

# Nutritional Manipulation of Primate Retinas, III: Effects of Lutein or Zeaxanthin Supplementation on Adipose Tissue and Retina of Xanthophyll-Free Monkeys

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**PURPOSE.** Macular pigment (MP) is composed of the xanthophylls lutein (L) and zeaxanthin (Z) and may help to prevent age-related macular degeneration or retard its progression. In this study the effects of L or Z supplementation on carotenoid levels was examined in serum, adipose tissue, and retina in rhesus monkeys with no previous intake of xanthophylls.

**METHODS.** From birth to 7 to 16 years of age, 18 rhesus monkeys were fed semipurified diets containing all essential nutrients but no xanthophylls. Six were supplemented with pure L and 6 with pure Z at 3.9  $\mu\text{mol/kg}$  per day for 24 to 101 weeks. At baseline and at 4- to 12-week intervals, carotenoids in adipose tissue were measured by HPLC. At study completion, carotenoids in serum and retina (central 4 mm, 8-mm annulus, and the periphery) were determined. Results were compared with data from control monkeys fed a standard laboratory diet.

**RESULTS.** Monkeys fed xanthophyll-free diets had no L or Z in serum or tissues. After L or Z supplementation, serum and adipose tissue concentrations significantly increased in the supplemented groups. Both L and 3R,3'S-Z (RSZ or *meso*-Z, not present in the diet) were incorporated into retinas of monkeys supplemented with L, with RSZ present only in the macula (central 4 mm). All-*trans* Z, but no RSZ, accumulated in retinas of monkeys supplemented with Z.

**CONCLUSIONS.** L is the precursor of RSZ, a major component of macular pigment. Xanthophyll-free monkeys can accumulate retinal xanthophylls and provide a valuable model for examining their uptake and conversion. (*Invest Ophthalmol Vis Sci.* 2005;46:692-702) DOI:10.1167/iov.02-1192

Lutein (L) and zeaxanthin (Z) are xanthophylls (carotenoids that contain one or more polar functional groups) that selectively accumulate in the retina and are particularly dense in the foveal region, or macula, where they are the main components of the macular pigment.<sup>1</sup> L and Z are known to function as antioxidants<sup>2,3</sup> and blue-light filters and thereby may protect the macular retina and retinal pigment epithelium from light-initiated oxidative damage.<sup>4</sup> Recent studies in quail exposed to bright light provide evidence that long-term zeaxanthin supplementation leads to increased retinal zeaxanthin and reduced photoreceptor death.<sup>5,6</sup>

Bone et al.<sup>7</sup> have studied the retinal distribution of L and Z in human retina. The L-to-Z ratio increased from an average of ~1:2.4 in the central macula (0-0.25 mm eccentricity) to >2:1 in the periphery (8.7-12.2 mm eccentricity).<sup>7,8</sup> These investigators have shown the components of human macular pigment to be L [(3R,3'R,6'R)- $\beta$ , $\epsilon$ -carotene-3,3' diol]; Z [3R,3'R)- $\beta$ , $\beta$ -carotene-3,3' diol or RRZ]; and RSZ or *meso*-Z, 3R,3'S-Z [(3R,3'S)- $\beta$ , $\beta$ -carotene-3,3' diol].<sup>9</sup> RSZ is primarily located in the center of the macula, where it is found to be in an approximate ratio of 1:1 with RRZ. RSZ is not generally present in the diet, but probably results from chemical processes occurring within the eye.<sup>9,10</sup> Some investigators have speculated that the origin of RSZ is dietary L.<sup>9,10</sup> However, until now, proving this suggestion was difficult, given the lack of both an appropriate animal model (i.e., primates with no macular pigment) and sufficient quantities of pure L and Z for controlled feeding studies.

There is epidemiologic evidence that intake of foods high in L and/or Z, as well as high serum levels of L+Z, are related to reduced risk of advanced age-related macular degeneration (AMD) (SanGiovanni JP, et al. *IOVS* 2004;45:ARVO E-abstract 2242).<sup>11,12</sup> This finding has raised the question of whether supplemental intake of L and Z may be effective in reducing the risk for AMD or slowing its progression.<sup>12-14</sup> However, there are many gaps in the knowledge about the uptake and metabolism of dietary L and Z and their effectiveness in raising macular pigment levels.

The present study was made possible by the existence of a group of xanthophyll-free adult rhesus monkeys. These animals were fed semipurified xanthophyll-free diets from birth and therefore had no detectable xanthophylls in serum and little or no macular pigment, as measured with an *in vivo* photographic method.<sup>15</sup> Thus, the effect of dietary supplementation with individual pure carotenoids could readily be followed. We examined the effects of dietary supplementation of these xanthophyll-free animals with pure L or pure Z. Pure sources of these two carotenoids were made available to us by DSM Nutritional Products, Ltd. (formerly Roche Vitamins, Ltd., Basel, Switzerland) to allow comparisons between the retinal

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Any opinion, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture.

Supported by DSM Nutritional Products, Ltd. (formerly Roche Vitamins Ltd., Basel, Switzerland); Grant 581950-9-001 from the U.S. Department of Agriculture; Grant DK-29930 from the Institute of Diabetes and Digestive and Kidney Diseases (MN); Grant RR-00163 from the Division of Research Resources, National Institutes of Health; and a grant from The Foundation Fighting Blindness (MN).

Submitted for publication November 21, 2002; revised July 1, 2003 and January 26 and October 6, 2004; accepted October 18, 2004.

Disclosure: **E.J. Johnson**, DSM Nutritional Products, Ltd. (F); **M. Neuringer**, DSM Nutritional Products, Ltd. (F); **R.M. Russell**, DSM Nutritional Products, Ltd. (F); **W. Schalch**, DSM Nutritional Products, Ltd. (E, F); **D.M. Snodderly**, DSM Nutritional Products, Ltd. (F)

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TABLE 1. Characteristics of Monkeys

Group, Animal ID	Sex	Age (y)	Body wt (kg)*	Supplement†	n-3 Fatty Acid Status
Xanthophyll-free					
Mean	2M, 4F	13.7	7.5	0	4, low
SEM		1.6	0.6	0	2, adequate
Zeaxanthin-fed					
642	F	9.0	7.1	14 (12, 2)	Low
567	F	10.9	7.9	14 (12, 2)	Low
224	F	18.3	7.6	24 (10, 14)	Low
217	F	18.4	6.9	24 (10, 14)	Adequate
586	M	11.6	12.7	8 (4, 4)	Low
398	M	15.4	11.1	8 (4, 4)	Adequate
Mean		13.9	8.9	11 (9, 3)	
SEM		1.6	1.0	2 (2, 1)	
Lutein-fed					
602	F	10.0	6.7	13 (12, 1)	Low
585	F	10.5	5.3	13 (12, 1)	Low
362	F	15.0	6.8	15 (10, 5)	Low
397	F	14.6	8.6	15 (10, 5)	Adequate
636	M	10.2	11.5	6 (4, 2)	Low
463	M	13.8	12.0	6 (4, 2)	Adequate
Mean		12.4	8.5	15 (9, 7)	
SEM		1.0	1.1	3 (2, 2)	
Control					
Mean	2M, 12F	13.8	7.0	0	Adequate
SEM		1.6	0.7	0	

\* Body weight at study's end.

† Numbers in parentheses indicate duration of supplementation (in months) at 7 days/wk and 4 days/wk, respectively.

response to L and to Z. Two prior papers in this series describe the time course of increases in serum xanthophylls and macular pigment optical density in vivo,<sup>15</sup> morphologic changes in the retinal pigment epithelium,<sup>16</sup> and the effects of acute blue-light exposure. In the present paper, we report longitudinal measures of adipose tissue xanthophylls and the analysis of serum and retinal carotenoids at the end of supplementation, including the levels of L and Z and their metabolites in the macula and periphery. This study provided a unique opportunity to determine the effectiveness of pure L and Z in increasing macular pigment and to identify the dietary origin of RSZ.

