Developmentally Regulated Production of meso-Zeaxanthin in Chicken Retinal Pigment Epithelium/Choroid and Retina

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Purpose. meso-Zeaxanthin is a carotenoid that is rarely encountered in nature outside of the vertebrate eye. It is not a constituent of a normal human diet, yet this carotenoid comprises one-third of the primate macular pigment. In the current study, we undertook a systematic approach to biochemically characterize the production of meso-zeaxanthin in the vertebrate eye.

Methods. Fertilized White Leghorn chicken eggs were analyzed for the presence of carotenoids during development. Yolk, liver, brain, serum, retina, and RPE/choroid were isolated, and carotenoids were extracted. The samples were analyzed on C-30 or chiral HPLC columns to determine the carotenoid composition.

Results. Lutein and zeaxanthin were found in all studied nonocular tissues, but no meso-zeaxanthin was ever detected. Among the ocular tissues, the presence of meso-zeaxanthin was consistently observed starting at embryonic day 17 (E17) in the RPE/choroid, several days before its consistent detection in the retina. If RPE/choroid of an embryo was devoid of meso-zeaxanthin, the corresponding retina was always negative as well.

Conclusions. This is the first report of developmentally regulated synthesis of meso-zeaxanthin in a vertebrate system. Our observations suggest that the RPE/choroid is the primary site of meso-zeaxanthin synthesis. Identification of meso-zeaxanthin isomerase enzyme in the developing chicken embryo will facilitate our ability to determine the biochemical mechanisms responsible for production of this unique carotenoid in other higher vertebrates, such as humans.

Keywords: carotenoid, macular pigment, meso-zeaxanthin, lutein, chicken

Meso-Zeaxanthin is a major carotenoid component of the human fovea,1,2 yet its physiological significance in ocular tissues remains elusive. This xanthophyll carotenoid is present only in ocular tissues, with peak concentrations at the fovea, but it is not detectable in the liver or in the serum.3,4 strongly suggesting specific production of meso-zeaxanthin in the eye. Natural dietary sources of meso-zeaxanthin are limited to rarely eaten foods, such as turtle fat, fish skin, and shrimp shells.5,6 Because these are not a significant part of a normal Western diet, the contribution of food intake toward meso-zeaxanthin is considered negligible. The biochemical process by which meso-zeaxanthin is produced in the retina is unknown. Feeding studies in Japanese quails and macaque monkeys have suggested that lutein is the precursor for meso-zeaxanthin.7,8 Meso-Zeaxanthin and lutein have identical stereochemistries at the 3 and 3′ positions and differ only in the position of one double bond (Fig. 1). Conversion of lutein to meso-zeaxanthin can be readily achieved under harsh industrial conditions of high temperature and strong base,1 but this reaction has not yet been identified in vitro under physiological conditions.

Identification of the biochemical mechanisms of this isomerization reaction in an isolated biological system could provide insights into the function of meso-zeaxanthin in the ocular tissues in health and disease. Zeaxanthins are better antioxidants than lutein,9 but lutein is much more abundant in dietary sources than zeaxanthin.10 Therefore, the isomerization reaction to produce meso-zeaxanthin from lutein may be an effective way to improve the antioxidant properties of macular carotenoids in the fovea. Defects of meso-zeaxanthin production in the eye could lead to abnormal macular pigment levels and distributions, which has led various investigators to speculate that deficiencies of a putative meso-zeaxanthin isomerase could underlie macular disease, such as AMD or macular telangiectasia type II (MacTel), but to make such an argument, it is imperative to unequivocally show that such a process is enzymatically mediated. Here, we demonstrate that meso-zeaxanthin is produced in a developmentally regulated manner in the embryonic chicken eye and that it is likely that the meso-zeaxanthin isomerase enzyme is expressed in the RPE/choroid.

Methods

Incubation of Eggs, Staging, and Isolation of Tissues

Fertilized eggs from White Leghorn chickens were obtained from a local hatchery. Embryonic day 0 (EO) eggs were incubated at 37°C under 50% humidity in dark conditions.
Eggs were removed and processed at various time points during development. Embryos were weighed and staged according to Hamburger-Hamilton Staging. Yolk, brain, eyes, blood, and liver were isolated from the embryo. Dissection of ocular tissues was carried out in saline with direct visualization under a dissecting microscope; 0.25% trypsin in saline was used to facilitate separation of RPE/choroid from retina. After dissection, tissues were spun down briefly to remove any excess saline; and wet weight was measured.

