LUTEIN AND ZEAXANTHIN: USE OF IN VITRO MODELS TO EXAMINE DIGESTIVE STABILITY, ABSORPTION, AND PHOTOPROTECTIVE ACTIVITY IN HUMAN LENS EPITHELIAL CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirement for
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ABSTRACT

Lutein and zeaxanthin are selectively accumulated in the lens and macular region of the retina. It has been suggested that these xanthophylls protect ocular tissues against ultraviolet radiation and reactive oxygen species that can cause cataracts and age-related macula degeneration. Insights regarding the absorption of dietary xanthophylls for delivery to ocular tissues are limited. My first objective was to examine factors affecting the transfer of lutein and zeaxanthin from foods to micelles during simulated digestion. Test foods included spinach, wolfberry, orange pepper, squash, mango and lutein supplement. Micellarization of lutein and zeaxanthin during the small intestinal phase of digestion exceeded that of β-carotene and was greater for xanthophylls in oil-based supplements than in spinach. Xanthophyll esters were decreased and non-esterified xanthophylls increased during simulated digestion as a result of cholesterol esterase activity. The efficiency of micellarization of free and esterified forms of the xanthophylls differed with micellarization of free zeaxanthin/lutein > zeaxanthin/lutein mono-esters > zeaxanthin/lutein di-esters. Apical uptake of lutein from micelles by human intestinal cells was linear for 4 hours. Stimulation of chylomicron synthesis was associated with secretion of only 7.6 ± 0.1% of cellular lutein. The activities of acquired xanthophylls within enterocytes merits investigation.
Although a photoprotective effect of xanthophylls in ocular tissue has been proposed, direct support is lacking. Therefore, the second objective of my study was to examine the effects of xanthophylls on lipid peroxidation and the mitogen-activated stress signaling pathways in human lens epithelial (HLE) cells following UVB irradiation. Pre-treatment of cultures with either 2 µmol/L lutein, zeaxanthin and astaxanthin for 4h before exposure to 300 J/m² UVB radiation decreased lipid peroxidation and attenuation of c-JUN NH₂-terminal kinase (JNK) and p38 by 47-57%, 50-60% and 25-32%, respectively. Significant inhibition of UVB-induced activation of JNK and p38 was observed for cells containing < 0.20 and 0.30 nmol xanthophyll/mg protein, respectively, whereas greater than 2.3 nmol α-TC/mg protein was required to significantly decrease UVB-induced stress signaling. These data suggest that physiological concentrations of xanthophylls protect cultured human lens epithelial cells against UVB irradiation. In vivo assessment of the photoprotective activities of xanthophylls is warranted.
This work is dedicated to my Mom and Dad who always have taught and encouraged their children to be a caring, sensitive and educated individual.
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ABSTRACTS


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CHAPTER 1

1.1 INTRODUCTION

Cataract remains the major leading cause of blindness in the world accounting for 40-70% of all cases. More than 20 million Americans aged 40 and older, or about one in every six people in this age range, are affected by cataract. It has been estimated that the federal government spends more than $3.4 billion each year treating cataract through the Medicare program (244). Approximately two thirds of cataracts are due to genetic factors, although the specific genes involved have not been identified. Environmental risk factors for cataracts include cigarette smoking and exposure to UVB radiation (70, 316). Aged-related macular degeneration (AMD) represents another important cause of blindness with more than 1.6 million Americans aged 50 and older having this condition. An increasing number of studies have suggested that dietary factors have the potential to prevent or alleviate the severity of these ocular abnormalities.

Epidemiological studies consistently have shown an inverse association between high intake of fruits and vegetables and the risks of cataract and AMD (43, 48, 217, 222, 295). The possibility that bioactive compounds such as carotenoids and polyphenols in fruits and vegetables can prevent diseases caused by oxidative stress has attracted much attention. The xanthophylls lutein (LUT) and zeaxanthin (ZEA) are of particular interest in regards to ocular health, since these compounds are selectively accumulated in the...
macular region and lens of human and non-human primates (192). Although there are now marketing claims that use of supplements containing xanthophylls decreases the risk of developing ocular diseases, definitive scientific evidence remains scarce.

In order to promote health, dietary compounds or their metabolites must be available to intestinal epithelial cells, absorbed, and delivered to target tissues. Details related to processes associated with the absorption of dietary xanthophylls and their subsequent transport to tissues are quite limited. Several animal and human studies (45, 54, 106, 341) suggest that lutein is more bioavailable than β-carotene and lycopene from food sources. The basis for this phenomenon is unclear. Likewise, the mechanisms responsible for selective accumulation of lutein and zeaxanthin in the lens and macular region remain unknown. Because the lens is not vascularized, it is unlikely that lens epithelial cells are directly exposed to plasma lipoproteins that serve as delivery vehicles of carotenoids to other tissues. The lens acquires nutrients from the surrounding aqueous humor which is secreted by ciliary body cells (CB). This suggests the existence of a unique mechanism for selective transfer of xanthophylls from plasma to the aqueous humor for transfer to the lens epithelium. Processes related to the absorption of xanthophylls and their selective delivery to the lens and macula merit detailed examination to better understand the relationship between dietary xanthophylls and ocular health.

General interest in the possible health-promoting effects of dietary xanthophylls stems from epidemiological studies that have shown increased intake of fruits and vegetables reduces the risk of chronic diseases including certain types of cancers, coronary heart diseases and ocular diseases (78, 319). However, definitive molecular
mechanisms by which xanthophylls protect cells against oxidative insult remain unclear. It is believed that photo-oxidative processes and endogenous H$_2$O$_2$ represent insults to the lens. Both ultraviolet radiation (UVR) and H$_2$O$_2$ activate NF-$\kappa$B in the mammalian lens, a process that appears to contribute to the development and progression of cataract (22, 72). Also, the classic mitogen-activated protein kinase (MAPK) pathways the and the growth arresting and DNA damage gene product 45 (gadd45) are activated after exposure of mammalian cells to UVR and H$_2$O$_2$ (23, 117, 197, 210). Low molecular weight antioxidants such as vitamin C, $\alpha$-tocopherol, glutathione, carotenoids and the antioxidant enzymes (Cu, Zn- and Mn-superoxide dismutase, glutathione peroxidase, catalase and ceruloplasmin) have all been proposed to contribute to the protection of the lens against oxidative stress (14). The xanthophylls may act to protect the eye from oxidative stress, and especially from UV phototoxicity and H$_2$O$_2$, by quenching singlet oxygen and other reactive oxygen species and inhibiting the activation of stress-reduced cascade pathways (e.g. NF-$\kappa$B and MAPK). Further research is necessary to examine the molecular roles of xanthophylls in protecting lens epithelial cells from UVR and oxidative insults.

My research has been directed towards two general questions. First, how are dietary xanthophylls absorbed from foods? Second, do xanthophylls protect the lens against UV and oxidative insults? My hypotheses and specific aims follow.
1.2 HYPOTHESIS

1. The enhanced absorption of lutein compared to β-carotene from foods is due to the more efficient micellarization of the xanthophylls during digestion.
2. Once micellarized, apical uptake of xanthophylls by intestinal absorptive cells is independent of dietary source.
3. Lutein and zeaxanthin attenuate oxidative damage and activation of the stress signaling pathway in human lens epithelial (HLE) cells exposed to ultraviolet irradiation.

1.3 SPECIFIC AIM 1

*Characterize the digestive stability and bioavailability of dietary xanthophylls using the in vitro digestion and Caco2-human intestinal cell models.*

Objective 1.1 To examine the digestive stability and micellarization of lutein and zeaxanthin and their esters during simulated digestion of selective foods and supplements.

Objective 1.2 To compare the uptake of xanthophylls from natural and synthetic micelles by Caco-2 human intestinal cells.

Objective 1.3 To investigate xanthophyll secretion from Caco-2 cells.
1.4 SPECIFIC AIM 2

Establish the role of lutein and zeaxanthin in preventing oxidative stress in HLE cells induced by ultraviolet radiation (UVR)

Objective 2.1 To assess the use of β-cyclodextrans as a vehicle for the delivery of lutein, zeaxanthin and α-tocopherol to human lens epithelial cells.

Objective 2.2 To examine the effectiveness of xanthophylls and α-TC as inhibitors of UVB-induced damage in human lens epithelial cells.

Objective 2.3 To determine if lutein, zeaxanthin and α-tocopherol diminish UV-induced activation of MAPK signaling kinases.

Objective 2.4 To compare the potency of xanthophylls and α-TC in attenuating UV-mediated activation of c-Jun NH₂-terminal kinase and p38.
CHAPTER 2

LITERATURE REVIEW

2.1 Chemical Properties and Biosynthesis

2.1.1 Structure and Nomenclature

*a. Speciation.* Carotenoids are yellow, orange, and red pigments that are present in many commonly consumed fruits and vegetables. More than 600 structurally distinct carotenoids have been identified (excluding isomers) in various photosynthetic organisms, and about 40 are regularly ingested by humans (113, 218). Most nutrition research has focused on the six carotenoids with the highest plasma concentrations in U.S. population. These include \(\alpha\)-carotene, \(\beta\)-carotene, lycopene, lutein, zeaxanthin and \(\beta\)-cryptoxanthin (*Figure 2.1*) (174,175). The term “hydrocarbon carotenoids” is used to describe carotenoids such as \(\beta\)-carotene and lycopene. The terms xanthophylls and oxy-carotenoids denote carotenoids derivatized with one or more oxygen containing functional groups and include lutein, zeaxanthin, astaxanthin and \(\beta\)-cryptoxanthin (6). Functional groups for xanthophylls are diverse and include hydroxyl, methoxy, carboxy, keto, oxo, or epoxy units.
FIGURE 2.1  Common carotenoids in human plasma (174, 175)
b. **Stereochemistry** refers to the study of the three-dimensional orientation of atoms in molecules. There are optical and geometric isomers of carotenoids.

i. **Optical isomers of xanthophylls.** Xanthophylls have two chiral or asymmetric centers located at carbons 3 and 3’ or the two rings in the structure. The two chiral centers at carbons 3 and 3’ in xanthophylls can exist either in the $R$ or the $S$ form. Thus, three stereoisomers are possible; $(3S,3’S)$, $(3R,3’S)$, and, $(3R,3’R)$. The $(3R,3’S)$ form is referred to as the “meso” configuration. In nature, the xanthophylls generally exist as a specific stereoisomer, whereas synthetic preparations of carotenoids represent racemic mixtures. Ocular tissues contain meso-zeaxanthin, an optical isomer of lutein. Meso-zeaxanthin is a conversion product derived from retinal lutein (24). The metabolism and biological activities of the stereoisomers may differ as exemplified by the use of L-, but not D-, forms of amino acids for protein synthesis.

ii. **Geometric isomers of xanthophylls.** Because there is no rotation of attached groups around double-bonded carbons, different spatial arrangements are possible. If the two largest groups are attached on the same side of the double bond, they are designated as being in a $cis$-($Z$) configuration. If the two groups are aligned on opposite sides of the carbon-carbon double bond, they are referred to as being in the $trans$-($E$) configuration. Conversions between $cis$- to $trans$- require that the double bond are broken and reformed. Because xanthophylls have multiple double bonds, there are numerous possibilities for $cis$- and $trans$-isomers (Figure 2.2). However, most carotenoids exist in the more stable $trans$-form rather than $cis$-form in nature. $Cis$-$trans$ conversions occur at elevated temperature and especially in the presence of light, iodine and acid (298). Since processing of foods often involves heating and decreases in pH, isomerization is known
FIGURE 2.2 Structure of the geometric isomers of lutein, zeaxanthin and β-carotene (modified from Ref. 41)
to occur. Stable cis-isomers of xanthophylls have been observed at positions 9, 13 and 15 and 9’, 13’ and 15’.

The cis- and trans- isomerism at carbon-carbon double bonds is an important feature of carotenoids because these geometric isomers may have different biological properties. For example, photoprotective and light-harvesting functions have been defined for cis-isomers of carotenoids different in photosynthetic bacteria (181). After heat treatment, 9-cis-βC is normally found in processed fruits and vegetables (47). This cis-isomer can be enzymatically cleaved to 9-cis-retinal or isomerized to trans-βC (90). These isomers are converted to their respective retinoic acid derivatives, each of which is the natural ligand for distinct nuclear receptors. All-trans-retinoic acid and 9-cis retinoic acid bind with high affinity to RAR and RXR receptors, respectively. These activated receptors function as hormone-activated trans-activating factors in the regulation of gene expression for the differentiation and development of cells (89). Moreover, RXR is a “master” regulator since it forms heterodimers with other nuclear receptors (e.g., VDR, PPAR and LXR) that modulate the expression of genes for carbohydrate, lipid, and mineral metabolism (Figure 2.3). Several cis-isomers of xanthophylls, i.e., 13-cis-lutein and 13-cis-zeaxanthin, also are present in the plasma of healthy subjects who consumed spinach and corn meal (182). Khachik and colleagues (173) also demonstrated that lutein may be oxidized in vivo in human to its 3’-keto derivative, isomerized from the 6’R to the 6’S form, or converted to 3’-epilutein and zeaxanthin. Specific roles of cis vs. trans-isomers of xanthophylls and their metabolites are unknown at this time.
FIGURE 2.3 All-trans-retinoic acid (all-trans-RA) and 9-cis-retinoic acid (9-cis-RA) bind and activate retinoic acid receptor (RAR) and retinoid X receptor (RXR) acted as trans-activating factors in the regulation of gene expression for the differentiation and development of cells (modified from Ref. 13a)
2.1.2 Biosynthesis in Plants

Photosynthesis is performed by many lower (e.g., photosynthetic bacteria and algae) and all higher plants. Chlorophylls and carotenoids are pigments that participate in the light-harvesting process, and provide protection against photo-oxidation (65). Chlorophylls $a$ and $b$ and carotenoids interact to trap light for the photosynthetic process. Thus, it is not surprising that the concentrations of carotenoids and chlorophylls in any given source are often proportional and edible dark green leaves are usually among the richest sources of provitamin A.

The genetics and molecular biology of carotenoid biosynthesis is relatively well established (6). As products of the isoprenoid pathway, carotenoids are derived from the five carbon isoprenoid precursor, IPP (isopentenyl diphosphate). The production of IPP from glyceraldehyde-3-phosphate and pyruvate is central for the synthesis of monoterpenes ($C_{10}$) such as limonene and menthol, diterpenes ($C_{20}$) such as taxol and casbene, and the phytol ($C_{20}$) conjugates such as tocopherol, phyloquinones and carotenoids. The first step in carotenoid biosynthesis is the condensation of two geranylgeranyldiphosphate (GGDP, $C_{20}$) moieties to yield the colorless carotenoid phytoene (Figure 2.4). Once phytoene with its nine double bonds is formed, it is further desaturated to lycopene which contains 13 double bonds. These four desaturations are catalyzed by the enzyme phytoene desaturaturase in bacteria. In plants, two enzymes, i.e., phytoene desaturase and zeta-carotene desaturase, each add two symmetrical double bonds. As the number of double bonds increase, the carotenoids become colored ranging from the pale yellow of zeta-carotene to the brilliant red of lycopene. Cyclohexene rings
FIGURE 2.4 Pathways of carotenoid synthesis
(Reactions: 1 = desaturation; 2 = cyclization; 3 = hydroxylation; 4 = epoxidation; and 5 = oxidation.) (modified from Ref 65)
can then be formed (cyclization) at one or both ends of the molecule. If two identical rings are formed, the product is β-carotene. If one β ring and one ε (epsilon) ring are formed, the product is α-carotene. These reactions are catalyzed by the enzymes β- and ε-cyclase. Both β- and α-carotene are orange-yellow in color.

Further steps in the pathways involve the addition of oxygen moieties to generate xanthophylls. The addition of hydroxyl groups to the 3 and 3’ positions of the rings on β-carotene gives rise to zeaxanthin, while the addition of two hydroxyls to α-carotene yields lutein. Lutein plays a central role in the photosynthetic apparatus. The hydroxylations of the β- and ε-cyclohexene rings are performed by two separate enzymes. Further addition of keto groups at the 4 and 4’ position of zeaxanthin via a ketolase yields astaxanthin, a prominent carotenoid in marine microalgae and the major pigment in salmon, trout and most crustaceans (e.g., shrimp, lobster, and crab). Epoxidation of β-carotene, β-cryptoxanthin, zeaxanthin, and lutein yields a large number of epoxy carotenoids.

2.2 Digestion, Absorption, Transport and Metabolism of carotenoids

2.2.1 Dietary Sources

Animals and humans are not capable of synthesizing carotenoids, but absorb them from the diet and use provitamin A carotenoids as a source of vitamin A. Structurally, retinol is essentially one half of the molecule of β-carotene with an added molecule of water at the end of the lateral polyene chain. Thus, β-carotene is a potent provitamin A carotenoid. An unsubstituted β ring with a C₁₁ polyene chain is the minimum
requirement for vitamin A activity. \(\gamma\)-carotene, \(\alpha\)-carotene, \(\beta\)-cryptoxanthin, \(\alpha\)-cryptoxanthin and \(\beta\)-carotene-5,6-epoxide all have one unsubstituted ring and therefore have one half the bioactivity of \(\beta\)-carotene. Acyclic carotenoids such as lycopene and the xanthophylls in which the \(\beta\)-rings have hydroxyl, epoxy, and carbonyl substitutes are not precursors of vitamin A.

Lutein and zeaxanthin are widely distributed in foods and can be found in the free form, bound to protein, or esterified at one (mono-) or both (di-) hydroxyl groups on the cyclohexene rings (113,177). The xanthophylls are located predominantly in the matrix of leaves of green plants. This matrix is usually complex, consisting of fiber, digestible polysaccharides, and protein. Food tables continue to be compiled for the Western diet. The USDA-NCC Carotenoid Database for U.S. Foods was reported by Holden et al. (146). Data on geometrical isomers of lutein and zeaxanthin in fruits and vegetables have been reported by Humphries and Khachik (132). Recently, O’Neill and his colleges (259) reported carotenoid content in fruits and vegetables consumed in European countries. Some countries in Latin American and Asia also have published carotenoid databases for their foods (99, 101, 152, 222, 320). The highest concentrations (normally in range 8,000-39,500 \(\mu\)g/100 g) for lutein and zeaxanthin are found in dark green leafy vegetables (Table 2.1). A mean of 1-2 mg/d of lutein are reportedly consumed in American and European countries (62, 259).

The carotenoid composition and content in fruits and vegetables are affected by many factors, including cultivar or variety, the part of the plant consumed, stage of maturity, climate or geographic site of production, harvest- and post-harvest processing, and
<table>
<thead>
<tr>
<th>Food</th>
<th>µg/ 100 g edible portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kale, raw</td>
<td>39,550</td>
</tr>
<tr>
<td>Kale, cooked</td>
<td>15,798</td>
</tr>
<tr>
<td>Spinach, raw</td>
<td>11,938</td>
</tr>
<tr>
<td>Turnip green, cooked</td>
<td>8,440</td>
</tr>
<tr>
<td>Collards, cooked</td>
<td>8,091</td>
</tr>
<tr>
<td>Spinach, cooked</td>
<td>7,043</td>
</tr>
<tr>
<td>Spinach soufflé, frozen, cooked</td>
<td>2,727</td>
</tr>
<tr>
<td>Lettuce, cos or romaine, raw</td>
<td>2,635</td>
</tr>
<tr>
<td>Broccoli, raw</td>
<td>2,445</td>
</tr>
<tr>
<td>Squash, summer, Zucchini with skin</td>
<td>2,125</td>
</tr>
<tr>
<td>Corn, sweet, cooked</td>
<td>1,800</td>
</tr>
<tr>
<td>Brussels sprouts, raw</td>
<td>1,590</td>
</tr>
<tr>
<td>Cornmeal, germinated</td>
<td>1,355</td>
</tr>
<tr>
<td>Peas, green, canned</td>
<td>1,350</td>
</tr>
<tr>
<td>Brussels sprouts, cooked</td>
<td>1,290</td>
</tr>
<tr>
<td>Corn, sweet, canned</td>
<td>884</td>
</tr>
<tr>
<td>Persimmons, Japanese, raw</td>
<td>834</td>
</tr>
<tr>
<td>Bean, snap green, cooked</td>
<td>700</td>
</tr>
<tr>
<td>Bean, snap green, canned</td>
<td>660</td>
</tr>
<tr>
<td>Bean, snap green, raw</td>
<td>640</td>
</tr>
<tr>
<td>Carrots, baby, raw</td>
<td>358</td>
</tr>
<tr>
<td>Lettuce, iceberg, raw</td>
<td>352</td>
</tr>
<tr>
<td>Cabbage, raw</td>
<td>330</td>
</tr>
<tr>
<td>Celery, raw</td>
<td>250</td>
</tr>
<tr>
<td>Papayas, raw</td>
<td>75</td>
</tr>
<tr>
<td>Squash, winter, cooked</td>
<td>66</td>
</tr>
<tr>
<td>Peaches, raw</td>
<td>57</td>
</tr>
<tr>
<td>Melons, cantaloupe, raw</td>
<td>40</td>
</tr>
<tr>
<td>Squash, winter, raw</td>
<td>38</td>
</tr>
<tr>
<td>Peaches, canned</td>
<td>33</td>
</tr>
<tr>
<td>Watermelon, raw</td>
<td>17</td>
</tr>
<tr>
<td>Grapefruit, raw, pink and red</td>
<td>13</td>
</tr>
</tbody>
</table>

**TABLE 2.1** Lutein and zeaxanthin content of common foods (µg/100 g edible portion) (modified from Ref. 146)
storage (123, 124, 286). For example, Mercadante and Rodriguez-Amaya (30) showed that the carotenoid content in mango increases as the fruit ripens. Enhanced carotenogenesis usually is associated with decomposition of chlorophylls and transformation of the chloroplasts into chromoplasts.

Carotenoids are not evenly distributed in the food. Carotenoids are usually more concentrated in the peel than in the pulp of fruits and fruit vegetables. Exceptions to the usual pattern are pink-fleshed guava, red pomelo and gac fruit, in which the high lycopene concentration in the pulp compensates for the greater amounts of other carotenoids in the peel (4, 158, 262).

The one factor that markedly affects the carotenoid content is the maturity of the plant foods when harvested and offered for consumption. For example, squash and pumpkin can be harvested over a long period and have a long shelf life during which time carotenoid biosynthesis continues. Carotenoid concentrations also can decrease during ripening of fruits in which the color at the ripe stage is due to anthocyanins (e.g., yellow cherry, red currant, strawberry, and olive fruit) and in fruits that retain their green color when ripe, such as kiwi.

Carotenoids are also available in the market as dietary supplements. The main supply of lutein is derived by extraction from marigold (Tagetes erecta) flower or from dried, powdered alfalfa (159, 252). Meanwhile, zeaxanthin is extracted from Chinese wolfberry (Lycium chinense Miller), tomatoes are the source of lycopene supplement, and astaxanthin is extracted from the algae Hematococcus pluvialis. In addition, extracts rich in β-carotene and containing other carotenoids are produced industrially from palm oil, algae (Dunaliella) and fungi (Blakeslea trispora).
2.2.2 Digestion and absorption of carotenoids (Figure 2.5)

2.2.2.1 Gastric ingestion

Carotenoids are processed during digestion in the same manner as other dietary lipophilic compounds. Thus, they must be released from the food matrix, emulsified in the lipid phase of chyme, and solubilized in mixed micelles. Digestion is initiated in the oral cavity as the food is mechanically sheared and lubricated with saliva before entering the stomach. Hydrochloric acid, pepsin and gastric lipase are secreted into the gastric lumen and mix with the ingested foods. This results in partial release of the carotenoids from the food matrix to emulsified oil droplets. Apolar carotenoids such as β-carotene reside in the core of the lipid droplet, whereas polar carotenoids are preferentially distributed at the surface (30).