## METHODS

### Animals and Diets

All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center and conformed to NIH guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eighteen rhesus monkeys (*Macaca mulatta*) were reared on one of two semipurified diets, both of which contained adequate levels of all known nutrients, including vitamin A (as vitamin A acetate) and  $\alpha$ -tocopherol, but no detectable xanthophylls, as analyzed by our laboratory. As described in more detail in Neuringer et al.,<sup>15</sup> the two diets differed only in their fat sources and therefore in fatty acid composition, with one containing low levels and one adequate levels of n-3 fatty acids in the form of  $\alpha$ -linolenic acid. Because no differences related to fatty acid status were found in any of the analyses reported herein, the data for these two diet groups were in this article. The animals also received limited amounts of very low xanthophyll foods such as wheat or rice cereals, white rice, sweetened drinks, gelatin, pineapple, and banana.

Beginning at 7 to 16 years of age, the diets of six of these monkeys were supplemented with pure L and six with pure Z, at 3.9  $\mu$ mol/kg per day (2.2 mg/kg per day). This dose represented 7.7 times the average daily xanthophyll intake from the standard laboratory diet (described later). The L and Z supplements were purified or synthe-

sized by DSM Nutritional Products, Ltd. (formerly Roche Vitamins Ltd., Basel, Switzerland) and formulated into gelatin beadlets. Doses of beadlets were determined for each animal based on our analysis of xanthophyll content and current body weight. The supplements were inserted into marshmallows, sweetened gelatin, or small pieces of fruit. Beadlets and individual supplement doses were stored at 4°C in the dark. Supplements were provided daily for 4 to 12 months and, due to limited supply of the pure xanthophylls, four times per week thereafter until the conclusion of the study. The duration of daily supplementation varied within each group but was matched between the two groups. However, because L had to be specially purified, its supply was more limited, resulting in a shorter duration of supplementation at four times per week for the L-fed group ( $3 \pm 1$  month compared to  $7 \pm 2$  months for the Z-fed group). The L- and Z-fed groups were balanced to the extent possible based on sex, n-3 diet group, and body weight. The remaining six animals continued on their semipurified diets but received no xanthophyll supplements (xanthophyll-free). Two L-fed and two Z-fed animals were killed after 6 to 8 months of supplementation, two from each group at 13 to 14 months and two from each group each at 15 to 24 months (see Table 1 for details).

Data from the L- and Z-fed monkeys were compared with data from normal control monkeys fed a standard stock diet (Purina 5047 Monkey Chow; Ralston Purina, Richmond, IN) providing a daily carotenoid intake of 0.26  $\mu$ mol/kg per day L, 0.24  $\mu$ mol/kg per day Z, and 0.035  $\mu$ mol/kg per day  $\beta$ -carotene (means of four analyses). These animals also received supplemental fruits and vegetables (typically, one fourth to one half an apple, or one half a carrot approximately three times per week), which contributed an estimated maximum additional daily average of  $\sim 3$  nmol/kg of L plus Z or  $<1\%$  of the intake from the stock diet.<sup>17</sup> They were housed under the same conditions as the experimental diet groups. Tissues analyzed from control monkeys included 17 serum samples and 10 adipose tissue samples. Retinal tissue was analyzed from 14 control monkeys (2 male, 12 female) including 7 for 4-mm macular samples, 11 for 8-mm annular samples, and 8 for peripheral samples (see description of retinal tissue dissection in the next section).

TABLE 2. Retinal Sample Wet Weights

	4 mm	8 mm
Xanthophyll-free	6.2 ± 0.1	13.1 ± 2.5
Zeaxanthin-fed	5.8 ± 0.3	14.7 ± 0.6
Lutein-fed	5.9 ± 0.1	13.3 ± 0.6
Control	4.7 ± 0.6	11.8 ± 0.9

Data are expressed as mean milligrams ± SE.

### Serum and Tissue Collection

Samples of subcutaneous adipose tissue (~40 mg) were taken from the subscapular region of the back under ketamine sedation (10 mg/kg) at 2, 4, 8, 12, 16, 20, 24, 36, and 48 weeks of supplementation. At the time of death, fasting blood samples (15 mL) were drawn from the saphenous or femoral vein into foil-wrapped tubes under dim light and centrifuged at 800g for 15 minutes to obtain serum.

Animals were perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde for morphologic studies.<sup>16</sup> Previous studies<sup>18</sup> have shown that the fixatives have no effect on measurement of carotenoids. However, fixation prevents the reliable separation of neural retina from the retinal pigment epithelium (RPE), so the two tissues were kept attached and analyzed together. Analyses of a separate set of individual fresh neural retinas and RPE from normal stock diet-fed rhesus monkeys showed that carotenoids in the small mass of RPE cells of our single samples were below the detection limit and were therefore negligible compared with the carotenoids in the much larger tissue mass of the retina. After hemisection of the globe, samples of three retinal areas were taken. Biopsy punches were used to dissect the central retina into a 4-mm diameter circle centered on the fovea and a concentric annulus of 8 mm outside diameter (referred to here as the 8-mm samples). Four-millimeter and 8-mm retinal tissue sample weights are given in Table 2. There were no significant differences in sample weight among the groups. A portion of the remaining peripheral retina that varied in area from one animal to another also was collected for xanthophyll analysis. For comparing data across samples, values are referenced to the sample weights.

All serum and tissue samples were protected from light and were stored at -70°C until analysis for carotenoids.

### Chemicals for Carotenoid Assays

HPLC grade methanol, water, hexane, and 2-propanol were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Methyl-*tert*-butyl ether and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Solvents were passed through a 0.45- $\mu$ m membrane filter and degassed before use. Echinenone, used as an internal standard, was from Hoffmann-La Roche, Inc. (Nutley, NJ), and all-*trans* lutein, RRZ, and RSZ standards were a gift from DSM Nutritional Products (Roche Vitamins, Ltd., at the time of the gift). All carotenoid standards were stored at -70°C. The *cis* isomers of xanthophylls were made by adding 2 to 3 drops of iodine in hexane solution (5 mg/100 mL) to a hexane solution of xanthophylls. This was then placed under an infrared heat lamp for 2 hours. The solution was dried under nitrogen gas and redissolved in ethanol. The resultant solution contained *cis* and *trans* isomers of xanthophylls, as identified by absorption spectra and mass spectrometry.

### Analysis of Diet and Lutein and Zeaxanthin Supplements

The carotenoid concentrations of the stock diet were determined by the official method of analysis of the Association of Official Analytical Chemists<sup>19</sup> and analyzed with the same reversed-phase HPLC system used for serum and tissue analysis.<sup>15</sup> For analysis of L and Z supplementation, an exact amount of the supplement beadlets (~0.5 mg) was dissolved in 1.0 mL of distilled water. This solution was extracted with 2 mL of chloroform-methanol (2:1) three times. The chloroform ex-

tract was evaporated to dryness under nitrogen. The residue was redissolved in 1 mL of ethanol, vortexed, and sonicated for 30 seconds and then taken up to 100 mL of ethanol. A 50- $\mu$ L aliquot was used for HPLC analysis. For each batch of xanthophylls the analysis was performed in triplicate and completed before the beadlets were fed. From batch to batch, the xanthophyll beadlets were found to contain 4% to 9% of the purified carotenoid, with the content in the Z beadlets being consistently higher. The L beadlets were found to contain only all-*trans* L and no Z. In the Z beadlets, approximately 90% was in the all-*trans* form, and 10% was present as a *cis* isomer of Z; no L was detectable. The *cis* isomer was tentatively identified as 13-*cis* Z based on comparison of absorption spectra and HPLC retention time with a known standard. The presence of *cis* isomer in only the Z beadlets may be explained by the different formulation processes. The L beadlets contained L that was specially purified in a noncommercial process that did not induce isomerization, whereas the Z beadlets were from a commercially synthesized product (DSM Nutritional Products Ltd.). The noncarotenoid portion of the beadlets was identical for the L and Z supplements.