**Extraction and Saponification of Carotenoids**

Yolk was homogenized on ice using three 30-second sonicator pulses; 0.5 mL homogenized yolk was removed and extracted using 1 mL tetrahydrofuran (THF) containing 0.1% butylated hydroxytoluene (BHT). Extractions were conducted by vortexing the samples for 2 minutes, followed by water bath sonication at 4°C for 10 minutes. Blood samples were centrifuged at 2000×g at 4°C for 2 minutes to isolate serum from blood cells. Sera were extracted using THF (+0.1% BHT) after vigorous vortexing. Liver and brain tissues were homogenized following the addition of 0.5 mL 0.85% saline; 700 μL 1-mm silica beads were added to 0.25 mL of the homogenate along with 1 mL THF (+0.1% BHT). Extraction was carried out following three 30-second pulses in a Mini-Beadbeater (BioSpec Products, Tulsa, OK, USA). Samples were rested on ice for at least 1 minute following each 30-second pulse. Ocular tissues from each embryo were pooled.

**FIGURE 1.** Structures of the macular carotenoids. The macular carotenoids are isomers with the same molecular formula: C_{40}H_{56}O_{2}. Lutein and meso-zeaxanthin differ in the position of the double bond in one of the ionone rings (4′-5′ vs. 5′-6′). Zeaxanthin and meso-zeaxanthin differ in the spatial arrangement of the 3′-OH group. 3′-Oxolutein is a xanthophyll carotenoid oxidation product with the molecular formula C_{40}H_{54}O_{2}.

**FIGURE 2.** Complete carotenoid profile of chicken embryonic tissues obtained by HPLC separation using a C-30 column. Embryonic day 21 retina, RPE/choroid, and liver samples contained lutein and zeaxanthin along with trace amounts of 3′-oxolutein. Embryonic day 21 brain showed only the presence of lutein and zeaxanthin. Embryonic day 0 yolk had lutein, 3′-oxolutein, and zeaxanthin. Trace amounts of β-carotene were detected in the yolk and liver samples. The peaks observed at 19.5 minutes in E21 RPE/choroid and retina samples remain unidentified because their visible spectra are not characteristic of carotenoids. The presence of other carotenoids, such as astaxanthin, canthaxanthin, and β-cryptoxanthin, were assessed in separate HPLC runs; none of these carotenoids that are occasionally added to chicken feed were present in our samples. O, 3′-oxolutein; L, lutein; Z, zeaxanthin; β-Cr, β-cryptoxanthin; α-Ca, α-carotene; β-Ca, β-carotene.
weighed, and extracted using 1 mL THF (+0.1% BHT), followed by bead beating as indicated above. Three extractions were conducted for each sample.

Extracts were combined and evaporated using a rotary evaporator under reduced pressure. All extracts, except sera, were subjected to saponification to hydrolyze esterified carotenoids and to break up fibrous tissues. Vertebrate serum contains only nonesterified carotenoids; therefore, saponification was not necessary for these samples. 3

Dried samples were treated with 0.1 mL methanol, 0.5 mL hexane, and 0.5 mL 20% KOH in methanol. The samples were saponified at 37°C for 45 minutes on a shaker. Following saponification, samples were washed with water, and 0.5 mL 90% hexane and 10% ethyl acetate was added to extract the carotenoids. The organic phase was removed following centrifugation at 2000g to remove any residual KOH and dried in a rotary evaporator under reduced pressure.

Preparation of Standards and HPLC Conditions

Two HPLC analysis methods were used. Method 1 was used for the separation of meso-zeaxanthin from zeaxanthin. In this method, a ChiralPak AD column (Daicel Technologies, West Chester, PA, USA) of 25 cm length × 4.6 mm internal diameter was used with a mobile phase that was composed of 95.5 (vol/vol) hexane:isopropanol. Method 2 was used to determine the complete carotenoid profile of samples. For this, a C-30 column (YMC Carotenoids, Allentown, PA, USA) of 25 cm length × 4.6 mm internal diameter was used. The mobile phase for the C-30 column was composed of a series of linear gradients of methanol:methyl tert-butyl ether (% methanol@min: 99 @0; 90 @10; 70 @20; 0 @30; 99 @35; 99 @40). The dried samples were mixed in 200 µL HPLC mobile phase. Mobile phase flow rates of 0.7 mL per minute for method 1 and 1 mL per minute for method 2 were used to separate the carotenoids. Columns were maintained at room temperature with peak monitoring at 450 nm. The identities of the carotenoid peaks were confirmed by photo-diode array spectra, and by co-elution with authentic standards. Previously published extinction coefficients were used to calculate the concentrations of the standard carotenoid solutions. These solutions were injected in amounts ranging from 0.1 ng to 100 ng to plot standard curves. The carotenoid concentrations in the samples were determined from these standard curves.