2.2.2.2 Small intestinal digestion

Entry of chyme into the small intestine is associated with release of pancreatic secretions and bile into the lumen. The acidity of chyme is neutralized by bicarbonate and hydrolytic enzymes continue to degrade the food matrix, releasing lipophilic compounds to lipid droplets. Lipases attack triglycerides, phospholipids and other lipids in the oil droplet and the products are partitioned in mixed micelles in the presence of bile salts. Cholesterol esterase and pancreatic triglyceride lipase hydrolyze carotenoid esters to free carotenoids (37,160). Once formed, mixed micelles diffuse across the unstirred water layer and deliver carotenoids and other lipophilic compounds to the apical surface of the mucosal epithelium. El-Gorab et al. (84) first showed that bile salts are required for the
FIGURE 2.5 Digestion and absorption of dietary carotenoids.
uptake of β-carotene by rat everted sacs.

The relative distribution of carotenoids in food matrix, lipid droplets and aqueous fraction within the stomach and duodenum was examined recently in human subjects (331). The mashed/pureed vegetable component of the test meal was either carrots, spinach and tomato providing a dose of approximately 10 mg of β-carotene, lutein and lycopene, respectively. The liquefied meal was delivered by nasogastric tube and samples were periodically collected from the gastric lumen and duodenal lumen for 2.5 hours. The aqueous fraction of the stomach contained only trace amounts of carotenoids, and isomerization of the carotenoids during digestion in the stomach and duodenum was minimal. The mean concentration of carotenoids during the sampling period was dependent on chemical species and/or food matrix with 5.6, 4.6 and 2.0% of lutein, all trans β-carotene, and lycopene, respectively, present in the aqueous (presumeably micellar) fraction of duodenum. The observation that substantial amounts of (13C)-all trans β-carotene and retinol appeared in plasma after ingestion of a meal containing greater than 99% (13C)-9-cis β-carotene in oil clearly demonstrates that isomerization can occur during and/or after processing (233). Faulks et al. (90) found that the ratio of all trans and 9-cis β-carotene in a test meal and stomal effluent collected from human subjects with an ileostomy were similar, suggesting that isomerization is minimal prior to delivery of the carotenoids to enterocytes. This and related reports suggest that isomerization is very limited during the digestion process.

### 2.2.2.3 Uptake and metabolism by absorptive epithelium

The transfer of carotenoids from micelles to the apical surface of epithelial cells lining the small intestine generally is assumed to occur by passive diffusion (100). The
possible influence of the physiocochemical properties of carotenoids on apical uptake of these compounds has received limited attention. Carotenes and polar carotenoids are likely to reside in the core and at the surface of micelles, respectively (83), but it is not known if this effects transfer to the epithelial cells. Despite the apparently greater efficiency of micellarization of the cis isomer of β-carotene (202), studies with intestinal cells (77), animals (68), and humans (107) consistently show that the mucosal uptake and absorption of cis isomers of β-carotene is markedly less than that of all trans β-carotene. Recent data suggest that apical uptake of β-carotene by Caco-2 human intestinal cells (77, 103) and absorption of lycopene by humans (68) may be saturable. This suggests that carotenoids, like cholesterol and fatty acids (139, 293), may be transported across the brush border membrane by a facilitated process. High affinity, carotenoid binding proteins have not been identified in the plasma membrane of higher animals.

Following apical uptake by the enterocyte, the pathway for intracellular transport of these hydrophobic compounds remains unknown. Central oxidative cleavage converts pro-vitamin A carotenoids to two molecules of retinal and generally yields two molecules of retinol. This process is known as bioconversion. To express the vitamin A activity of carotenoids in diet on a common basis, a joint FAO/WHO Expert Consultation Committee (276) introduced the concept of the retinol equivalent (RE) and established the following relationships among food sources of vitamin A:

\[
\begin{align*}
1 \text{ µg Retinol} & = 1 \text{ µg RE} \\
1 \text{ µg β-carotene} & = 0.167 \text{ µg RE} \\
1 \text{ µg other provitamin A carotenoids} & = 0.084 \text{ µg RE}
\end{align*}
\]

Provitamin A cleavage is catalyzed by β,β-carotene 15,15’-monooxygenase.
(BCO1), which was previously referred to as β,β-carotene 15,15’-dioxygenase. BCO1 is a cytosolic, nonheme iron-containing enzyme which has the ability to cleave the 15,15’-double bond of a variety of carotenoids in addition to β-carotene, including α-carotene, β-apocarotenenols, and β-apocarotenals (189, 190). β-carotene 9’10’-monooxygenase (BCO2) is a distinct enzyme that catalyzes eccentric cleavage of β-carotene to β-apocarotenals and β-ionone. The apo-carotenals can be converted to retinal and β-apocarotenoic acids, which may serve as precursors for retinoic acid (241, 359).

Examination of the expression of BCO1 and BCO2 in rodent, chicken and human tissues has been facilitated by the identification and cloning of the respective genes (345, 372). The sequence of BCO1 is highly homologous between animal species. The predicted amino acid sequences of BCO1 and BCO2 are approximately 40% homologous and the activity of BCO2 appears to be much lower in tissues than that of BCO1. Eccentric cleavage products of β-carotene have been reported to affect the activity of transcription factors and modulate cell proliferation in cultured cells (e.g., 323). β-carotene 9’10’-monooxygenase (BCO2) also is expressed in several of the same organs as BCO1. In contrast with rodents and chickens, BCO1 mRNA levels in human enterocytes are lower than in liver, retina, and kidney. Lindqvist and Anderson (208) recently reported that BCO1 protein is present in epithelial cells throughout the body including the mucosal layer of the gastrointestinal tract, hepatic parenchymal cells, exocrine pancreatic cells, kidney tubules, adrenal gland, Sertoli and Leydig cells in testis, endometrium in uterus, and the ovary. β-carotene 9’10’-monooxygenase (BCO2) also is expressed in several of the same organs as BCO1. Expression of the BCO genes in many
tissues has led to speculation that localized synthesis of retinoids are important, especially during times when dietary intake of provitamin A carotenoids and retinoids is inadequate.

While the expression of BCO2 appears to be constitutive, BCO1 activity is subject to regulation by both vitamin A status and other nutrients. Intestinal, but not hepatic, activity is increased in vitamin A deficient rats and decreased in response to supplementation with β-carotene, retinyl acetate, apo-8’-carotenal, and all \textit{trans}- and \textit{9-cis}-retinoic acid (9, 266, 350). Effects of vitamin A status on the activity of BCO1 in intestinal homogenates were well correlated with the ratio of retinyl ester to β-carotene in lymph collected for 8h after administration of the carotenoid to groups of rats with different vitamin A status (346). Likewise, Lemke \textit{et al.} (198) found a decreased ratio of $^{13}$C-retinyl ester to $^{13}$C-β-carotene in the plasma of human subjects supplemented with vitamin A. Intestinal activity of BCO1 also was decreased in rats fed diets deficient in either protein or iron, an essential cofactor for catalytic activity (75, 266). In contrast, BCO1 activity was increased in the intestine of rats fed diets enriched in unsaturated fatty acids (74). These data suggest that diet modulates BCO1 activity by both transcriptional and post-translational processes.

In addition to carotenoid cleavage and the possibility of isomerization within the enterocyte, oxidative products of dietary carotenoids have been identified in plasma and several tissues (238). It is not clear if these metabolites are generated by enzymatic or non-enzymatic reactions, where the reactions occur, and what, if any, physiological processes they modulate. The possibility that oxidized products are generated enzymatically is supported by reports that high intake of carotenoids increased expression
of several cytochrome P₄₅₀ proteins (164).

Enterocytes containing carotenoids not incorporated into chylomicron or metabolized will be sloughed from the villus tip during normal turnover of the mucosal epithelia. Possible roles for carotenoids acquired by enterocytes but not transferred to the lymph have not received attention. Likewise, subsequent absorption of carotenoids that may be released into the lumen from sloughed enterocytes is possible, although supporting data are lacking.

**2.2.2.4 Intestinal transport and delivery of carotenoids and cleavage products to extraintestinal tissues.**

Carotenoids and retinyl esters synthesized after cleavage of provitamin A carotenoids are incorporated into nascent chylomicrons in the golgi apparatus of the enterocytes and secreted into lymphatic circulation. Recent studies with human intestinal cells have shown that the extent of incorporation of carotenoids into chylomicrons is dependent on carotenoid species and geometric isomer (77). The differential incorporation of carotenoids into chylomicrons suggests that high affinity binding proteins may participate in intracellular partitioning of these compounds. Chylomicrons are secreted into lymph, converted to chylomicron remnants by lipoprotein lipase, and endocytosed by the liver where carotenoids may be utilized, stored, or re-secreted into plasma in very low density (VLDL) and high density (HDL) lipoproteins. The distribution of β-carotene, α-carotene, and lycopene are 58-73% in LDL, 17-26% in HDL, and 10-16% in VLDL, whereas β-cryptoxanthin is distributed with 40% in LDL, 40% in HDL and the remainder in VLDL. The dihydroxy carotenoids lutein and zeaxanthin are found predominately in HDL (53%) with 31% in LDL and 16% in VLDL (264). Furr and Clark (100) also
suggested that the xanthophylls can be transferred directly from chylomicrons to HDL resulting in more rapid disappearance of polar carotenoids than nonpolar carotenoids from circulation.

Peripheral tissues accumulate carotenoids by LDL receptor-mediated endocytosis. It is not known if HDL can serve as a donor of carotenoids to cells. Tissues with high density of LDL receptors generally contain relatively high concentrations of the provitamin A carotenoids.

2.2.2.5 Excretion

The absorption of carotenoids from foods is incomplete. Once absorbed, carotenoids are not excreted in urine (32), although some oxidized metabolites of retinoids are likely to be eliminated by this route. Also, very small quantities of endogenous carotenoids are lost by exfoliation of skin, and low concentrations of carotenoids are present in bile (199). Thus, fecal elimination represents the primary route of elimination from the body. Several groups have reported that β-carotene is stable during in vitro incubation of the carotenoid with intestinal aspirates or homogenized stool from rats and humans (121, 313). It is unknown if the lack of modification of the carotenoid was due to in part to its addition as water-dispersable beadlets and in ethanol vehicle as opposed to a food matrix.

2.2.3 Distribution of carotenoids in blood and tissues

Although about 40 carotenoids appear to be absorbed from diet, only six carotenoids (α-carotene, β-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin) are predominant and nine metabolites have been detected in human blood and milk (175). The ratio of lutein to zeaxanthin in the plasma is consistently between 4 or 5 : 1 (62, 267, 304)
More recently, investigators have focused on the plasma and tissue concentration of different carotenoid isomers. Holloway et. al. (150) showed that cis-isomers, and predominantly 5-cis-, 13-cis- and 9-cis lycopene were significantly increased in plasma after healthy volunteers ingested either a lycopene supplement or tomato puree containing mostly all trans-lycopene. In contrast, You et. al. (374) reported that 9-cis-β-carotene was converted to all-trans β-carotene after oral administration in humans. Interestingly, selective accumulation of cis-lycopene was observed in both benign and malignant prostatatic tissue, whereas all-trans lycopene was predominant in plasma (55). The basis for this difference and its possible biological significance are unknown.

Carotenoids are ubiquitous in mammalian tissues, but the accumulation of carotenoids among animals is species specific and highly variable. In humans, dietary carotenoids accumulate primarily in adrenal and testes followed by liver, adipose, kidney and skin (41). Some tissues, like the corpus luteum, have very high concentration of carotenoids (3.3-33.7 nmol/g), whereas other major organs like muscle and brain have very low concentrations (<0.04 nmol/g) (156). Investigators also have quantified the concentrations of carotenoids in cells and tissues that are readily available to assess the relationship between dietary intake and accumulation of these compounds in health and disease. For example, buccal mucosal cells contain 0.64 nmol/g wet weight, a concentration which is similar to the amount found in heart, thyroid, and spleen. Colonic and rectal epithelial cells obtained at biopsy contained about 0.3 nmol/g. (66, 156, 216, 267). Of particular relevance to my dissertation is the interesting finding that human macula and lens only contain lutein, zeaxanthin and several of their metabolites (17, 24).
2.2.4 Factors affecting the bioavailability of carotenoids (Table 2.2)

The generally accepted definition of bioavailability is the portion of a substance in a food, meal or diet that is utilized for normal body function. Thus, a direct measurement of bioavailability can be made if it possible to monitor utilization. This definition is too restrictive for vitamin A, carotenoids and other essential nutrients. The specific end point (target tissues) for assessing utilization of carotenoids has not been clearly identified, carotenoids such as lutein and zeaxanthin are selectively accumulated in the ocular tissue (24, 41). Provitamin A carotenoids are required for activities such as replication of cells in villus crypts, maturation of cells migrating along the villus, and the integrity of epithelial barrier function. Carotenoids also may act as both antioxidants and pro-antioxidants in tissues. Thus, it is more appropriate to define functional bioavailability as the efficiency of transfer of carotenoids or provitamin A carotenoid from the food matrix to epithelial cells lining the mucosa of the gastrointestinal tract. The numerous exogenous and endogenous factors that influence carotenoid bioavailability have been discussed in detail previously and are summarized below (29, 285, 335, 340, 363).

2.2.4.1 Effects of chemical speciation, food matrix, and processing. It is well recognized that the absorption of dietary carotenoids and their metabolites is not simply a function of the amount ingested. The physicochemical properties of the carotenoid, plant food matrix, pre- and post-harvesting practices, food preparation, composition of the meal, and host-related factors affect carotenoid bioavailability (Table 2.2). For example, the relative bioavailability of lutein from spinach is greater than that of β-carotene (341) and β-carotene bioavailability from carrots > broccoli > spinach (152, 284). Likewise, lutein was absorbed more efficiently than β-carotene when the carotenoids were
administered in oil (45, 180, 341). The observation that carotenoids are more bioavailable from oil-based supplements than from foods further demonstrates the influence of the food matrices on absorption. Moderate cooking, mashing and juicing also increase carotenoid bioavailability (e.g., 81, 260, 342). Such processing destroys plant tissue structure, thereby increasing surface area and interactions of hydrolytic enzymes and emulsifiers with food particles during the gastric and small intestinal phases of digestion. Processing also can induce conversion of some of the all trans to cis isomers of carotenoids. For example, processed kale, spinach, and corn and broccoli had increased levels of cis-lutein and and cis-zeaxanthin than the fresh vegetables (334). Esterified lutein normally are found in lutein supplement. However it was showed that esterified lutein from many fruits and vegetables was low bioavailability (36, 171). In contrast, lutein supplement in the form of lutein-dipalmitate was 62 % more bioavailable than the free form of lutein supplement in healthy subjects consumed single dose of supplement (33). There is limited information of digestion and absorption of other xanthophyll esters in either in vivo or in vitro studies.
Amount ingested
• ↑ intake → ↑ plasma carotenoid and perhaps retinol

Physiochemical properties
• speciation
• crystalline vs liquid/oil
• trans vs. cis isomers
• free vs esterified vs protein bound

Food sources, matrix and processing
• subcellular localization (chloroplasts vs. chromoplasts)
• leaf vs. flower/seed
• particle size (e.g., puree > shopped > leaf/whole)
• raw vs processed foods
• foods/meals vs supplements

Diet
• amount and type of fat, protein and fiber
• interactions with other carotenoids

Host factors
• gut health
• nutritional status
• genotype

TABLE 2.2  Factors affecting the bioavailability of carotenoids
2.2.4.2. Dietary factors.

i. Fat. Investigators have examined the roles of dietary fat, fiber, and other carotenoids on carotenoid bioavailability. Dietary fat increases carotenoid bioavailability by providing a depot for the release of hydrophobic compounds from the food matrix, stimulating the secretion of bile salts and pancreatic lipases for micelle formation, and inducing chylomicron synthesis (29). Approximately 5-10g fat in a meal is required for efficient absorption of carotenoids, although more fat was required when the diet contained lutein ester instead of free lutein (287). The type of fat also affects absorption of carotenoids. Absorption of carotenoids by rats was more efficient when the carotenoids were administered in olive oil than in corn oil (54). Similarly, the presence of unsaturated fatty acids, and particularly oleate, in micelles stimulated β-carotene absorption from the perfused rat intestine (147). Hu et al. (153) reported that β-carotene was absorbed more efficiently when ingested with a meal rich in sunflower oil compared to beef tallow. Dietary triglycerides with long-chain rather than medium-chain fatty acids also enhance β-carotene and retinyl palmitate absorption (27). As expected, inhibitors of lipid absorption, including olestra (364) and plant sterols (280), decrease carotenoid bioavailability primarily by decreasing micellarization. Phospholipids represent another class of lipids that affect carotenoid bioavailability. Lyso-phosphatidylcholine has been shown to stimulate the absorption of lutein by mice (13).

ii. Fiber. The water soluble fibers pectin, guar and alginate decrease the apparent absorption of β-carotene, lycopene and lutein (281, 284). Possible mechanisms responsible for the fiber-mediated decrease in carotenoid bioavailability include decreased micellarization due to binding of bile acids and phospholipids, inhibition of
lipase activity, increased viscosity and volume of luminal contents, and decreased residence time of enterocytes on the villus (281).

iii. Other carotenoids. There also is interest in the possibility that carotenoids can influence the absorption of one another from the same food or meal. For example, β-carotene was reported to decrease lutein absorption, while lutein either decreased and increased β-carotene absorption in different subjects (180). In another study, lutein impaired β-carotene absorption, but did not affect the secretion of retinyl esters in chylomicrons (336). The interaction between β-carotene and lutein appeared to be somewhat specific, since β-carotene absorption was not affected by lycopene. Additional reports of interactions between pure carotenoids affecting post-prandial appearance in plasma of humans and animals were reviewed by van den Berg (337). More recently, Tyssandier et al. (332) reported that the absorption of β-carotene, lutein and lycopene from a single vegetable was greater than when the food was co-administered with either a second carotenoid-rich vegetable or the purified carotenoid from the second vegetable. Possible sites for pre-absorptive interactions between carotenoids include competition for incorporation into micelles, cellular uptake from the micelle, binding to BCO1 and incorporation into chylomicrons.

2.2.4.3. Physiological and pathophysiological factors

i. Gut “health”. The absorption of dietary carotenoids and their bioactive products also is modulated by phenotypic characteristics of the host that affect processes associated with digestive and absorptive events. These include the composition and activity of luminal fluids and the morphological and functional integrity of the absorptive epithelium. For example, plasma response to a single dose of β-carotene was significantly
lower in subjects administered omeprazole to increase gastric pH to the neutral range compared the same subjects when gastric pH was normal, i.e., acidic (313). Cholestasis, pancreatic insufficiency, biliary cirrhosis, cystic fibrosis and other syndromes responsible for fat malabsorption decrease carotenoid bioavailability and can induce vitamin A deficiency, especially in children (257).

Intestinal parasitism can impair carotenoid absorption or utilization. Metabolism of the carotenoid by parasites residing in the intestinal lumen, parasite-associated changes in the numbers and maturation of absorptive cells along the villi, and cytokine-mediated decreases in lipid absorption associated with the infection may all contribute to the decline in carotenoid absorption and conversion. Absorption and utilization of β-carotene were enhanced after de-worming children infected with *Ascaris* (163). In contrast, plasma retinol concentrations in pre-school children in Ghana fed a stew with dark green cassava and kapok supplemented with fat and β-carotene were not further elevated by administration of antihelmenthics (314).

**ii. Nutritional status.** Nutritional status can affect the bioavailability of provitamin A carotenoids. The plasma vitamin A response curve was decreased after administration of β-carotene in protein deficient rats compared to controls (266). This suppression was due to a decline in the activity of BCO1. Because of the central role of retinoic acid in cellular differentiation, vitamin A deficiency compromises the integrity of epithelial barriers. Mild vitamin A deficiency reduced the numbers of duodenal goblet cells per villus and luminal mucus, and decreased cellular division in intestinal crypts villus in rats (227). These alterations can affect intestinal permeability. Gastrointestinal integrity as assessed by the dual-sugar gastrointestinal permeability test was markedly improved
when vitamin A deficient young children in Gambia and India ingested β-carotene rich mango and received vitamin A supplementation, respectively (321). Erdmann and associates (21, 234) observed decreased uptake of micellarized β-carotene in synthetic mixed micelles by brush border membrane vesicles isolated from vitamin A-deficient Mongolian gerbils and rats compared to membrane preparations from animals fed vitamin A adequate diets. It is unknown if the differences were due to immaturity of plasma membranes from donor cells or other biochemical alterations associated with dietary inadequacy. Decreased uptake of micellarized β-carotene across the brush border membrane may offset the greater activity of BCO1 associated with vitamin A deficiency.

Since the catalytic activity of BCO1 appears to require iron (76), it is expected that iron status of the host will affect the absorption and cleavage of pro-vitamin A carotenoids. The specific activity of BCO1 in the soluble fraction of homogenized intestinal mucosa was positively correlated with iron content of the tissue prepared from rats fed diets with different levels of the trace metal (75). Munoz et al. (236) found that iron supplementation of Mexican children with iron significantly increased plasma retinol and that the effect was more robust for children who were deficient in vitamin A and iron at the initiation of the intervention. It is unknown if iron-mediated improvement of vitamin A status was due to effects of the trace metal on provitamin A uptake from the lumen, increased conversion to retinoids, absorption of the provitamin A carotenoids and retinyl esters, or post-absorptive metabolism.

Plasma concentrations of carotenoids are affected by adiposity, gender, consumption of alcohol, smoking status, and menstrual cycle (34, 95). Possible influences of such factors on the accessibility, conversion efficiency, and absorption of dietary carotenoids
are unknown.

**iii. Genotype.** Recent studies using tracer isotope techniques have confirmed earlier observations concerning the marked variability in the absorption of β-carotene by human subjects after feeding β-carotene-rich foods for several weeks or after administering a single oral dose of β-carotene (140, 206). Moreover, plasma β-carotene and vitamin A were neither predictive of absorption of β-carotene nor its conversion to retinol by healthy women and men. These differences originally resulted in the classification of individuals as “responders” or “non-” or “low-responders”. Suggestions for the observed variation among healthy subjects tested under well controlled conditions have included differences in absorption efficiency, incorporation into chylomicrons, rates of cleavage to retinal, and clearance from circulation. Lin *et al.* (206) suggested that differences in the ability to transfer the carotenoid from a complex matrix to the absorptive cell may be the basis for the reported variability, since all individuals were “responders” when administered high doses of β-carotene in oil (e.g., 28). Differences in conversion of β-carotene to retinyl esters also will affect the quantity of the carotenoid appearing in lymph and plasma.

Genetic factors also likely affect the efficiency of carotenoid absorption and conversion. Polymorphisms in genes required for the many reactions affecting the transfer of carotenoids from food matrix to micelles during digestion, secretion of chylomicrons, and the kinetics of post-absorptive delivery of carotenoids and retinoids to tissues may contribute to the observed variations in the absorption and conversion efficiency of provitamin A carotenoids by individuals. However, knowledge about the characteristics and regulation of carotenoid transport and metabolism remains quite
limited, precluding consideration of specific polymorphisms at this time.