### Serum and Adipose Tissue Extraction for Carotenoids

Serum carotenoids were measured as described previously.<sup>15</sup> Adipose tissue samples (38 ± 2 mg wet weight) were lyophilized (20 hours at -20°C, <100 psi, 29 ± 2 mg dry weight). To the sample was added 100  $\mu$ L 12% pyrogallol in ethanol, 200  $\mu$ L 30% KOH, and 1 mL ethanol. The mixture was vortexed and incubated at 37°C for 2 hours. After incubation, the sample was cooled to room temperature, 1 mL H<sub>2</sub>O was added, and the mixture was vortexed. Echinenone in ethanol (100  $\mu$ L) was added as an internal standard. The mixture was extracted by using 3 mL ether-hexane (2:1, vol/vol). The mixture was vortexed and then centrifuged at 800g at 4°C for 5 minutes. The upper layer was removed. The extraction with ether-hexane was repeated and the upper layers combined. To the extract was added 1 mL H<sub>2</sub>O. The mixture was vortexed, 1 mL ethanol was added to make the solution clear, and the mixture was centrifuged at 800g for 5 minutes. The H<sub>2</sub>O layer (lower layer) was removed and discarded. Another 1 mL H<sub>2</sub>O was added and removed as just described. The extract was evaporated to dryness under nitrogen. The residue from adipose tissue was redissolved in 100  $\mu$ L of ethanol, vortexed, and sonicated for 30 seconds. A 50- $\mu$ L aliquot was used for HPLC analysis. Adipose tissue concentrations of carotenoids are expressed as picomoles per milligram dry weight.

### Retinal Extraction and General Procedure for Determination of Lutein and Zeaxanthin Stereoisomers

The retinal samples (4-mm central punch, 8-mm annulus, and the periphery) were weighed and ground with a glass rod while on ice. To the sample was added 3 mL chloroform-methanol (2:1), 1 mL 0.85% saline and 150  $\mu$ L echinenone in ethanol (as the internal standard). The mixture was vortexed for 30 seconds and centrifuged at 800g for 15 minutes at 4°C. The chloroform layer was removed and evaporated to dryness under nitrogen. A second extraction was performed on the mixture by using 3 mL hexane, and the mixture was vortexed and centrifuged as described earlier. The hexane layer was combined with the first extraction and evaporated to dryness under nitrogen. The residue from retina samples was redissolved in 75  $\mu$ L of ethanol, vortexed, and sonicated for 30 seconds. A 60- $\mu$ L aliquot was used for HPLC analysis.

The extracted samples were analyzed for carotenoids with a reversed-phase, gradient HPLC system and method of separating L and Z that has been described.<sup>20</sup> In the reversed-phase HPLC system, RRZ and RSZ eluted in one peak. The L and Z peak samples (retention times, 7 to 10 minutes) of each retinal sample were collected from the reversed-phase HPLC system and dried under N<sub>2</sub> and the residue redissolved in 75  $\mu$ L hexane. A 60- $\mu$ L aliquot was injected into a



normal-phase HPLC system to separate the Z stereoisomers (RRZ, RSZ) and L. The L and Z content of the 4-mm macula, 8-mm annulus, and peripheral samples were expressed as picomoles per milligram wet weight for easy comparison of the retinal regions.

### Reversed-Phase HPLC Analysis

The reversed-phase, gradient HPLC system consisted of a pump (616 LC; Waters Corp., Milford, MA), an autosampler (model 717 plus; Waters Corp.), a C-30 column (carotenoids S-3, 4.6 × 150 mm; YMC, Kyoto, Japan) and a detector (model 490E; Waters Corp.). This gradient method allows adequate separation of L, the *cis* isomer of Z, all-*trans* Z (RRZ + RSZ), cryptoxanthin,  $\alpha$ -carotene, 13-*cis*  $\beta$ -carotene, all-*trans*  $\beta$ -carotene, and 9-*cis*  $\beta$ -carotene, as well as four geometrical isomers of lycopene (15-*cis*, 13-*cis*, 9-*cis*, and all-*trans* lycopene).<sup>20</sup> Carotenoids were quantified at 455 nm by determining peak areas in the HPLC chromatograms calibrated against known amounts of standards. Concentrations were corrected for extraction and handling losses by monitoring the recovery of the internal standards. The lower limit of detection was 0.2 pmol for carotenoids. A reversed-phase HPLC chromatogram of standards of L, Z, and RSZ (*meso*-Z) is shown in Figure 1A.

### Normal-Phase HPLC System

The normal-phase HPLC system consisted of a pump and autosampler from the reversed-phase system (Waters Corp.), a column (amlose derivative coated on silica-gel, Chiralpak AD; Daicel Chemical Industries, Ltd., Tokyo, Japan) and a programmable photodiode array detector (model 994; Waters). The HPLC mobile phase was hexane (solvent A) and hexane-isopropanol (1:1, solvent B). The procedure began at 90% solvent A and 10% solvent B at 0.8 mL/min for 55 minutes. This was followed by a 1-minute gradient to 100% solvent A at 1.5 mL/min. The system was held at 100% solvent A for 10 minutes, followed by a 4-minute gradient to 90% solvent A and 10% solvent B at 1.5 mL/min. The system was held at this condition for 15 minutes followed by a 1-minute gradient to 0.8 mL/min. The system was held at 90% solvent A and 10% solvent B at 0.8 mL/min for 10 minutes for equilibration back to initial conditions. This method separated RSZ, RRZ, and L. The lower limit of detection was 0.2 pmol for each xanthophyll. A normal-phase HPLC chromatogram of standards for L, RRZ, and RSZ is shown in Figure 1B.

For retinal samples, the identifications of RRZ, RSZ, and L were confirmed by comparing absorption spectra of samples with those of known standards. Further confirmation was obtained by coelution of sample peaks from extracts of two samples from each xanthophyll-supplemented group with known standards of RRZ, RSZ, or L. In our quantitation of the retinal xanthophylls, we followed the same rationale as Bone et al.<sup>21</sup> That is, no internal standard was necessary for the normal-phase system because the total quantity of Z stereoisomers was obtainable from the reversed-phase chromatography, and the normal-phase separation permitted measurement of their relative proportions. This procedure was carefully worked out using tissue from control monkeys.

### Statistics

All data are presented as the mean  $\pm$  SEM. The significance of differences in serum, adipose tissue, and retinal xanthophyll levels among groups (L-fed, Z-fed, and stock-diet controls) were tested with one-way ANOVAs ( $\alpha$  level  $P < 0.05$ ) followed when appropriate by post hoc pair-wise Bonferroni-Dunn tests ( $\alpha$  level  $P \leq 0.05$  for comparisons between two groups, or  $\leq 0.017$  for pair-wise comparisons among three groups). Significant differences in xanthophyll concentration in serum and adipose tissue at the end of the seven-times-per week (7 $\times$ /wk) supplementation schedule and at the time of death were evaluated using two-way repeated-measures ANOVA followed, when appropriate, by post hoc comparisons for effect of time in each group and effect of diet at each time point. Significant differences from baseline xanthophyll concentrations in adipose tissue were evaluated

with a repeated-measures ANOVA. Although the L- and Z-fed groups were balanced with respect to sex and fatty acid status, two-way ANOVAs were used to test for effects of these variables or interactions with supplement type. Linear regression was used to explore whether tissue xanthophyll concentrations were related to age, body weight (at the beginning or end of the study), or the duration of supplementation (total duration or the duration of 7 $\times$ /wk four-times-per week [4 $\times$ /wk] supplementation). In addition, analyses of covariance (ANCOVAs) were used to test the effects of L versus Z supplementation after adjusting for these factors.

