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Mark L. Failla and Chureeporn Chitchumroonchokchai

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In vitro Models as Tools for Screening the Relative Bioavailabilities of Provitamin A Carotenoids in Foods

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I | Introduction: The rationale for investigating the bioavailability of provitamin A carotenoids

Vitamin A is required for vision, reproduction, and immune function. The biological activities of this micronutrient are mediated by metabolites that have important roles in cellular proliferation, differentiation, maturation, and signal transduction. With the exception of the essential role of 11-*cis*-retinaldehyde in vision, vitamin A-dependent activities are mediated by retinoic acids that serve as ligands for two subfamilies of nuclear retinoic acid receptors, namely RXR and RAR. The characteristics of vitamin A metabolism and the molecular mechanisms by which retinoids regulate cellular functions are discussed in recent reviews (Clagett-Dame and DeLuca 2002; Stephensen 2001; Ross 1999).

Because humans lack the ability to synthesize vitamin A, they are dependent on dietary intake to provide adequate levels of this vitamin. Dietary sources of vitamin A include the provitamin A carotenoids in plant foods and preformed vitamin A primarily in the form of retinyl esters in animal products. Vitamin A fortified foods (e.g., milk) and pharmaceutical supplements containing provitamin A and preformed vitamin A are readily available in affluent countries. Thus, the provitamin A carotenoids in fruits and vegetables generally account for less than one-third of the total retinol intake in nutritionally diverse diets consumed in developed countries (Rodriquez-Amaya 1997). In contrast, many of the poor in developing countries lack access to animal products and pharmaceutical supplements and are dependent on good access to carotenoid-rich vegetables and fruit, which are typically seasonal.

Inadequate intake of vitamin A results in numerous physiological abnormalities. Vitamin A deficiency continues to be a major public health problem that affects more than 100 million children and as many as 7 million pregnant women residing in more than 100 countries (World Health Organization 2003; West 2002). The incidence of vitamin A deficiency in women and children is particularly high in South and Southeast Asia and sub-Saharan Africa. More than 250,000 children become blind as a result of vitamin A deficiency each year, and half of the affected children die within a year of losing their sight. Vitamin A deficiency in young children is a consequence of low concentrations of vitamin A in maternal breast milk, inadequate intake after weaning, and loss of endogenous vitamin A due to chronic illness (Miller et al. 2002). In Nepal, pregnant women with chronic moderate-to-severe vitamin A deficiency were shown to be at increased risk of infection and mortality during pregnancy and up to one year post-partum (Christian et al. 2000). In partnership with bilateral donors, the World Health Organization and UNICEF have been actively promoting programs to control vitamin A deficiency through the encouragement of both exclusive and prolonged breastfeeding, the distribution of pharmacological vitamin A supplements to children and postpartum mothers, the fortification of food, and the implementation of programs aimed at increasing home gardening so as to increase the availability of fruits and vegetables rich in provitamin A carotenoids (World Health Organization 2003).

The home gardening strategy is particularly interesting because it offers the potential for cost-effective, long-term sustainability. However, it is based on the assumption that provitamin A from plant food sources is absorbed and utilized efficiently. This assumption was challenged by the findings of a study by de Pee et al. (1995) in which lactating Indonesian women were administered either an additional daily portion of stir-fried vegetables containing 3.5 mg β-carotene or a similar amount of β-carotene in a wafer. In that study, vitamin A status (as assessed by serum retinol concentration) did not improve in the group receiving the vegetables, but did improve in the group receiving the supplement in a wafer. This result led de Pee et al. to conclude that the bioavailability of β-carotene in green leafy vegetables was poor compared with the purified compound in a simple matrix. These findings provided the impetus for the systematic investigation of carotenoid bioavailability. As a result of the considerable efforts of many investigators over the past decade, it now is recognized that the absorption and conversion of provitamin A carotenoids by humans are affected by

numerous factors. This makes the reliable prediction of carotenoid bioavailability most challenging.

The selection and breeding of micronutrient-dense crop foods has been proposed as a sustainable strategy for combating the widespread deficiencies of iron, zinc, and vitamin A in developing countries (Graham et al. 2001). Increased concentrations and relatively high bioavailability of these nutrients are required for foods to be nutritionally superior. It is also essential that the cultivars are well adapted to local growing conditions and produce yields equal to or better than those achieved by currently utilized cultivars. While standard technologies are available to quantify the levels of provitamin A and trace metals in cultivars, the assessment of bioavailability poses a more difficult problem. Judicious coupling of chemical analyses with cost-effective methods for estimating bioavailability and evaluating environmental fitness is expected to facilitate identification of those cultivars that merit further testing in appropriate cellular and animal models. Such studies in turn are expected to yield a subset of cultivars for investigation in free-living human subjects.

Here we propose that the efficiency of micellarization of provitamin A carotenoids during simulated digestion of plant foods is an effective tool for the initial screening of the relative bioavailabilities of carotenoids from candidate cultivars. This *in vitro* model can also be used to evaluate the effect of different food processing and meal preparation methods. We further propose that the coupling of simulated digestion with the Caco-2 human intestinal cell line can be useful for confirming that micellarized provitamin A carotenoids are indeed accessible for uptake by absorptive small intestine cells. Results from such studies are expected to facilitate the selection of appropriate cultivars for *in vivo* studies with gerbils or ferrets. Animal studies in turn will generate data that can be used to identify plants and processing methods that merit examination in human intervention trials in local communities.

This review is organized as follows. First, the characteristics of the digestion and absorption and the metabolism of vitamin A and carotenoids are briefly discussed in Sections 2 and 3, respectively. The numerous factors that affect carotenoid bioavailability are considered in Section 4. Section 5 first presents an overview of the techniques used to determine the relative bioavailabilities of carotenoids *in vivo*, and then describes the biochemical and cellular methods that are used to investigate the gastrointestinal processes associated with the accessibility and cellular transport of carotenoids. Key results from studies employing *in vitro* methods are systematically reviewed in Section 6. Finally Section 7 directly compares the findings of *in vivo* and *in vitro* studies and, on the basis of this comparison, proposes the use of simulated digestion and Caco-2 cells as tools for the initial screening for the relative bioavailability of provitamin A carotenoids from staple foods prepared according to local methods.

II | Dietary vitamin A

a. Digestion and absorption (Figure 1).

Retinyl esters represent the form in which vitamin A is stored in animal tissues and, therefore, are the primary source of vitamin A for individuals consuming diets that include meats and meat products. These compounds are released from the food matrix during digestion and partition into lipid droplets. In the transit of chyme[1] from the stomach to the small intestine, the lipid droplets are exposed to pancreatic enzymes and bile. The retinyl esters are efficiently hydrolyzed by pancreatic triacylglycerol lipase and possibly by pancreatic lipase-related proteins (Harrison and Hussain 2001). Free retinol and the medium- and long-chain fatty acids produced by the enzymatic cleavage, as well as residual retinyl esters, partition into mixed micelles[2] (Breithaupt et al. 2002; Borel et al. 2001; Harrison and Hussain 2001).

[1] A mixture of partially digested food, electrolytes, secreted enzymes and other digestive factors present in the gastrointestinal lumen.

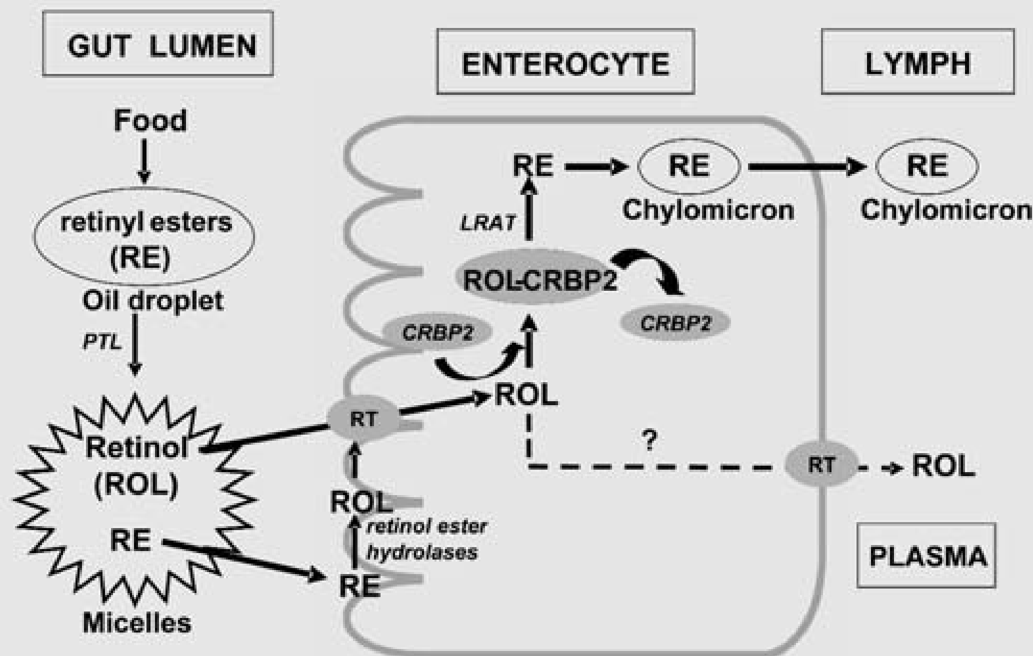
[2] Water soluble, spherical aggregates of bile salts, monoacylglycerides, phospholipids, cholesterol and other fat-soluble compounds in the small intestine.