2.2.5. **Approaches to studying the bioavailability of carotenoids (Table 2.3)**

2.2.5.1. **Methods for investigating carotenoid bioavailability in human subjects.**

There is universal agreement that carefully controlled investigations using human subjects represent the ideal standard for determination of the bioavailability of carotenoids. Balance studies and plasma response curves have been used to determine relative bioavailability of carotenoids.

**i. Balance** studies (335). Metabolic balance studies represent a traditional method for estimating the absorption and excretion of compounds that are not metabolized in the gastrointestinal tract. A primary advantage of this approach is that it is not invasive. Dietary carotenoids are not excreted in urine. Small quantities of endogenous carotenoids are lost with exfoliated skin and bile (199). Since elimination in feces represents the major excretory route for ingested carotenoids, it has been suggested that absorption can be estimated by carefully monitoring intake and fecal output. Fecal sampling (32, 343), collection of stomal effluent from ileostomists (90, 260), and gastrointestinal lavage (32) have been used to estimate carotenoid absorption. Collected materials contain ingested materials that were not transferred to cells in mucosal epithelium, as well as compounds that may have been absorbed and subsequently returned to lumen of gastrointestinal tract with bile and pancreatic secretions, effluxed across the apical surface of mucosal epithelium, or retained within cells sloughed from intestinal and colonic villi. Stability of carotenoids in the lower gut remains unknown despite several reports that purified β-carotene was stable during incubation of purified carotenoids with fecal suspensions (121) and jejunal washings (313).
**In vivo methods**

- Balance techniques
  - metabolic mass balance
  - ileostomy mass balance
  - gastrointestinal lavage

- Plasma response techniques
  - changes in carotenoid concentration in plasma
  - appearance-disappearance of carotenoids in plasma triglyceride-rich fraction after dosing
  - isotopic methods

- Sampling from gastrointestinal lumen after ingestion

- Intestinal perfusion techniques

**In vitro methods**

- Simulation of gastric and small intestinal phases of digestion

- Uptake by isolated intestinal segments

- Uptake and metabolism by Caco-2 human intestinal cell line

- Coupled *in vitro* digestion/Caco-2 cell model

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**TABLE 2.3** Approaches for studying factors affecting the bioavailability of carotenoids
ii. **Plasma assessment techniques** (265, 335). Relative bioavailability also has been estimated by monitoring changes in plasma concentration of carotenoids after feeding purified compounds or enriched test foods for a period of days or weeks. This method lacks sensitivity due to relatively high level of endogenous carotenoids in plasma and assumes that different carotenoids have similar rates of both clearance from plasma and redistribution between tissues and plasma. The problem is somewhat offset by monitoring the temporal rise and removal (AUC, area under the curve) of recently absorbed carotenoid and its metabolites in plasma following administration of test dose of purified compound or meal. Periodic collection of the triglyceride rich fraction from plasma after administering the dose provides investigators with a qualitative profile for newly absorbed material (347).

Administration of physiologic doses of purified carotenoids or plant foods that are labeled with stable isotopes (²H and ¹³C) facilitates the study of *in vivo* absorption and metabolism. Published studies using this powerful approach have been reviewed recently by van Lieshout *et al.* (344). The authors provide useful insights about experimental design, choice of isotopic tracer, dosing regimen, sample collection, chemical analysis and mathematical modeling for the use of stable isotope technology to assess the bioavailability and bioefficacy of carotenoids. Yao *et al.* (376) were the first to examine the absorption of ¹³C-lutein purified from algal biomass by human subjects. More recently, several groups studies the kinetics of 13C-lutein from intrinsically labeled vegetables (187, 205).

iii. **Digestive stability and micellarization.** Borel and associates (5, 27) examined the characteristics of digestion of fat and other dietary lipophiles in the human stomach and
Results from an experimental study with pureed vegetables rich in either β-carotene, lutein and lycopene were discussed in 2.2.2.2. Carotenoids were stable during the gastric and duodenal phases of digestion and the efficiency of micellarization in the duodenal lumen was dependent on the carotenoid species and/or food matrix.

2.2.5.2 Animal models.

The primary advantages of animal models for investigating nutritional problems relevant to humans include the ability to induce dietary deficiencies and excess, administer radioisotopes, access tissues of interest, and induce acute and chronic diseases. Approaches used to investigate carotenoid bioavailability in human subjects are applicable for animal models. The central issue concerns the selection of an animal that absorbs and metabolizes carotenoids in a comparable manner to human subjects. Erdman and associates (195) have provided a critical review of this subject.

Carotenoid absorption, metabolism and function have been investigated to varying degrees in the mouse, rat, gerbil, ferret, preruminant calf, nonhuman primate and the chicken. Because mice and rats efficiently convert ingested provitamin A carotenoids to vitamin A in the intestine, they do not absorb intact carotenoids unless supraphysiologic doses are administered. Thus, these rodents are not useful for investigating the bioavailability of provitamin A carotenoids from foods. Since vitamin A deficiency can be induced in rats, they are a preferred animal model for investigating the pathophysiological consequences of an inadequate supply of this essential nutrient.

Ferrets, gerbils and preruminant calves, like humans, absorb a portion of dietary provitamin A carotenoids and produce retinyl esters in enterocytes. The high cost of maintaining calves precludes their widespread use for assessing problems related to the
bioavailability of provitamin A carotenoids. β-carotene bioavailability in the ferret was shown to be greater from carrot juice than from carrot (365), less for the 13-cis isomer than the all-trans isomer (88), and antagonized by canthaxanthin (364). The ferret also is the appropriate size for collection of mesenteric lymph during intestinal perfusion for examining problems related to carotenoid absorption and conversion (357). Gerbils also absorb all trans β-carotene more efficiently than cis isomers of β-carotene, and conversion of β-carotene to vitamin A appeared to be decreased when gerbils were fed diets with low fat and citrus pectin (67, 68). These effects of dietary components and the differences in absorption of β-carotene isomers are similar to those reported in humans and demonstrate the utility of the gerbil and ferret for investigating the bioavailability of provitamin A carotenoids. Because vitamin A deficiency has not been induced in either the ferret or gerbil, it is not feasible to investigate the impact of vitamin A nutritional status on bioavailability and conversion efficiency in these animal models at this time.

2.2.5.3. In vitro models as tools for investigating carotenoid bioavailability

Isolated intestinal cells, membrane vesicles, and simulated digestive processes represent models for studying the characteristics and regulation of complex processes associated with digestion and absorption. These cost effective models can be used to assess the effects of physiochemical properties of carotenoids, food matrices, methods of processing, and other components of meals on the digestive stability and bioavailability of carotenoid containing foods and supplements. However, these simplified models are “static”, since they are not influenced by the many factors that can affect digestive and absorptive processes in vivo. These include, but are not limited to, rate of transit of the matrix along the mucosal epithelium, alterations in luminal content of digestive enzymes
and bile salts in response to the quantity and composition of ingested foods, stirring of the mucous layer separating the epithelium and the luminal contents, and humoral factors secreted by cells in lamina propria and peripheral tissues that modulate transport and metabolic processes in enterocytes. Differences between the intact organism and simple biochemical and cellular models dictate the need for cautious interpretation of results from \textit{in vitro} studies as applied to the more complex situation \textit{in vivo}. Nevertheless, \textit{in vitro} models represent useful tools for defining key questions that merit investigation \textit{in vivo}.

\textit{i. Biochemical models.} Simulation of the gastric and small intestinal phases of digestion, individually and particularly in combination, is commonly used to evaluate the impact of the digestive process on nutrients, drugs, and, more recently, transgenes and their products (Table 2.4). Failla and associates (91, 104, 105) have investigated the stability and micellarization of carotenoids and chlorophylls during the simulated gastric and small intestinal digestion of foods, meals and supplements. The carotenoid profile before and after digestion is determined to assess stability of the carotenoids during the digestive process. The final product, referred to as digesta, is centrifuged at high speed to remove residual particulate material and oil droplets to yield the aqueous fraction (Figure 2.6). Conditions are optimized for complete digestion of oil droplets, \textit{i.e.}, micellarization of lipophiles. The aqueous fraction is passed through a filter with 0.22 umeter pores to separate possible microcrystalline aggregates from micelles. Carotenoids are extracted from digesta and aqueous filtrate and analyzed by HPLC to determine recovery and isomeric profile (\textit{i.e.}, digestive stability) and efficiency of micellarization, respectively. Others have examined the effects of processing, dietary components and luminal
<table>
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<tr>
<td>DNA (transgene)</td>
<td>genetically modified soy</td>
<td>98</td>
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<tr>
<td>ellagic acid arabinoside</td>
<td>strawberries</td>
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<td>red, white and Sherry wines</td>
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<td>orange juice</td>
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<td>broccoli</td>
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<td>heterocyclic amines</td>
<td>cooked chicken</td>
<td>185</td>
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<td>human milk, infant formulae</td>
<td>31</td>
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<tr>
<td>Se</td>
<td>bovine milk</td>
<td>296</td>
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<td>Phosphorus</td>
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<td>368</td>
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<tr>
<td>vitamin C</td>
<td>broccoli</td>
<td>348</td>
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</tbody>
</table>

**TABLE 2.4** Simulated gastric and small intestinal digestion for evaluating the chemical stability and release of nutrients and bioactive phytochemicals from food matrix.
FIGURE 2.6 Schematic of *in vitro* digestion method for examining digestive stability and micellarization of carotenoids from foods and supplements.
conditions on transfer of carotenoids from the food matrix to oil droplets (gastric digestion) and from the oil droplet to micelles (small intestinal digestion).

ii. Caco-2 cells. Three colonic cell lines of tumor origin, viz., Caco-2, HT-29 and T84, exhibit some morphological and functional characteristics of differentiated cells lining the mucosa of the gastrointestinal tract (291). The Caco-2 cell line is of particular interest for this review. The characteristics of Caco-2 are summarized in Table 2.5 and now will be described in more detail. These cells spontaneously begin the process of differentiation to an enterocyte-like phenotype when monolayers reach confluency and are maintained using conventional culture conditions (141, 269). During the early phases of the differentiation, the cells express both colonocyte- and enterocyte-specific proteins (86). As differentiation proceeds, colonocyte-specific gene expression decreases and morphological and biochemical characteristics of enterocytes develop. By approximately 2 weeks post-confluency, the monolayer is composed of highly polarized columnar cells with both tight junctions and desmosomes that separate the microvillar (apical) membrane from the basolateral membrane. Moreover, hydrolases such as sucrase-isomaltase, lactase, and dipeptidylpeptidase IV are localized in the apical membrane. These enzymes are normally present in the brush border membrane of enterocytes, but not colonocytes. Other biochemical characteristics of differentiated Caco-2 cells that are similar to normal small intestinal enterocytes include the expression of apical sodium-dependent glucose and amino acid transporters and the PEPT1 peptide transporter, lipoprotein synthesis and secretion, and the ability to induce phase I and phase II drug detoxification enzymes and the ABC effluxers (63). The high correlation between the extent of oral drug absorption in humans and transport across the Caco-2 monolayer has
• Originated from human colon adenocarcinoma

• Differentiate spontaneously into enterocyte-like cells under normal culture conditions

• Polarized cells are characterized by:
  • Tight junctions between cells
  • Basolateral Na+,K+-ATPase
  • Phase I and II drug detoxification enzymes inducible
  • ABC effluxers expressed
  • Apical brush border surface enriched with hydrolytic enzymes
  • Synthesis and vectoral secretion of chylomicrons

TABLE 2.5 Characteristics of Caco-2 human intestinal cells
resulted in the widespread use of Caco-2 cells as a model system for high throughput screening of transport and metabolism of numerous drugs and their derivatives (63, 308). Nutritional scientists have adopted the Caco-2 model to study the characteristics and regulation of processes associated with the apical uptake, metabolism and transepithelial transport of nutrients and other dietary compounds. Table 2.6 presents a partial listing of dietary compounds studied using this cell model. The application of this cell model to the study of carotenoid bioavailability will be discussed in detail below. Before doing so, it is important to mention that some characteristics of differentiated Caco-2 cells differ from small intestinal enterocytes (Table 2.7). First and most obvious, the cells originate from a human colonic carcinoma rather than normal small intestine (269). Second, the cell line is genetically and phenotypically heterogeneous. A third concern is that the transepithelial resistance associated with assembly of tight junctions in Caco-2 cells is more characteristic of colonic epithelium than small intestinal epithelium (63). Finally, Caco-2 cells and small intestinal epithelium use the glycerol 3-phosphate pathway and the monoacylglycerol pathway, respectively, for synthesis of triglycerides (328).
<table>
<thead>
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<th>Refs.</th>
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<td>carotenoids</td>
<td>(see text)</td>
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<td>cholesterol</td>
<td>239</td>
<td>quercetin</td>
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<td>choline</td>
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<td>copper</td>
<td>3, 275</td>
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<td>141, 274</td>
<td>selenium</td>
<td>194</td>
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<td>334a, 353</td>
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<td>vitamin B&lt;sub&gt;6&lt;/sub&gt;</td>
<td>217, 290</td>
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<td>isoflavonoids</td>
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<td>manganese</td>
<td>93</td>
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**TABLE 2.6** Use of Caco-2 human intestinal cell model for investigations of nutrient uptake and metabolism
• Transformed cell line

• Genetic and phenotypic heterogeneity

• Transepithelial resistance more characteristic of colonic epithelium than small intestinal epithelium

• Glycerol-3-phosphate, not monoacylglycerol, pathway used for triglyceride synthesis

• Low synthesis and secretion of chylomicrons

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**TABLE 2.7** Criticisms of Caco-2 cell model
Some of the indicated differences are offset by standardization of procedures associated with growth, maintenance and design of studies using the Caco-2 cell line. Factors that must be strictly controlled to minimize genetic and phenotypic “drift” and facilitate comparison of results within and between laboratories include source of cells, range of passage numbers used for investigations, composition of incubation medium, pH used for uptake studies, degree of maturation of cells at time of experimentation, and composition and porosity of support material for cells for transport studies (10). The parent Caco-2 cell line (HTB 37) is available at passage 18 from the American Type Culture Collection and should be used between passages 20 and 45 for investigations (10, 63). The cells require a minimum of 10-12 days post-confluency for maturation to the enterocyte-like state (10, 141, 269) and 21-25 days for effective synthesis and secretion of lipoproteins (228).

A past criticism of the Caco-2 model was that lipid secretion differed from normal enterocytes, since VLDL rather than chylomicrons represented the primary lipoprotein particle. Recent studies have shown that Caco-2 cells secrete chylomicrons rich in apo-B48 in response to prandial-like conditions in the intestinal lumen, i.e., the addition of micelles containing oleate and taurocholate to the apical compartment (215, 244). Since the absorption of carotenoids and their metabolites occurs by a transcellular process, the highly restricted paracellular flux across monolayers of differentiated Caco-2 cells is not necessarily a concern for investigating carotenoid transport and metabolism. Finally, the ability to synthesize triacylglycerides rather than the specific pathway responsible for the process seems to be the key issue for investigating the incorporation of carotenoids into chylomicrons and subsequent secretion across the basolateral membrane.
Caco-2 cells also provide a useful model for investigating processes associated with the absorption of nutrients and drugs, and the effects of compounds and microorganisms on barrier integrity. Such investigations require the use of a three compartment model. Cells are grown and maintained on a permeable, relatively inert membrane support that is attached to the base of a plastic ring suspended in a standard cell culture well containing medium (Figure 2.7). Thus, the apical and basolateral surfaces of cells face the upper and lower compartments, respectively. Flux of compounds across the monolayer by transepithelial vs. paracellular routes is determined by comparing transport rates of compound of interest with known markers of paracellular flux (e.g., phenol red, inulin, and dextrans).

Collectively, available data support the utility of differentiated cultures of Caco-2 cells as a model for investigating the characteristics and regulation of the transport and metabolism of dietary compounds by absorptive epithelial cells.

**iii. Coupled in vitro digestion/Caco-2 model.** Glahn and associates (102, 110-112) developed a coupled digestion/Caco-2 human intestinal cell model to investigate iron accessibility from dietary salts, chelates, foods, and even germplasm for some staple crops. Differentiated cultures of Caco-2 cells are given access to solubilized compounds and ions generated during simulated small intestinal digestion of test materials. The availability of iron is assessed by quantifying the ferritin protein content in cells. Garrett et al. (91, 105) developed a similar two component system to examine cellular acquisition of carotenoids and other lipophiles from foods, supplements, and meals that are micellarized during simulated digestion. After completing digestion of
FIGURE 2.7 Culture of Caco-2 cells for studying cellular uptake, metabolism and transport of carotenoids and other dietary components.
carotenoid-containing foods and meals in vitro, the aqueous fraction containing micelles is isolated from the digesta and filtered to remove microcrystalline aggregates of carotenoids and microbial contamination. The micellar fraction is diluted with basal medium and added to the apical compartment of wells with cells adhered to either to the plastic surface of the culture vessel or to the membrane insert. Exposure of the monolayer of differentiated cells to diluted micellar fraction for as long as 4-6 hours does not adversely affect cellular morphology and metabolic integrity.

2.3 Functional Properties

2.3.1 Plants

Biosynthesis of carotenoids occurs in all photosynthetic organism-bacteria, algae, and plants, as well as in some non-photosynthetic bacteria and fungi (144). Carotenoids are capable of absorbing light in the blue region of spectrum (400 to 600 nm) and subsequently transfer the absorbed energy to the chlorophylls (251). That is, the chlorophylls do not directly absorb light. This action of the carotenoids occurs by the xanthophyll cycle, a pathway that is present in thylakoid membranes of all higher plants, ferns, mosses and several algal groups. Within the thylakoid membrane, carotenoids are bound mostly to specific chlorophyll/carotenoid-binding protein complexes of the two photosystems. In excessive light, zeaxanthin is rapidly formed in the xanthophyll cycle from violaxanthin via the intermediate antheraxanthin (65). This reaction is reversed in the dark. The proposed roles of the xanthophyll cycle includes protection against light-induced oxidative stress, participation in the blue light response, modulation of the membrane fluidity, and regulation of abscisic acid synthesis (231).
2.3.2 Animals and Humans

2.3.2.1 Coloration

Many carotenoids provide both dramatic and protective coloration in birds and fish. Xanthophyll pigmentation of fish skin is important for reproduction and survival. Unlike birds, fish have chromatophores, which are pigment-containing cells in the dermis that carry carotenoids and allow for color change (113). Extracted natural carotenoid pigments are used as food colors. For example, paprika extract provides capsanthin, and extracts of alfalfa and marigold flower are sources of xanthophylls and primarily lutein. These natural extracts are gradually being replaced by synthetic carotenoids (14). Carotenoids are commonly added to food to enhance color for human consumption or added to animal feed to color body tissues (skin and fat) or products such as milk, eggs, butter, and cheese. Most people prefer the yellow color of the egg yolk that results from the inclusion of xanthophylls in poultry diets (253).

2.3.2.2 Provitamin A

Epidemiologic evidence has strongly linked higher levels of carotenoid intake with reduced risk for many cancers, cardiovascular diseases, cataracts, and age-related macular degeneration. The most well established biological function of carotenoids is the enzymatic conversion of some carotenoids, and especially β-carotene, to vitamin A in humans and animals. Forty carotenoids are listed as vitamin A precursors by Bauernfiend (14) and include the provitamin A activity of astaxanthin and canthaxanthin in freshwater fish (122). Ten carotenoids in vegetables possess provitamin A activity. These include β-carotene, α-carotene, γ-carotene, β-zeacarotene, β-carotene-5,6-epoxide, β-carotene-5,8-epoxide, β- cryptoxanthin, cryptoxanthin-5,6-epoxide, 3’-
hydroxy-α-carotene and cryptocapsin.

2.3.2.3. Antioxidant activity.

An antioxidant is defined as “a substance in foods that significantly decreases the adverse effects of reactive oxygen and nitrogen species, or both on normal physiological function. (96). Generally, vitamin C, vitamin E and carotenoids are considered the major dietary antioxidants. Carotenoids act as antioxidants both by quenching single oxygen (\( ^{1}{O_2} \)) and by scavenging free radicals, but pro-oxidant activity may occur in some conditions. In biological systems, molecules such as porphyrins, chlorophylls and riboflavin can sensitize single oxygen production and this can lead to deleterious effects, including DNA damage and lipid peroxidation. Electron exchange energy transfer quenching is the principle mechanism of carotenoid photoprotection against \( ^{1}{O_2} \), although chemical quenching also occurs leading to destruction of the carotenoid. Energy transfer produces the carotenoid triplet state (\(^{3}\text{CAR}\)) via electronic energy transfer: \\
\[
^{1}{O_2} + \text{CAR} \rightarrow ^{3}\text{O}_2 + ^{3}\text{CAR}.
\]
Once produced, \(^{3}\text{CAR}\) can easily return to the ground state dissipating the energy as heat or it can quench physically via enhanced intersystem crossing by \(^{3}\text{O}_2^-\). Thus, the carotenoid acts as a catalyst for deactivating \(^{3}\text{O}_2^-\). As the number of conjugated double bonds increase, the energies of the excited state decrease. This is reflected in the dependence of the \(^{1}{O}_2\) quenching rate constant on carotenoid chain length (329). However, this may be an oversimplification since Conn et al. (58) demonstrated that 9-\(\text{cis}\)- and 15-\(\text{cis}\)-β-carotene have slightly lower reactivities towards quenching single oxygen than that of all-\(\text{trans}\)-β-carotene. Moreover, Edge and co-workers (79) showed that lutein and zeaxanthin have
very different quenching rate constants with respect to $^{1}\text{O}_2$. Lutein appears to be twice as effective as zeaxanthin. DiMascio et al. (68a) showed that lycopene is the naturally occurring carotenoid that quenches $^{1}\text{O}_2$ most efficiently. However, lycopene is more susceptible to oxidation than other carotenoids and may be degraded more rapidly.

The ability of carotenoids to scavenge reactive oxygen species has been discussed as one biochemical mechanism that underlies their protective influences in cells. Carotenoids can react with free radicals in a number of ways, namely i) electron transfer, ii) hydrogen atom transfer and iii) addition.

\[
\begin{align*}
\text{i)} & \quad \text{ROO}^\cdot + \text{CAR} \rightarrow \text{ROO}^- + \text{CAR}^{\cdot^\ast} \\
\text{ii)} & \quad \text{ROO}^\cdot + \text{CAR} \rightarrow \text{ROOH} + \text{CAR}^\cdot(-\text{H}^\cdot) \\
\text{iii)} & \quad \text{ROO}^\cdot + \text{CAR} \rightarrow [\text{ROO-CAR}]^\cdot
\end{align*}
\]

The antioxidative capacity of carotenoids appears to depend on the number of conjugated double bonds and functional groups. It has been proposed that the antioxidant capacity of lycopene > β-carotene = β-cryptoxanthin > lutein = zeaxanthin > α-carotene > echinenone > canthaxanthin > astaxanthin (302)

### 2.3.2.4 Gap junction intercellular communication

In addition to its antioxidant activity, carotenoids and their metabolites are known to enhance gap junction intercellular communication (GJIC). Gap junctions are water-filled pores called connexons that link adjacent cells in most organs of the body (18). These pores allow direct cytoplasmic-to-cytoplasmic communication via ions and small hydrophilic molecules (<1 kDa). The structural element of a gap junction is a trans-membrane protein called connexin. It was suggested that GJIP is involved in controlling the growth of precancerous cells, since increased GJIP is correlated with growth.
inhibition of transformed cells (305). A numbers of researchers (142, 145, 211, 379) found that β-carotene, canthaxanthin, lutein, lycopene and astaxanthin derivatives induce GJIC through increased expression of connexin 43. Enhanced expression of connexin 43 and increased GJIP inhibits in vitro neoplastic transformation of 10T1/2 cells and human fetal skin fibroblasts, as well as surpressing the growth of human tumor in xenografts (211, 307, 384).