Results

Complete Carotenoid Profile of Chicken Embryos

To determine the range of carotenoids that may be present in the tissues of chicken embryos, we first analyzed the extracted carotenoids from liver, yolk, brain, RPE/choroid, and retina on a reverse-phase C-30 column. However, this column cannot be
used for the detection of meso-zeaxanthin because dietary 3R,3’S-zeaxanthin and 3R,3’S-meso-zeaxanthin co-elute.

Analysis by C-30 column of E0 yolk samples showed the presence of only zeaxanthin, lutein, β-carotene, and 3’-oxolutein (Fig. 2). 3’-Oxolutein is a well-known oxidative product of dietary carotenoids.11 Embryonic day 21 retina, RPE/choroid, and liver samples, when analyzed on the C-30 column, showed the presence of 3’-oxolutein, zeaxanthin, and lutein, but only liver had detectable amounts of β-carotene. Brain contained only lutein and zeaxanthin. We repeated all of these HPLC analyses on a chiral column that adequately separates zeaxanthin stereoisomers, and only ocular tissues had detectable meso-zeaxanthin (Fig. 3).

**Carotenoid Profiles of Nonocular Tissues During Chicken Embryonic Development**

Yolk contained significant amounts of lutein and zeaxanthin throughout embryonic development. At E0, 5.8 μg lutein and 3.3 μg zeaxanthin were present per milliliter of yolk. By E15, the yolk lutein concentration decreased to 4.3 μg/mL whereas the zeaxanthin amount remained closer to E0 levels (Fig. 4A). Lutein concentrations decreased at a higher rate relative to zeaxanthin as the embryo neared the time of hatching. Decreasing amounts of 3’-oxolutein were detected in the egg yolk. At E0, the ratio of lutein to zeaxanthin in the egg yolk was close to 2.0, but between E15 and E21, the ratio had dropped to approximately 1.5 (Fig. 5A). No meso-zeaxanthin was observed in egg yolk at any time points.

In the brains of developing chicken embryos, we detected low amounts of lutein and zeaxanthin. As opposed to microgram levels of lutein and zeaxanthin that were observed in the yolk, brain contained only nanogram levels of these carotenoids (Fig. 4B). We observed an increasing trend in the concentration of lutein as well as zeaxanthin in the brain tissue with embryonic development. Only trace amounts of 3’-oxolutein were detected in the brain. In the time points under consideration, there were equal amounts of lutein and
zeaxanthin in the brains (Fig. 5B). No meso-zeaxanthin was observed in brain samples at any time point.

Embryonic day 15 liver tissues contained 55.5 ng/g lutein and 43.2 ng/g zeaxanthin. By E21, the carotenoid concentrations were 4.5 µg/g and 3.0 µg/g, respectively (Fig. 4C). Small and increasing amounts of 3'-oxolutein were detected in the liver during development. Even though the carotenoid content in the liver of embryonic chicken increased 10-fold as the embryos neared hatching, the ratio of lutein to zeaxanthin remained close to 1.5 (Fig. 5C). No meso-zeaxanthin was observed in the liver samples.

Serum samples of chicken embryos showed considerable variation in lutein and zeaxanthin concentrations during development (Fig. 4D). It was challenging to remove blood samples from embryos without yolk contamination, which may be the reason for the fluctuation in lutein and zeaxanthin levels in our samples. Importantly, there was no meso-zeaxanthin present in the serum at any time points, and lutein:zeaxanthin ratios remained in the 1.3 to 1.6 range between E15 and E21 (Fig. 5D).