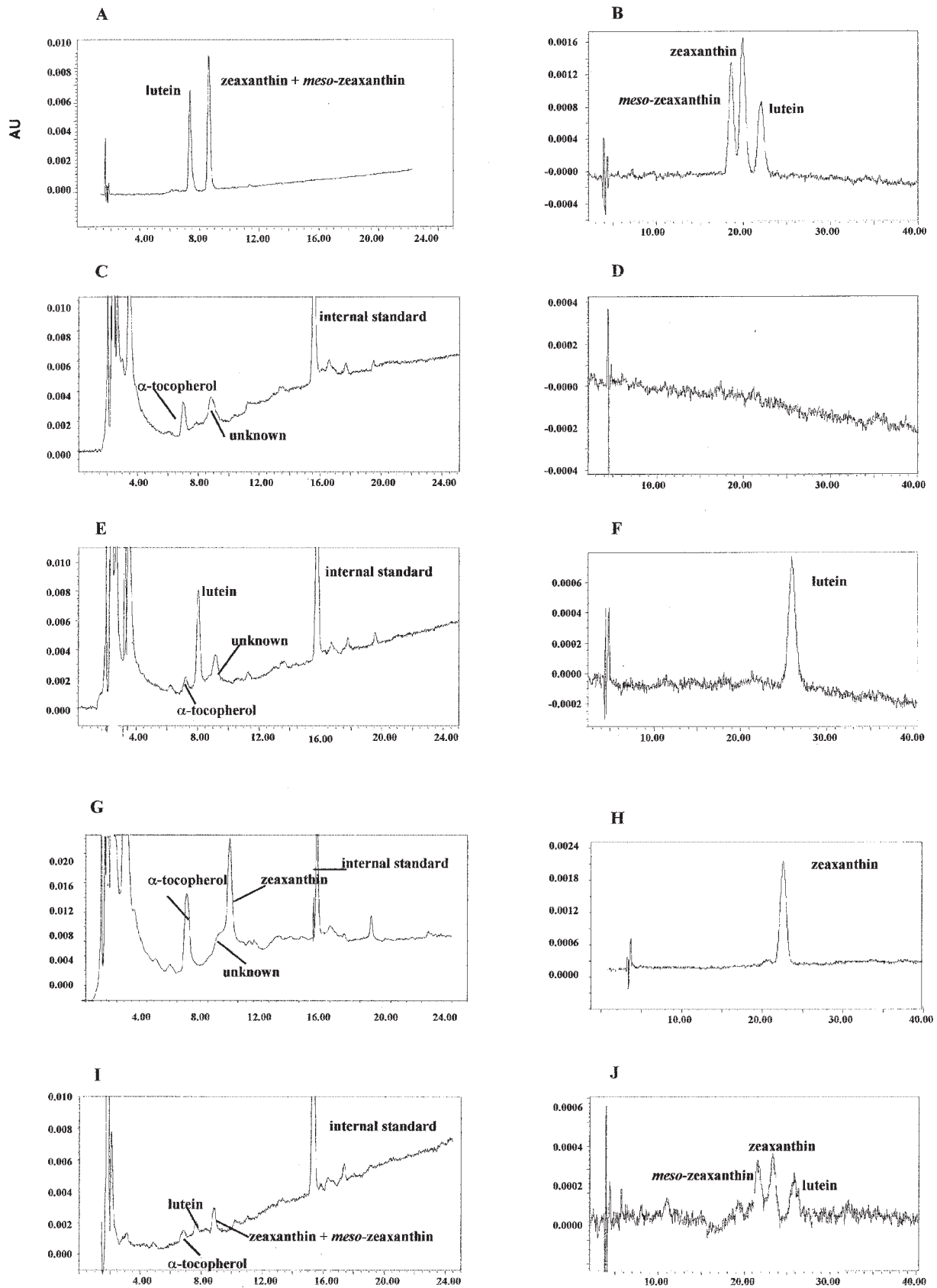
## RESULTS

### Serum Carotenoids

The serum concentrations of xanthophylls at the end of the 7 $\times$ /wk supplementation schedule and at the time of death are presented for comparison, with postmortem tissue concentrations shown in Table 3. Longitudinal measures of serum xanthophylls in these monkeys over the course of L and Z supplementation have been reported.<sup>15</sup> The unsupplemented xanthophyll-free monkeys had no detectable xanthophylls in serum and are not included in the table. There was a trend toward higher total xanthophyll concentrations in the L-fed group ( $P = 0.052$ ) at the end of the 7 $\times$ /wk supplementation period but no difference at study's end ( $P = 0.2$ ). In the L-fed monkeys, serum concentrations of L were significantly lower at the study's end than at the end of the 7 $\times$ /wk supplementation period ( $P < 0.015$ ), but in the Z-fed group there was no difference between the two time points. Serum xanthophyll concentrations at the end of the study were significantly higher in both the L- and Z-fed groups than in the stock-diet control group ( $P < 0.014$  and  $P < 0.0001$ , respectively). Serum L in the L-fed monkeys was entirely in the *trans* form. Serum Z in the Z-fed monkeys was in both the *trans* (72%) and *cis* (27%) forms. There was also a small amount of 3' dehydrolutein (1%) present in the serum of these animals. The identification of this metabolite as 3' dehydrolutein was suggested by coelution with a known standard, absorption spectra comparison with a known standard, and LC/MS (data not shown). Final serum xanthophyll concentration was not significantly related to sex or n-3 fatty acid status (by two-way ANOVA) or to body weight or the duration of supplementation (by linear regression), and the difference between the Z- and L-fed groups remained non-significant when adjusted for these factors by ANCOVA. Although serum xanthophyll concentrations initially decreased after the reduction in the frequency of supplementation,<sup>15</sup> the duration of 4 $\times$ /wk supplementation was not related to final serum xanthophyll levels ( $P = 0.366$ , overall;  $P = 0.857$  for the L-fed group and 0.790 for the Z-fed group).

### Adipose Tissue Carotenoids

The adipose tissue concentrations of individual and total xanthophylls at the end of the 7 $\times$ /wk supplementation schedule and at study end are presented in Table 3. Before supplementation, monkeys fed the xanthophyll-free semipurified diets had no measurable L or Z in the adipose tissue. Adipose tissue carotenoid concentrations increased by 2 weeks of supplementation but were highly variable thereafter in both groups. Therefore, there were no significant differences between the two supplement groups at any time point. However, adipose tissue concentrations of total xanthophyll were significantly greater at the end of the study than at the end of the 7 $\times$ /wk supplementation period ( $P = 0.017$  overall;  $P = 0.087$  in the L-fed group alone,  $P = 0.038$  in the Z-fed group alone). By the end of the study, adipose total xanthophyll concentrations in both supplement groups had risen to the level found in normal control monkeys. Adipose total xanthophyll concentrations at



**FIGURE 1.** HPLC chromatograms. (A) Standards for L, Z (RRZ), meso-Z (RSZ): reversed phase; (B) standards for L, Z (RRZ), meso-Z (RSZ): normal phase; (C) xanthophyll-free animal, 4-mm retinal punch: reversed phase; (D) xanthophyll-free, 4-mm retinal punch: normal phase; (E) L-fed animal, 8-mm retinal punch: reversed phase; (F) L-fed, 8-mm retinal punch: normal phase; (G) Z-fed, peripheral punch: reversed phase; (H) Z-fed, peripheral punch: normal phase; (I) Stock-diet control animal, 4-mm retinal punch: reversed phase; and (J) stock-diet control, 4-mm retinal punch: normal phase.