Micellarized retinyl esters can be cleaved by retinyl ester hydrolase located in the brush border membrane of rat and human enterocytes[3] (Rigtrup et al. 1994). Retinol is generally transported into enterocytes by a facilitated process although nonsaturable, passive uptake has also been observed in cell and animal models administered pharmacologic doses of the compound. Once inside the cell, retinol associates with the high affinity retinol binding protein (CRBP2) for presentation as a substrate to lecithin:retinol acyltransferase (LRAT), which re-esterifies the alcohol primarily to retinyl palmitate during the postprandial state. The newly synthesized retinyl esters are incorporated into chylomicra[4] for secretion into lymph. Studies in Caco-2 human intestinal epithelial cells suggest that some free retinol can be secreted into portal circulation during the fasting state (Nayak et al. 2001; Levin 1993). It has been suggested that this retinol absorption pathway becomes important when chylomicron secretion is limited or absent (Harrison and Hussain 2001).

b. Tissue distribution. Once secreted, chylomicra are transported from the lymph to the plasma, where the triglycerides undergo rapid lipolysis by lipoprotein lipase to produce chylomicron remnants (Harrison and Hussain 2001). These chylomicron remnants are endocytosed by hepatic parenchymal cells and the acquired retinyl esters are hydrolyzed to retinol and fatty acids by retinyl ester hydrolases. Retinol either associates with retinol binding protein for secretion and delivery to peripheral tissues or is transported to stellate cells in the liver for storage as retinyl ester. As vitamin A becomes limiting, retinyl esters are hydrolyzed and free retinol is transferred back to the hepatocytes for

FIGURE 1

Overview of digestion and absorption of vitamin A.
CRBP2, cellular retinol-binding protein type 2; LRAT, lecithin:retinol acyltransferase; PTL, pancreatic triacylglycerol lipase; RT, retinol transport protein (modified from Harrison and Hussain, 2001).



[3] Absorptive epithelial cells lining the villi in the small intestine.

[4] Large, water soluble lipoprotein complexes secreted by enterocytes into lymph for the mass transfer of lipids throughout the body.

secretion into the plasma via association with retinol binding protein. Extrahepatic tissues such as adipose tissue, testis, and retinal epithelium also synthesize and store retinyl esters that appear to serve as local reserves for producing free retinol as required.

III | Metabolism of dietary carotenoids

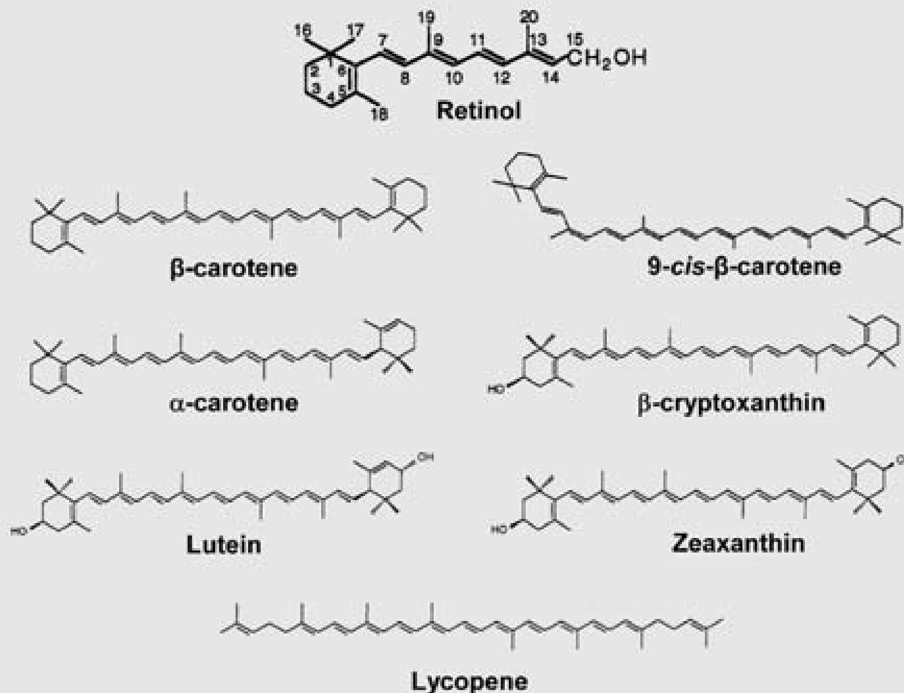
a. Carotenoid chemistry and biology.

Approximately 40 carotenoids are present in plant foods consumed by humans (Goodwin and Britton 1988). The plasma profile of carotenoids typically reflects the types of carotenoids present in recently consumed fruits and vegetables. In general, the most abundant carotenoids in plasma include α-carotene, β-carotene, lutein, zeaxanthin, and β-cryptoxanthin, and relatively high plasma concentrations of lycopene are found in populations that regularly ingest tomatoes and tomato products (Figure 2). Carotenoids are C_{40} compounds that contain numerous conjugated double bonds (Britton 1995). They are generally classified as either the hydrocarbon (or apolar) carotenoids or the oxy- (or polar) carotenoids. Polar carotenoids contain one or more oxygen-containing functional groups and include lutein, zeaxanthin, and β-cryptoxanthin. Carotenoids are also classified as provitamin A or nonprovitamin A compounds. The former serve as dietary sources of vitamin A because they can be enzymatically cleaved to yield either one (e.g., β-cryptoxanthin and α-carotene) or two (β-carotene) molecules of retinal. β-carotene is the most potent source of vitamin A in the diet.

Most carotenoids in plants exist in the all-*trans* configuration, although *cis* isomers may form during food processing (Rodriguez-Amaya 1997). *Cis* isomers of both dietary carotenoids and their retinoid metabolites are found in tissues (Ross 1999). The molecular functions of two isomers of retinoic acid have been identified.

FIGURE 2

Structures of retinol and abundant carotenoids in human plasma.



These isomers, the 9-*cis* and all-*trans* configurations, are activating ligands for the RXR and RAR nuclear receptor proteins, respectively, which regulate the transcription of target genes (Ross 1999). In addition, 11-*cis* retinal has been shown to be an essential cofactor for rhodopsin, the protein that participates in phototransduction in the retina (Ross 1999).

In addition to serving as precursors for vitamin A, the literature supports roles for provitamin A and nonprovitamin A carotenoids as antioxidants, photoprotective agents, immunoenhancers, inducers of intercellular gap junction communication, and modulators of transcriptional processes. These activities have been discussed in detail in several recent reviews (Sharoni et al. 2004; Stahl et al. 2002; Basu et al. 2001; Bertram, 1999; Olson, 1999).

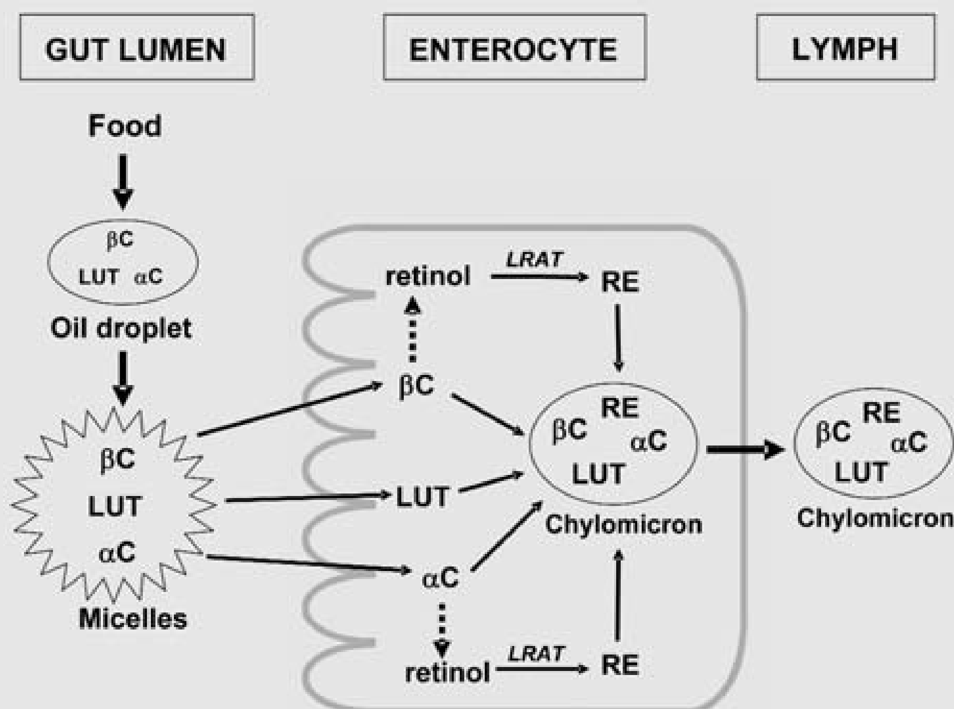
b. Digestion, absorption, tissue distribution, and excretion of carotenoids (Figure 3).

i. Gastric digestion. Carotenoids are processed during digestion in the same manner as retinyl esters and other fat-soluble compounds. Thus, they must be released from the food matrix, emulsified in the lipid phase of chyme, and solubilized in mixed micelles to be accessible for uptake by absorptive epithelial cells. 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 mixed with the ingested foods, resulting in partial release of the carotenoids from the food matrix into the emulsified oil droplets. Apolar carotenoids such as β-carotene reside in the core of lipid droplets, whereas polar carotenoids are preferentially distributed at the surface (Borel et al. 1996).

