating the metabolism of carotenoids in the eye is that the same carotenoid by-products that are observed in the human serum are also observed in human ocular tissues. Therefore, metabolic studies must be able to distinguish between those carotenoid metabolites formed locally in the eye and the ones that may be simply transported to the eye tissues from the circulating blood. Because of the invasive nature of such studies, appropriate non-primate animal models that metabolize carotenoids similar to humans must be selected. We initially examined eyes from mammals such as cows, rats, mice, pigs, rabbits, and ground squirrels and found that endogenous ocular carotenoids were either absent or else were present at unsuitably low levels, and we were therefore compelled to explore nonmammalian vertebrate systems. Based on the qualitative and quantitative distribution of carotenoids in the plasma, liver, and ocular tissues of human (Table 1) in comparison with the same data obtained from quail and frog (Table 2), these animals appear to serve as suitable models for metabolic studies. During the preparation of this report, Toyoda et al.³⁸ published a well-designed study in which they elegantly demonstrated that xanthophyll profiles in quail mimic those in primates. This was accomplished by supplementation of quails with zeaxanthin, which resulted in a significant increase in the concentration of this carotenoid in serum, retina, liver, and fat. Because of the robust response to zeaxanthin supplementation, the authors concluded that quail serves as an animal model for exploration of factors regulating delivery of dietary carotenoids to the retina.

In quails and frogs, carotenoids are accumulated as yellowish oil droplets in the cone inner segments or RPE, respectively,³⁹⁻⁴² whereas in primates carotenoids are mostly concentrated in the Henle fiber layer.¹⁶ Because of this pattern of accumulation, it can be argued that quail and frog may not serve as appropriate models for investigating the role of carotenoids in prevention of diseases such as macular degeneration in humans. Nonetheless, the data presented herein clearly indicate that these animals are likely to possess similar families of enzymes that may be responsible for the biochemical transformation of dietary $(3R,3'R,6'R)$ -lutein and $(3R,3'R)$ -zeaxanthin in humans. Based on several human supplementation

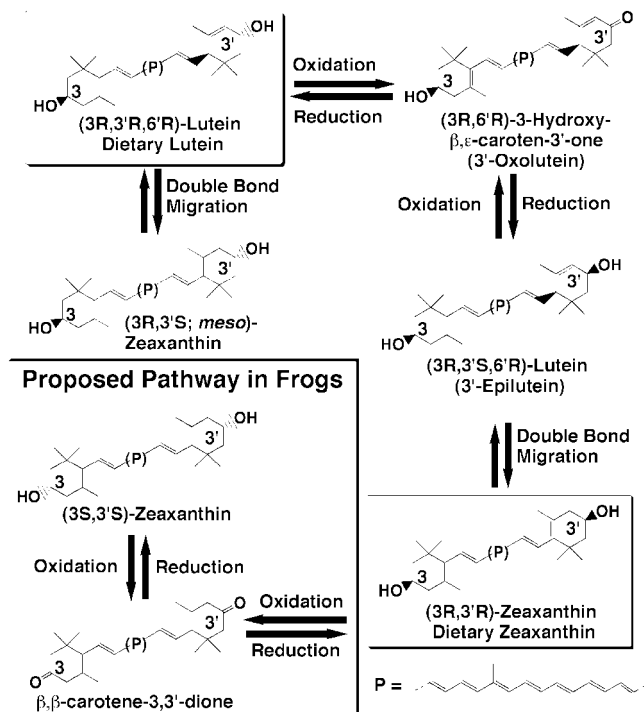


FIGURE 6. Proposed transformation processes of dietary (3*R*,3'*R*,6'*R*)-lutein and (3*R*,3'*R*)-zeaxanthin in human, quail, and frog retinas.