TABLE 3. Serum and Adipose Tissue Xanthophyll Concentrations

	Zeaxanthin-Fed				Lutein-Fed		
	Control	7×/wk End	Study End	P	7×/wk End	Study End	P
Serum (nmol/L)							
Lutein	74 ± 9	0 ± 0*	0 ± 0*	NS	838 ± 38*	529 ± 73*	0.015
Zeaxanthin							
<i>trans</i>	59 ± 9	391 ± 44*†	577 ± 125*†	NS	0 ± 0	0 ± 0	NS
<i>cis</i>	22 ± 4	263 ± 32*†	213 ± 60*†	NS	0 ± 0	0 ± 0	NS
Total zeaxanthin	81 ± 12	654 ± 68*†	791 ± 178*†	NS	0 ± 0	0 ± 0	NS
3' Dehydrolutein	ND	10 ± 2*†	11 ± 4*†	NS	ND	ND	NS
Total xanthophyll	154 ± 20	665 ± 69*	801 ± 180*	NS	838 ± 38*	529 ± 73*	0.015
Adipose tissues (nmol/g dry wt)							
Lutein	1.18 ± 0.44	0 ± 0*	0 ± 0*	NS	0.44 ± 0.17	1.89 ± 0.82	0.087
Zeaxanthin							
<i>trans</i>	0.57 ± 0.13	0.33 ± 0.04†	1.33 ± 0.30*†	0.040	0 ± 0	0 ± 0	NS
<i>cis</i>	0.15 ± 0.04	0.06 ± 0.02†	0.32 ± 0.07*†	0.038	0 ± 0	0 ± 0	NS
Total zeaxanthin	0.72 ± 0.16	0.39 ± 0.06†	1.64 ± 0.37*†	0.040	0 ± 0	0 ± 0	NS
3' Dehydrolutein	ND	0.02 ± 0.01	0.06 ± 0.00	0.093	ND	ND	
Total xanthophyll	1.90 ± 0.57	0.41 ± 0.06	1.70 ± 0.39	0.038	0.44 ± 0.06	1.89 ± 0.82	0.087

\* For each row: significantly different from control ( $P < 0.05$ ).

† For each timepoint (7×/wk, study end): significantly different from lutein-fed ( $P < 0.05$ ).

ND, not detectable. Data were recorded at the end of the 7×/wk supplementation period and at time of death (study end) for lutein-fed and zeaxanthin-fed monkeys and at single time point for control monkeys fed stock diets.  $P$  is 7×/wk versus end of study. For control, serum groups  $n = 17$ ; For adipose tissue control group  $n = 10$ . For adipose groups at study's end  $n = 4$  (Z-fed). For all other  $n = 6$ .

the end of the 7×/wk supplementation period did not correlate significantly with the duration of this period. In addition, final concentrations did not correlate significantly with the total duration of supplementation or the duration of 4-week supplementation. Final concentrations also were not significantly related to sex, body weight, or n-3 fatty acid status, and the absence of a difference between the Z- and L-fed groups was not altered by adjusting for any of these factors by ANCOVA.

In the L-fed group, all the L was in the all-*trans* form, and no Z was detected. In the Z-fed group, 81% of the total Z was all-*trans*, and 19% was in the *cis* form. In addition, 3' dehydrolutein (presumptive) was detected from 12 to 48 weeks in one or two Z-fed monkeys per time point (not always the same monkey) at concentrations of  $0.04 \pm 0.01$  nmol/g dry weight ( $8.1\% \pm 1.2\%$  of the total xanthophyll, 9/30 adipose tissue samples). Thereafter, 3' dehydrolutein was detected in the two remaining Z-fed monkeys at 86 to 103 weeks at similar concentrations ( $0.04 \pm 0.01$  nmol/g dry weight;  $3.9\% \pm 1.1\%$  of the total xanthophyll content, 5/6 adipose tissue samples). In the adipose tissue biopsy specimens sampled at time of death, 3' dehydrolutein was detected in all samples at a concentration of  $0.06 \pm 0.01$  nmol/g dry weight ( $3.2\% \pm 0.3\%$  total xanthophyll). For the control group, all the L was in the all-*trans* form and the Z was in the all-*trans* (79% of total Z) and *cis* (21% of total Z) forms.

## Retinal Carotenoids

**Control Monkeys.** Representative reversed-phase and normal-phase chromatograms from the 4-mm macular sample of a stock-diet control monkey are shown in Figures 1I and 1J. For control monkeys, the amount of total xanthophylls (L, RRZ, RSZ, and *cis* Z) in the 4-mm macular sample was  $2.91 \pm 0.84$  pmol/mg (Table 4, bottom two rows). Approximately 88% of this was as Z (RRZ + RSZ) with the remainder being L, resulting in an L-to-Z ratio of 0.12. Only one monkey had a small amount of detectable *cis* Z ( $0.32$  pmol/mg, 9% of total xanthophyll). The normal-phase HPLC results demonstrated that the ratio of RRZ to RSZ in the 4-mm macular sample of the control monkeys was  $1.0:0.91 \pm 0.2$ .

In the 8-mm annulus samples, the total amount of xanthophyll in the control monkeys was  $0.27 \pm 0.04$  pmol/mg (Table 4), only 10% of the concentration in the 4-mm macular samples. Approximately 63% of this was RRZ with the remainder as L, for an L-to-Z ratio of 0.59. Unlike the 4-mm punch, no RSZ or *cis*-Z was detectable in these samples.

In the peripheral retina of the control monkeys, the amount of xanthophyll was  $0.22 \pm 0.04$  pmol/mg (Table 4), very similar to the value for the 8-mm sample. Approximately 64% of the peripheral xanthophylls was L, and the remainder was RRZ (36%) and *cis* Z (<1%), so that the L-to-Z ratio was 1.75.

**Xanthophyll-Free and Supplement-Fed Monkeys.** *Incorporation of L into the Retina.* There was no detectable L or Z in retinal samples from xanthophyll-free, unsupplemented monkeys. Furthermore, there was no L in retinal samples from Z-fed monkeys. These results show that retinal L must be derived from the diet and it is not derived from Z. Supplementation was very successful in inducing the incorporation of L into the retina of animals that previously had no L in the diet. The mean L content of the 4-mm macular area from the L-fed animals ( $2.44 \pm 0.34$  pmol/mg) was nearly 8 times the amount found in control monkeys ( $0.31 \pm 0.08$  pmol/mg;  $P < 0.0001$ ). In the 8-mm sample from the L-fed monkeys, the L content ( $0.81 \pm 0.07$  pmol/mg) was eight times the amount found in control monkeys ( $0.10 \pm 0.01$  pmol/mg;  $P < 0.0001$ ), and in the periphery it was nearly five times the amount in the control animals ( $0.69 \pm 0.07$  vs.  $0.14 \pm 0.03$  pmol/mg,  $P = 0.0001$ ).

*Formation of RSZ (meso-Z) in the Retina.* In the 4-mm macular samples of the L-fed group, Z was present entirely in the form of RSZ. RSZ was not found outside the 4 mm sample in the L-fed animals, and it was not present in any samples from the Z-fed animals. Thus, in rhesus monkeys, RSZ appears to be formed from dietary L, and only in the macular region.