FIGURE 3

Overview of digestion and absorption of dietary carotenoids.

αC, α-carotene; βC, β-carotene; LRAT, lecithin:retinol acyltransferase; LUT, lutein; RE, retinyl esters.



ii. 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 further degrade components of the food matrix. Released fat-soluble compounds partition into lipid droplets. Luminal lipases hydrolyze triacylglycerols, phospholipids, and other esters in emulsified oil droplets. Cholesterol esterase and pancreatic triglyceride lipase are capable of hydrolyzing polar carotenoid esters to free carotenoids (Breithaupt et al. 2002; Jacobs et al. 1982). Bile salts are required for the partitioning of the lipophilic products into mixed micelles (El-Gorab et al. 1975; Olson 1964). Once formed, the mixed micelles diffuse across the unstirred water layer and deliver carotenoids and other fat-soluble compounds to the apical surface of the mucosal epithelium. Early studies showed that intestinal uptake of micellarized β-carotene is influenced by a number of intraluminal factors including bile salt composition, pH, sodium concentration, and length and degree of saturation of fatty acids (Hollander and Ruble, 1978; El-Gorab et al. 1975; Olson, 1964).

The relative distribution of carotenoids in the food matrix, lipid droplets, and aqueous fraction within the stomach and duodenum was recently examined in human subjects (Tyssandier et al. 2002). In that study, subjects were given mashed/pureed carrot, spinach, or tomato, where the amount of vegetable was chosen such that each meal contained approximately 10 mg of β-carotene, lutein, or lycopene, respectively. The liquefied meal was delivered by nasogastric tube and samples were collected periodically from the lumen of the stomach and duodenum for 2.5 hours. The aqueous fraction of the stomach contained only trace amounts of carotenoids. The proportion of carotenoids presenting the aqueous (presumably micellar) fraction of the duodenum during the sampling period corresponded to 5.6%, 4.6%, and 2.0%, respectively. This suggests that the transfer of carotenoids to micelles is affected by chemical speciation and/or the food matrix.

The presence of substantial amounts of (^{13}C)-all-*trans*-β-carotene and -retinol in plasma after ingestion of a meal containing greater than 99% (^{13}C)-9-*cis* β-carotene (You et al. 1996) suggested possible isomerization of the carotenoid during digestion. However, Tyssandier et al. (2002) found minimal carotenoid isomerization during digestion of carotenoid-rich meals in the stomach and duodenum of human subjects. Likewise, Faulks et al. (1997) found a similar ratio of all-*trans* and 9-*cis* β-carotene in a test meal and intestinal effluent collected from human subjects with ileostomy. These data suggest that the marked isomerization of 9-*cis* β-carotene observed by You et al. (1996) likely occurred in enterocytes and/or after absorption.

iii. Uptake and metabolism by absorptive epithelium. The transfer of carotenoids from micelles to the apical surface of epithelial cells lining the small intestine is generally assumed to occur by passive diffusion (Furr and Clark 1997; Parker 1996). A recent study examining lycopene absorption by humans suggested that the absorption of this carotenoid is saturable (Diwadkar-Navsariwala et al. 2003). Similarly, β-carotene uptake by intestinal cells has been reported to be saturable (see Section 6). These observations suggest that carotenoids, like cholesterol and fatty acids (Davis et al. 2004; Schaffer, 2002), 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 human and nonhuman primates. The potential influence of the physicochemical properties of carotenoids on uptake of these compounds across the brush border surface of enterocytes has received limited attention. Hydrocarbon and polar carotenoids are likely to reside in the core and at the surface of micelles, respectively (Borel 2003), but it is not known if this affects transfer to the epithelial cells. Despite the apparently greater efficiency of micellarization of *cis* isomers of β-carotene compared to the all-*trans* isomer (Levin and Mokady 1995), studies in animals (Deming et al. 2000) and humans (Gaziano et al. 1995) have consistently shown markedly less mucosal uptake and absorption of the *cis* isomers of β-carotene compared to the all-*trans* isomer. In contrast, the *cis* isomers of lycopene appear to be substantially more bioavailable than all-*trans* lycopene (Boileau et al. 2002).

Once taken into the enterocyte, β-carotene can be converted to vitamin A. The mechanism for this cleavage reaction remained controversial for many years, because attempts to purify the enzyme to homogeneity were unsuccessful and both retinaldehyde and apo[5]-carotenals were identified as products *in vitro* and *in vivo* (e.g., Wang et al. 1991, Olson 1961). Several recent developments have clarified the mechanisms of β-carotene cleavage. Barua and Olson (2000) demonstrated that central cleavage of dietary β-carotene is the predominant mechanism for retinaldehyde formation in rats. In addition, two distinct recombinant cleavage enzymes have been purified and characterized, namely β,β-carotene 15,15'-monooxygenase (BCO1) and β-carotene 9'10'-monooxygenase (BCO2) (Wyss 2004; von Lintig and Vogt 2004). BCO1 catalyzes central cleavage. It is a nonheme iron enzyme located in the cytoplasm that hydrolyzes the 15,15'-double bond of β-carotene, α-carotene, β-apo-carotenols, and β-apo-carotenals (Lakshmanan et al. 1972; Lakshmanan et al. 1968) to generate one or two molecules of retinol. In contrast, BCO2 catalyzes the eccentric cleavage of β-carotene to β-apo-carotenals and β-ionone. Each apo-carotenal molecule can be subsequently converted to a single molecule of retinaldehyde or the corresponding β-apo-carotenoic acid. β-Apo-carotenoic acids may serve as precursors for retinoic acid (Wang et al. 1991; Napoli and Race 1988) and have been shown to modulate cell proliferation in cultured cells (Tibaduiza et al. 2002).

Examination of the expression of BCO1 and BCO2 in rodent, chicken, and human tissues has been facilitated by the cloning of the respective genes (von Lintig and Vogt 2004; Wyss 2004). The sequence of BCO1 is highly homologous among animal species. The predicted amino acid sequences of BCO1 and BCO2 are approximately 40% homologous and the activity of BCO2 in tissues appears to be much lower than that of BCO1. In contrast to rodents and chickens, BCO1 mRNA levels are lower in human enterocytes than in liver, retina, and kidney. Lindqvist and Anderson (2004) recently showed that BCO1 protein is present in many types of epithelial cells, 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. BCO2 is also expressed in several of the same organs as BCO1. The expression of the BCO genes in many tissues has led to speculation that localized synthesis of retinoids is important, especially during periods when dietary intake of provitamin A carotenoids and retinyl esters is inadequate.

While the expression of BCO2 appears to be constitutive, BCO1 activity is subject to regulation by vitamin A and other nutrients. Intestinal, but not hepatic, activity is increased in vitamin A deficient rats and decreased in response to dietary supplementation with β-carotene, retinyl acetate, apo-8'-carotenal, and all-*trans*- and 9-*cis*-retinoic acid (Bachmann et al. 2002; Parvin and Sivakumar 2000; van Vliet et al. 1996; Villard and Bates 1986). Likewise, Lemke et al. (2003) found that vitamin A supplementation was associated with a decreased ratio of ^{14}C-retinyl ester to ^{14}C-β-carotene in the plasma of two human subjects; surprisingly, apparent absorption of the administered dose of ^{14}C-β-carotene actually increased in response to supplementation Intestinal activity of BCO1 was also decreased in rats fed diets deficient in either protein or iron (During et al. 2000; Parvin and Sivakumar, 2000). In contrast, BCO1 activity was increased in the intestines of rats fed diets rich in unsaturated fatty acids (During et al. 1998). The mechanisms responsible for such diet-mediated changes merit investigation.

Oxidized products of dietary carotenoids have been identified in plasma and several tissues (Nagao 2004). The possibility that these oxidized products are generated enzymatically is supported by reports that high intake of carotenoids increases expression of several cytochrome P_{450} proteins (Jewell and O'Brien 1999). The tissue sites at which the oxidation reactions occur and the physiological activities that may be modulated by the metabolites formed in such reactions remain largely unknown.