2.3.3.5 Structure organization of membranes

Carotenoids interact with lipids in membranes and cell proteins such as tubulin in the retina (129). Some of the observed activities of carotenoids may be due to effects that carotenoids have on membrane organization. There is no general rule for the orientation of β-carotene or lycopene within a lipid bilayer. Hydrophobic interactions with acyl fatty acid chains would be expected to orient the carotenoid molecule within the hydrophobic region of the bilayer (126) (Figure 2.8). The orientation of lutein and zeaxanthin in the membrane appears to differ from the hydrocarbon carotenids and from one another. These molecules differ in the position of the ring double bond which is located between carbon atoms 4’ and 5’ in lutein and 5’ and 6’ in zeaxanthin. also, the terminal ring of lutein can rotate around the 6’-7’ single bond allowing the possible interaction of hydroxyl groups located at 3 and 3’ positions with the same polar zone of the membrane. Such a difference results not only in the altered spectroscopic properties of the both pigments related directly to the length of the conjugated double bond system, but also in the stereochemical conformations of the molecules. Lutein may orient itself either parallel or perpendicular to the plane of the membrane. Zeaxanthin is found to be located roughly perpendicular orientation to the plane of the membrane. Moreover, the
FIGURE 2.8 Schematic representation of the hydrophobic core of the lipid bilayer and the carotenoid pigment oriented differently depending on their stereochemical structure (126).
orientation of the xanthophylls is affected by lipid composition.

### 2.3.2.6 Cell transformation and differentiation

The results of clinical trials indicate that β-carotene supplements can reduce the rate of cell proliferation in rectal crypts (156). In cell culture, carotenoids can prevent carcinogen-induced transformation and induce cell differentiation (271). The main ligands for retinoid-responsive nuclear receptors (RAR and RXR) are all-

\[ \text{trans} \] - and 9-

\[ \text{cis} \] -retinoic acid. Provitamin A carotenoids may produce retinoic acid from the retinal formed by central cleavage of the carotenoid or excentric cleavage. Thus β-apo-14’-carotenoic acid, like retinoic acid, induces the nuclear receptor RARβ either directly or by conversion to retinoic acid (358).

### 2.3.2.7 Immune Response

The immune system is comprised of the innate (natural) and acquired (adaptive) defenses. Studies on the role of carotenoids on immune response have generally used several functional assays, including immunoglobulin (Ig) production, lymphoblastogenesis, lymphocyte cytotoxic activity, cytokine production, delayed type hypersensitivity (DTH) and flow cytometric and molecular techniques (49). Early studies demonstrated that dietary β-carotene prevented bladder, kidney, ear and gut infection in vitamin A deficiency rats (118) and reduced ear infection in children (56). Both β-carotene and non-provitamin A carotenoids including lutein, canthaxanthin, astaxanthin, and lycopene have been shown to protect phagocytic cells from autooxidative damage, enhance T and B lymphocyte proliferative response, stimulate effector T cell functions and enhance macrophage, cytotoxic T cell and natural killer cell, tumoricidal capacities,
and increase the production of certain interleukins in animal and human subjects (50, 51, 114, 360). Chew and Park (49) recently reviewed their studies that have demonstrated the immuno-enhancing effects of dietary lutein, astaxanthin and canthaxanthin in rodents, canine and feline.

2.3.2.8 Skin

Acute and chronic exposures to UVR lead to variety of changes in skin. The most significant of these changes are a premature aging of skin, UV-induced hyperkeratosis, skin disorders and neoplasms. Furthermore, the local and systemic immune response can be negatively modulated, e.g. UV-induced erythema (125). Dietary antioxidants including carotenoids have been shown to reduce these response in human. Stahl & Sies (306) found that subjects who consumed a variety of carotenoids, including lutein, maintained skin health by reducing UV-induced erythema. Dietary lutein or lutein plus zeaxanthin supplementation of mice also diminished acute inflammatory response, hyperproliferation, and immunosuppression in mouse skin exposed to UVB (115, 196).

2.3.2.9 Chronic diseases

i. Cardiovascular diseases (CVD)

Epidemiological studies and generally support the theory that fruit and vegetables promote cardiovascular health (247). An increment of one serving of fruits and vegetables per day was associated with a 6% lower risk of ischemic stroke and a 4% lower risk of CVD (15, 166, 167). High carotenoid intake may protect against coronary heart diseases, and atherosclerosis. The Nurses Health Studies, which monitored 121,000 nurses for eight years, found that there was a 22% reduction in the risk of coronary heart disease for the women in the top quintile for \(\beta\)-carotene consumption,
compared with those in the bottom quintile (80). The concentration of lutein and β-cryptoxanthin in plasma in subjects in Toulouse was twice as high as in people in Belfast and this was correlated with the incidence of coronary heart disease in the two cohorts (151). The Los Angeles Atherosclerosis Study also showed that subjects who had high serum lutein (0.42 µmol/L) had 80% less arterial wall thickening relative to those at the low quintile of serum lutein (0.15 µmol/L), whereas there was no relationship with serum β-carotene (8, 78). A possible beneficial effect of a lutein-rich diet was also reported in the Atherosclerosis Risk in Communities (ARIC) study (184).

**ii. Cancer**

More than two decades ago, Peto et al. (268a) originally proposed that β-carotene, and perhaps other carotenoids, might decrease the incidence of cancer by provitamin A–independent mechanisms. This hypothesis provided the impetus for numerous studies to evaluate such a possibility. For example, β-carotene, α-carotene and fucoxanthin were shown to suppress tumorigenesis in mouse skin (250). β-carotene intake also was inversely correlated with the incidence of lung cancer in non-smoking men and women in a case-controlled study (225). Also, supplementation of mice with low, but not high, quantities of lutein lowered mammary tumor incidence and tumor growth in mice inoculated with a mammary tumor cell line (263). Narisawa et al. (242) reported that lutein, but not B-carotene, inhibited the development of aberrant crypt foci on colon of rats administered intra-rectal N-methylnitrosourea. Khachik et al. (173) identified oxidized metabolites of lutein and lycopene in plasma and suggested that these compounds might possess chemopreventive activity. While the number of reports that
xanthophylls, like other carotenoids, exhibit chemopreventive properties in cell and animal models continues to increase, definitive evidence for such a role in humans is lacking.

iii. Ocular Health

The eye is exposed to a variety of metabolic and environmental oxidative challenges, both metabolically and from the external environment. Results from several epidemiological studies suggest that individuals with high degree of exposure to UVR have an increased risk of cataracts later in life (143, 226). A particularly strong association has been observed between cataract development and exposure to radiation wavelengths of 290-320 nm (designated UVB) (317). UVB radiation is thought to contribute to cataract formation by directly damaging DNA (179, 297), producing reactive oxygen species (ROS) (116), and generating cytotoxic products from actively translating ribosomes (157). In addition to UVB, hydrogen peroxide is chronically present in the aqueous environment surrounding the anterior lens and may contribute to cataract development (72, 303, 349). Like all tissues, the lens is equipped with antioxidant defense mechanisms that generally protect against the harmful effects of UVB- and H$_2$O$_2$-induced ROS formation. Because many of the enzymatic cofactors and chemical constituents necessary for antioxidant activity are obtained only through the diet, adequate nutrition is likely to be important in preventing ROS-induced oxidative damage and maintaining the overall health of the eye (161, 214, 288) Indeed, some epidemiological and experimental studies suggest that increased consumption of dietary antioxidants such as vitamin C, vitamin E, zinc, and carotenoids may reduce the incidence or progression of ocular diseases (43, 48, 162, 220). Hankinson et al. (135)
used a prospective cohort to show that consumption of spinach which is high in luten and zeaxanthin was inversely related to cataract extraction. Recently, Brown et al. (43) and other groups (48, 216) showed that increased intake of lutein and zeaxanthin were inversely associated with a 20-50% lower risk of cataract extraction.

Lutein, zeaxanthin and *meso*-zeaxanthin are the only dietary carotenoids that are present in the macula region of the retina; lutein and zeaxanthin are the only carotenoids in lens (17, 192, 376). Lutein and zeaxanthin are obtained from diet, whereas *meso*-zeaxanthin is hypothesized to arise from the conversion of lutein to *meso*-zeaxanthin in the retina (25). Dietary supplementation with these xanthophylls increases macula pigment density in human subjects (130, 193), primates (201), and quail (322, 324). Moreover, it has been reported that chronic intake of high amounts of lutein (15 mg lutein diesters) improved visual acuity and glare sensitivity in several studies with small numbers of subjects with age-related cataracts and macular degeneration whereas the patients using the *α*-tocopherol supplement experienced no improvement (255). In addition, retinal cell culture treated with antioxidant including lutein and zeaxanthin exhibit markedly decreased levels of lipid peroxidation and apoptosis in response to oxidative stress (44, 312, 369). The above data have served as impetus for addition of lutein to a number of multi-vitamin and mineral preparations and the marketing of numerous lutein and zeaxanthin supplements for healthy vision.

While considerable efforts are being directed towards defining the potential roles of the xanthophylls in the macula, information about the uptake and possible function of lutein and zeaxanthin in lens is extremely limited. In addition, the low lenticular
concentrations of lutein and zeaxanthin (17, 376) challenge the feasibility that these xanthophylls are capable of contributing to the protection of this organ against environmental and endogenous stressors.

**CONCLUSION.** Studies examining the intestinal absorption, the selective delivery of xanthophylls to the lens epithelium, and the photoprotective activity of these carotenoids in lens and other ocular tissues merit systematic investigation. Below, I summarize my efforts related to the *in vitro* investigation of the bioavailability and photoprotective activity of lutein and zeaxanthin.
CHAPTER 3

Assessment of Lutein Bioavailability from Meals and Supplement using Simulated Digestion and Caco-2 Human Intestinal Cells

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Abbreviations used:  BHT, butylated hydroxytoluene; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GC, glycocholate, GDC, glycodeloxycholate; Lyso-PC, lyso-phosphatidylcholine; MO, monoolein; MTBE, methyl-tert-butyl-ether; OA, sodium olate; PBS, phosphate buffer saline; PC, phosphatidylcholine; TC, taurocholate; TDC, taurodeoxycholate; TRL, triglyceride-rich lipoprotein.
3.1 ABSTRACT

Lutein and zeaxanthin are selectively accumulated in the lens and macular region of the retina. It has been suggested that these xanthophylls protect ocular tissues against free-radical damage that can cause cataracts and age-related macula degeneration. Insights regarding the absorption of dietary xanthophylls for delivery to ocular tissues are limited. Our primary objective was to examine factors affecting transfer of lutein from foods to absorptive intestinal epithelial cells during digestion. Lutein and other carotenoids present in spinach puree and lutein from commercial supplement were relatively stable during in vitro digestion. Micellarization of lutein and zeaxanthin during the small intestinal phase of digestion exceeded that of β-carotene, was higher for xanthophylls in oil-based supplements than in spinach, and was enhanced by substituting a mixture of bile salts for crude bile extract or taurocholate alone. Apical uptake of lutein from micelles by Caco-2 human intestinal cells was linear for at least 4 hours and accumulation from synthetic micelles exceeded that from micelles generated during simulated digestion. Stimulation of chylomicron synthesis was associated with secretion of 7.6 ± 0.1% of cellular lutein into the triglyceride-rich fraction in the basolateral chamber. These data support the use of simulated digestion and Caco-2 cell model as effective tools for identifying factors affecting absorption of dietary carotenoids.
3.2 INTRODUCTION

The dihydroxy-xanthophylls lutein and zeaxanthin are the predominant carotenoids that accumulate in the lens and the macular region of the retina (300, 378). Because lutein and zeaxanthin efficiently absorb blue-light and quench photochemically-induced singlet oxygen, it has been proposed that these pigments protect lens and macula against insults that can induce development of cataracts and age-related macula degeneration (120, 183, 182). Accumulation of xanthophylls and other bioactive compounds from foods and supplements in peripheral tissues depends on intestinal absorption and first-pass metabolism by the intestinal epithelium and/or liver. Available data clearly demonstrate that carotenoid bioavailability is affected by numerous factors, including their physiochemical properties, food matrix and processing, a variety of dietary components, nutritional status, gut health and genotype (46, 382). Thus, reliable prediction of their bioavailability from foods and meals remains problematic. Accurate assessment of carotenoid absorption from a single test food or meal requires isotopic tracer techniques (187, 205). Carotenoid absorption also can be estimated by measuring the concentration of carotenoids and their metabolites in triglyceride-rich fractions of plasma at various times after ingestion of the test dose (153, 259). However, these methods are expensive, labor-intensive and require sophisticated instrumentation.

We previously reported the development of an in vitro method for examining the micellarization of carotenoids and chlorophylls during simulated digestion of commercial baby foods and a stir-fried meal (91, 104, 105). Caco-2 human intestinal cells were shown to accumulate carotenoids and chlorophyll derivatives from medium
containing micelles generated during in vitro digestion. Recent studies by During, Harrison and associates (74, 77, 245) demonstrated that Caco-2 human intestinal cells can convert β-carotene to retinol and secrete β-carotene, retinol and retinyl esters. Moreover, secretion of all-trans-β- and α-carotene exceeded that of lutein, 9-cis-β-carotene and lycopene following their delivery to cells in Tween micelles (77). Other simple models have been used to define factors that affect the transfer of carotenoids from the food matrix to oil droplets and subsequent transfer into mixed micelles (30, 277, 278, 330). Thus, in vitro methods are providing important insights about the impact of gastrointestinal processes on carotenoid bioavailability.

The present study extends previous work by comparing micellarization of lutein and other carotenoids in spinach with commercially available xanthophyll supplements during simulated digestion and their subsequent uptake and transport by Caco-2 human intestinal cells. Also, we have further characterized the influence of bile salts and phospholipid composition on micellarization of lutein, and examined uptake, intracellular stability and secretion of lutein by Caco-2 cells to better understand pre-absorptive events as related to the bioavailability of this common dietary carotenoid.

3.3 MATERIALS AND METHODS

Chemicals and standards:

Unless stated otherwise, all reagents and materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Fisher Scientific Co. (Fair Lawn NJ USA). ³H-retinol (specific activity 2072 GBq/mmol) was purchased from Perkin-Elmer Corp.
Purified all-*trans*-lutein for use as standard was a generous gift from Dr. Zoraida DeFreitas, Kemin (Des Moines, IA).

**Preparation of test foods for in vitro digestion:**

Fresh spinach (*Spinacia oleracea*) was purchased at a local supermarket. After removing stems and ribs, the leaves were washed with tap water, rinsed with deionized water and drained. Spinach was weighed (125 g) and microwaved (750 watts) for 3 min in the presence of deionized water (50 mL) in a tray with lid. Microwaving at moderate intensity has been shown to destroy plant cell integrity without altering the carotenoid content and profile of spinach (172). The sample was cooled and homogenized with a kitchen blender (Osterizer Galaxie)” for 4 min to produce a consistent puree. Aliquots of homogenized spinach were transferred to 50 mL screw cap polypropylene tubes, and stored at -80°C under a blanket of nitrogen for use within four weeks. The carotenoids profile of the preparation was quantitatively and qualitatively stable during this storage period.

Soft-gel capsules containing 20 mg free lutein in corn oil were purchased from Vitamin World Inc, Ronkonkoma, NY. The content of the capsule were diluted 30-fold with corn oil. Lutein-rich corn oil was then diluted 50-fold in 25 g/L skim milk and homogenized (Ultra Turrax) on ice at 13,500 rpm for 7 min to prepare a stabilized emulsion (16).

**In vitro digestion:**

The procedure described by Garrett et al. (104) was modified to investigate digestive stability and micellarization of lutein during simulated digestion of the test materials. Each reaction (50 mL final volume) generally contained either 3.57 g pureed spinach...
with 75 mg virgin olive oil or 100 mg lutein diluted supplement (corn oil with 1.3 µmol lutein) emulsified with 5 mL 2.5% skim milk. To simulate the gastric phase of digestion, test foods were diluted with 120 mmol/L NaCl containing 150 µmol/L butylated hydroxytoluene (BHT), pH was decreased to 2.0 ± 0.1, and porcine pepsin was added to final concentration of 2 mg/mL. Samples were incubated at 37°C at 85 rpm for 1 h in a reciprocal shaking water bath (Allied Fisher Scientific, Versa-Bath Model 24, Columbus, OH, USA). The small intestinal phase of digestion was initiated by neutralization of the digesta with NaHCO₃ before addition of porcine pancreatin (0.4 mg/mL final concentration), porcine pancreatic lipase (0.2 mg/mL final concentration), and bile salts. Samples (pH 6.9 ± 0.1) were incubated in shaking water bath at 37°C, 85 rpm for 2h. The product after completion of simulated digestion is referred to as “digesta”. Digesta was centrifuged (Ti 50 rotor, Beckman Model L7-65, Palo Alto, CA, USA) at 167,000 x g at 4°C for 35 min to separate the aqueous fraction containing mixed micelles from residual solids and oil. Aqueous fraction was collected, and filtered (cellulose acetate, 0.22 µm pore size; Gelman Science, Ann Arbor, MI USA) to remove microcrystalline carotenoid aggregates and other insoluble dispersed materials. Homogenized food, digesta and aliquots of filtered aqueous fraction containing natural micelles were stored at -80°C under a blanket of nitrogen and analyzed within one week.

**Evaluation of Bile Extract Replacement:**

Previously, we have used crude porcine bile extract at a final concentration of 2.4 mg/mL during the small intestinal phase of digestion (91, 104, 105). Bile salts represent approximately 50% of this material (250), resulting in an estimated concentration of 2.5 mmol/L bile salts per reaction tube. To evaluate the effect of bile extract replacement
with one or more pure bile acid on micellarization of carotenoids from spinach during simulated digestion the following substitutes for the bile extract were assessed: 2.0 mmol/L sodium taurocholate (TC) only; a mixture of 0.8 mmol/L glycodeoxycholate, (GDC), 0.58 mmol/L glycocholate (GC), 0.45 mmol/L taurodeoxycholate (TDC), and 0.16 mmol/L TC; and, a mixture of 0.8 mmol/L GDC, 0.45 mmol/L TDC and 0.75 mmol/L TC. These bile acids are among the most abundant in human bile (299).

**Preparation of synthetic micelles (SM) containing lutein**

Preparation and composition of synthetic micelles was based on that described for several previous in vitro studies (214, 310, 330, 373). Aliquots of stock solutions of monoolein (MO), phosphatidylcholine (PC), and lyso-phosphatidylcholine (Lyso-PC) in chloroform, and α-tocopherol (α-TC) and all-trans-lutein in ethanol were combined in a 35 mL glass vial. Solvents were removed under a stream of nitrogen at room temperature. Basal medium containing 0.8 mmol/L GDC, 0.45 mmol/L TDC, 0.75 mmol/L TC, and 1.5 mmol/L sodium oleate (OA) was added to the vial and the mixture was sonicated in a bath at room temperature for 30 min. The clear solution was filter sterilized (0.22 µmeter pores). Final concentrations of lipids were 1.5 mmol/L OA, 500 µmol/L MO, 200 µmol/L PC, 200 µmol/L lyso-PC, 10 µmol/L α-TC and 1 µmol/L lutein.

**Uptake and secretion of lutein by Caco-2 human intestinal cells:**

Stock cultures of Caco-2 cells (HTB37, American Type Culture Collection, Rockville, MD USA; passage 26-34) were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 150 mL/L heat-inactivated
fetal bovine serum (FBS), nonessential amino acid (10 ml/L), L-glutamine (2 mmol/L), amphotericin B (0.5 mg/mL), penicillin-streptomycin (10 ml/L), sodium bicarbonate (44 mmol/L), and HEPES (15 mmol/L) in a humidified atmosphere of 95% air 5% CO₂ at 37°C. Caco-2 cells were seeded in 6 well dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) for experiments at density 3.5 x 10⁵ cells/well. Cultures were used 11-14 day post-confluency for investigating the uptake of lutein from natural and synthetic micelles. When the cultures achieved confluence, the FBS content of medium was decreased to 75 ml/L and medium was changed every other day.

To characterize uptake of micellarized lutein, spent medium was removed and monolayers were washed twice with basal medium at 37°C. DMEM containing 250 ml/L of either aqueous fraction from simulated digestion (referred to as natural micelles) or synthetic micelles was added to wells containing the washed monolayers (2 ml/well). At indicated times, test medium was removed and monolayers were washed twice with ice-cold phosphate buffer saline (PBS) containing albumin (2 g/L) to remove residual lutein adhering to the trans face of the cell surface (326) before washing twice with cold PBS. Cells were collected in 1.5 mL ice-cold PBS, centrifuged (1,800 rpm, 4°C, 5 min) and cell pellets were stored at -80°C under nitrogen for a maximum one week. Exposure of monolayers to micellar medium as described above does not adversely affect general cell morphology or metabolic integrity (104).

To examine the secretion of lutein accumulated by Caco-2 cells, inserts in 6 well dishes (24 mm diameter, 0.4 μm pore size) were seeded with 3.0 x 10⁵ cells. Cultures were used for experiments 21-25 d after reaching confluence since lipoprotein synthesis and secretion by Caco-2 cells are maximal at this time (229). DMEM (1.5 mL)
containing ~2.0 µmol/L lutein in synthetic micelles was added to the apical chamber and
DMEM (2.5 mL) containing 10 mL/L lipid-free FBS was added to the basolateral
chamber. Apical medium was removed after 6h and monolayers were washed twice with
warm PBS containing albumin (2g/L). Either DMEM (1.5 mL) containing 0.5 mmol/L
phenol red or DMEM with 0.5mmol/L phenol red plus 1.6 mmol/L OA, 0.5 mmol/L TC,
0.05 mmol/L glycerol was added to the apical chamber. The addition of indicated
concentrations of OA, TC and glycerol to apical chamber has been shown to stimulate
synthesis and secretion of chylomicrons (26, 30). Fresh phenol red-free DMEM with 10
mL/L lipid-free FBS was added to the basolateral chamber. After incubation for 20h,
medium in the apical and basolateral chambers and washed monolayer were collected and
stored at -80°C. Lutein was quantified in all samples within one week, as was the phenol
red content of basolateral medium.