Ocular Tissues Show the Presence of meso-Zeaxanthin in a Developmentally Regulated Manner

In the RPE/choroid of chicken embryo, there was an increase in concentration of lutein as well as zeaxanthin with age. At E15, RPE/choroid samples contained 2.03 ng lutein and 4.03 ng zeaxanthin in a pair of eyes (Fig. 6A). By E21, the concentrations increased to 19.9 ng lutein and 18.6 ng zeaxanthin per pair of eyes. 3'-Oxolutein was first detected at E21 in the embryos. The ratio of lutein to zeaxanthin was 0.5 in E15 embryo, and it increased to 1.1 by E21 (Fig. 6C). Retinas of chicken embryos showed similar trends in carotenoid concentration. At E15, 0.84 ng lutein and 0.93 ng zeaxanthin per pair of eyes were observed (Fig. 6B). By E21, there were 8.51 ng lutein and 18.17 ng zeaxanthin per pair of eyes. Trace amounts of 3'-oxolutein were detected in the retina starting at E20. In the retina, the ratio of lutein to zeaxanthin was at 0.9 at E15 but decreased to 0.5 by E21 (Fig. 6C).
Among the various tissues that were analyzed, retina and RPE/choroid were the only tissues that showed the presence of meso-zeaxanthin (Figs. 3, 6). Interestingly, we observed the presence of this carotenoid in a tissue-specific, developmentally regulated manner. In the RPE/choroid, we first detected meso-zeaxanthin at E17 (Fig. 7A). The concentration of this carotenoid steadily increased from 0.25 ng per pair of eyes at E17 to 1.34 ng per pair of eyes at E21 (Fig. 7A). In the retina, meso-zeaxanthin was detected in 80% of the analyzed samples at E19, and the concentration ranged from 0.63 ng per pair of eyes at E19 to 3.37 ng/pair of eyes at E21 (Fig. 7B). By E20 and E21, 100% of the analyzed retina and RPE/choroid samples contained meso-zeaxanthin (Fig. 7B). With the exception of just one E19 embryo, meso-zeaxanthin was consistently detected in the RPE/choroid starting at E17, several days before it was consistently detected in the retina (Figs. 6, 7). In addition, if RPE/choroid of an embryo was devoid of meso-zeaxanthin, the corresponding retina was always negative as well. Conversely, we never observed a case in which the retina was positive for meso-zeaxanthin when the corresponding RPE/choroid was negative.

**DISCUSSION**

The macular pigment consists of three carotenoids: lutein, zeaxanthin, and meso-zeaxanthin. These carotenoids are highly concentrated at the fovea, and their concentration decreases toward the periphery of the retina. In nonocular tissues and serum, the ratio of lutein:zeaxanthin:meso-zeaxanthin is approximately 4:1:0; however, in the peripheral retina it becomes 2:1:0, and in the fovea the ratio changes to 1:1:1. The lack of meso-zeaxanthin in the serum has led to the hypothesis that this isomer may be formed as a result of metabolic transformations within the retina. The macular carotenoids are isomers with a common C40H56O2 composition. Dietary zeaxanthin and meso-zeaxanthin differ in the stereochemistry of just the hydroxyl group at the 3 position, but biological interconversion reactions at a single chiral center rarely occur in nature.
In vivo studies conducted by Johnson and coworkers\(^8\) indicated that lutein rather than zeaxanthin may be the precursor of meso-zeaxanthin in primates. In their study, carotenoid-deficient animals were maintained on lutein or zeaxanthin supplements. The animals that were fed with lutein showed the presence of meso-zeaxanthin in their retinas. This suggests a nondietary origin of meso-zeaxanthin in primates. Studies conducted in our laboratory using deuterium-labeled carotenoids produced similar results in Japanese quails.\(^7\) These studies suggest that ocular-specific transformation of lutein to meso-zeaxanthin by an enzyme-mediated double-bond shift reaction is the source of the latter carotenoid.

Identification of the isomerase enzyme is complicated in primates and quails. This is because experiments in primates are difficult to execute and are expensive. Quail retina contains several rare carotenoids that may confound downstream enzymology assays. Therefore, in the present study, we chose to determine whether chicken embryos might be a better system to study the production of meso-zeaxanthin because they have a simpler ocular carotenoid profile, and their eggs are easily incubated in a laboratory setting.