[5] Apo - apolipoprotein - cholesterol-lipid-protein complex that transports cholesterol and lipid in the blood.

iv. Intestinal transport and delivery of carotenoids and cleavage products to peripheral tissues. Carotenoids and retinyl esters synthesized after cleavage of provitamin A carotenoids are incorporated into nascent chylomicra in the golgi of enterocytes (Parker 1996). Conversion of chylomicra to remnants is associated with uptake of the particles by liver, where the carotenoids may be utilized, stored, or re-secreted into plasma in very low density lipoproteins (VLDL) and high density lipoproteins (HDL). Relatively high concentrations of provitamin A carotenoids are commonly found in tissues expressing a high density of LDL receptors such as adipose and adrenal tissue, liver, testis, and ovary (Olson 1999). Although carotenoids are also present in HDL, it is not known if these lipoprotein particles donate carotenoids to extrahepatic cells.

v. Excretion. The absorption of carotenoids from foods is incomplete. It has been assumed that urinary losses of carotenoids are extremely low (Bowen et al. 1993). Lemke et al. (2003) recently showed that human subjects excreted 25–30% of ^{14}C from a tracer dose of orally administered ^{14}C-β-carotene in urine within 72 hours. Although not mentioned by the investigators, it is likely that urinary ^{14}C represented oxidized and conjugated metabolites of β-carotene. In addition, very small quantities of endogenous carotenoids are lost by exfoliation of skin and low concentrations of carotenoids have been identified in bile (Leo et al. 1995). Thus, fecal excretion represents the primary route of elimination from the body. Although several groups have reported that β-carotene is stable during *in vitro* incubation with intestinal aspirates or homogenized stools from rats or humans (Grolier et al. 1998; Tang et al. 1996), it is unknown if the lack of modification of the carotenoid was due in part to its introduction to the mixture in either water-dispersible beadlets or ethanol instead of a partially digested food matrix.

vi. Summary. Absorption of dietary carotenoids generally requires their 1) transfer from the food matrix to micelles, a process assumed to require initial partitioning into emulsified oil droplets, 2) uptake from micelles by absorptive epithelial cells, and 3) secretion of intact carotenoids or retinyl esters derived from cleavage of provitamin A carotenoids into circulation via chylomicra.

IV | The bioavailability of carotenoids

Bioavailability refers to the efficiency of absorption of an ingested compound and its bioactive metabolites for delivery to and utilization by target tissues. Events preceding acquisition of carotenoids by absorptive epithelial cells are largely influenced by the physicochemical properties of the carotenoid species, the matrix of the dietary source of the ingested carotenoid and other components of the meal, and dietary and physiological factors that affect the digestive processes. Once an enterocyte acquires carotenoids from a micelle, processes related to conversion, intracellular retention, and secretion in chylomicra are influenced by a variety of host factors including nutritional status. These factors are summarized in Table 1

TABLE 1

FACTORS AFFECTING THE BIOAVAILABILITY OF CAROTENOIDS

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 > chopped > 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

and briefly discussed below. Interested readers are encouraged to refer to several reviews for additional details (Borel 2003; van den Berg et al. 2000; van het Hof et al. 2000; West and Castenmiller 1998).

a. Chemical speciation, food matrix, and processing.

The absorption of dietary provitamin A carotenoids is influenced by numerous factors in addition to the amount ingested. The physicochemical properties of the carotenoid of interest, its subcellular location in the plant tissue that constitutes the food, the method of food preparation, and the chemical composition of the meal may affect carotenoid bioavailability. For example, the bioavailability of lutein from spinach compared with that from a supplement containing lutein in oil was reported to be greater than that of β-carotene from spinach compared with a supplement containing β-carotene in oil (van het Hof et al. 1999). Likewise, lutein was absorbed more efficiently than β-carotene when the carotenoids were administered in oil to human subjects (Castenmiller et al. 1999; van het Hof et al. 1999; Kostic et al. 1995). The bioavailability of β-carotene has also been reported to be influenced by the food matrix, with absorption from carrots > broccoli > spinach (Castenmiller et al. 1999; Micozzi et al. 1992). These observations suggest that carotenoid bioavailability is affected by both chemical speciation and food matrix. However, interpretation is confounded by a lack of information about the extent of β-carotene conversion, potential interactions between carotenoids during digestion and uptake and transport across the mucosal epithelium, and the rates of plasma clearance for individual carotenoids.

Moderate cooking, mashing, and juicing increase carotenoid bioavailability (Livny et al. 2003; Edwards et al. 2002; van het Hof et al. 1998). 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 may also induce conversion of the all-*trans* isomers of some carotenoids to *cis* isomers (see Section 3.b.iii.).

b. 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 hydrophobic compounds released from the food matrix, stimulating the secretion of bile salts and pancreatic lipases required for micelle formation, and inducing chylomicron synthesis (Borel 2003). Approximately 5–10 g fat in a meal is required for efficient absorption of carotenoids (Reddy and Mohanran 1980), although a greater amount of fat is required when the dietary source is lutein ester instead of free lutein (Roodenberg et al. 2000). The type of fat may also affect carotenoid absorption. For example, absorption of carotenoids by rats was more efficient when the carotenoids were administered in olive oil than in corn oil (Clark et al. 2000). Similarly, the presence of unsaturated fatty acids, particularly oleate, in micelles stimulated β-carotene absorption from the perfused rat intestine (Hollander and Ruble 1978). Hu et al. (2000) reported that the efficiency of β-carotene absorption by human subjects increased when the meal was rich in sunflower oil compared with beef tallow. Also, dietary triacylglycerols with long-chain rather than medium-chain fatty acids enhanced the absorption of β-carotene and retinyl palmitate (Borel et al. 1998b). As expected, inhibitors of lipid absorption such as olestra (Cooper et al. 1997; Weststrate and van het Hof 1995) and phytosterols (Richelle et al. 2004) decrease carotenoid bioavailability primarily by decreasing micellarization. The potential for phospholipids to affect carotenoid bioavailability is supported by the observation that lyso-phosphatidylcholine stimulates carotenoid absorption by mice (Baskaran et al. 2003).

ii. Fiber. The water soluble fibers pectin, guar, and alginate decrease the absorption of β-carotene, lycopene, and lutein (Riedl et al. 1999; Rock and Swendseid, 1992). 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 increased rate of transit of enterocytes along the villus (Riedl et al. 1999).

iii. Interactions between carotenoids. Carotenoids in the same food or meal may influence the absorption of one another. For example, β-carotene was reported to decrease lutein absorption, whereas lutein decreased β-carotene absorption in some human subjects but increased it in others (Kostic et al. 1995). In another study, lutein impaired β-carotene absorption by human subjects, but did not affect the secretion of retinyl esters in chylomicra (van den Berg and van Vliet 1998). In contrast, β-carotene absorption was not affected by lycopene in these subjects. Additional reports of interactions between pure carotenoids that affect their postprandial appearance in plasma of humans and animals have been reviewed by van den Berg (1999). More recently, Tyssandier et al. (2003) reported that the absorption of β-carotene, lutein, and lycopene from a single vegetable was greater when the food was administered alone than when it was coadministered with either a second carotenoid-rich vegetable or the purified carotenoid from the second vegetable. Possible sites for preabsorptive interactions between carotenoids include their competition for incorporation into micelles, uptake from the micelle by intestinal cells, competitive binding to BCO1, and incorporation into chylomicra (van den Berg 1999).

c. Physiological and pathophysiological factors

i. Gut health. The absorption of dietary carotenoids and their bioactive products is also 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 with the same subjects when gastric pH was acidic (Tang et al. 1996). In addition, 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 (Olson 1999).

Intestinal parasitism can impair carotenoid absorption or utilization. Metabolism of carotenoids 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 parasite infection may all contribute to a decline in carotenoid absorption. Absorption and utilization of β-carotene were enhanced after deworming children infected with *Ascaris* (Jalal 1998). In contrast, plasma retinol concentrations in helminth-infected preschool 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 antihelminthics (Takyi 1999).

ii. Nutritional status. Nutritional status can affect the bioavailability of provitamin A carotenoids. The plasma vitamin A response curve following the administration of β-carotene to protein deficient rats was decreased compared with that for rats fed control diet (Parvin and Sivakumar 2000). 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 the crypts of intestinal villi (McCullough et al. 1999). Gastrointestinal integrity, assessed by the dual-sugar gastrointestinal permeability test, was markedly improved when vitamin A-deficient children in Gambia and India ingested β-carotene-rich mango and received vitamin A supplementation, respectively (Thurnham et al. 2000). Erdman and associates (Boileau et al. 2000; Moore et al. 1996) observed decreased uptake of micellarized β-carotene by brush border membrane vesicles isolated from vitamin A-deficient Mongolian gerbils and rats compared with 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. Finally, the specific activity of BCO1 in the soluble fraction of homogenized intestinal mucosa was positively correlated with the iron content of the tissue prepared from rats fed diets with different quantities of the trace metal (During et al. 2000).

iii. Genotype. Recent studies using tracer isotope techniques have confirmed earlier observations of a marked variability in the absorption of β-carotene by human subjects (Hickenbottom et al. 2002; Lin et al. 2000). Moreover, plasma β-carotene and vitamin A were not predictive for the absorption or conversion of β-carotene. These differences in absorption efficiency originally resulted in the classification of individuals as "responders", "low responders", and "nonresponders". Explanations for the observed variation among healthy subjects tested under well-controlled conditions have included differences in the rate of cleavage of β-carotene to retinal, the efficiency of incorporation of the carotenoid into chylomicra, and the rate and extent of clearance from circulation (Borel 2003). Lin et al. (2000) also 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, because all individuals were "responders" when administered high doses of β-carotene in oil (Borel et al. 1998a).