*Deriving Retinal Z Concentrations.* Quantitative analysis of retinal Z data from the experimental monkeys was more complex, because an unknown peak overlapping with Z appeared in the reversed-phase HPLC system in all retinal extracts from animals fed the semipurified diet, including animals that were not supplemented (Fig. 1C). This was unexpected because no xanthophyll was detected in the diet, serum, or

TABLE 4. Xanthophyll Concentrations in Retina

Group, Animal ID	4 mm						8 mm						Periphery		
	L	RRZ	RSZ	Total*	Unknown	L	RRZ	Total	Unknown	L	RRZ	Total	Unknown		
Xanthophyll-free unsupplemented															
Mean	0	0	0	0	0.41	0	0	0	0	0	0	0	0		
SEM	0	0	0	0	0.03	0	0	0	0.03	0	0	0	0.10		
Zeaxanthin-fed															
642	0	2.30†	0	2.58†	0.44‡	0	0.53†	0.53†	0.32‡	0	0.53†	0.53†	0.35‡		
567	0	0.47†	0	0.47†	0.44‡	0	0.26†	0.26†	0.32‡	0	0.43†	0.43†	0.35‡		
224	0	3.58†	0	3.81†	0.44‡	0	0.16†	0.16†	0.32‡	0	0.99†	0.99†	0.35‡		
217	—§	—	—	—	—	0	0.24†	0.24†	0.32‡	0	0.73†	0.73†	0.35‡		
586	0	2.09†	0	2.09†	0.44‡	0	0.87†	0.87†	0.32‡	0	0.81†	0.81†	0.35‡		
398	0	2.93†	0	3.06†	0.44‡	—	—	—	—	0	1.01†	1.01†	0.35‡		
Mean	0¶	2.28†	0¶	2.40†	0.44‡	0	0.41†  ¶	0.41†  ¶	—	0	0.75†  ¶	0.75†  ¶	—		
SEM	0	0.52†	0	0.56†	0.07	0	0.13†	0.13†	0.03	0	0.10†	0.10	0.10		
Lutein-fed															
602	2.89	0	3.20†	6.09†	0.50	0.97	0	0.97	0.52	0.75	0	0.75	0.38		
585	0.94	0	1.11†	2.04†	0.46	0.79	0	0.79	0.24	0.60	0	0.60	0.35		
362	2.19	0	1.62†	3.81†	0.52	0.71	0	0.71	0.17	0.74	0	0.74	0.12		
397	3.25	0	2.72†	5.98†	0.38	0.55	0	0.55	0.44	0.49	0	0.49	0.24		
636	3.05	0	1.53†	4.58†	0.74	1.05	0	1.05	0.24	1.00	0	1.00	0.59		
463	2.33	0	0.98†	3.31†	0.18	0.80	0	0.80	0.30	0.57	0	0.57	0.36		
Mean	2.44¶	0¶	1.86†	4.30†	0.46	0.81¶	0	0.81¶	0.32	0.69¶	0	0.69¶	0.34		
SEM	0.34	0	0.37†	0.64†	0.07	0.07	0	0.07	0.03	0.07	0	0.07	0.07		
Control	<i>n</i> = 7					<i>n</i> = 11				<i>n</i> = 8					
Mean	0.31	1.34	1.21	2.91	0	0.10	0.17	0.27	0	0.14	0.08	0.22	0		
SEM	0.08	0.39	0.37	0.84	0	0.01	0.04	0.04	0	0.03	0.01	0.04	0		

Data are expressed in picomoles per milligram

\* Total xanthophylls = L + RRZ + RSZ + *cis* zeaxanthin.

† Corrected for contribution from an unknown component (xanthophyll-free, lutein-fed, and zeaxanthin-fed samples only). For the xanthophyll-free and lutein-fed, individual values were used. For the zeaxanthin-fed, the mean of the xanthophyll and lutein-fed was used.

‡ For each retinal area, unknown concentration was derived from the mean unknown concentration from the xanthophyll-free and lutein fed samples.

§ —, sample lost due to instrument failure.

|| Significantly different from lutein-fed ( $P < 0.017$  for post-hoc comparisons among three groups).

¶ Significantly different from control ( $P < 0.017$  for post hoc comparisons among three groups).



adipose tissue of the unsupplemented animals. Furthermore, no Z or other xanthophyll was detected in the subsequent analysis by normal-phase HPLC (Fig. 1D), indicating that the unknown peak was not Z. Similarly, this unknown peak was also detected in the 8-mm and peripheral retinal samples of L-fed monkeys in the reversed-phase HPLC analysis (Fig. 1E), but not in the normal-phase HPLC analysis (Fig. 1F), even though the peak was well within the detection capability of the normal-phase system.

In the retinal samples of Z-fed monkeys, the unknown peak appeared as a left shoulder on the Z peak (Fig. 1G) that was not present in control monkey tissue. The retention time of this shoulder relative to  $\alpha$ -tocopherol was the same as that of the unknown peak in the L-fed and xanthophyll-free groups ( $1.9 \pm 0.1$  minute), indicating that the same compound was present in all groups fed the semipurified diet. Subsequent normal-phase HPLC analysis revealed no additional information about its identity (Fig. 1H).

The unknown compound was not a *cis* isomer of L or Z, based on its retention time relative to RRZ in the reversed-phase system and known retention times of *cis* isomers. Furthermore, the unknown was found in the retina of the xanthophyll-free and L-fed monkeys. These animals had no dietary intake of Z. Therefore, it would be extremely unlikely that *cis* Z, but not *trans* Z, would be measured. In other analyses we conducted in chicken retina, which contained extremely high concentrations of xanthophylls and therefore *cis* Z,<sup>22</sup> our chromatograms clearly show baseline separations of *cis* and *trans* isomers of L and Z. Identification of the unknown peak was not feasible, because of the small amount present, and attempts to obtain its spectrum with a photodiode array detector were unsuccessful.

For all samples from xanthophyll-free animals, and for 8-mm and peripheral samples in the L-fed animals, there was a direct measure of the unknown peak in the reversed-phase because normal-phase analysis showed no RRZ or RSZ. The mean amount of the unknown compound was nearly identical in xanthophyll-free monkeys and in those with L supplementation. This fact, as well as the consistency of the outcomes described below, indicates that the unknown peak was derived from the semipurified diet, and not the supplements.

Whenever Z was present in the samples, the contribution of the unknown peak meant that the normal-phase Z peaks could not be quantitated by reference to the reversed-phase results. Direct quantitation in the normal phase was not possible, because the volatility of the solvent in which the sample was dissolved, combined with the long run times, made standard curves unreliable. However, use of the reversed-phase values for Z clearly overestimated its concentration and indeed, indicated its presence spuriously, when the normal-phase analysis confirmed its absence. Therefore, a strategy was devised to estimate the contribution of the unknown component. This strategy was based on the available data and the explicit assumptions outlined herein.

For the 4-mm sample of the L-fed monkeys, in which normal-phase analysis confirmed the presence of RSZ, an estimate of the contribution of the unknown peak was derived by using data from both the reversed-phase and normal-phase HPLC systems. This estimate was based on the observation that the ratio of the peak areas for L and Z from each system was the same. This conclusion was based on data from 10 samples (4 mm,  $n = 6$ ; 8 mm,  $n = 4$ ) from control monkeys that were analyzed on both the normal-phase and the reversed-phase systems. Three of the samples were spiked with standards to assure that the elution times were correct. The L-to-Z ratios (including RSZ) for the 10 samples for the reversed-phase and normal-phase systems were  $0.986 \pm 0.300$  and  $0.932 \pm 0.285$ , respectively. The correlation between the L-to-Z ratios in the

reversed-phase and the normal-phase chromatograms was 0.99.

Using this information, estimates of the unknown peak in the 4-mm samples from L-fed animals were derived as follows: In the reversed-phase (rp) HPLC system, RRZ, RSZ, and the unknown elute in one peak, but in the normal-phase (np) HPLC system the unknown peak is absent, and RRZ and RSZ are separated. Therefore, if the unknown is absent:  $(L_{rp}/Z_{rp}) = (L_{np}/[RRZ_{np} + RSZ_{np}])$ . However, if there is a contribution to  $Z_{rp}$  in the reversed-phase system from the unknown peak, then the equation becomes:  $(L_{rp}/[Z_{rp} - \text{unknown}_{rp}]) = (L_{np}/[RRZ_{np} + RSZ_{np}])$ . The equation was then solved for the unknown peak. Because the unknown peak contributed to the peak area of  $Z_{rp}$  measurements, the extinction coefficient ( $E_{1\%}^{1\text{cm}}$ ) for RRZ [ $2350 \text{ g/dL}^{-1} \cdot \text{cm}^{-1}$ ] was used to quantitate the unknown peak. The concentrations of the unknown peak calculated for the 4-mm samples from the L-fed group are summarized in Table 4. Note that the mean estimated value of the unknown peak in the 4-mm samples from the L-fed animals (0.46) is nearly the same as the mean value measured directly in the 4-mm samples from the unsupplemented animals (0.41). This outcome is consistent with the conclusion that the unknown is derived from the semipurified diet rather than from the supplement. There was no indication that this peak was present in the control animals, because even though it was large and relatively evenly distributed across the retina in the experimental animals, it was not apparent in the control 8-mm and peripheral samples (Table 4). As a check on this observation, the unknown peak was also estimated using the same assumptions for the 4-mm samples from the control monkeys, and the negligible calculated value of  $0.03 \pm 0.06 \text{ pmol/mg}$  is consistent with the assumption that animals fed the stock diet did not have the unknown compound in their retinas.