Distribution of secreted lutein in the basolateral compartment was also determined
using a modification of the procedure described by During et al. (77). Filtered DMEM
(1.5 mL) with 0.5 mmol/L phenol red, synthetic micelles containing 2 µmol/L and 74
kBq 3H-retinol, 1.6 mmol/L OA and 0.5 mmol/L TC was added to the apical
compartment. The 3H-retinol tracer was included in order to confirm the synthesis of
retinyl ester and its incorporation and secretion in a triglyceride-rich lipoprotein fraction
(see below, ref. 245). The basolateral compartment contained phenol red-free DMEM
(2.5 mL) with 10 mL/L lipid-free FBS. After continuous exposure of the monolayer to
micellar lutein for 20h, media from both compartments and washed cells were collected
for analyses. The triglyceride-rich lipoprotein (TRL) fraction in freshly collected
basolateral medium was isolated according to van Vliet et al. (347). Briefly, spent
medium from 4 wells was pooled (10 mL), transferred to polyallomer tubes coated with polyvinyl alcohol (149), and overlayed with 3 mL NaCl solution (density = 1.006 kg/L). The samples were centrifuged at 100,000 x g (SW 41T1, Beckman Palo Alto, CA, USA) at 20°C for 35 min. The bottom of each tube was punctured and 0.5 mL fractions were collected. The uppermost fraction had a creamy appearance and is referred to as the TRL fraction. Fractions were stored under a blanket of nitrogen at -80°C until analysis of lutein, $^3$H-retinol, and $^3$H-retinyl ester. $^3$H-retinol and $^3$H-retinyl esters in cells, basolateral medium and the TRL fraction were separated using alumina column chromatography (244). To measure $^3$H, aliquots were solubilized in ScintiVerse (Fisher Scientific Company) and analyzed by liquid scintillation spectrometry (Beckman LSCounter Model 3801).

**Extraction and analysis of carotenoids in test meals, media and cell pellet:**

Thawed samples (1-3 mL) of homogenized food, digesta, and aqueous fraction from simulated digestion were extracted by addition of 3.0 mL petroleum ether:acetone (2:1) containing 4.5 mmole/L BHT, vortexing for 1 min, and centrifuging (2000 x g for 5 min) to hasten phase separation. Thawed samples of apical and basolateral medium, and fractions collected after ultracentrifugation of the basolateral medium were treated similarly. The extraction procedure was repeated a total of three times and petroleum ether fractions were combined and dried at room temperature under a stream of nitrogen. The film was resolubilized in methyl-tert-butyl-ether (MTBE): methanol (MeOH)(1:1) and analyzed immediately.

Cell pellets were thawed before addition of phosphate buffered saline, pH 7.4 containing 10 mg/mL protease from *Streptomyces griseus* (Sigma product 5147). After
samples were incubated at 37°C for 30 min, 0.5 mL of EtOH containing 34.6 mmol/L sodium dodecyl sulfate (SDS) and 4.5 mmol/L BHT was added before vortexing for 1 min. Lutein was extracted by addition of 1 mL of petroleum ether:acetone (2:1). Samples were vortexed and centrifuged as above to facilitate separation of phases. The extraction was repeated a total of three times. Combined petroleum ether fractions were dried under a stream of nitrogen at room temperature, resolubilized in MTBE:MeOH (1:1) and analyzed immediately.

Carotenoids were quantified by high performance liquid chromatography (HPLC) according to Ferruzzi et al. (92) with slight modification. HPLC (Hewlett Packard model HP 1050) with UV/visible detector set at 450 nm generally was used for analysis. Carotenoids were separated using a YMC C₃₀ analytical scale (4.5 x 250 mm) reversed phase column (Waters, Milford, MA) with a C₁₈ stationary-phase guard column. Separations were achieved using a gradient elution with a binary mobile phase of methanol:ammonium acetate (98:2) in reservoir A and MTBE in reservoir B. Initial conditions were set at 85:15 A/B with a linear gradient to 70:30 A/B over 15 min. The gradient was held 15 min before following a 5.0 min linear gradient back to 15% B for a total run time of 25 min. The concentration of carotenoids was calculated from comparison of area under the curve with known concentrations of the all-trans-isomers of lutein, zeaxanthin and β-carotene standards at retention times of 10.3, 13.5, and 29.5 min, respectively. The employed extinction coefficients, $E_{1cm, 1\%}$, at 450 nm in hexane were 2550 for all-trans and cis-lutein, 2540 for all-trans and cis-zeaxanthin, and 2592 for all-trans and cis-β-carotene (41). All-trans-carotenoids and their geometrical isomers were identified by UV-visible absorbance spectra recorded using a Waters 996 photodiode.
array detector (Waters, Milford, MA) and comparison of retention times and absorbance spectra to previous separations with a C<sub>30</sub> column (20, 85)

**Miscellaneous assays:**

Protein content of cell samples was determined by the bicinchoninic acid assay (Pierce, Rockford, IL USA) using bovine serum albumin as a standard. \(^3\)H-retinol and \(^3\)H-retinyl ester in cells, basolateral medium and the fractions collected after centrifugation were determined according to Nayak et al. (245). The diffusion of phenol red across the monolayer from the in apical to the basolateral compartment was determined by measuring absorbance at 546 nm 15 sec after addition of 20 µL of 1mol/L NaOH to 150 µL basolateral medium at room temperature (127).

**Statistical analysis of data:**

All data were analyzed using Stata 8.0 (Stata Corporation, Texas, USA). Descriptive statistics including mean and standard deviation were calculated for the efficiency of micellarization of carotenoids from digested foods, the stability of micellarized carotenoids in cell culture medium, and the uptake and secretion of carotenoids by Caco-2 cells. Means were compared using one-way ANOVA followed by Turkey’s or Bonferroni correction. Differences were considered significant at \( p<0.05 \). All tests were conducted in triplicate for each experiment was repeated at least once to provide a minimum of six independent observations.
3.4 RESULTS

Composition of test materials

Spinach contained approximately 75 µg carotenoids per g fresh weight with all-trans-lutein and β-carotene representing 94% of the total (Table 3.1). Small amounts of 13-cis-lutein, 9-cis-β-carotene, and all-trans-zeaxanthin were detected. All-trans-lutein accounted for 95% of carotenoids in the supplement with the remainder detected as all-trans-zeaxanthin (Table 3.1).

Composition of bile salts and efficiency of micellarization during simulated digestion of spinach.

Digestive stability is defined as the percentage of the carotenoid in test food recovered in the digesta, whereas efficiency of micellarization represents the percentage of the carotenoid in test food that is present in the filtered aqueous fraction after simulated digestion. Previously, we have used porcine bile extract during the small intestinal phase of digestion (e.g., 12). Here, we compared the effect of substituting either TC, a bile salt commonly used for preparation of simple micelles in vitro (e.g., 26), or mixtures of bile salts abundant in small intestinal lumen of humans during pre-prandial state (19) with porcine bile extract on the efficiency of carotenoid micellarization during simulated digestion of spinach.

Concentration of all-trans-lutein was determined to be 210 ± 4 µg/50 mL digestion reaction. Transfer of all-trans-lutein from the food matrix to aqueous (micellar) fraction increased from 27% to approximately 50% (p< 0.001) when mixtures of three and four bile salts were substituted for crude porcine extract during the small intestinal phase of
TABLE 3.1 Carotenoid content of fresh spinach and commercial lutein supplement

<table>
<thead>
<tr>
<th></th>
<th>Spinach</th>
<th>Supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/100g fresh wt</td>
<td>% total</td>
</tr>
<tr>
<td>all-trans-lutein</td>
<td>5871 ± 8</td>
<td>77.8</td>
</tr>
<tr>
<td>13-cis-lutein</td>
<td>111 ± 6</td>
<td>1.5</td>
</tr>
<tr>
<td>all-trans-zeaxanthin</td>
<td>142 ± 3</td>
<td>1.9</td>
</tr>
<tr>
<td>all-trans-β-carotene</td>
<td>1220 ± 43</td>
<td>16.2</td>
</tr>
<tr>
<td>9-cis-β-carotene</td>
<td>199 ± 56</td>
<td>2.6</td>
</tr>
</tbody>
</table>

N=3 independent analyses. Data are mean ± SD. ND = not detected.
digestion (Figure 3.1). In contrast, only 15% of all-trans-lutein in spinach was micellarized when sodium taurocholate replaced the porcine bile extract during simulated digestion ($p<0.01$). This pattern also was similar for other carotenoids in the digested spinach meal. Micellarization efficiency of 13-cis-lutein, all-trans-zeaxanthin, and all-trans- and 9-cis $\beta$-carotene was 28, 29, 15 and 17%, respectively, when crude bile extract was used. Substitution of the mixtures of bile salts for bile extract increased ($p<0.001$) micellarization of 13-cis-lutein and all-trans-zeaxanthin to approximately 50 and 60%, respectively. Similarly, replacement of bile extract with GDC, TDC and TC increased ($p<0.01$) micellarization of all-trans-$\beta$-carotene and 9-cis-$\beta$-carotene to approx. 27%, and addition of the fourth bile salt, GC, further enhanced micellarization of the carotenoids to 37 and 38%, respectively. Conversely, replacement of bile extract with only TC decreased ($p<0.01$) the efficiency of micellarization of all the carotenoids to 10-15%. Mixture of 0.8 mmol/L GDC, 0.45 mmol/L TDC and 0.75 mmol/L TC was used for simulated small intestinal digestion in all subsequent experiments.
FIGURE 3.1 Mixture of bile salts abundant in small intestine enhances micellarization of carotenoids during in vitro digestion of spinach. Spinach puree containing 3.2% olive oil was prepared and digested as described in Materials and Methods. Indicated bile salt(s) were added at the beginning of the small intestinal phase of simulated digestion. Data are the mean ± SD for three independent experiments per treatment. The presence of different letters above the error bar indicates that the mean efficiency of micellarization of all-trans-lutein differed significantly ($p<0.001$). Abbreviations: GC, glycocholate; GDC, glycodeoxycholate; TC, taurocholate; and, TDC, taurodeoxycholate.
Digestive stability and micellarization of lutein and other carotenoids from spinach and supplement.

Recovery of carotenoids after simulated gastric and small intestinal phases of digestion of the spinach meal was 70-88% (Figure 3.2A). Mean efficiency of micellarization for carotenoids in digested spinach ranged from 25-53% and differed significantly (p<0.05) for xanthophylls and β-carotene with all-trans-lutein and zeaxanthin > 13-cis-lutein > all-trans- and 9-cis-β-carotene (Figure 3.2B). Quantities of all-trans-lutein and all-trans-zeaxanthin from commercial lutein supplements used for each digestion (50 mL final volume) were 80.6 ± 1 and 4.4 ± 0.3 µg, respectively. Both digestive stability and micellarization efficiency of all-trans-lutein and all-trans-zeaxanthin from the commercial supplement were significantly (p<0.001) greater than that of xanthophylls in digested spinach (Figure 2A and 2B).
FIGURE 3.2 Recovery (panel A) and efficiency of micellarization (panel B) of carotenoids in spinach and lutein supplement after simulated digestion. Spinach puree and an aliquot of the contents of the soft-gel capsule were prepared and digested \textit{in vitro} as described in Materials and Methods. Carotenoids were extracted from test food, digesta and aqueous fraction and analyzed by HPLC. Digestive stability represents the percentage of carotenoid recovered after simulated gastric and small intestinal digestion. The efficiency of micellarization represents the percentage of the carotenoid in the test food that was transferred to the filtered aqueous fraction during simulated digestion. Data are means ± SD for three independent experiments each having three replicate digestion reactions per treatment. The presence of letters above the error bar indicates significant ($p<0.05$) differences in the remaining levels of carotenoids after simulated digestion (panel A) and significant ($p<0.001$) differences in the transfer of indicated carotenoids from the food matrix to the aqueous fraction, i.e., micellarization, during digestion (panel B).
**Uptake of micellar lutein and β-carotene by Caco-2 cells.**

Cellular acquisition of lutein from the apical compartment was examined by incubating monolayers of Caco-2 cells in serum-free DMEM containing micelles either generated during simulated digestion of spinach and lutein supplement or prepared synthetically. Lutein content of the medium was standardized to 1.0 ± 0.05 µmol/L to facilitate comparison of the effect of source and micellar composition on accumulation. Assuming an average intake of 1 mg lutein/d (62) and delivery of 2-3 liters of water to the small intestine per meal (178), this micellar concentration of lutein is physiologic. All-trans-lutein, but not cis-lutein or all-trans-zeaxanthin, was detected in Caco-2 cells incubated in test media. Accumulation of lutein from micelles was proportional to length of incubation and dependent on the type of micelle (Figure 3.3). Cellular content of lutein after 4, 8 and 20h incubation in medium with micelles generated during digestion of spinach represented 15, 28 and 35%, respectively, of that in the medium at initiation of the experiment. Similarly, 14, 29, and 46% of micellarized lutein in medium prepared with the digested oil supplement was accumulated by cells after 4, 8 and 16h. Cultures exposed to micelles generated during digestion of spinach and the oil supplement containing all-trans-lutein for 4 and 8h did not contain detectable amounts of cis-lutein in cells or medium after incubation in carotenoid-free medium for 16-20h. This suggests that minimal isomerization of lutein occurs within Caco-2 cells under the defined conditions.

Pilot studies demonstrated that the efficiency of incorporation of lutein into synthetic micelles was affected by the relative concentrations of lyso-PC and PC.
FIGURE 3.3 Effect of length of incubation and source of micelles on lutein accumulation by Caco-2 cells. Differentiated monolayers of Caco-2 cells were incubated with 2 mL medium containing micelles with $1.0 \pm 0.1 \mu$mol/L lutein for indicated times before determination of cellular lutein content. Micelles were either generated during simulated digestion of spinach or supplement or prepared synthetically as described in Materials and Methods. Data (mean ± SD) are representative of three independent experiments with 3-4 replicate samples per experiment ($N = 9-12$). Means within each treatment that do not share a common letter above the bar differ significantly ($p < 0.01$) as determined by one way ANOVA followed by Bonferroni correction. Cell lutein content was significantly greater ($p < 0.001$) with increasing length of incubation for each source. Cell accumulation of lutein from synthetic micelles was significantly ($p < 0.001$) greater than from natural micelles containing similar concentration of lutein at each time of incubation.
Micellarization of lutein was 12% when PC, but not lyso-PC, was present. The efficiency of micellarization of lutein increased to 30% and >90% when the lyso-PC to PC ratio was 0.1 and 1.0, respectively. Therefore, micelles containing 200 µmol/L lyso-PC and 200 µmol/L PC were prepared to maximize the efficiency of incorporation of lutein for assessing cellular accumulation of the carotenoid without other lipophilic compounds transferred from spinach or supplement to micelles during digestion. Cellular content of all-trans-lutein after incubation in medium with synthetic micelles for 4, 8 and 20h represented 21%, 40% and 67%, respectively, of that in starting medium. Accumulation at each time was significantly ($p<0.001$) greater than that from medium with an equivalent concentration of lutein in micelles generated during simulated digestion. It is unknown if this difference in lutein accumulation from the natural and synthetic micelle preparations is due to differences in the composition, number or size of micelles, or perhaps some combination of these factors, in the various preparations. It is noteworthy that analysis of lyso-PC and PC in the micellar fraction after digestion of the spinach revealed that the ratio of lyso-PC to PC was 1.02 (data not shown).

Cellular accumulation of micellarized all-trans-β-carotene from digested spinach was similar to that of all-trans-lutein with 16 and 31% of lutein initially present in medium present in cells after 4 and 20h of exposure, respectively. *Cis*-isomers of β-carotene were not detected in cells or medium following overnight incubation after cultures were pre-incubation in medium containing micelles from digested spinach for 4 and 8h.
Stability of carotenoids in natural and synthetic micelles.

Medium containing carotenoids in natural and synthetic micelles was incubated in cell culture incubator dishes without cells for as long as 20h to examine if isomerization and/or degradation occurred. The concentrations of all-trans-lutein, 13-cis-lutein, all-trans-zeaxanthin or all-trans-β-carotene in medium containing micelles prepared by digestion of spinach did not change significantly \((p>0.05)\) during the 4h incubation period. However, the amount of all-trans-lutein decreased by 19 ± 2% \((p<0.05)\) of the initial concentration after 20h. There was a slight (16%), but significant \((p<0.05)\), increase in the concentration of 13-cis-lutein during this period suggesting some conversion of all-trans to the cis isomer. This change appeared to be somewhat specific to isomerization of lutein since recovery of all-trans- and 9-cis-β-carotene at 20h was 99% and 91%, respectively, after 20h. Micellarized all-trans-lutein from digested lutein supplement was less stable than from spinach meals. Concentration of all-trans-lutein declined linearly with 75% remaining after 16h incubation in the cell-free environment (Figure 4). This decline was associated with the appearance of equivalent amounts of 13-cis-lutein at each time (Figure 3.4). All-trans-zeaxanthin in micelles generated from digestion of the lutein supplement was detected, but the extent of isomerization was impossible to determine as zeaxanthin isomer content was below the limit of quantification. In contrast with the partial isomerization of all-trans-lutein in natural micelles in the cell culture environment, recovery of all-trans-lutein from medium containing synthetic micelles exceeded 95% for incubation periods as long as 20h.

Since isomerization of carotenoids can be induced by oxidants (298), α-tocopherol
FIGURE 3.4 Spontaneous generation of cis-isomers during incubation of medium containing all-trans-lutein in micelles produced during simulated digestion of lutein from supplement. Filtered aqueous fraction isolated after simulated gastric and small intestinal digestion of commercial lutein supplement were diluted 1:4 with DMEM cell culture medium and added to well without cells. Dishes were incubated in humidified incubator at 37°C with 95% air: 5% CO₂ for indicated time before collection. Recovery of the starting concentration of lutein exceeded 95% at all times tested. Data are mean ± SD for 12 independent samples at each time. ANOVA revealed an effect of time of incubation ($p<0.05$) on the isomerization of lutein.
concentration in medium was measured. The mean concentration of \( \alpha \)-tocopherol present in medium containing synthetic micelles (10.0 ± 0.2 \( \mu \)mol/L) was significantly higher \((p<0.001)\) than that measured in medium containing aqueous fraction from digested spinach (0.3 ± 0.1 \( \mu \)mol/L) and milk with emulsified lutein supplement (0.1 ± 0.04 \( \mu \)mol/L). The added \( \alpha \)-tocopherol may have prevented degradation and isomerization of lutein.

\textit{Secretion of cellular LUT.}

Synthetic micelles were used as the delivery vehicle for lutein to investigate secretion. Medium with micellar lutein (approx. 3 nmol) was added to the apical compartment for 6h to load Caco 2 cells on membrane inserts with the carotenoid. Cultures were then incubated overnight to determine retention and secretion of lutein. Caco cells retained 99 ± 2\% of accumulated lutein when incubated in DMEM medium with 10mL/L lipid-free FBS for 20h. Lutein was not detected in the apical compartment and less than 1\% was found in the basolateral chamber. The addition of 1.6 mmol/L OA, 0.5 mmol/L TC and 0.05 mmol/L glycerol to the apical compartment to stimulate chylomicron synthesis and secretion resulted in transfer of 6.3 ± 0.4\% of cellular lutein to the basolateral compartment after 20h. Lutein was not detected in the apical compartment.

To examine the distribution of lutein within the basolateral fraction, medium containing synthetic micelles with approx. 4.3 nmol lutein plus OA, TC, glycerol and \( ^{3} \)H-retinol was added to the apical chamber and cultures were incubated for 20h. \( ^{3} \)H-retinol tracer was present to confirm the presence of retinyl esters in chylomicrons and VLDL located in the TRL fraction (245). The amount of lutein in the apical compartment
decreased by 38% after 20h and the quantities of lutein present in the cells and basolateral chamber were 1.58 nmol and 0.13 nmol, respectively i.e., 7.6 ± 0.1% of the lutein acquired by cells was transferred to the basolateral compartment (Table 3.2). Similarly, the quantity of $^3$H-retinol in apical medium decreased by 36%. The majority of the $^3$H lost from the apical compartment was present in the monolayer (92%) with 7.6 ± 0.5% transferred to the basolateral compartment. Within the cells and the basolateral compartment, retinyl esters accounted for 60% of total $^3$H. After centrifugation, all detectable lutein (98% recovery) and 65% of $^3$H were present in the TRL fraction. Chromatographic analysis of $^3$H in fractions after centrifugation showed that retinyl ester and retinol accounted for >95% of $^3$H in TRL and other fractions, respectively (Table 3.2). Mean rate of phenol red flux from the apical to the basolateral compartment was 0.011 ± 0.003%/cm$^2$/h and independent of the composition of the medium in the apical chamber, demonstrating that monolayer integrity was not affected by the various treatments.

3.5 DISCUSSION

Here, we demonstrate that the micellarization of lutein and zeaxanthin is more efficient than that of β-carotene during simulated digestion of spinach puree. Micellarization of xanthophylls in an oil-containing supplement exceeds that from the more complex matrix of spinach. Moreover, we show that bile acid composition and
<table>
<thead>
<tr>
<th>Compartment</th>
<th>Lutein</th>
<th>Retinol</th>
<th>Retinyl ester</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pmol</td>
<td>pmol</td>
<td>pmol</td>
</tr>
<tr>
<td>0h Apical</td>
<td>4,310 ± 106</td>
<td>27.4 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>20h Apical</td>
<td>2,657 ± 35</td>
<td>17.6 ± 0.8</td>
<td>0</td>
</tr>
<tr>
<td>Cells</td>
<td>1,576 ± 86</td>
<td>3.6 ± 0.3</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>Basolateral</td>
<td>131 ± 2</td>
<td>0.3 ± 0.01</td>
<td>0.5 ± 0.03</td>
</tr>
<tr>
<td>TRL fraction</td>
<td>129;131</td>
<td>0.01; 0.02</td>
<td>0.45; 0.47</td>
</tr>
<tr>
<td>Non-TRL fraction</td>
<td>0</td>
<td>0.24; 0.27</td>
<td>0.01; 0.03</td>
</tr>
</tbody>
</table>

* Post-confluent cultures of Caco2 were incubated with synthetic micelles containing 2 µmol/L lutein, 74 kBq ³H-retinol, 1.6 mmol/L OA, and 0.5 mmol/L TC for 20h in a humidified atmosphere of 95%, air 5% CO₂ at 37°C. At 20h, media from apical and basolateral chambers and washed cells were collected for analyses. TRL fraction was freshly isolated from basolateral medium to quantify lutein, retinol and retinyl ester. Data represent mean ± SD from 4-6 individual samples, except for the TRL fraction which was collected and analyzed from two pooled samples.

**TABLE 3.2** Secretion of lutein and retinol by Caco-2 cells*
lyso-phosphatidylcholine affect incorporation of carotenoids into micelles. We also demonstrate that some all-trans-lutein may isomerize within micelles, but not once accumulated by Caco-2 cells cultured under standard conditions. We show that a small percentage of micellarized lutein accumulated within Caco-2 cells is secreted into the triglyceride-rich fraction when lipoprotein synthesis is stimulated. Collectively, the results support the possibility that differences in the efficiency of micellarization of ingested carotenoids have a marked impact on the extent to which the compounds are absorbed. The quantity of ingested carotenoid absorbed is dependent on its partitioning in micelles, delivery to enterocytes and incorporation into chylomicrons (100). Results from numerous human studies have shown that carotenoids in oil-based supplements are absorbed more efficiently than from foods (42, 45, 180). Our demonstration that lutein in the corn oil supplement is micellarized more efficiently than that in spinach offers a possible explanation for the increased absorption of lutein from supplements. Several human studies have suggested that apparent absorption of lutein is more efficient than β-carotene. Van het Hof et al. (341) reported higher relative changes in plasma lutein than β-carotene after feeding a high vegetable diet for several weeks when compared with responses to pure carotenoid supplements. Similarly, serum lutein concentrations increased to a greater extent than β-carotene after feeding subjects spinach products for 3 weeks (45). Mean area under the curve for serum lutein was approximately twice that for a single equimolar dose of β-carotene after administering carotenoid-rich oils (180). Gartner et al. (106) reported that the efficiency of absorption of lutein and zeaxanthin into the triglyceride-rich fraction exceeded that of β-carotene following a single dose of Betatene, an algal extract in soybean oil enriched in β-carotene. The more efficient
micellarization of lutein than β-carotene in foods during digestion may contribute to the above observations in humans.