The chicken has been a widely used model to study carotenoid transport and retention.\(^3,13,14\) High-density lipoprotein (HDL)-deficient Wisconsin Hypoalpha Mutant chickens were used in studies in which the role of HDL-mediated transport of lutein into the retina was identified.\(^14\) Work done by Connor et al.\(^13\) showed that HDL deficiency led to decreased content of both lutein and zeaxanthin in the retina. In another study, it was determined that chickens maintained on a xanthophyll-free diet resulted in a decreased accumulation of lutein and zeaxanthin in tissues, consistent with a dietary origin of these carotenoids in birds.\(^13\)

Interestingly, it was shown that although plasma and other tissues of birds on a xanthophyll-free diet exhibited the loss of more than 90% of lutein and zeaxanthin, the retina selectively retained these carotenoids.\(^13\) This is consistent with a profound physiological significance for lutein and zeaxanthin in the cone-rich retinas of birds.

Zeaxanthins are better antioxidants than lutein.\(^9\) Because dietary sources typically contain higher concentrations of lutein,\(^10\) it is not surprising that tissues have selective uptake/retention mechanisms optimized to maintain higher zeaxanthin concentrations. Our data show consistently lower lutein:zeaxanthin ratios in the retinas of developing embryos when compared with nonocular tissues (Figs. 5, 6).

Data presented by Wang and coworkers\(^13\) documented the presence of meso-zeaxanthin in the retinas of 1-day-old chickens. The ocular concentration of this carotenoid was maintained in the birds fed with the xanthophyll-free diet, suggesting a potential physiological relevance. Consistent with their observations, our study shows the presence of meso-zeaxanthin in a developmentally regulated manner. In addition, our study reveals that meso-zeaxanthin is an ocular-specific carotenoid in the chicken embryos. More importantly, our data show that RPE/choroid is the likely site of meso-zeaxanthin production. At E17, we first observe the presence of meso-zeaxanthin in all of the RPE/choroid samples that were analyzed; however, only 20% of the retina samples showed the presence of this carotenoid. At E19, we identified meso-zeaxanthin in 80% of both retina and RPE/choroid samples. Whenever RPE/choroid was negative for meso-zeaxanthin, this carotenoid was absent in the retina as well, and we never had an instance in which an embryo’s retina was positive for this carotenoid when its RPE/choroid was negative. Considering that we consistently observe the presence of meso-zeaxanthin in the RPE/choroid several days ahead of when it is present in retina and that we never

![Figure 7](image-url)
identified this carotenoid in the retina of an embryo when RPE/choroid was devoid of it, we can conclude that the RPE/choroid is the primary site of synthesis of meso-zeaxanthin. Because the eggs were kept in the dark during incubation, we can exclude the role of light in the production of meso-zeaxanthin. Previous studies have shown that in the RPE/choroid, carotenoids are nonesterified,7 which provides higher accessibility of the carotenoids in this tissue to enzymes that may be responsible for the production of meso-zeaxanthin.

If the RPE/choroid is the primary site for meso-zeaxanthin production, then transporter proteins may be involved in carrying the newly formed meso-zeaxanthin from the RPE into the retina. A similar situation is observed for retinoid metabolism in which the visual chromophore 11-cis-retinal is enzymatically produced in the RPE and then transported to the photoreceptors.15 Retinoids are closely associated to carotenoids, and retinoid metabolism involves coordination among choroid, RPE, and the photoreceptors mediated by transporter proteins.15 Interphotoreceptor retinoid-binding protein (IRBP) is one such transport protein implicated in retinoid transport between RPE and retina.15,16 It is an abundant transport protein in the interphotoreceptor matrix. Studies from our laboratory have shown that IRBP can bind macular carotenoids with moderate affinity, characteristic of a nonselective transporter.15,16 with equilibrium binding constants comparable to IRBP-retinoid interactions. We therefore hypothesize that IRBP may be involved in transporting meso-zeaxanthin from the RPE into the retina in chicken and human eyes as well (Fig. 8).

This is the first report of developmentally regulated synthesis of meso-zeaxanthin in a vertebrate model system. Our study has determined that the RPE/choroid is the primary site of meso-zeaxanthin synthesis. Because the carotenoids in the RPE are nonesterified, they are accessible to enzymes that may mediate the production of meso-zeaxanthin in this tissue. Future studies will determine candidate proteins that can function as a meso-zeaxanthin isomerase.

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