Obtaining the correction for the unknown peak allowed us to estimate the mean amount of RSZ in the 4-mm macular samples of the L-fed group (Table 4). It was greater than the amount of RSZ in the corresponding samples from control animals, although the difference was not statistically significant with this small number of animals.

**Incorporation of Z into the Retina.** The only xanthophylls detected in Z-fed monkeys were RRZ (~95% of the total) and *cis*-Z (~5% of the total) with no detectable amounts of L or RSZ. The low ratio of *cis*-Z in the retina contrasts with the data from adipose tissue described earlier. *cis*-Z was found only in the 4-mm sample. The only xanthophyll measured in the 8-mm and the peripheral samples of the Z-fed monkeys was RRZ.

In the Z-fed group, a direct method of estimating the amount of the unknown peak was not possible, because no L was present to solve the equation. Instead, we relied on the consistency of the data from the L-fed and the unsupplemented animals, and we used the means of the values for the unknown peak in each retinal region of these two groups to correct the estimates of Z concentrations in the corresponding retinal regions of the Z-fed group. This fact introduces an inherent limitation in the precision of our estimates, but as explained later, it affects only the estimates of RRZ in the Z-fed group.

Bearing in mind the limitations of our estimates, we found that all samples from the Z-fed animals had higher concentrations of RRZ than did the control subjects, and the differences between Z-fed and control animals for the 8-mm and the peripheral samples were statistically significant (Table 4).

**Total Xanthophylls in Different Retinal Regions.** In the L-fed monkeys, the total xanthophyll content of the 4-mm punch ( $4.30 \pm 0.64 \text{ pmol/mg}$ , Table 4) was higher than the content in the control and Z-fed animals ( $2.91 \pm 0.84$  and  $2.40 \pm 0.56 \text{ pmol/mg}$ , respectively; Table 4), but the differences between groups were not significant. In the 8-mm annulus samples, the total amount of xanthophyll for control



monkeys ( $0.27 \pm 0.04$  pmol/mg) was significantly lower than that for either the L-fed group ( $0.81 \pm 0.07$  pmol/mg,  $P < 0.001$ ) or the Z-fed group ( $0.41 \pm 0.13$ ,  $P = 0.008$ ; Table 4). Although the mean value for the L-fed group was higher than for the Z-fed animals ( $0.41 \pm 0.13$  pmol/mg), this difference did not reach the criterion for multiple comparisons ( $P = 0.031$ ). In the peripheral retina, the total xanthophyll concentration in the L- and Z-fed groups were similar ( $0.69 \pm 0.07$  and  $0.75 \pm 0.10$  pmol/mg, respectively), and both were significantly higher than in the control group ( $0.22 \pm 0.04$  pmol/mg, both  $P < 0.0001$ ; Table 4).

In the L-fed, Z-fed, and control groups the concentration of xanthophylls was higher in the 4-mm macular sample than in the 8-mm sample ( $P < 0.05$ ). The differences in xanthophyll concentration between the 8-mm and peripheral samples were not significant in any of the groups.

For each retinal region, 4 mm, 8 mm, and periphery, total xanthophyll content was not related to sex, n-3 fatty acid group, age, or body weight. It also did not correlate with the durations of total supplementation or 7×/wk or 4×/wk supplementation. In no case did adjusting for any of these factors alter the pattern of results.

There were no significant correlations between xanthophyll concentration in the 4- or 8-mm samples and concentration in serum, using either the values at the time of death or the averages of values over the previous 3 months. However, total xanthophyll concentration in the peripheral retina was significantly correlated with the final serum total xanthophyll concentration ( $P = 0.0012$ ,  $r = 0.82$  for L- and Z-fed groups combined;  $P = 0.0085$ ,  $r = 0.92$  for Z-fed group alone; not significant ( $P = 0.143$ ) for L-fed group). There were no correlations that approached significance between total xanthophyll in any of the retinal samples and final adipose tissue total xanthophyll concentrations, except for a negative correlation of peripheral retina total xanthophylls with the final adipose level in the Z-fed group only [ $P = 0.025$ ,  $r = -0.98$ ; not significant for L-fed group ( $P = 0.09$ ) or both groups combined ( $P = 0.78$ )]. Thus, serum and adipose tissue Z concentration showed opposite relationships to concentrations in the peripheral retina.

## DISCUSSION

### Serum and Adipose Tissue Xanthophylls

In the L-fed group, virtually all the serum L was in the 3*R*,3'*R*,6*R* (all-*trans*) form and no RRZ or RSZ was detected. In the Z-fed group, about three fourths of Z in the serum was RRZ, of which approximately one fourth was in the *cis* form, and no RSZ was detected. The proportion of *cis* Z was considerably higher than the 10% present in the beadlets, which confirms results in two other monkey species<sup>23</sup> indicating preferential serum accumulation of *cis* Z, in vivo isomerization and/or preferential tissue accumulation of *trans* Z. The serum of the Z-fed group also contained small amounts of a compound tentatively identified as 3' dehydrolutein. This metabolite of zeaxanthin may be the result of acid dehydration in the stomach and has been reported to appear in human serum.<sup>24</sup> The significance of these conversions of RRZ is not known.

In both L- and Z-fed groups the individual serum response was quite varied, although the average concentrations in each group were similar. This variation in response is similar to that observed in humans,<sup>25</sup> but the reasons for the variation are not known. In our study, we found no significant relationships in serum response to gender, age, or the fatty acid composition of the diet,<sup>15</sup> although the samples sizes may have been too small to detect such effects.

The xanthophyll content of adipose tissue is considered to be a better marker of long-term intake of xanthophylls than concentrations in the serum, which reflect more recent intakes.<sup>26</sup> However, in this study levels in adipose tissue were even more varied than serum levels. It should be noted for the control monkeys consuming the stock diet with approximately equal amounts of L and Z, the L/total Z ratio in adipose tissue was 1.6 compared to 0.9 for serum, confirming the selective accumulation of L in adipose tissue reported for quail by Thomson et al.<sup>5</sup>

### Retinal Xanthophyll Metabolism

Rhesus monkeys with life-long xanthophyll exclusion from the diet had no detectable xanthophylls in the retina. Supplementation with pure L or Z led to the accumulation of these xanthophylls in the serum and adipose tissue and retina. A major new finding of this study is that retinal RSZ (or *meso*-Z) is derived from L and not from 3*R*,3'*R*-Z (RRZ or all-*trans* Z). This conclusion is based on the finding that in the 4-mm macular area of the L-fed monkeys, approximately half the xanthophyll content was RSZ, whereas no RSZ was detected in animals fed RRZ. These results are consistent with the proposal of Bone et al.,<sup>9</sup> who first suggested L to be the source of RSZ after demonstrating conversion of L to RSZ under nonphysiological conditions. The observation that RSZ is derived from L may help explain, in part, the individual variation in macular pigment response to L supplementation. That is, there may be individual variation in the efficiency of converting L to RSZ. The conversion of L to RSZ (but not to RRZ) is consistent with the higher ratio of L to Z (including RSZ) in serum versus retina in humans.<sup>9</sup> We did not detect RSZ in the diet, serum, or adipose tissue of any of the monkeys. Therefore, it seems likely that the retina is the site for the enzymatic or photochemical conversion of L to RSZ. Another definitive finding was that the presence of RSZ also was limited to the 4-mm diameter macular area—that is, no RSZ was detected in the 8-mm annulus or peripheral samples. Consequently, the conversion from L to RSZ, and/or the mechanisms for its binding or incorporation, also are limited to the macular area, and may be related to the distribution of cone photoreceptors.<sup>27</sup>