Many factors contribute to the relative efficiency of carotenoid micellarization during digestion. The higher efficiency of micellarization of lutein and zeaxanthin from emulsified oil supplement compared to spinach is likely due to the absence of factors limiting transfer from chloroplasts to oil droplets (277). The observed differences between micellarization of the xanthophylls and β-carotene from spinach are likely related to factors affecting transfer efficiency from the food matrix to the oil droplet and subsequent flux to the mixed micelle. Tyssandier et al. (330) observed that the transfer of carotenoids from oil droplets to mixed micelles is inversely proportional to the hydrophobicity of the pigments. Carotenes and lycopene are located deep within the lipid droplet, whereas xanthophylls reside at the surface and are kinetically active (30). Moreover, transfer of β-carotene from the oil droplet to the mixed micelle was impaired when lutein also was present in the droplet (330). Lutein in disrupted spinach chloroplasts also has the potential to be transferred directly to micelles without the need for oil droplets to serve as an intermediate reservoir (278). In contrast, transfer from β-carotene in carrot chloroplast to micelles required passage to oil droplets as an intermediate step.

Recently, Tyssandier et al. (332) examined carotenoid stability and micellarization after delivery of a liquefied meal containing vegetable puree by nasogastric tube. The relative quantity of lutein in micelles in the duodenum during the 3h sampling period (5.6%) was higher than that of β-carotene (4.7%). Although the percentages of micellarized lutein and β-carotene in duodenum were well below that we observed in
vitro, it is important to recognize that carotenoids are delivered to the brush border surface of enterocytes once micellarized within the small intestine. In contrast, carotenoids continue to accumulate in micelles in the in vitro digestion model since they are not delivered to target cells.

In the current study spinach was processed and the bile composition modified in order to increase carotenoid content in micelles to facilitate examination of uptake and stability in Caco-2 cell culture. Spinach was microwaved and pureed to destroy tissue structure and increase surface area (45, 339). Micellarization of lutein and other carotenoids in spinach was increased two to three-fold when physiological levels of several bile salts replaced crude bile extract and TC. Previous investigators also have reported effects of various bile salts on micellarization and intestinal uptake of β-carotene (84, 147). Cell accumulation of lutein and β-carotene from micelles generated during digestion of spinach was similar to that we previously reported (91, 104). In contrast, Sugawara et al. (310) reported that apical uptake of carotenoids from synthetic micelles is proportional to the hydrophobicity of the carotenoid. Differences in the composition of the micelles may contribute to this discrepancy

Lutein accumulation by Caco-2 cells was greater when cells were exposed to synthetic micelles instead of micelles generated during digestion of spinach or supplement. The different composition of natural versus synthetic micelles may affect particle size and surface charge and, therefore, ability to interact with the brush border surface (100). While it is possible that the presence of other carotenoids in the micelle affect apical uptake (377), this does not appear to contribute to the observed difference since micelles generated from digestion of the lutein supplement contain very low
amounts of zeaxanthin. Phospholipid composition represents another possible factor. Lyso-PC has been shown to stimulate lutein and β-carotene uptake from micelles by Caco-2 cells and absorption by mice (13, 310). We observed that lutein incorporation into synthetic micelles was markedly increased by addition of equivalent concentrations of lyso-PC and PC compared to PC only. Effects of the lyso-PC to PC ratio on uptake were not tested, since the number of micelles in the apical compartment would have varied to introduce equimolar amounts of lutein. However, analysis of lyso-PC and PC in the micellar fraction after digestion of the spinach revealed that the ratio of lyso-PC and PC was almost identical to that in the synthetic micelles. This suggests that increased cellular uptake of lutein from synthetic micelles was due to factors other than phospholipid composition.

Micelles within the intestinal lumen and lipoproteins in circulation represent the physiological carriers for carotenoids (100). Investigators have reported that carotenoids are degraded when introduced into cell culture medium using organic solvents, liposomes and water-dispersable beadlets as the vehicle (361, 366, 373). We (this study) and others (373) have found that carotenoids are relatively stable in medium containing synthetic micelles. However, there was some degradation of lutein when micelles formed during digestion of spinach were incubated in culture medium (this paper and ref. 104). Oxidizing compounds in spinach and the relatively low concentration of α-TC may have contributed to the loss of some of the micellarized lutein. Isomerization of all-trans-lutein was observed in micelles generated during digestion of the supplement. Durning et al. (77) also reported that all-trans-β-carotene in Tween 40 micelles was partially isomerized to cis-β-carotene in the apical compartment of Caco-2 cultures after 16 h. It
is well recognized that light, acid, heat, photosensitizers, and oxidants can induce isomerization of carotenoids (298). Since we handled all samples in an identical manner, it is likely that unidentified oxidants in the digested spinach and oil supplements contributed to the partial degradation and isomerization of lutein.

Lutein secretion from Caco-2 cells required the presence of oleate and taurocholate in the apical compartment. The extent of secretion was similar when cells were either exposed to micellar lutein before or at the same time as oleate and taurocholate. Lutein within the basolateral compartment was localized in the TRL fraction, as were retinyl esters. These results are quite similar to those reported by Harrison and associates (77, 245) who introduced carotenoids in Tween micelles and retinol in the presence of oleate and taurocholate.

The above in vitro observations provide additional support for the use of model systems of digestion and differentiated intestinal epithelial cells for investigating factors affecting the digestive stability, micellarization, and the uptake and transport of lutein and other carotenoids from foods and supplements. Comparison of results using the identical test material in the in vitro system and fed to human subjects to estimate bioavailability are needed to evaluate the predictive power of coupling simulated digestion with the Caco-2 cell model.

3.6 ACKNOWLEDGMENTS

We thank Dr. Mario Ferruzzi for helpful conversations and his critical reading of the manuscript and Dr. Martha Belury for her suggestions for phospholipids analysis. The
authors also thank Dr Zoraida DeFreitas for the gift of all-\textit{trans}-lutein.
CHAPTER 4

Free and esterified zeaxanthin and lutein are micellarized during in vitro digestion of wolfberry, orange pepper, squash and mango

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Abbreviations used: ARAT, acyl CoA retinol acyltransferase; BHT, butylated hydroxytoluene; CEase, cholesterol esterase; LRAT, lecithin retinol acyltransferase; MTBE, methyl-\textit{tert}-butyl-ether
4.1 ABSTRACT

Zeaxanthin and lutein are the only dietary carotenoids that accumulate in the macula region of the retina and in lens. It has been proposed that these two carotenoids protect these tissues against photo-oxidative damage. Despite this potential health promoting activity, few plant foods enriched in zeaxanthin have been identified and there is no information about the bioavailability of zeaxanthin from these foods. Total zeaxanthin content and the relative amounts of the free form, mono-esters and di-esters for several plant foods known to contain zeaxanthin were determined. Wolfberry had the greatest concentration of zeaxanthin with di-esters accounting for 95% the total. Free, mono- and di-esters of zeaxanthin were present in orange pepper, whereas only mono-esters of zeaxanthin were present in squash. Lutein esters also were present in squash and mango. When subjected to simulated digestion, total zeaxanthin recovery exceeded 85%. The concentrations of free xanthophylls increased during digestion as a result of the partial cleavage of zeaxanthin esters by cholesterol esterase. Fatty acid esters of the xanthophylls, like free xanthophylls, were transferred to micelles during in vitro digestion. However, the efficiency of micellarization of free and esterified forms of the xanthophylls differed with micellarization of free zeaxanthin/lutein > zeaxanthin/lutein mon-esters > zeaxanthin/lutein di-esters. The metabolism of micellarized xanthophylls esters merits examination in order to compare their bioavailability to non-esterified xanthophylls.
4.2 INTRODUCTION

The oxycarotenoid zeaxanthin (ββ-carotene3,3’-diol), like lutein, is present in ocular tissues. The highest concentration of zeaxanthin in human and animal primates is found in the retina and particularly the fovea region of the macular which is located within the optical axis of the eye (26, 134). It is hypothesized that the accumulation of zeaxanthin and lutein protects ocular tissue against photo-oxidative damage by absorbing damaging “blue range” (400-550 nm) and scavenging free radicals (176, 318). This antioxidant activity is associated with the polyene chain in zeaxanthin (225).

Zeaxanthin must be obtained from the diet. However, it is a minor carotenoid in green leafy vegetables consumed by humans. Several foods have been identified as good sources of zeaxanthin (301, 362). Generally, zeaxanthin is present in these foods as mon- and di-esters rather than the non-esterified or “free” xanthophylls (362). For example, red pepper contains zeaxanthin mono-palmitate and four zeaxanthin di-esters, viz, laurate/myristate, di-myristate, myristate/palmitate, and di-palmitate (362). Wolfberry (Lycium Chinense) is an Asian fruit that has the highest concentration of zeaxanthin found in all analyzed foods. This fruit is used in traditional Chinese medicine as a herb to promote visual health (155, 204). The major carotenoid in wolfberry is dipalmitate zeaxanthin with lesser amounts of mono-palmitoyl zeaxanthin and free zeaxanthin also present (381). The zeaxanthin content in ripe wolfberry generally exceeds 100 mg/100g dry weight.

Recently, purified xanthophyll esters and free xanthophylls have been reported to have similar bioavailability when administered in oil (39, 40). Xanthophylls esters are
either not detected (39, 367) or detected in exceedingly low concentrations (38, 119, 255) in plasma. This indicates that the esters are hydrolyzed to the free carotenoid during digestion or after uptake from the lumen by enterocytes. Generally, transfer of dietary esters must be hydrolyzed to the alcohol and free fatty acid in the small intestine to facilitate partitioning in mixed micelles for delivery to absorptive epithelial cells (100). It has been assumed that carotenoid esters are similarly processed during digestion. The objectives of this study were to i) characterize the profile of zeaxanthin and lutein esters in several plant foods, ii) examine the extent of hydrolysis of xanthophylls esters from several foods during simulated gastrointestinal digestion, and iii) to determine the efficiency of micellarization of non-esterified and esterified xanthophylls during the digestive process.

4.3 MATERIALS AND METHODS

Chemicals:

Unless stated otherwise, all reagents and materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Fisher Scientific Co. (Fair Lawn NJ USA). Purified all trans-zeaxanthin for use as standard was a generous gift from Dr. Minhthy Nguyen (Ross Nutrition Products, Columbus, OH).

Preparation of test foods for in vitro digestion:

Test fruit and vegetables, viz., dried wolfberry, orange pepper, mango and commercially processed acorn squash were purchased from local food markets. Wolfberry (250 g) was added to 250 mL deionized water and left to stand 12h for
rehydration. Orange pepper and mango were washed and the edible portions were prepared. Olive oil (3.5% v:w) was added to weighed samples of the three plant foods before homogenizing (Osterizer Galaxie) for 4 min to produce a consistent puree. Aliquots of homogenized tested foods were transferred to 50 mL screw cap polypropylene tubes, and stored at -80°C under nitrogen for use within several weeks. The jarred squash was opened and homogenized before use.

**Preparation of zeaxanthin-rich extract from wolfberry:**

A sample (10g) of homogenized wolfberry (see above) was transferred to a 1 L separatory flask. Two hundred mL methanol : ethyl acetate : petroleum ether (1:1:1) was added to the sample and the flask was shaken for 5 min. After standing for 2 min to allow the upper (organic) layer was collected and the lower layer was extracted three additional times. Pooled organic layers were passed through a Sep-pak C18 cartridge, the lower layer was collected and the filtrate was passed through the cartridge again. The final filtrate was transferred to 11 mL screw cap vials and dried under nitrogen. The extract was stored at -80°C.

**In vitro digestion:**

The procedure described by Garrett et al. (104) was followed. Each reaction (25 mL final volume) generally contained 500 mg of test food and 18 mg virgin olive oil diluted with 120 mmol/L NaCl containing 150 µmol/L butylated hydroxytoluene (BHT). The gastric and small intestinal phases were performed as previously described with one exception. Either porcine pancreatin (0.4 mg/mL final concentration) and porcine pancreatic lipase (0.2 mg/mL final concentration), or pancreatin, lipase and bovine cholesterol esterase (1 unit/mL final concentration) were present during the small
intestinal phase of digestion. The product after completion of simulated digestion is referred to as “digesta”. Digesta was centrifuged (Ti 50 rotor, Beckman Model L7-65, Palo Alto, CA, USA) at 167,000 x g at 4°C for 35 min to separate the aqueous fraction containing mixed micelles from residual solids and oil. Aqueous fraction was collected and filtered (cellulose acetate, 0.22 µm pore size; Gelman Science, Ann Arbor, MI USA) to remove microcrystalline carotenoid aggregates and other insoluble dispersed materials. Homogenized food, digesta and aliquots of filtered aqueous fraction containing natural micelles were stored at -80°C under nitrogen and analyzed within one week.

**Hydrolysis of zeaxanthin esters by bovine cholesterol esterase:**

Cholesterol esterase (CEase) is secreted by the exocrine pancreas and hydrolyzes dietary cholesterol esters to free cholesterol and fatty acids (148). CEase also previously has been shown to hydrolyze esterified astaxanthin, lutein diesters, capsanthin esters and β-cryptoxanthin esters (37, 160). The possibility that zeaxanthin esters from wolfberry extract were a substrate for CEase was tested using the method described by Tyssandier et al. (333) with cholesteryl linoleate serving as a positive control. Briefly, either wolfberry extract containing either 70 µmol zeaxanthin esters in chloroform or 542 µmol cholesteryl linoleate in chloroform, plus 5 mg egg yolk phosphatidylcholine, and 30 mg olive oil were added to a 5 mL glass vial. A stream of nitrogen was passed over the mixture to remove chloroform. Saline (0.9% NaCl, 1.6 mL) at 70°C was added to the reaction vial and the mixture was sonicated in a bath at room temperature for 30 min. Mixed bile salts (8 mmol/L) and test enzymes were added to reaction tubes. Enzymes tested included pancreatin alone (10 mg/mL), pancreatic lipase plus co-lipase alone (4.4 mg/mL and 9.4 mg/mL, respectively), bovine CEase (1 unit/mL) alone, or a mixture with
pancreatin, lipase, co-lipase and CEase. A replicate set of tubes that did not have any of
the enzymes added served as a control. After adjusting the final volume to 2 mL with
saline, vials were incubated in a shaking water bath at 37°C for 1, 2 and 3h. Reactions
were terminated by the addition of organic solvents for extraction of substrates and
products as described below.

**Extraction and analysis of xanthophylls:**

Thawed samples (1-3 mL) of homogenized food and the digesta and aqueous
fraction after simulated digestion were extracted by addition of 3.0 mL methanol : ethyl
acetate : petroleum ether (1:1:1 v/v/v) containing 4.5 mmol/L BHT, vortexing for 1 min,
and centrifuging (2000 x g for 5 min) to hasten phase separation. The extraction
procedure was repeated a total of three times and petroleum ether fractions were
combined and dried at room temperature under a stream of nitrogen. The film was
resolubilized in methyl-tert-butyl-ether (MTBE): methanol (MeOH)(1:1) and analyzed
immediately. Zeaxanthin and its esters were extracted from reaction vials for assessing
cleavage by digestive enzymes in an identical manner.

Non-esterified and esterified xanthophylls were quantified by high performance
liquid chromatography (HPLC) a slight modification of the procedure described by
Weller and Breithaupt (362). HPLC (Waters 2695 Separation model) with a Waters 996
photodiode array detector (Waters, Milford, MA) was used to analyze carotenoids.
Xanthophylls were separated with a YMC C30 analytical scale (4.5 x 250 mm) reversed
phase column (Waters, Milford, MA) with a C18 stationary-phase guard column. Guard
column and column were maintained at 35°C, whereas sample was maintained at 10°C
in the autosample tray. Separations were achieved using a gradient elution with a binary
mobile phase of methanol: MTBE:water (81: 15: 4 v/v/v) in reservoir A and methanol: MTBE:water (6: 90: 4 v/v/v) in reservoir B. Initial conditions were set flow rate of 1 mL/min at 100% A 10 min., followed by a linear gradient to obtain 50:50 A/B over 40 min, 100% B at 50 min, 100% A at 55 min and maintained until 60 min. The concentration of xanthophylls was calculated from comparison of area under the curve with known concentrations of the all-\textit{trans}-isomers of lutein and zeaxanthin. The employed extinction coefficients, $E_{1\text{ cm}, 1\%}$, at 450 nm in hexane were 2550 for all-\textit{trans} and \textit{cis}-lutein and 2540 for all-\textit{trans} and \textit{cis}-zeaxanthin (41). Xanthophyll esters were identified by UV-visible absorbance spectra and comparison of retention times to separations with a $C_{30}$ column according to Weller and Breithaupt (362).

\textit{Extraction and analysis of cholesterol and cholesterol ester:}

Cholesterol was extracted by first adding (3 mL) chloroform : methanol (2:1) to 2 mL reaction mix described. Mixtures were vortexed for 1 min and centrifuged (2000 x g for 5 min) to hasten phase separation. The extraction was repeated a total of three times and chloroform fractions were combined and dried at room temperature under nitrogen. The film was resolubilized in the mixture of 1% methanol. Free cholesterol was assayed enzymatically according to Robyt and White (283). Briefly, 100 $\mu$L of sample was added to 2.0 mL of reagent containing 10 units cholesterol oxidase and 2.0 mL water. Tubes were incubated at 37°C for 25 minutes. Absorbance was measured at 500 nm and compared with standard curve prepared using cholesterol in the range of 0.1-4.0 mmol/L. For analysis of total cholesterol, 10 units cholesterol esterase also was added to the reaction tube.
Statistical analysis of data:

All data were analyzed using Stata 8.0 (Stata Corporation, Texas, USA). Descriptive statistics including mean and standard error were calculated for the recovery and efficiency of micellarization of xanthophylls from digested foods. Means were compared using one-way ANOVA followed by Tukey’s correction. Differences were considered significant at \( p<0.05 \). All tests were conducted in triplicate for each experiment to provide at a minimum of three independent observations.

4.4 RESULTS

Xanthophyll content of foods.

The analytical procedure provided excellent separation of the non-esterified zeaxanthin, zeaxanthin mono-esters, and zeaxanthin di-esters (Figure 4.1). Zeaxanthin di-palmitate was previously reported to be the predominant form of this carotenoid (381) and the major peak eluting from the column was arbitrarily assigned this structure. The structures were confirmed by spectral analysis and comparative retention times. Moreover, saponification of the wolfberry extract with 15% KOH at 37°C for 30 min resulted in complete hydrolysis to free zeaxanthin (Figure 4.2). The relative amounts of zeaxanthin in orange pepper and squash were 17% and 6% that in wolfberry (Table 4.1). Free zeaxanthin, zeaxanthin mono-esters and zeaxanthin di-esters were present in orange pepper, whereas only zeaxanthin mono-esters were found in squash. Lutein mono- and di-esters, as well as a trace of free lutein, were present in squash. Only lutein mono-esters were found in mango.
Stability of xanthophylls esters in foods subjected to standard simulated digestion procedure.

Homogenized samples of wolfberry, orange pepper and squash were digested in vitro with pancreatin, pancreatic lipase and bile salts presence during the small intestinal phase. Recovery of xanthophylls after simulated digestion exceeded 85%. Surprisingly, there was very limited cleavage of zeaxanthin or lutein esters during this process (Table 4.2).
FIGURE 4.1 HPLC separation of non-esterified zeaxanthin, zeaxanthin mono-esters, and zeaxanthin di-esters.
FIGURE 4.2 Saponification of the wolfberry extract with 15 %KOH at 37°C for 30 min resulted in complete hydrolysis to free zeaxanthin.
<table>
<thead>
<tr>
<th>Food</th>
<th>Zeaxanthin equivalents² (µg/g wet wt.)</th>
<th>Free and esterified xanthophylls (µg/g wet wt.)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>non-esterified</td>
<td>mon-esters</td>
</tr>
<tr>
<td>Wolfberry</td>
<td>628.0 ± 12³</td>
<td>4.9 ± 1.2</td>
<td>20.4 ± 2.1</td>
</tr>
<tr>
<td>Orange pepper</td>
<td>106.5 ± 7</td>
<td>47.8 ± 1</td>
<td>47.2 ± 2</td>
</tr>
<tr>
<td>Squash</td>
<td>37.8 ± 2</td>
<td>ND⁴</td>
<td>37.8 ± 3</td>
</tr>
<tr>
<td>Squash</td>
<td>25.6 ± 2</td>
<td>0.7 ± 0.1</td>
<td>18.5 ± 2</td>
</tr>
<tr>
<td>Mango</td>
<td>13.4 ± 2</td>
<td>ND</td>
<td>13.4 ± 2</td>
</tr>
</tbody>
</table>

¹ N = 5 independent analysis per food.
² Xanthophyll equivalents = quantity of xanthophyll present independent of esterified fatty acids
³ Data are mean ± SEM
⁴ ND = not detected

**TABLE 4.1** Xanthophyll content and profile in selected foods.¹
<table>
<thead>
<tr>
<th>Source</th>
<th>Pre-digestion, μg/mL</th>
<th>%recovery after digestion</th>
<th>Post-digestion, μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>non-esterified</td>
<td>monoesters</td>
<td>diesters</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wolfberry</td>
<td>0.2 ± 0.001</td>
<td>0.8 ± 0.04</td>
<td>24.1 ± 0.5</td>
</tr>
<tr>
<td>Orange pepper</td>
<td>1.7 ± 0.1</td>
<td>1.9 ± 0.05</td>
<td>0.7 ± 0.02</td>
</tr>
<tr>
<td>Squash</td>
<td>ND</td>
<td>1.5 ± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>Lutein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squash</td>
<td>0.03 ± 0.002</td>
<td>0.7 ± 0.02</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>Mango</td>
<td>ND</td>
<td>0.5 ± 0.06</td>
<td>ND</td>
</tr>
</tbody>
</table>

1 In vitro digestion performed according to Garrett et al. (104)
2 Data are mean ± SEM for N = 3 independent digestion per food
3 ND = not detected

**TABLE 4.2** Xanthophyll esters are stable during simulated digestion of test vegetables 1
Micellarization of xanthophylls during simulated digestion.

Micellarization of zeaxanthin equivalents during digestion differed significantly ($p < 0.05$) for the three test foods with that in orange pepper > squash > wolfberry (Table 4.3). The mean efficiency of micellarization of lutein equivalents during digestion of both squash and mango was 26%. Mono-ester derivatives of both zeaxanthin and lutein in digesta from the four test foods also were micellarized. A low percentage of zeaxanthin di-esters in wolfberry and orange pepper also were micellarized. The presence of xanthophylls esters in the aqueous fraction was confirmed by saponification of aliquots resulting in the quantitative recovery of all zeaxanthin and lutein in the free form (not shown)

Pancreatin lacks cholesterol esterase (CEase) activity.