Genetic factors also likely affect the efficiency of carotenoid absorption and conversion. Polymorphisms in genes whose products are required for the many reactions affecting the transfer of carotenoids from food matrix to micelles during digestion, assembly and secretion of chylomicra, and the kinetics of postabsorptive 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, a lack of knowledge about the characteristics and regulation of carotenoid transport and metabolism precludes consideration of specific polymorphisms at this time. Also, variability within an individual over time and effects of lifestyle factors on carotenoid absorption remain ill-defined.

V | Approaches for investigating the bioavailability of carotenoids

In vivo and *in vitro* approaches are used to determine and predict, respectively, the relative bioavailability of provitamin A carotenoids from complex food matrices. Methods involving human subjects, animals, cells and biochemical models are summarized in Table 2. The advantages and disadvantages of using these diverse approaches as screening tools for assessing the bioavailability of carotenoids are discussed below.

TABLE 2

APPROACHES FOR STUDYING FACTORS AFFECTING THE BIOAVAILABILITY OF CAROTENOIDS.

***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

a. Human studies. Carefully controlled investigations using human subjects are necessary for accurate determination of the relative bioavailability and conversion of provitamin A carotenoids. Balance studies and plasma response curves have been used to estimate relative bioavailability, whereas functional improvement in vitamin A status (e.g., restoration of night vision) has been used to assess bioefficacy of intervention programs in vitamin A deficient populations (Congdon and West 2002; Christian et al. 2000). Sampling of gastrointestinal contents during the digestive process provides insights into the stability of carotenoids and their transfer from the food matrix to oil droplets and micelles (Tyssandier et al. 2002). The inherent limitations in experimental design, data interpretation, cost of instrumentation, and labor intensiveness of these *in vivo* methods limit their utility for screening the bioavailability of carotenoids for large numbers of cultivars that may be prepared for ingestion in many different ways.

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 noninvasive. Because elimination in feces represents the major excretory route for ingested carotenoids (Section 3.b.v), it is assumed that absorption can be estimated by carefully monitoring intake and fecal output. Collections of feces (van Lieshout et al. 2003b; Bowen et al. 1993), intestinal effluent from subjects with ileostomy (Livny et al. 2003; Faulks et al. 1997), and gastrointestinal washes (Bowen et al. 1993) have been used to estimate carotenoid absorption. These collections contain ingested carotenoids that were not transferred from the food matrix to absorptive cells, as well as carotenoids that were absorbed and subsequently returned to the lumen of the gastrointestinal tract with bile and pancreatic secretions, *retro*-transported across the apical surface of the mucosal epithelium, and retained within cells sloughed from intestinal and colonic villi. Also, the stability of carotenoids in the lower gut remains unclear and sample collection and extraction are labor intensive. These factors limit the utility of balance studies for determination of carotenoid bioavailability.

Bioavailability has also 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 approach lacks sensitivity due to a relatively high level of endogenous carotenoids in plasma, failure to account for the cleavage of provitamin A carotenoids, and the assumption that different carotenoids have similar rates of plasma clearance (van den Berg et al. 2000; Parker et al. 1999). These problems are partially offset by monitoring the temporal rise and removal (AUC, area under the curve) of carotenoids in the triacylglycerol-rich fraction from plasma after administration of a single dose or test meal containing the carotenoids of interest (van Vleit et al. 1995). Parker et al. (1999) have discussed the limitations of the postprandial chylomicron response model for assessing the absorption efficiency of carotenoids.

Administration of physiologic doses of purified carotenoids or plant foods that are intrinsically labeled with stable isotopes (^{2}H and ^{13}C) facilitates the study of *in vivo* absorption and metabolism of carotenoids. Published studies using this powerful approach have been reviewed recently by van Lieshout et al. (2003a). The authors provide useful insights into the importance of experimental design, choice of isotopic tracer, dosing regimen, sample collection, chemical analysis, and mathematical modeling when using stable isotope technology to assess the bioavailability and bioefficacy of carotenoids. Burri and colleagues have pioneered the use of accelerator mass spectrometry for examining the absorption and excretion of trace amounts of ^{14}C-β-carotene in human subjects (Burri and Clifford, 2004; Lemke et al. 2003; Dueker et al. 2002). This powerful approach is yielding important new insights into the absorption and whole body metabolism of dietary carotenoids. At this time, however, the cost of instrumentation and labor intensiveness of stable isotope technology precludes its consideration as a tool for screening the bioavailability of provitamin A carotenoids from staple crops.

The collection and analysis of aspirates from stomach and small intestine of human subjects provides investigators with the ability to investigate the stability and partitioning of dietary compounds within the gastrointestinal lumen during digestion (Borel et al. 2001; Armand et al. 1996). This method has been effectively used to examine the gastrointestinal processing of β-carotene, lutein, and lycopene from pureed vegetables (see Section 3.b.ii.), but does not lend itself to the initial screening of multiple types of foods prepared in diverse manners.

b. Animal models. The primary advantages of animal models for investigating nutritional problems relevant to humans include the ability to induce dietary deficiencies and excesses, administer radioisotopes, collect tissues of interest, and induce acute and chronic diseases. The central issue concerns the selection of an animal that absorbs and metabolizes carotenoids in a comparable manner to human subjects. Lee et al. (1999) have critically reviewed this subject.

Carotenoid absorption, metabolism, and function have been investigated to varying degrees in mouse, rat, gerbil, ferret, preruminant calf, nonhuman primates, aves, and amphibians. Because mice and rats efficiently convert ingested provitamin A carotenoids to retinol in the intestine, they do not absorb intact carotenoids unless supraphysiologic doses are administered. Thus, these rodents are not particularly relevant to the human situation regarding the bioavailability of provitamin A carotenoids from foods. The rat, however, is a preferred model for investigating the pathophysiological consequences of an inadequate supply of this essential nutrient because vitamin A deficiency can be induced.

Preruminant calves, ferrets, and gerbils, like humans, absorb a portion of dietary provitamin A carotenoids intact and also produce retinyl esters in enterocytes. The high cost of maintaining calves limits their utility for assessing issues related to the bioavailability of provitamin A carotenoids. Ferrets and gerbils represent more practical models. The size of these animals facilitates *in vivo* perfusion of intestinal segments for studying the uptake, conversion and absorption of provitamin A carotenoids and their metabolites (e.g., Wang et al. 1994). Studies in ferrets have been effectively used to show that β-carotene in carrot juice is more bioavailable than that in intact carrot (White et al. 1993b), that 13-*cis* β-carotene is less bioavailable than all-*trans* β-carotene (Erdman et al. 1998), and that the absorption of β-carotene is antagonized by canthaxanthin (White et al. 1993a). Gerbils also absorb all-*trans* β-carotene more efficiently than *cis* isomers of β-carotene, and apparent conversion of β-carotene to vitamin A decreased when gerbils were fed diets low in fat or containing citrus pectin (Deming et al. 2002; Deming et al. 2000). These effects of dietary components and the differences in absorption of β-carotene isomers are similar to those observed in humans (see Sections 3.b.iii. and 4.b.) and demonstrate the general utility of the gerbil and ferret for investigating problems related to the bioavailability of provitamin A carotenoids. It is important to recognize that these species differ from humans in some characteristics of vitamin A metabolism. Gerbils store high levels of vitamin A in the liver, making it difficult to induce vitamin A deficiency, and ferrets have markedly higher amounts of retinyl esters in their plasma compared with human subjects. These differences do not, however, diminish the overall usefulness of the two animal species for investigating many problems related to the bioavailablity of provitamin A carotenoids.

c. *In vitro* models. Simulated digestive processes, isolated intestinal cells and intestinal segments, and brush-border and basolateral membrane vesicles represent models for studying specific characteristics and the regulation of complex processes associated with digestion and absorption. These models have been used to investigate the effects of chemical speciation, food matrix and processing, and dietary components on the digestive stability, accessibility, and intestinal transport and metabolism of carotenoids from foods and supplements. However, it is important to note that these relatively simple models are "static" in the sense that they are not influenced by the many factors that can affect digestive and

absorptive processes *in vivo*. These factors include appropriate mixing, grinding and 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 mucosal layer separating the epithelium and the luminal contents; and influence of humoral factors secreted by cells residing in the lamina propria and peripheral tissues on the transport and metabolism of various compounds by enterocytes. A multicompartmental, computer-controlled system that accurately reproduces various physiological factors during gastric and small intestinal digestion has been developed to offset some of the limitations of the commonly used static methods (Minekus et al. 1995). A preliminary report on the use of this system for examining the effects of processing on the bioaccessibility of carotenoids in vegetables is available (Minekus et al. 2001). However, the method requires information about the effects of meal composition on gastric pH, transit rates, and mean concentrations of bile and digestive enzymes to program the computer-operated system to accurately mimic the luminal environment after the ingestion of a specific food or meal. Also, the complex design of the system precludes the efficient screening of numerous test foods. Differences between the intact organism and simple biochemical and cellular models dictate the need for caution when using results from *in vitro* studies to conceptualize the more complex environment *in vivo*. Nevertheless, *in vitro* approaches are useful tools for defining key questions that merit more rigorous investigation *in vivo*, and also yield insights into the mechanisms underlying observations in humans and animals.