We also analyzed the retinal xanthophyll content of control monkeys. To date, there has been limited information on the macular pigment content and composition of rhesus monkeys. Our results for xanthophyll concentrations in the retina are in the range of previous measures for rhesus monkeys,<sup>28</sup> but the mean content of xanthophylls in the 4-mm macular sample, expressed per unit area, is 1.11 pmol/mm<sup>2</sup>, approximately half the mean of corresponding values for the closely related species *Macaca fascicularis* (2.1 pmol/mm<sup>2</sup>, compiled from data in references 19,31). This difference in retinal xanthophyll concentration may be related to the generally lower serum xanthophylls found in rhesus monkeys compared with other nonhuman primate species.<sup>23</sup> The L-to-Z ratio increased progressively from center to periphery (0.12, 0.59, and 1.75, respectively, for the 4-mm macular area, 8-mm annulus, and periphery). Thus, Z predominated in the macular area and L in the periphery, as found previously in human and macaque retinas.<sup>7,29</sup>

We detected no xanthophylls in the RPE, a finding that probably reflects our analysis of small samples, that is, RPE from individual monkeys. Previous reports<sup>24,30,31</sup> have also found relatively small amounts of xanthophylls in the RPE, indicating that they should make only a small contribution to the macular xanthophylls measured in our study.

In the central 4-mm sample of the control monkeys, 88% of the total xanthophyll content was RRZ and RSZ, in approximately equal concentrations, and 11% was L. Bone et al.<sup>7</sup>

reported Z (including RSZ) to be the major pigment in the central 0 to 0.25-mm portion of the human retina, accounting for approximately 70% of the total xanthophylls. When one considers that RSZ is derived from L, the contribution of dietary L and Z to the xanthophyll content of the macular region was similar. However, in the 4-mm macular sample, RSZ was four times higher than L, indicating a clear preference of the macula to accumulate Z (RRZ and RSZ) rather than L.

Even for the L-fed monkeys, Z in the form of RSZ preferentially accumulated in the 4-mm macular area. The Z content (RSZ or RRZ) of the 4-mm sample of both the L- and Z-fed monkeys was similar to the total Z of the stock-diet control animals, even though the latter group consumed approximately eight times less L and Z and had three to six times less total xanthophyll in serum than either supplement group. This suggests the possibility that the amount of Z in the macula is locally regulated<sup>19</sup> or that a limited number of deposition sites may be essentially saturated. In contrast, retinal levels of L appeared to be more reflective of dietary levels. That is, the control monkeys consumed approximately 15 times less L than the L-fed monkeys and had approximately eight times less L in their retina. The Z-fed monkeys consumed no L and none was detected in their retinas.

It should be noted that the bioavailability of xanthophylls from the stock diet and from the pure beadlets may have been different. Xanthophylls contained in the fibrous plant materials in the stock diet may have been less bioavailable or, alternatively, the purified xanthophylls in the beadlets may have been proportionately less bioavailable because they were not contained in the usual food matrix or because large doses were given at one time (7.7 times the levels of total xanthophylls in the control diet, or 15 to 16 times the levels of the individual xanthophylls).

Another new finding in this study is the increase in xanthophylls in the 8-mm and peripheral samples for both supplement groups compared with the stock-diet control animals. This suggests that high levels of supplementation and/or the animal's life-long history of xanthophyll-free diets, and therefore retinas without macular pigment, resulted in abnormally high accumulation outside the macula. Elevated levels outside the macula have implications for the measurement of macular pigment optical density *in vivo* by reflectometry or psychophysical methods, because these methods depend on a comparison between foveal and extrafoveal sites. Thus, abnormally high levels outside the fovea would result in underestimation of retinal macular pigment content by *in vivo* methods. This could explain why the greater xanthophyll content in retina in L-fed compared with the stock-diet control animals measured by HPLC was not found in the reflectometric measurements described in the companion paper.<sup>15</sup>

It should be noted that the L- and Z-fed animals were maintained on a different supplementation schedule (Table 1). The duration of 7×/wk supplementation varied among individuals but was matched between the two supplement groups; however, the subsequent duration of 4×/wk supplementation tended to be longer for the Z-fed animals. The fact that the serum and adipose tissue concentrations of total xanthophyll were not different between the two groups at the end of the 7×/wk phase suggests that L and Z have similar bioavailabilities for rhesus monkeys, although this does not appear to be the case for other species.<sup>23</sup> In most cases, there were no differences at study end between the total xanthophyll concentrations in serum and tissues of these two groups. However, the experimental design and sample sizes do not permit a robust examination of whether the duration of the 4×/wk supplementation influenced the serum and tissue xanthophyll concentrations.

A major limitation of these data is the presence of an unknown peak in extracts of retinas from animals fed the semipurified diets that coeluted with Z in the reversed-phase system, but was not present in the normal-phase system. The correction for this unknown was significant, but most important for nonmacular regions of the Z-fed group, as it accounted for only 9% to 15% of the uncorrected 4-mm retinal zeaxanthin concentrations, but ~44% of the 8-mm and ~32% of the peripheral uncorrected zeaxanthin concentrations. Therefore, the most significant uncertainties relate to our estimation of the Z concentrations in the parafoveal and peripheral retina. We attempted to minimize this error using data from the L-fed and xanthophyll-free groups, based on the assumption that the consistency of estimates of the unknown across these two groups supported the extension of these estimates to the Z-fed group. Even with this limitation, it was clear that Z supplementation resulted in increased Z concentrations throughout the retina, with concentrations being greatest in the 4-mm region, and that Z supplementation of animals with no previous xanthophyll exposure, at least in this concentration, led to elevated levels in the perimacular and peripheral retina.

The unknown peak did not affect the *quantitation* of L or the ability to determine *presence or absence of RRZ or RSZ* in the retinal samples. Therefore, the presence of the unknown peak did not affect the following conclusions of the study: (1) L is converted to RSZ; (2) this conversion occurs exclusively in the 4-mm macular area; (3) L is not converted to RRZ nor is RRZ converted to L; (4) L supplementation results in increased L concentrations throughout the retina, with concentrations being the greatest in the central 4 mm; (5) L supplementation, at least in these concentrations, to animals with no previous xanthophyll exposure, leads to elevated levels in the perimacular and peripheral retina relative to normal animals fed a standard stock diet; and (6) there is no measurable macular pigment by HPLC in monkeys reared on diets free of xanthophylls. The main effects observed for L in conclusions 4 and 5 are likely to apply to Z supplementation as well.

Our study in xanthophyll-depleted monkeys provided a unique opportunity to examine the individual metabolism of L and Z. Studies such as these may help to determine the importance of these nutrients in macular health.

### Acknowledgments

The authors thank Noelle Landauer, Josephine Gold, and Audrey Trupp for care of experimental animals, preparation and feeding of supplements, and assistance with blood sampling and tissue processing; John Fanton and Anne Lewis for veterinary and pathology services; Noelle Landauer for assistance with data analysis; Guangwen Tang for assistance with mass spectroscopy; Norman Krinsky for discussions regarding xanthophyll metabolism; and Josel Schierle for providing the chiral column for retina analysis; and at DSM Nutritional Products, Ltd., Regina Goralczyk for logistic assistance (in addition to author Wolfgang Schalch), Alfred Giger for providing purified zeaxanthin-free lutein that would not otherwise have been available, and Oliver Froeschis for information on methodology for separation of xanthophylls.

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