Several investigators (37, 160) have shown that the fatty acid esters of several xanthophylls are cleaved by CEase. The stability of the xanthophyll esters during in vitro digestion suggested that the crude pancreatin lacked CEase activity. This was confirmed by monitoring conversion of cholesterol linoleate to cholesterol in the presence of pancreatin in a reaction simulating small intestinal digestion (Figure 4.3). Addition of CEase alone or CEase along with pancreatin, lipase and co-lipase was associated with a time dependent generation of free cholesterol. Similarly, free zeaxanthin was produced when zeaxanthin esters extracted from wolfberry were incubated with CEase or CEase and the normal complement of pancreatic enzymes. Approximately 50% of cholesterol linoleate and zeaxanthin esters were hydrolyzed to cholesterol and zeaxanthin, respectively, during the 3 h incubation period. In vitro
<table>
<thead>
<tr>
<th>Source</th>
<th>% xanthophylls in digesta present in aqueous fraction</th>
<th>Efficiency of micellarization of xanthophylls&lt;sup&gt;1&lt;/sup&gt;</th>
<th>% micellarization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>non-esterified</td>
<td>mono-esters</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wolfberry</td>
<td>7.6 ± 0.9&lt;sup&gt;2&lt;/sup&gt;</td>
<td>90.5 ± 1</td>
<td>56.2 ± 3</td>
</tr>
<tr>
<td>Orange pepper</td>
<td>54.2 ± 0.5</td>
<td>104.4 ± 3</td>
<td>30.1 ± 2</td>
</tr>
<tr>
<td>Squash</td>
<td>35.2 ± 1.1</td>
<td>74.9 ± 1</td>
<td>33.0 ± 3</td>
</tr>
<tr>
<td>Lutein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squash</td>
<td>26.7 ± 1.2</td>
<td>106.2 ± 3</td>
<td>30.6 ± 3</td>
</tr>
<tr>
<td>Mango</td>
<td>26.7 ± 1.3</td>
<td>ND</td>
<td>23.1 ± 2</td>
</tr>
</tbody>
</table>

<sup>1</sup> % Micellarization = ([xanthophylls] in aqueous fraction/[xanthophylls] in digesta) x 100
<sup>2</sup> Data are mean ± SEM for N=3 digestions
<sup>3</sup> ND = not detected

**TABLE 4.3** Efficiency of micellarization of xanthophylls during simulated digestion
FIGURE 4.3 Zeaxanthin esters, like cholesterol linoleate, are hydrolyzed by cholesterol esterase (CEase). Cholesterol linoleate and zeaxanthin esters in wolfberry extract were incubated i) in the absence of enzymes (◊), or with either ii) pancreatin (□), iii) pancreatic lipase + co-lipase (▲), iv) cholesterol esterase (○), or v) pancreatin + pancreatic lipase + co-lipase + cholesterol esterase (■). The quantities of free cholesterol (panel A) and free zeaxanthin (panel B) at 0-3 h are shown. Each point is the mean for N=3 reactions. Standard errors of the mean of ranged from 8-12 and 1.3-2.8 umol/L for cholesterol and non-esterified zeaxanthin, respectively.
digestion of test foods was repeated in the presence of CEase to re-examined to determine xanthophyll ester stability and micellarization.

**Recovery and micellarization of xanthophylls after in vitro digestion with CEase present.**

Recovery of zeaxanthin and lutein after simulated gastric and small intestinal digestion of wolfberry, orange pepper and squash exceeded > 80% (Table 4.4). There were evident, but limited, increases in the concentrations of free xanthophylls for each digested test food with the corresponding decreases in the concentrations of both di- and mono-esters. The presence of CEase activity during the small intestinal phase of digestion significantly ($p < 0.05$) increased the mean efficiency of xanthophyll micellarization (compare second columns in Tables 4.3 and 4.5). Micellarization of free zeaxanthin and lutein was exceedingly efficient (> 70%) in samples digested with CEase activity (Table 4.5), as observed when samples were digested in the absence of bovine CEase (Table 4.5). The efficiency of micellarization of zeaxanthin and lutein mono-esters ranged from 17-63%, whereas micellarization of the abundant di-esters in digested wolfberry was approximately 5%. The small quantity of zeaxanthin di-esters in orange pepper were micellarized with 17% efficiency.

### 4.5 DISCUSSION

Xanthophylls are often present as fatty acid esters in fruits and vegetables. The profiles of mono- and di-esters were examined in several plant foods known to contain zeaxanthin and/or lutein. The results confirm previous reports that wolfberry contains
<table>
<thead>
<tr>
<th>Source</th>
<th>Pre-digestion, µg/mL</th>
<th>%recovery after digestion</th>
<th>Post-digestion, µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Orange pepper</td>
<td>1.7 ± 0.1</td>
<td>1.9 ± 0.05</td>
<td>0.7 ± 0.02</td>
</tr>
<tr>
<td>Squash</td>
<td>ND</td>
<td>1.5 ± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>Lutein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squash</td>
<td>0.03 ± 0.002</td>
<td>0.7 ± 0.02</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>Mango</td>
<td>ND</td>
<td>0.5 ± 0.06</td>
<td>ND</td>
</tr>
</tbody>
</table>

1 1 unit cholesterol esterase/mL was added to standard *in vitro* digestion described by Garrett et al. (104)
2 Data are mean ± SEM for N = 3 independent digestion per food
3 ND = not detected

**TABLE 4.4** CEase activity during simulated digestion increases the amount of free xanthophylls in digesta.
<table>
<thead>
<tr>
<th>Source</th>
<th>% total xanthophylls in digesta present in aqueous fraction(^1)</th>
<th>Efficiency of micellarization of xanthophylls(^2)</th>
<th>% micellarization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>non-esterified</td>
<td>mono-esters</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wolfberry</td>
<td>9.5 ± 0.7(^3)</td>
<td>82.3 ± 1.1</td>
<td>17.5 ± 1.5</td>
</tr>
<tr>
<td>Orange pepper</td>
<td>69.0 ± 1.4</td>
<td>92.4 ± 3.5</td>
<td>33.4 ± 2.3</td>
</tr>
<tr>
<td>Squash</td>
<td>41.0 ± 1.1</td>
<td>74.0 ± 1.7</td>
<td>36.2 ± 3.2</td>
</tr>
<tr>
<td>Lutein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squash</td>
<td>43.7 ± 2.2</td>
<td>112.6 ± 5</td>
<td>32.6 ± 1.9</td>
</tr>
<tr>
<td>Mango</td>
<td>76.6 ± 1.6</td>
<td>92.9 ± 3.3</td>
<td>62.7 ± 2.3</td>
</tr>
</tbody>
</table>

\(^1\) All indicated means are significantly (p<0.05) higher than % total xanthophylls present in the aqueous fraction after simulated digestion without CEase activity (Table 4.3)

\(^2\) Defined as (xanthophylls in aqueous fraction divided by xanthophylls in digesta) x 100%

\(^3\) Mean ± SEM

\(^4\) ND = not detected

**TABLE 4.5** Efficiency of micellarization of xanthophylls increases when CEase activity is present during simulated digestion.
high concentrations of zeaxanthin with the predominant form being a di-ester. Likewise, both zeaxanthin and lutein esters were found in squash as reported by Khachik et al. (171). Orange pepper was found to contain similar amounts of free zeaxanthin and its mono-esters, as well as lower concentrations of zeaxanthin di-esters. Several recent human studies have shown that the absorption of lutein and zeaxanthin administered in oil as the ester is equal to or greater than that of the non-esterified xanthophylls administered in the same vehicle (33, 40). The relative bioavailability of free vs. esterified xanthophylls from natural sources has not been investigated. Results from the in vitro studies reported above reveal that cholesterol esterase catalyzes the hydrolysis of some of the xanthophylls esters during simulated digestion of plant foods. Also, mono- and di-esters of xanthophylls were incorporated into micelles, although less efficiently than free xanthophylls.

Micelles represent vehicles for the delivery of lipophiles to the brush border surface of absorptive epithelial cells. It is unknown if fatty acids must be cleaved from xanthophylls for efficient uptake by cells. One possible site for such cleavage is within the micelle itself, perhaps by CEase or other enzymes with esterase activity within the small intestinal lumen. It has been shown that CEase is required for the absorption of cholesterol when cholesterol esters are present in micelles (238). It also is possible that enzymes with xanthophyll esterase activity are located within or anchored to the brush border membrane. Indeed, retinyl ester hydrolase activity has been shown to cleave dietary retinyl ester with the free retinol being transferred into the cell (282). A third possibility is that the intact ester is transferred from the micelle to the enterocyte and
subsequently cleaved by an intracellular esterase. The free xanthophyll could then be
incorporated into chylomicrons for secretion into lymph. Because low concentrations of
xanthophyll esters have been reported in the plasma of birds (168) and humans (245)
after feeding high levels of these compounds, a small percentage of the acquired ester
may be incorporated directly into chylomicrons. Alternatively, xanthophylls may be
hydrolyzed and the free xanthophyll may be re-esterified by lecithin retinol
acyltransferase (LRAT) or acyl CoA retinol acyltransferase (ARAT) prior to
incorporation into the chylomicron. Indeed, dietary retinyl esters are hydrolyzed within
the lumen or at the brush border surface and cellular retinol is esterified by LRAT
and secreted across the basolateral surface in chylomicrons (136, 245). Investigators
generally do not consider the possibility of xanthophyll esters in plasma since these
extremely hydrophobic compounds have long retention times with the standard solvent
systems used for analyzing carotenoids. The Caco-2 cell model lends itself to addressing
these important questions about the site(s) of hydrolysis and the efficiency of uptake of
xanthophyll esters by absorptive epithelial cells.

An important observation of this study was the absence of CEase activity in crude
porcine pancreatin. This commercial preparation is used by many investigators for in
vitro digestion. It is interesting that the addition of 1 unit CEase/mL cleaved 40-50% of
zeaxanthin esters in the wolfberry extract solubilized in oil after 2 h (Figure. 4.3),
whereas only 6% of the zeaxanthin esters where hydrolyzed during the 2 h period of
simulated small intestinal digestion (Table 4.4). This suggests release of the zeaxanthin
esters from the food matrix for partitioning into the oil droplet is limited. The relative
bioavailability of \( \beta \)-carotene and lutein from oil is known to exceed that for these
carotenoids when administered as a food (42). The observation that the absorption of lutein after administration of lutein esters required more fat in the meal than when subjects were dosed with free lutein is likely due to the need for a larger depot of fat droplets to enhance release of the xanthophyll esters from the matrix.
CHAPTER 5

Xanthophylls and \( \alpha \)-tocopherol decrease UVB-induced lipid peroxidation and stress signaling in human lens epithelial cells.

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Jayme E. Glamm\(^2\) & Mark L. Failla\(^{1,2}\)

\(^1\)OSU Interdisciplinary PhD Program in Nutrition and
\(^2\)Department of Human Nutrition, The Ohio State University.

Supported in part by: The Ohio State Agriculture Research and Development Center and The Virginia Vivian Scholarship Fund of the OSU Human Ecology College (to C.C.)

Abbreviations used: AST, astaxanthin; DMEM, Dulbecco’s modified Egale’s medium; HAE, 4-hydroxyalkenals; HLE, human lens epithelial cells; JNK, c-JUN NH\(_2\)-terminal kinase; LDH, lactate dehydrogenase; LUT, lutein; MDA, malonyldialdehyde; M\(\beta\)CD, methyl-\(\beta\)-cyclodextrin, MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; \(\alpha\)-TC, \(\alpha\)-tocopherol; UVB, ultraviolet B light; ZEA, zeaxanthin.
5.1 ABSTRACT

Epidemiological studies suggest that consumption of vegetables rich in the xanthophylls lutein (LUT) and zeaxanthin (ZEA) reduce the risk for developing age-related cataract, a leading cause of vision loss. While LUT and ZEA are the only dietary carotenoids present in the lens, direct evidence for their photoprotective effect in this organ is not available. The present study examined the effects of xanthophylls and α-tocopherol (α-TC) on lipid peroxidation and the mitogen-activated stress signaling pathways in human lens epithelial (HLE) cells following UVB irradiation. When presented with LUT, ZEA, astaxanthin (AST) and α-TC as methyl-β-cyclodextrin (MβCD) complexes, HLE cells accumulated the lipophiles in a concentration- and time-dependent manner with uptake of LUT exceeding that of ZEA and AST. Pre-treatment of cultures with either 2 µmol/L xanthophyll or 10 µmol/L α-TC for 4h before exposure to 300 J/m² UVB radiation decreased lipid peroxidation by 47-57% compared with UVB-treated control HLE cells. Pre-treatment with the xanthophylls and α-TC also inhibited UVB-induced activation of c-JUN NH₂-terminal kinase (JNK) and p38 by 50-60% and 25-32%, respectively. Significant inhibition of UVB-induced JNK and p38 activation was observed for cells containing < 0.20 and approximately 0.30 nmol xanthophylls/mg, respectively, whereas greater than 2.3 nmol α-TC/mg protein was required to significantly decrease UVB-induced stress signaling. These data suggest that xanthophylls are more potent than α-TC for protecting human lens epithelial cells against UVB insult.
5.2 INTRODUCTION

Approximately 20 million people in the United States have their vision obstructed by cataract and approximately 500,000 new cases are diagnosed annually. Current treatment involves surgical extraction, an expensive procedure that is performed over 1.5 million times annually in the United States at an estimated cost of $3.4 billion dollars per year (244). Furthermore, the incidence and costs associated with this disease are certain to increase with the rapidly increasing number of individuals over 65 years of age. Effective strategies aimed at preventing and/or delaying the development of age-related cataract are needed.

Ultraviolet radiation (UVR) from sunlight and oxidative stress appear to be the most relevant contributors to age-related cataractogenesis. Results from several epidemiological studies suggest that individuals with high exposure to UVR have an increased risk of cataracts later in life (143, 227). A particularly strong association has been observed between cataract development and exposure to radiation wavelengths of 290-320 nm (designated UVB) (317). UVB radiation is thought to contribute to cataract formation by directly damaging DNA (179, 297), producing reactive oxygen species (ROS) (116, 128), and generating cytotoxic products from actively translating ribosomes (157). In addition to UVB, hydrogen peroxide is chronically present in the aqueous environment surrounding the anterior lens and may contribute to cataract development (72, 302, 349). Like all tissues, the lens is equipped with antioxidant defense mechanisms that generally protect against the harmful effects of UV- and ROS. Because many of the enzymatic cofactors and chemical constituents necessary for antioxidant...
activity are obtained only through the diet, adequate nutrition is likely to be important in preventing ROS-induced oxidative damage and maintaining the overall health of the eye (161, 214, 288). Indeed, some epidemiological and experimental studies suggest that increased consumption of dietary antioxidants such as vitamin C, vitamin E, zinc, and carotenoids may reduce the incidence or progression of ocular diseases. (43, 48, 162, 221).

LUT and ZEA are the only dietary carotenoids that are present in the macula region of the retina and the lens (17, 192, 376). Dietary supplementation with these xanthophylls increases macula pigment density in human subjects (131, 193), primates (201), and quail (322, 324). Moreover, it has been reported that chronic intake of high amounts of LUT improved visual acuity and glare sensitivity in several studies with small numbers of subjects with age-related cataracts and macular degeneration (256, 257). The above data have served as impetus for addition of LUT to a number of multi-vitamin and mineral preparations and the marketing of numerous LUT and ZEA supplements for healthy vision.

While considerable efforts are being directed towards defining the potential roles of the xanthophylls in the macula, information about the uptake and possible function of LUT and ZEA in lens is extremely limited. In addition, the low lenticular concentrations of LUT and ZEA (17, 376) challenge the feasibility that these xanthophylls are capable of contributing to the protection of this organ against environmental and endogenous stressors. The present study examined the ability of several xanthophylls to protect cultures of immortalized human lens epithelial cells (HLE) against UVB insult. Epithelial cells comprise the outermost cellular layer of the human lens and are exposed to UV
irradiation not filtered by the cornea. UV-induced oxidative damage to these cells is mediated via production of ROS and characterized by alterations in cell growth and morphology, changes in membrane potentials, oxidization of proteins, unscheduled DNA synthesis, DNA strand breakage and lipid peroxidation (179, 297, 302). The results reported below demonstrate that LUT and ZEA decrease UVB-induced lipid peroxidation and mitogen-activated protein kinase (MAPK) stress signaling in the HLE cell line, and that this protective effect of the xanthophylls is much more potent than α-TC.

5.3 MATERIAL AND METHODS

Chemicals

Unless stated otherwise, all reagents and materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Fisher Scientific Co. (Fair Lawn NJ USA). Purified all trans-LUT and all trans-ZEA were generous gifts from Drs. Zoraida DeFreitas, Kemin (Des Moines, IA), and Minhthy Nguyen, Ross Products Division, Abbott Laboratories (Columbus, OH), respectively.

Preparation of xanthophylls and α-tocopherol:methyl-β-cyclodextrin complexes

Stable water soluble complexes of xanthophylls and α-TC were prepared by complexation with methyl-β-cyclodextrin (MβCD) using a modification of the procedure described by Pfitzner et. al. (270). Briefly, either 35 μmol xanthophylls or 174 μmol α-TC was solubilized in 2 mL dichloromethane and diluted with 48 mL ethanol containing 417 μmol MβCD. The mixture was stirred for 24 h at 37°C and then dried in a vacuum concentrator. Residue was solubilized, filter-sterilized (cellulose acetate 0.22 μm pores) and aliquots stored under a blanket of nitrogen at -80°C. Complexation efficiency, i.e.,
the percentage of the lipophile passing through filters with 0.22 µm pores, was greater than 90% for LUT, ZEA, AST and α-TC. Molar ratios of MβCD to LUT, ZEA, AST and α-TC were 12:1, 12:1, 13:1, and 2.5:1, respectively. Stock solutions of complexed xanthophylls and α-TC remained stable (99 ± 2%) without isomerization or degradation for as long as one year. Prior to experiments, the purity and the concentration of the xanthophylls and α-TC complexed with MβCD were verified by HPLC as described below.

**Cell Culture**

The HLE cell line SRA 01-04 was provided by Dr Venket Reddy (Kellog Eye Institute, University of Michigan, Ann Arbor, MI). HLE cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 mL/L heat-inactivated fetal bovine serum (FBS), amphotericin B (0.5 mg/mL), penicillin-streptomycin (10 mL/L), sodium bicarbonate (44 mmol/L), and HEPES (15 mmol/L) in a humidified atmosphere of 95% /air 5% CO2 at 37ºC. HLE cells (3.5 x 10⁵ cells) were seeded in 60 mm² dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) for experiments. Medium was renewed every second day and cultures were used for experiments when confluency was 80-95%.

**Accumulation of xanthophylls and α-tocopherol from CD complexes by HLE cells**

Pre-confluent cultures of HLE cells were incubated in DMEM containing varying concentration of xanthophylls or α-TC as cyclodextrin complexes. After 4 h, spent medium was removed and monolayers were washed once with ice cold phosphate buffered saline (PBS) containing 2g/L albumin and twice with ice cold PBS. Monolayers were scraped and transferred to 2.0 mL screw cap conical tubes and centrifuged at 4000 x
g for 5 min. Cell pellets were stored under N₂ and stored at -80°C. Similarly, pre-confluent cultures of HLE cells were incubated in medium containing either 2 µmol/L xanthophyll or 10 µmol/L α-TC as cyclodextrin complexes and incubated for varying times to examine cell accumulation of lipophiles with increasing length of exposure. Monolayers were washed, collected and stored as above.

To assess possible cytotoxicity of MβCD itself and MβCD complexed with xanthophylls and α-TC, lactate dehydrogenase (LDH) release was determined as described by Clynes (57). The results are expressed as percentage of the total LDH released to medium, with total LDH activity = released LDH activity + cellular LDH activity.

**UVB radiation of HLE cells**

Cultures of HLE were washed twice with warm PBS containing 2 g/L albumin. After removing buffer, cultures of HLE were washed twice with warm PBS containing 2 g/L albumin. After removing buffer, tissue culture dishes without lids were inverted on a support at a fixed distance above a transilluminator (3UV, UVP, Upland, CA) and irradiated at 3.0 mW/cm² for approximately 10 sec to achieve a UVB dosage of 300 J/m² as measured with a calibrated radiometer. Replicate monolayers serving as controls were handled identically except that the UVB light was off. Immediately after treatment, 4 mL DMEM containing 10 mL/L FBS was added to the dish and cultures were returned to the incubator for 30 min. Pilot studies showed that cell viability and morphology were not changed for at least 2 h following exposure to the dose of 300 J/m².
Lipid peroxidation

HLE cells were washed several times with cold PBS, before sonication and centrifugation at 3000 x g at 4°C for 10 min. Lipid peroxidation was assessed in the supernatant as a marker for UV-induced damage. The concentrations of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were quantified simultaneously by their reaction with N-methyl-2-phenylindole at 45°C. The stable chromophore was measured at 586 nm (Lipid Peroxidation Kit, F-12, Oxford Biochemical Research, Oxford, MI).

Western blot analysis of MAPK proteins

Monolayers were harvested for determination of MAPK signaling proteins by washing twice with cold PBS before addition of 60 µL lysis buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L Tris-HCl, 1% Triton X-100, 0.1% SDS, 50 mM sodium fluoride, 10 mmol/L sodium pyrophosphate, 10 µL protease inhibitor cocktail and 1.2 mL water). Cells were sonicated on ice, and centrifuged at 20,000 x g for 5 min. Protein concentration of supernatent was measured by bicinchoninic acid method (BCA, Pierce, Rockford, IL). Aliquots of supernatant were diluted into modified Laemmli sample loading buffer and heated at 95°C for 3 min. Equal amounts of protein (25 µg/ sample) from each sample was loaded into each lane of a 7.5% sodium dodecyl sulfate polyacrylamide gel and electrophoresed at 120 volts for 1.5 h. Protein was transferred to a nitrocellulose membrane and processed for immunoblotting. Membranes were blocked with 50g/kg non-fat milk in TBS (20 mmol/L Tris [pH 7.5] and 150 mmol/L NaCl) containing 0.1% Tween-20 (TTBS) for 1 h and incubated with primary antibody (diluted 1:1000) to either human phospho-JNK or human p38 kinase (Cell Signaling Technology,
Inc. Beverly, MA) in 50g/kg non-fat milk in TTBS overnight at 4°C. Unbound primary antibody was removed by washing membranes six times with 20 mL TTBS on a rotating shaker for 5 min. Secondary antibodies (i.e., HRP-conjugated anti-mouse IgG and anti-rabbit IgG for phospho-JNK and phospho-p38, respectively) were diluted 1:2000 (in 20 mL of 5% milk in TTBS), and incubated with membranes on a rotating shaker at room temperature for 1 h. Unbound secondary antibody was removed by washing as above. Immunoreactivity was detected with an enhanced chemiluminescence kit (Super-Signal, Pierce, Rockford, IL). Excess reagent was drained before exposure of membrane to photographic film. Total JNK and p38 (both phosphorylated and dephosphorylated forms of protein) were analyzed by probing stripped nitrocellulose membrane with antibodies that recognized both phosphorylated and dephosphorylated forms of JNK (murine antisera to human –JNK MAPK, Santa Cruz Biotechnology, Inc) and p38 (Rabbit antisera to human-p38, MAPK, Cell Signaling Technology, Inc. Beverly, MA)) and processing as above. After exposure, films were scanned and band density was quantified by using an Image Station 2000R and ID Image Analysis Software version 3.6 (Eastman Kodak Company, Rochester, NY). Density units for each gel band are corrected for variations in loading using the reactivity of total JNK and p38.

**Extraction and analysis of xanthophylls and α-tocopherol.**

Cell pellets were resuspended in 500 µL of 35 mmol/L sodium dodecyl sulfate (SDS) in ethanol containing 4.5 mmol/L butylated hydroxytoluene (BHT) and sonicated for 30 sec on ice. Samples were extracted at least twice with 3 mL petroleum-ether:acetone (2:1) containing 4.5 mmol/L BHT. Petroleum-ether layers were combined
and dried under a stream of nitrogen. Dried residue was resolubilized in methyl-tert-butyl-ether (MTBE): methanol (50:50, v/v) for analysis by HPLC.