i. Biochemical models of digestion. Simulated gastric and small intestinal digestion has been widely used to investigate the digestion of proteins (Lindberg et al. 1998), starch (Englyst et al. 1999), lipids (Fouad et al. 1991), polyphenols (Gil-Izquierdo et al. 2002), transgenic plant DNA (Netherwood et al. 2004), and recombinant proteins (Richards et al. 2003) in complex food matrices. This method has also been used to examine carotenoid stability and partitioning during the digestion of foods, meals, and supplements (Chitchumroonchokchai et al. 2004; Ferruzzi et al. 2001; Garrett et al. 2000; Garrett et al. 1999a). Comparison of the carotenoid profile before and after simulated digestion provides information about the stability of the carotenoids during the gastric and small intestinal phases of digestion. After completion of the simulated process, digesta is centrifuged to collect the aqueous fraction (Figure 4). Conditions are optimized for complete digestion of emulsified oil droplets, i.e., micellarization of fat-soluble compounds transferred to the droplets. The aqueous fraction is passed through a filter with 0.22 micrometer pores to separate possible microcrystalline aggregates from micelles. Carotenoids are extracted from the starting material, digesta, and aqueous filtrate and analyzed by HPLC to determine digestive stability (i.e., recovery and isomeric profile) and efficiency of micellarization. Other investigators have examined the effects of processing, dietary components, and luminal conditions on transfer of carotenoids from the food matrix to oil droplets (gastric digestion) and from oil droplets to micelles (small intestinal digestion). Results from such studies are discussed in Section 6.

ii. Caco-2 human intestinal cells. Caco-2 is a cell line originating from human colonic carcinoma that exhibits some morphological and functional characteristics similar to those of differentiated epithelial cells that line the intestinal mucosa (Sambruy et al. 2001). The general characteristics of Caco-2 cells are listed in Table 3. These cells spontaneously differentiate to an enterocyte-like phenotype when monolayers reach confluency and are maintained using conventional culture conditions (Hildalgo et al. 1989; Pinto et al. 1983). During the early phases of differentiation, the cells express both colonocyte- and enterocyte-specific proteins (Engle et al. 1998). As differentiation proceeds, colonocyte-specific gene expression decreases and morphological and biochemical characteristics of enterocytes develop. After approximately 2 weeks, the monolayer is characterized by highly polarized columnar cells with tight junctions and desmosomes that separate the microvillar (apical) membrane from the basolateral membrane. Moreover,

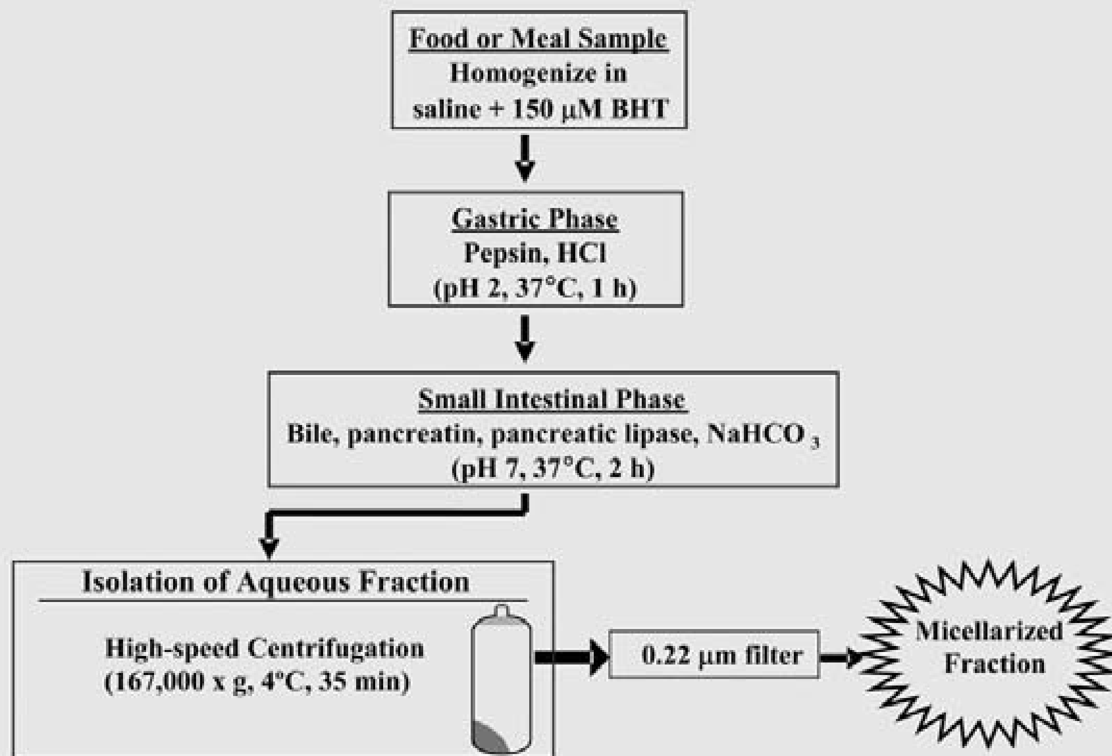
hydrolases such as sucrase-isomaltase, lactase, and dipeptidylpeptidase IV are localized in the apical membrane. These enzymes are normally present in the brush border membrane of enterocytes, but not colonocytes. Other biochemical characteristics of differentiated Caco-2 cells that are similar to those of normal small intestinal enterocytes include the following: expression of apical sodium-dependent glucose and amino acid transporters, and the di- and tripeptide transporter PepT1; synthesis and secretion of chylomicra and lipoproteins; and the ability to induce phase I and II drug detoxification enzymes and the ATP-dependent family of multidrug resistant effluxers (Delie and Rubas 1997). The high correlation between the extent of oral drug absorption in humans and transport across the Caco-2 monolayer has 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 (Delie and Rubas, 1997; Lau et al. 2004; Stewart et al. 1995).

It is important to note that some characteristics of differentiated Caco-2 cells differ from those of small intestinal enterocytes. First and most obvious, the cells originate from a human colonic carcinoma rather than normal small intestine (Pinto et al. 1983). Second, the cell line is genetically and phenotypically heterogeneous. Third, the transepithelial resistance associated with assembly of tight junctions in Caco-2 cells is more characteristic of colonic epithelium than small intestinal epithelium (Delie and Rubas 1997). Finally, Caco-2 cells

FIGURE 4

General procedure for simulated digestion of foods, meals and supplements for determination of digestive stability and efficiency of micellarization of carotenoids.

BHT, butylated hydroxytoluene, is an antioxidant often added to processed foods.



use the glycerol 3-phosphate pathway for the synthesis of triacylglycerols, whereas the small intestinal epithelium uses the monoacylglycerol pathway (Trotter et al. 1996). Some of the indicated differences are offset by standardization of procedures associated with the growth and maintenance of Caco-2 cells and the design of studies using this cell line. Factors that must be strictly controlled to minimize genetic and phenotypic "drift" and to facilitate comparison of results within and among laboratories include source of cells, range of passage numbers[6] 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 (Bailey et al. 1996). The parent Caco-2 cell line (HTB 37) is available at passage 18 from the American Type Collection and should be used between passages 20 and 45 for investigations (Delie and Rubas, 1997; Bailey et al. 1996). The cells require a minimum of 10–12 days after the monolayer reaches confluency to mature to the enterocyte-like state (Hildago et al. 1989) and 21–25 days for effective synthesis and secretion of lipoproteins (Mehran et al. 1997).

TABLE 3

Characteristics of Caco-2 human intestinal cells

- Originated from human colon adenocarcinoma
- Differentiate spontaneously into enterocyte-like cells under normal culture conditions
- Differentiated cells are characterized by:
  - Tight junctions between cells
  - Basolateral Na+,K+-ATPase
  - Inducible drug detoxification enzymes
  - ABC effluxers expressed
  - Apical brush border surface enriched with hydrolytic enzymes
  - Synthesis and vectoral secretion of chylomicra

A past criticism of the Caco-2 model was that lipid secretion by Caco-2 cells differed from that by normal enterocytes, because the primary lipoprotein particles identified in the basolateral compartment of Caco-2 cells were VLDL rather than chylomicra. Recent studies have shown that Caco-2 cells secrete chylomicra that are rich in apo-B48[7] in response to prandial-like conditions in the intestinal lumen, i.e., the addition of micelles containing oleate and taurocholate to the apical compartment (Nayak et al. 2001; Lunchoomum and Hussain, 1999). Because 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 triacylglycerols, even if by the glycerol-3-phosphate pathway, is essential for investigating the incorporation of carotenoids into chylomicra and subsequent secretion across the basolateral membrane.