studies involving purified lutein, zeaxanthin, and lycopene, we have hypothesized a series of metabolic reactions that may explain the conversion of these dietary carotenoids to their presumed metabolites in humans.^{43,44} In accordance with these findings and the data presented herein, the possible transformations for dietary lutein and zeaxanthin in humans, quails, and frogs may be summarized as a series of oxidation-reduction and double-bond isomerization reactions, as shown in Figure 6. The allylic oxidation of dietary lutein can result in the formation of (3*R*,6'*R*)-3-hydroxy- β,ϵ -carotene-3'-one (3'-oxolutein), which may undergo reduction either to revert back to dietary lutein or else epimerize at C-3' to form 3'-epilutein. Both of these nondietary carotenoids have been identified in most ocular tissues of humans (Table 1). Alternatively, stereospecific double-bond isomerization of dietary zeaxanthin can also result in the formation of 3'-epilutein that may subsequently undergo allylic oxidation to yield 3'-oxolutein. Because 3'-oxolutein and 3'-epilutein are found in human plasma, it is not known whether their presence in ocular tissues is due to their transport through the circulatory system or whether these carotenoids may be formed locally in the eye by an independent process. However, the data presented in Table 1 provides the most compelling evidence for metabolic conversion of dietary (3*R*,3'*R*,6'*R*)-lutein to (3*R*,3'*S*; meso)-zeaxanthin in the human ocular tissues through double-bond isomerization. This is because no detectable concentration of (3*R*,3'*S*; meso)-zeaxanthin is found in human plasma and liver, despite the presence of this carotenoid in human eye tissues. Unfortunately, the dietary history and plasma carotenoid profile of the subject whose liver was analyzed in the current study is not known. Nonetheless, high levels of dietary lutein and zeaxanthin were present in this liver sample, whereas no (3*R*,3'*S*; meso)-zeaxanthin could be detected. The highest concentration of (3*R*,3'*S*; meso)-zeaxanthin relative to dietary (3*R*,3'*R*)-zeaxanthin was found in human macula and RPE-choroid (Table 1) where carotenoid metabolism would be expected. The conversion of dietary lutein and zeaxanthin to their presumed

metabolites as depicted in Figure 6, may be light induced and/or enzymatically mediated. Bone et al.⁴ have demonstrated that the ratio of (3*R*,3'*R*)-zeaxanthin to (3*R*,3'*S*; meso)-zeaxanthin for regions of human retina is subject to the choice of the area used in sectioning the retina. This ratio was shown by these investigators to reach approximately 1:1 if a 3-mm diameter punch of macula is selected and analyzed. We have chosen a 5-mm section of the macula and have found that the ratio of (3*R*,3'*R*)-zeaxanthin to (3*R*,3'*S*; meso)-zeaxanthin is nearly 3:1. Therefore, selection of a larger diameter of the macula for the analysis of zeaxanthins may explain the difference between our data and those reported by Bone et al.⁴

Another interesting aspect of carotenoids identified in human eye tissues is the presence of *all-E*-lycopene and its 5*Z* isomer in RPE-choroid, ciliary body, and iris. The ratio of *all-E*-lycopene to 5*Z*-lycopene in human RPE-choroid and ciliary body reflects that in plasma, whereas in iris, the *all-E* isomer predominates. The methodology described for separation of *all-E*-lycopene from its 5*Z* isomer in serum, liver, and ocular tissues of human and animals is novel and will enable future investigators to determine whether the light-sensitive lycopene can undergo *E/Z* isomerization in ocular tissues. Our detailed qualitative and quantitative distribution of carotenoids in plasma, liver, and the eye tissues of quail and frog as shown in Table 2 suggest that these animals can serve as suitable models of primate ocular carotenoid metabolism. The selection of these animals was based on two major criteria. First, high concentrations of dietary lutein and zeaxanthin as well as the nondietary carotenoids observed in humans appear to accumulate in their ocular tissues from their regular diet. Second, both types of animal possess unique carotenoid profiles in plasma, liver, and ocular tissues that allows for the distinction between certain carotenoid byproduct that may be originating from plasma and those formed locally in the eye. For example, relatively high concentrations of (3*R*,3'*S*; meso)-zeaxanthin are found in quail retina (11% of total zeaxanthin) and RPE-choroid (8% of total zeaxanthin), whereas this carotenoid is absent in plasma, and only minute quantities are found in the liver. In the human eye tissues, (3*R*,3'*S*; meso)-zeaxanthin constitutes 15% of total zeaxanthin in the retina, 26% in RPE-choroid, and 25% in macula. Consequently, quail would be an ideal model for identifying the family of enzymes that may be involved in mediating the double-bond isomerization of dietary (3*R*,3'*R*,6'*R*)-lutein to (3*R*,3'*S*; meso)-zeaxanthin. In frogs, however, dietary (3*R*,3'*R*)-zeaxanthin appears to transform predominantly to (3*S*,3'*S*)-zeaxanthin. This carotenoid is probably formed from oxidation of dietary zeaxanthin to β,β -carotene-3,3'-dione followed by stereoselective reduction to (3*S*,3'*S*)-zeaxanthin as shown in Figure 6. Although we were unable to detect β,β -carotene-3,3'-dione in plasma, liver, and ocular tissues of human, quail, and frog, this carotenoid is the most likely and logical precursor to the (3*S*,3'*S*)-zeaxanthin identified in frog.