Xanthophylls and α-TC were quantified by high performance liquid chromatography (HPLC) according to Ferruzzi et al. (92) with slight modification described elsewhere (52). Concentrations of LUT and ZEA were calculated from comparison of area under the curve with known concentrations of the all-trans-isomers of LUT and ZEA standards at retention times of 10.3 min and 13.5 min, respectively. The gradient was modified for determination of AST by setting starting mixture of 95:5 A/B with a linear gradient to 40:60 A/B over 20 min. The retention times of cis- and all-trans-AST were 6.8 and 9.3 min, respectively. Isocratic system of reservoir A at a flow rate 0.8 mL/min was used for separation of α-tocopherol with a retention time of 11 min. The extinction coefficients, $E_{1cm, 1\%}$, were 2550 for all-trans and cis-LUT in ethanol at 450 nm, 2540 for all-trans and cis-ZEA in ethanol at 450 nm, 2100 for all-trans and cis-AST in hexane at 475 nm, and 71 for α-TC in ethanol at 292 nm (41, 294).

**Statistical analysis**

Each experiment was repeated independently at least twice with 2-4 replicate cultures per treatment per experiment. Results are expressed as the mean ± SE. Data were evaluated by two-tail unpaired Student’s t-test and one-way ANOVA and post-hoc Turkey test using Stata 8 statistic program. The level of statistical significance was taken as $p < 0.05$.  

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5.4 RESULTS

**Cyclodextrin complexes of xanthophylls and α-TC are not cytotoxic.**

MβCD complexes of xanthophylls and α-tocopherol were stable (mean recovery of 99 ± 3%) after incubation for as long as 8 h in DMEM in cell-free dishes in the incubator. Because cyclodextrins can disrupt cell integrity by removal of cholesterol from the plasma membrane (213), the potential toxicity of MβCD complexed with either 0-4 µmol/L xanthophylls or 0-15 µmol/L α-TC was tested; the maximum concentration of MβCD in test medium containing the highest concentrations of test lipophiles was 52 µmol/L. HLE cells exhibited no evident change in morphology and cell number per well was not altered after incubation with MβCD-complexes for as long as 8 h. Furthermore, release of LDH into medium was similar (p > 0.05) in control cultures (5.9 ± 0.1%) and cultures treated with MβCD complexes (6.5 ± 0.1%). However, the morphology of cells changed from their normal fiber-like appearance (382) to spheres with more than 50% of cells detaching from the surface within 4h when the concentrations of complexed LUT and α-TC were increased to 8 and 40 µmol/L, respectively. Maximum concentrations of xanthophylls and α-TC complexed to MβCD were 2 µmol/L and 15 µmol/L, respectively, and the period of exposure of cells to the complexes did not exceed 4h in the experiments described below. These condition did not affected cell morphology or cell number per culture.

**Cell accumulation of xanthophylls and α-TC from MβCD complexes.**

Cellular content (nmol/mg protein) of xanthophylls increased proportionally as medium concentrations were increased from 1 to 4 µmol/L (Figure 5.1A), and with
increasing length of exposure (Figure 5.1B). However, the amount of each xanthophyll accumulated by HLE cells from the MβCD complex differed with LUT uptake approximately twice that of ZEA ($p<0.001$) for each concentration and incubation period tested (Figures 1A and 1B). Accumulation of AST was intermediate between LUT and ZEA. Cell concentrations of α-TC also increased linearly when monolayers were incubated in medium containing 3 to 15 µmol/L α-TC complexed to MβCD and uptake was proportional to length of incubation (Figure 5.2).

**Xanthophylls and α-TC decrease UVB-induced lipid peroxidation.**

Since lipid peroxidation is a well-established consequence of UVB-exposure (116, 302), we examined if pre-incubation of HLE cells with xanthophylls and α-TC affected the extent of lipid peroxidation caused by UVB treatment. Lipid peroxide degradation products (i.e., malonyldialdehyde and 4-hydroxyalkenals) were $214 \pm 18$ pmol/mg protein in control cultures of HLE cells incubated for 4h in medium containing 10 mL/L FBS. Incubation of cells with xanthophylls and α-TC for 4h did not significantly ($p>0.05$) alter the concentration of lipid peroxidation products ($263.6 \pm 9.0$ pmol/mg protein) after incubation with 2 µmol/L LUT, ZEA or AST and 10 µmol/L α-TC ($265.1 \pm 31$ pmol/mg protein). UVB exposure (300 J/m²) of control cells or cells pre-treated with vacant MβCD increased the concentrations of lipid peroxide degradation products 525% (Figure 5.3). In contrast, incubation of monolayers in medium containing either 2 µmol/L xanthophyll or 10 µmol/L α-TC for 4h before exposure to UVB significantly ($p<0.01$) decreased the mean concentrations of malonyldialdehyde and 4-hydroxyalkenals by $52 \pm 4\%$ (range of 47-57%) compared to UVB-exposed cultures that were not pre-treated (Figure 5.3).
FIGURE 5.1 Accumulation of xanthophylls delivered as cyclodextrin complexes to HLE cells is proportional to medium concentration and length of exposure. Panel A. Pre-confluent HLE cell cultures were incubated with 1, 2, and 4 µmol/L of either LUT, ZEA or AST as MβCD complexes for 4h. Panel B. Cultures were incubated in medium containing 2 µmol/L test xanthophyll for either 2 or 4 h. Data are mean ± SE for 9-12 cultures at indicated concentrations for each xanthophyll in panel A. and 6-9 cultures at each time in panel B. Accumulation of LUT>AST>ZEA at each dose and time (p<0.05).
FIGURE 5.2 Accumulation of \( \alpha \)-tocopherol from M\( \beta \)CD complexes by HLE cells is proportional to medium concentration and duration of exposure. Test conditions and sample analyses were the same as indicated in legend to Figure 1. Data are mean ± SE for \( n = 8 \) cultures at each medium concentration and time. The presence of different letters above the error bars indicates that means differ significantly (\( p < 0.05 \)).
Xanthophylls and α-TC suppress UVB-induced stress signaling in HLE cells.

The MAPK stress signaling pathways are present in lens epithelium (203) and HLE cell line SRA 01-04 (23). To evaluate if xanthophylls and α-TC decrease UVB-induced activation of stress signaling, total and activated (phosphorylated) JNK and p38 were measured in control and pre-treated cultures by western blot. ERK was not studied since this protein was activated in the pre-confluent cultures and that exposure to UVB exposure did not enhance the degree of activation (23). Phospho-JNK protein (p-p46 and p-p54) was not detected in either control cultures or cultures incubated with 2 µmol/L xanthophyll or 10 µmol/L α-TC (Figure 5.4A). UVB irradiation markedly activated JNK in cells that had not been exposed to test compounds (Figure 5.4A). Pre-incubation of cells in medium with 2 µmol/L xanthophyll and 10µmol/L α-TC complexed to MβCD suppressed UVB-induced JNK activation by 50-60% (Figures 4A and 4B). The decline in JNK activation was similar in cultures pre-treated with equimolar concentrations of LUT, ZEA and AST (Figure 5.4B), although cell content of ZEA was only one-half that of LUT (Figure 5.1A). Total JNK (non-phosphorylated plus phosphorylated) was similar in all test cultures (data not shown).
FIGURE 5.3 Pre-treatment with xanthophylls and α-TC decreases UVB-induced generation of lipid peroxidation products in HLE cells. Control pre-confluent cultures of HLE cells were not exposed to vehicle, test compounds or UVB. Test cultures were incubated in medium with either vacant MβCD or xanthophylls or α-TC complexed to MβCD for 4h prior to exposure to UVB (300 J/m²). The quantity of malondialdehyde plus 4-hydroxyalkenals in cells after 30 min was measured. Data represent mean ± SE for 6-12 for each treatment. Different letters above error bars indicate significant differences at \( p < 0.01 \).
Phosphorylated p38 was not detected in either control cultures or cultures incubated in medium with 2 μmol/L xanthophylls or 10 μmol/L α-TC (data not shown). Similar to the results for JNK, p38 was phosphorylated when control cultures were exposed to UVB (Figure 5.5A). Pre-treat of cultures with LUT, ZEA, AST and α-TC prior to exposure to UVB irradiation significantly ($p < 0.05$) reduced p38 activation by 34, 14, 30 and 18% respectively (Figure 5.5B).

**LUT and ZEA are more potent than α-TC at attenuating UVB induced activation of stress signaling.**

HLE cells were incubated in medium containing 0-2 μmol/L LUT or ZEA (concentration of MβCD was constant at 25 μmol/L) for 4 h to vary the intracellular concentrations of the two xanthophylls. Cells were then exposed to UVB irradiation and the levels of phospho-JNK and phospho-p38 determined. The extent of UVB-induced phosphorylation of JNK was inversely proportional to cellular concentrations of the xanthophylls (Figure 5.6). Significant ($p<0.05$) decreases in UVB-induced activation of JNK were observed when cell LUT and ZEA concentrations were 0.16 ± 0.02 and 0.15 ± 0.01 nmol/mg cell protein, respectively; these were the cellular concentrations of xanthophylls following incubation in medium with 0.125 μmol/L LUT and 0.25 μmol/L ZEA, respectively. Similarly, UVB-induced activation of p38 decreased significantly ($p<0.05$) when cell concentrations of LUT and ZEA were 0.29 ± and 0.32 ± nmol/mg protein, respectively; these cell concentrations were achieved after incubation in medium with 0.25 μmol/L LUT and 0.50 μmol/L ZEA for 4 h.
FIGURE 5.4 Pre-treatment of HLE cells with xanthophylls (LUT, ZEA and AST) and α-TC decrease UVB-induced activation of JNK 1(p46) and JNK 2 (p54) in HLE cells. Test cultures were treated as in Figure 3 before exposure to UVB (300 J/m²). After 30 min, total and phospho-JNK 1 and 2 were measured. (A) Representative immunoblots for phosphorylated JNK 1 and JNK 2. (B) Mean (± SE) densitometric analysis of blots from four separate experiments each with samples from three replicate cultures per treatment. Asterisk above the bar indicates that the mean for treated cells is significantly (p< 0.05) lower than for cultures not pre-treated with xanthophylls or α-TC.
FIGURE 5.5 Xanthophylls and α-TC decrease UVB-induced phosphorylated of p38 in HLE cells. HLE cells were treated and phosphor-p38 and total p38 were determined. (A) Representative immunoblots. (B) Mean (± SE) densitometric analysis of the blots from four independent experiments with three replicate samples per treatment. The presence of different letters above bars indicates that the mean is significantly (p<0.05) less than that for UVB-exposed cells that were not pre-treated with test compounds.
Incubation of cells in medium containing 10 µmol/L α-TC increased cellular α-TC to 5.9 ± 0.11 nmol/mg protein, whereas cells without supplementation had <20 pmol/mg protein α-TC. The increased concentration of cell α-TC was associated with 51 ± 6 and 32 ± 4% declines in UVB-induced activation of JNK (Figure 5.4) and p38 (not shown), respectively. Incubation of monolayers with 5 µmol/L α-TC for 4h increased cell concentration of α-TC to 2.3 ± 0.06 nmol/mg protein but failed to decrease UVB-induced phosphorylation of JNK or p38 compared to that in cells not pre-treated with α-TC (data not shown).

5.5 DISCUSSION

Among the many environmental, lifestyle, and genetic risk factors associated with cataracts, exposure to UV radiation from sunlight and oxidative stress appear to be the most relevant in the development of this disease (143, 226, 302). H₂O₂ is a major oxidant in the human eye and increased levels are observed in patients with cataracts (72). The lens is equipped with antioxidant defense mechanisms designed to protect against the harmful effects of UV radiation- and ROS (161). These mechanisms consist of enzymes (e.g., superoxide dismutase, catalase and glutathione peroxidase) and low molecular weight compounds (e.g., vitamins C and E and glutathione) that metabolize and/or conjugate ROS, thereby rendering them inactive. Of particular relevance for this report is the suggestion that the low concentrations of LUT and ZEA in the lens contribute to the protection of this organ against UVB radiation (183). Indirect support for this possibility
FIGURE 5.6 Low cellular concentrations of LUT and ZEA decrease UVB-induced activation of JNK and p38. HLE cells were incubated in medium containing 0 -2 μmol/L LUT or ZEA complexed with MβCD before exposure to UVB (300J/m²). After 30 min, quantities of phospho-JNK and phosphor-p38, as well as total-JNK and p38, were measured. The intensity of the bands for activated JNK and activated p38 in cells that were not pre-treated before exposure to UVB were arbitrarily assigned a value of 100% for comparison with samples from cells pre-treated with xanthophylls. JNK and p38 were not activated in cells exposed to either xanthophyll without UVB exposure and the relative quantities of total JNK and p38 were not affected by treatment (not shown). LUT and ZEA were quantified in replicate sets of cells incubated in medium with indicated concentrations of xanthophylls, but not exposed to UVB. Data are means ± SE for N= 6 for each treatment for both the quantity of xanthophylls in cells and the activation of JNK and p38. Different letters above or below the error bars indicate that means for relative degree of stress signaling activation differ significantly (p<0.05) from untreated control.
is provided by recent reports that ZEA supplementation protected quail photoreceptor cells against light-induced death (322) and LUT supplementation diminished acute inflammatory responses, hyperproliferation, and immunosuppression after exposure of mouse skin to UVB (115, 196). Our results provide the first data demonstrating that LUT and ZEA decrease UVB-induced lipid peroxidation and attenuate activation of the stress signaling pathways in HLE cells.

Xanthophylls are extremely hydrophobic compounds, making their delivery to the HLE cells difficult. While a delivery system that is most similar to the physiologic process is preferred, the mechanism for the transfer of xanthophylls and other lipophilic compounds to the non-vascularized lens remains unknown. Carotenoids often are introduced into cell culture models in organic solvents (e.g., ref. 59) or water-dispersible beadlets (356) and liposomes (82), although problems associated with actual solubility and stability are encountered. Several investigators have reported that liposomes (82), micelles (52, 373) and cyclodextrin complexes of carotenoids (270, 191) provide effective and non-toxic vehicles for delivery of the pigments to cultured cells and organelles. The complexes remained stable for more than one year at -80°C and for 8 h in culture medium. HLE cells accumulated the xanthophylls and α-TC in a dose and time dependent manner at medium concentrations of the complexes that were not cytotoxic. The efficiency of cell uptake of the xanthophylls from the cyclodextrin particles varied two-fold with LUT>AST>ZEA. Such differential transfer of carotenoids complexed with cyclodextrin has been observed previously (191). Transfer of β-carotene, LUT and canthaxanthin from MBCD to plasma membranes, mitochondria, and microsomes from pig liver was found to be dependent on the specific carotenoid and membrane
characteristics. This may be due to differences in binding affinities to the hydrophobic core of the cyclodextrin and perhaps to the distinct orientations of LUT and ZEA within the bilayers (311).

α-TC is effective at protecting cells against UV-induced oxidative damage (169, 240). Therefore, this compound was selected as an appropriate control for evaluating the photoprotective activities of carotenoids in HLE cells. Carotenoids are lipophilic antioxidants that quench singlet oxygen and scavenge lipid peroxy-radicals (183). Induction of carotenoid deficiency in plants by mutation causes photo-oxidative stress characterized by damage to pigments, proteins, lipids and DNA (12). LUT has been shown to decrease UVB-induced lipid peroxidation in human skin fibroblasts (82) and ROS generation in murine skin (192). Therefore, it was not surprising that pre-treatment of HLE cells with LUT, ZEA or AST decreased UVB-induced generation of end products of lipid peroxidation by approximately 50%. This protective effect was observed when medium concentration of the xanthophylls was only 20% that of α-TC. It also was interesting that while the xanthophylls provided a similar degree of protection against UVB-induced lipid peroxidation, the cellular concentration of ZEA was only one-half that of LUT, suggesting that ZEA is more effective than LUT in protecting lipid membranes against UV-mediated oxidative damage. This observation is supported by Sujak et al. (311), who demonstrated that ZEA protects membranes against lipid peroxidation to a greater extent than LUT.

There is considerable evidence that UV-induced oxidative stress is associated with activation of MAPK and other protein kinase cascades (188). In order to ascertain if the decline in lipid peroxidation in HLE cells treated with xanthophylls and α-TC reflected a
more generic reduction in UV-induced photo-oxidative stress, activation of MAPK was assessed. Three major MAPK cascades have been identified, including the extracellular signal-regulated kinase (ERK 44/42) cascade, which preferentially regulates cell growth and differentiation, and the c-Jun N-terminal kinase (JNK) and p38 cascades that mediate cellular stress responses. All three of these MAPK signaling pathways are expressed in mammalian lens with activity dominant in the epithelial layer (203). Our data show that UV-induced activation of JNK and p38 in HLE cells is attenuated by pre-treatment with LUT, ZEA, AST and α-TC. We next titrated cellular concentrations of LUT and ZEA to determine if protection occurred at physiologically relevant levels of these compounds. Yeum et al. (376) reported that concentrations of LUT and ZEA, like α-TC, were several fold higher in the epithelial/cortical layer than in the nuclear layer of human cataractous lens. The mean quantity of LUT plus ZEA in the outer layer was 44 ng/g wet weight; α-TC content was 2227 ng/g wet wt. Assuming that the lens is approximately 65% water and that protein represents 60% of the total dry weight (1), the estimated means for LUT/ZEA and α-TC are 0.23 and 18.6 nmol/mg protein. We found that UVB-induced activation of JNK was significantly decreased in HLE cells when the concentrations of LUT and ZEA were approximately 0.16 nmol/mg protein. Similarly, UVB-induced activation of p38 was suppressed when the cell content of the xanthophylls was approximately 0.30 nmol/mg protein. It is noteworthy that UVB-induced lipid peroxidation in human skin fibroblasts was attenuated 30-50% when cell LUT content was 0.1-0.9 nmol/mg protein (82). In contrast, UVB-mediated activation of JNK and p38 was observed when cell content of α-TC was 5.9 nmol/mg protein, but not 2.3 nmol/mg protein. These data support a photoprotective role for LUT and ZEA in lens
epithelial cells. Wrona and associates (370, 371) have reported that xanthophylls, α-TC and ascorbate act synergistically to protect liposomes and retinal pigmented epithelial cells against oxidative stress. Evaluation of such interactions in the HLE cell line merits attention.

5.6 ACKNOWLEDGMENTS

We thank Dr. Venket Reddy for providing us with the HLE SR01-04 cell line. Special thanks to Dr. Zoraida DeFreitas, Kemin Foods, Inc., and Dr. Minhthy Nguyen, Ross Nutrition Products Division, Abbott Laboratories, for the gifts of lutein and zeaxanthin, respectively.
Bioavailability of carotenoids is affected by many factors which is not really understand due to limitation of knowledge especially xanthophylls. The process of digestion and absorption of xanthophylls bases on the understanding of digestion and absorption of β-carotene. Also biological function of xanthophylls on ocular health is claim without any scientific evident support. The research described in the previous sections was designed to evaluate digestion, absorption of xanthophylls lutein and zeaxanthin either from food and supplement or from food source. Moreover, photoprotective capacity of xanthophylls in human epithelial cells was investigated.

The first study was conducted to examine lutein bioavailability from food and supplement using simulated digestion and Caco-2 human intestinal cells. The results which associated with human studies demonstrated that absorption of xanthophylls was greater than carotene even digestive stability of xanthophylls and carotene were the same. The different is associated with the ability of transfer the different carotenoids into the mixed mcelles. In addition, lutein and zeaxanthin from supplement was greater than that in the digested foods. Numerous in the literature showed that absorption of supplement that carotenoids are already in the oil droplet will be more efficiently micellarization than it must go from food matrix to the oil droplet to the mixed micelles. Once Caco-2 cells accumulated carotenoids small fraction of these compounds were secreted into
basolateral chamber which agreed with Harrison and colleges who had done arithmetic and suggested that human truly absorb small fraction of the carotenoids that transfer into the enterocytes in the intact gut.

The second study was performed to investigate the micellarization of zeaxanthin during simulated digestion of food source of zeaxanthin. It was found that pancreatin usually used in the standard \textit{in vitro} digestion lacks of cholesterol esterase activity which zeaxanthin esters are also substrate of this enzyme. Interestingly, xanthophylls esters as well as free xanthophylls, are incorporated into mixed micelles.

The final study, focus on the photoprotective capacity of xanthophylls in human lens epithelial cells using lipid peroxidation and MAPK stress signaling pathways as indicators after cells were exposed to UVB. The presence of xanthophylls and $\alpha$-tocopherol decreased UVB-induced lipid peroxidation and also attenuated UVB-induced activation of JNK and p38. Furthermore, UVB-induced activation of JNK and p38 was decreased by physiological relevant concentration of lutein and zeaxanthin.

The overall conclusion of this study was that bioavailability of xanthophylls depends upon the efficiency of micellarization during simulated digestion. Esterified xanthophylls are incorporated into micelles which the efficiency of micellarization of non-esterified $>$ nomoester $>$ diesters xanthophylls. The final was xanthophylls decrease lipid peroxidation and attenuate activation of JNK and p38 in human lens epithelial cells.

Although this work move us forward in our understanding the absorption and the photoprotective capacity of xanthophylls, the gap of the knowledge of xanthophylls still need to explore more in the area of mechanism and biological function of xanthophylls. Caco-2 cells secrete small fraction of accumulated carotenoids. It is possible that the
accumulated carotenoids will play an important role within enterocytes because they are exposed to many potentially invasive microorganisms and also gut-associated lymphatic tissues (GALT) viz antibody-secreting plasma cells, macrophages, and a large number of lymphocytes. These are causes of stress in the enterocytes.

Even this study was the first time to demonstrate the xanthophyll esters are micellared, however, the level of carotenoid esters in the plasma is extremely low. Therefore carotenoid esters must be hydrolyzed before they are transferred into the lymphatic tissues. The hydrolysis may occur within the enterocytes after accumulation of these compounds by intracellular esterase. Similar, retinol ester which is hydrolyzed by retinal hydorase at the apical surface of enterocytes before transferring into the cells, xanthophyll eaters may be cleavaged by esterase at the brush border membrane then transfer into enterocytes. All these possibilities merit to study and this unquestionable will enhance our knowledge of the mechanistic process of carotenoid esters absorption.

Lens is surrounded by aqueous humor and nursed by ciliary body, however, lens is a vascularized tissues. How lipophilic compound especially lutein and zeaxanthin are transferred into lens tissue is needed to study. It may have specific xanthophyll binding protein as α-tocopherol transfer protein within the liver. Due to xanthophyllys are lipophilic compound, it may require unique lipoprotein to transfer this compounds into the lens. A number of studies revealed that lutein and zeaxanthin are the only dietary carotenoids in the lens and associated with epidemiological studies that high consumption of fruits and vegetables that contain carotenoids xanthophylls decrease risk of cataract major caused from chronic exposure to UVB. Although this study successes to demonstrated that pretreated HLE cells with luten and zeaxanthin before UVB exposure
decreased lipid peroxidation and reduced activation of MAPK stress signaling pathway it is needed to confirm by *in vivo* study and also the mechanism of photoprotective capacity of xanthophylls on UVB stress is needed to be investigated.


37. Same as ref. 35


186. Same as ref. 187