Many investigators have used the Caco-2 model to study the characteristics and regulation of processes associated with the apical uptake, metabolism, and transepithelial transport of diverse nutrients and other dietary components. These include amino acids (Costa et al. 2000; Nicklin et al. 1995), cholesterol (Nagaoka et al. 2002), fatty acids (Ranheim et al. 1994), monosaccharides (Blais et al. 1987), nucleosides (He et al. 1994), calcium (Giuliano and Wood 1991), iron (Glahn et al. 2002; Au and Reddy 2000), zinc (Oikeh et al. 2003; Han et al. 1994), retinol (Nayak et al. 2001; Puyol et al. 1995), vitamins B6 (Mackey et al. 2004), B12 (Ramanujam et al. 1991), and E (Traber et al. 1990), and bioactive polyphenols (Steensma et al. 2004; Vaidyanathan and Walle 2003; Walgren et al. 1998) and saponins (Hu et al. 2004). Thus, the literature supports 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.

[6] The number of times that a specific type of replicating cell previously isolated from a tissue is transferred from one culture vessel to another for the purpose of continued growth.

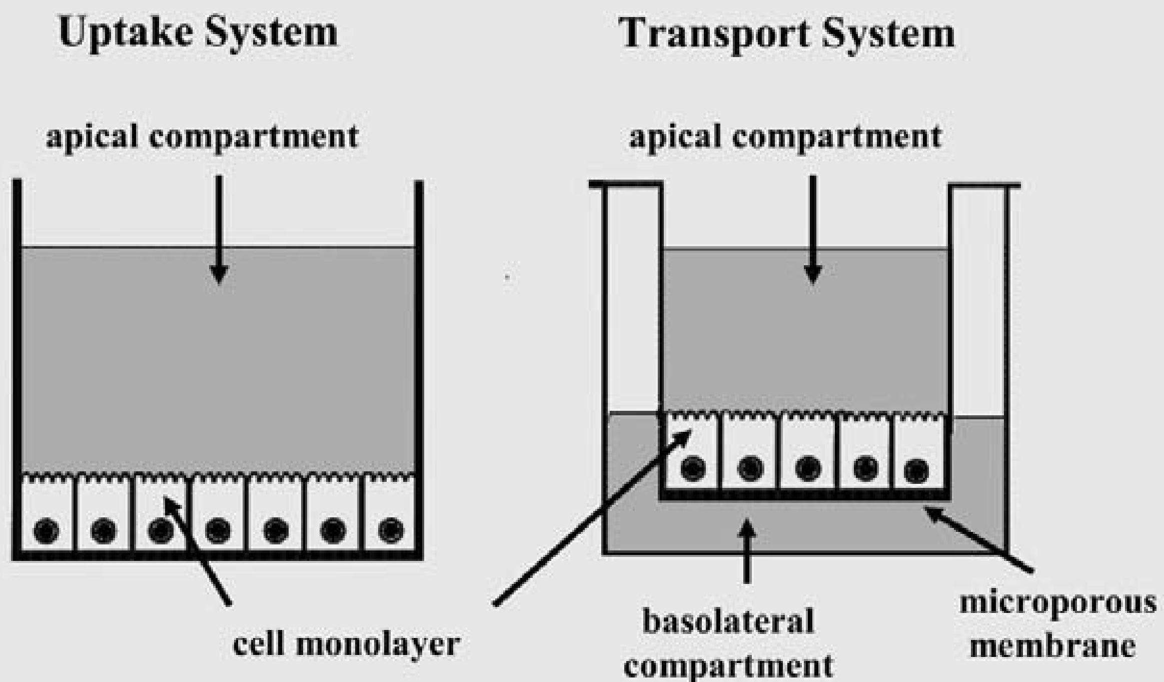
[7] Apolipoprotein B48 - A protein synthesized in enterocytes and incorporated into chylomicra.

The investigation of transepithelial transport requires the use of a three compartment model. Cells are grown and maintained on a permeable, inert membrane support that is attached to the base of a plastic ring suspended in a standard cell culture well containing medium (Figure 5). 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 compounds of interest with known markers of paracellular flux (e.g., phenol red, Lucifer yellow, inulin, and dextrans).

iii. Coupling in vitro digestion with the Caco-2 cell model. Garrett et al. (2000, 1999a) developed a two component coupled digestion/Caco-2 human intestinal cell system to examine cellular acquisition of micellarized carotenoids and other lipophiles from digested foods, supplements, and meals. After completing digestion of carotenoid-containing foods and meals *in vitro*, the micellar fraction is isolated, filtered, and diluted with basal medium. This solution is added to the apical compartment of wells with cells adhered to either the plastic surface of the culture vessel or the membrane insert for investigating the uptake and transport of micellar carotenoids, respectively. Exposure of the monolayer of differentiated cells to diluted micellar fraction for 4–6 hours does not adversely affect cellular morphology and metabolic integrity.

FIGURE 5

Culturing Caco-2 cells for investigating cellular uptake, metabolism and transport of carotenoids.



VI | *In vitro* investigations of digestive stability, micellarization, and intestinal transport and metabolism of carotenoids.

a. Gastric digestion. The stability of carotenoids during the gastric phase of digestion has been investigated because purified carotenoids are unstable in acidic solutions. Re et al. (2001) reported that total lycopene content did not change when lycopene from a commercially available supplement or tomato puree were incubated for as long as 3 hours at pH 1. However, when the lycopene from the supplement was added to either water or artificial gastric juice, a marked increase in the percentage of *cis* isomers of the carotenoid was observed. In contrast, isomerization from all-*trans* to *cis* isomers of lycopene was minimal when intact tomato puree was used instead of the extract, suggesting that the carotenoid associated with the food matrix is stable in the gastric lumen. Rich and associates modeled conditions in the gastric lumen to examine factors affecting the transfer of β-carotene from carrot juice to olive oil (Rich et al. 1998) and from chromoplasts prepared from raw and cooked carrots to oil droplets (Rich et al. 2003a). Transfer to oil droplets in the simple model was incomplete, but was enhanced by blanching the carrots, lowering the pH, or adding pepsin.

b. Small intestinal digestion. Tyssandier et al. (2001) investigated the *in vitro* transfer of carotenoids from lipid droplet emulsions to micelles. Single and mixed carotenoids in lipid droplets were incubated with lipase, co-lipase, and bile salts to simulate the environment of the small intestinal lumen. Maximum transfer of β-carotene and lutein was observed at pH 6–7 with bile salt concentrations of 2–8 mM. Moreover, the efficiency of micellarization of β-carotene was significantly less than that of the tested xanthophylls, but greater than lycopene, suggesting that transfer to the micelle during the simulated small intestinal process was inversely proportional to hydrophobicity of the carotenoid. Thus, the association between carotenoid species and the efficiency of micellarization in the *in vitro* model was similar to that observed in aspirates from human duodenum (Tyssandier et al. 2002). The investigators also found that the presence of either lutein or lycopene along with β-carotene in the lipid droplet decreased transfer of β-carotene to the micelle. This observation suggests that reported interactions between carotenoids in some human studies assessing bioavailability can occur within the small intestinal lumen.

Rich et al. (2003b) simulated the duodenal environment to determine if the transfer of β-carotene and lutein from their respective plant organelles to micelles during the small intestinal phase of digestion requires intermediate partitioning in oil droplets. Transfer of β-carotene from chromoplasts to both oil droplets and mixed micelles was very limited in conditions simulating those in the small intestinal lumen. Although the transfer of lutein from chloroplasts to oil droplets was also inhibited at neutral pH, direct transfer of the xanthophyll from chloroplasts to micelles was observed. These results further demonstrate the influence of carotenoid species and plant matrix on pre-absorptive events.