Regarding the distribution of lutein, zeaxanthin, and 3'-epilutein, quail and frog showed interesting patterns of distribution. In quail plasma, the average concentration of dietary lutein was nearly the same as that of zeaxanthin. A similar distribution was also found in quail liver. The average ratio of dietary (3*R*,3'*R*)-zeaxanthin to dietary (3*R*,3'*R*,6'*R*)-lutein in retina and RPE-choroid of quail is 8.6 and 4.4, respectively. Although 3'-epilutein is absent in the quail liver and present only in minute quantities in plasma, a high concentration of this presumed metabolite relative to dietary lutein is found in quail retina and RPE-choroid. As a result, 3'-epilutein is more likely to result from the *in vivo* transformation of dietary lutein and/or zeaxanthin in the quail eye rather than to originate from the low levels found in the circulating plasma. Opposite to quail, frog accumulates a higher concentration of dietary lutein in their plasma and liver relative to dietary zeaxanthin. Similar

to quails, frogs also show the presence of a high concentration of 3'-epilutein in retina and RPE-choroid, regardless of the very low concentrations of this carotenoid found in their plasma and liver. No detectable amount of 3'-oxolutein was found in the ocular tissues of quail and frog, even though low levels of this carotenoid were detected in their plasma. As expected, quail and frog plasma, liver, and ocular tissues did not show the presence of any lycopene, a finding that may be related to the absence of this carotenoid in their diets. Dietary manipulation of quail and frog with lycopene may be useful in understanding the E/Z isomerization of lycopene and the possible conversion of this carotenoid to 2,6-cyclolycopene-1,5-diol, which has been identified in human plasma.^{2,33,44}

In summary, because (3R,3'S; meso)-zeaxanthin and 3'-epilutein are not present in quail plasma and liver in an appreciable amount but are found in retina and RPE-choroid in significant levels, this animal is particularly useful for investigating the metabolic pathways of dietary lutein and zeaxanthin as shown in Figure 6. Although the presence of high concentrations of carotenoids and their nondietary by-products in the ocular tissues of quail and frog readily allows for metabolic studies, quail is the preferred animal model, because obtaining sufficient quantities of blood from frog is extremely difficult. There is no doubt that metabolic studies of this nature should be conducted with isotopically labeled carotenoids to establish unequivocally the proposed transformations shown in Figure 6. However, the absence of appreciable amounts of the presumed metabolites of dietary lutein and zeaxanthin in quail and frog plasma and liver can significantly reduce interference from those carotenoid by-products that may be originating from the circulatory system of these animals. The major disadvantage of quail and frog as appropriate nonprimate animal models for metabolic studies is that dietary lutein and zeaxanthin are esterified in quail and frog retinas and RPE-choroid but are unesterified in ocular tissues of humans. Future studies with these animal models should be carefully designed, not only to investigate the bioavailability, absorption, and ocular tissue uptake of dietary lutein, zeaxanthin, and even perhaps lycopene, but also to monitor the possible conversion of these carotenoids to their presumed metabolites.

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