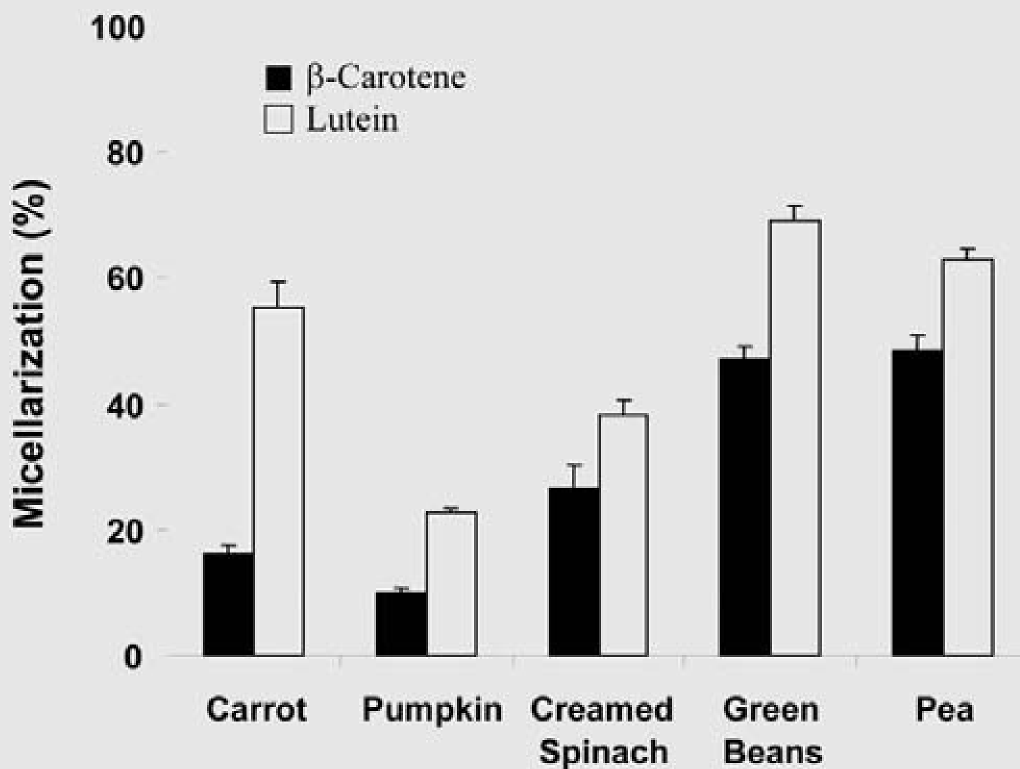
Deletion of bile extract during the small intestinal phase of *in vitro* digestion inhibited transfer of carotenoids from a complete meal (Garrett et al. 1999a, 1999b) and microwaved spinach (Chitchumroonchokchai et al. 2004) to the aqueous fraction of the digesta. Crude porcine bile extract often serves as the source of bile salts for simulation of small intestinal digestion; approximately 50% of the weight of the dry extract is bile salts. Addition of equimolar quantities of several of the most abundant bile salts present in human intestinal lumen in place of the crude bile extract increased the efficiency of micellarization of all-*trans* and 13-*cis* lutein, all-*trans* zeaxanthin, and all-*trans* and 9-*cis* β-carotene in digested spinach (Chitchumroonchokchai et al. 2004). In contrast, replacement of bile extract with sodium taurocholate, the bile salt often used for *in vitro* biochemical studies, resulted in a significant decline in

carotenoid micellarization during simulated digestion of spinach. Because the composition of bile salt(s) similarly affected micellarization of all the carotenoids present in spinach, crude bile extract represents an inexpensive reagent for general studies focused on screening the stability and extent of micellarization of carotenoids in foods and supplements during simulated digestion. Reduction of the standard quantity of pancreatic enzymes during the small intestinal phase of simulated digestion also decreased micellarization of α- and β-carotene from a meal (Garrett et al. 1999a).

c. Combined gastric and small intestinal digestion. The influences of chemical speciation, food matrix, and food processing on the digestive stability and micellarization of carotenoids and other health-promoting bioactive compounds during simulated digestion have been investigated (Hedren et al. 2002b; Ferruzzi et al. 2001; Garrett et al. 2000; Garrett et al. 1999a). Recovery of carotenes, xanthophylls, and lycopene from foods, meals, and supplements digested *in vitro* exceeds 75%. In addition, the isomeric profiles of α- and β-carotene, lutein, and lycopene showed no marked changes during simulated digestion (Pusateri et al. 2003; Ferruzzi et al. 2000). Micellarization of α- and β-carotene was found to be similar during the *in vitro* digestion of processed foods (Garrett et al. 2000, 1999a). In contrast, the micellarization of lutein exceeded that of β-carotene when processed green and orange vegetables were digested *in vitro*, as shown in Figure 6. For each of these carotenoids, the efficiency of micellarization varies depending on the type of vegetable subjected to the *in vitro* digestive procedure. Moreover, lutein and zeaxanthin were more efficiently

FIGURE 6

Comparison of efficiency of micellarization of β-carotene and lutein during simulated digestion of processed vegetables.



micellarized during simulated digestion of an oil-based supplement compared with microwaved spinach (Chitchumroonchokchai et al. 2004). The efficiency of micellarization of lycopene was markedly less than that of lutein and carotenes during digestion of a stir-fried meal containing spinach, carrot, and tomato paste (Garrett et al. 2000). Micellarization of *cis* isomers of lycopene was found to be several-fold greater than that of all-*trans* lycopene during simulated digestion, and the micellarization of both the *cis* and all-*trans* isomers of lycopene increased after tomatoes were cooked in the presence of oil.

Hedren et al. (2002a, 2002b) used a slight modification of the *in vitro* digestion procedure described by Garrett et al. (1999a) to estimate the release of α- and β-carotene from carrot matrix. Relatively low speed centrifugation was used after simulated gastric and small intestinal digestion to prepare a supernatant that was not filtered. Although the extent of micellarization of carotenoids present in the supernatant fraction was not assessed directly, the quantity of carotenes present in the supernatant was increased by homogenizing the vegetable to increase particle size (Hedren et al. 2002a). Cooking the homogenized pulp increased carotene release to the supernatant and adding oil during cooking further increased release from the food matrix. Similarly, cooking with sunflower oil increased the *in vitro* release to the supernatantof carotenes from five green leafy vegetables commonly used in Tanzania (Hedren et al. 2002b).

Collectively, the results of experiments using simulated digestion demonstrate that micellarization is markedly affected by the plant matrix and carotenoid speciation. The *in vitro* findings additionally suggest that differences in micellarization efficiency may contribute to the differential absorption of carotenoids from particular foods and meals.

d. Coupling of simulated digestion and Caco-2 models. Cell uptake of carotenoids (Ferruzzi et al. 2001; Garrett et al. 2000, 1999a) and natural chlorophylls (Ferruzzi et al. 2001) from micelles generated during simulated digestion of foods was proportional to their concentration in cell culture medium and length of exposure. Liu et al. (2004) recently reported that undifferentiated cultures of Caco-2 cells accumulated greater concentrations of β-carotene than α-carotene from digested carrots and accumulated more zeaxanthin from digested cooked corn than from raw corn. Although sufficient information was not provided for a detailed analysis, these results can be readily explained by a) the higher concentration of β-carotene than α-carotene in both carrots and micelles following simulated digestion (Garrett et al. 1999a), and b) the likely increased efficiency of micellarization of zeaxanthin from the cooked corn. Differences in apical uptake of all-*trans* vs. *cis* isomers of β-carotene by Caco-2 cells have been reported (During et al. 2002). Carotenoid uptake also depends on micellar composition. For example, apical uptake of carotenoids by Caco-2 cells was greater from micelles containing *lyso*-phosphatidylcholine instead of phosphatidylcholine (Sugawara et al. 2001). β-Sitosterol inhibited the uptake of β-carotene by immature Caco-2 cells (Fahy et al. 2004), an observation that is in line with the need for increased intake of carotenoids to maintain plasma carotenoid concentrations when hypercholesterolemic individuals consume products enriched with plant sterols and stanols (Noakes et al. 2002).

e. *In vitro* stability of micellar and intracellular carotenoids. Carotenoids are not stable when added to cell culture medium in organic solvents and water-dispersible beadlets (Williams et al. 2000; Wei et al. 1998). In contrast, carotenes and lutein in micelles generated during simulated digestion of a meal containing pureed carrots, spinach, and chicken or only spinach remained unaffected after 4 hours of incubation in basal medium in the conventional cell culture environment, i.e., 37°C in an atmosphere of 95% air/5% CO_2 with saturated humidity (Chitchumroonchokchai et al. 2004; Garrett et al. 1999a).

In contrast, all-*trans* lutein partially isomerized to 13-*cis* lutein when micelles generated during simulated digestion of a lutein in corn oil supplement were incubated in the absence of cells for 4 hours (Chitchumroonchokchai et al. 2004). Because the concentrations of α-tocopherol in micelles from the digested spinach and lutein supplement were similar, other dietary components have the potential to affect carotenoid stability within micelles. This possibility is supported by the observation that purified carotenoids were stable in synthetic mixed micelles (Chitchumroonchokchai et al. 2004; Garrett et al. 1999b) and Tween[8] micelles (O'Sullivan et al. 2004) also containing α-tocopherol.

f. Carotenoid metabolism by Caco-2 cells.

Quick and Ong (1990) first reported partial conversion of β-carotene in early, but not later, passages of Caco-2 cells. During et al. (1998) confirmed the lack of BCO1 activity in the parental line of Caco-2 cells, but observed conversion of β-carotene to retinyl esters in TC7 and PF11 subclones of Caco-2 cells. BCO1 activity in the TC7 cells was increased when confluent monolayers were cultured in serum-free medium. Nagao et al. (2000) reported that the parent line of Caco-2 cells also exhibits BCO1 activity after cultures have been maintained in serum-free medium. BCO1 activity in TC7 cells was stimulated by iron salts and decreased by exposure of cells to the iron chelator desferrioxamine (During et al. 2001).

Nayak et al. (2001) demonstrated that differentiated Caco-2 cells synthesize retinyl esters that are stored intracellularly when culture conditions simulate the fasted state, but secreted in chylomicra in response to prandial-like conditions. These findings were recently confirmed by Chitchumroonchokchai et al. (2004).

Transfer of carotenoids from the apical to the basolateral compartment of cultures of highly differentiated Caco-2 cells has been investigated (During et al. 2002). Carotenoids delivered to the apical compartment in Tween 40 micelles were secreted in large and small chylomicra when prandial conditions were simulated, i.e., after addition of micellarized oleate to the apical compartment. Moreover, the extent of secretion in chylomicra differed for specific carotenoids with the relative quantities of all-*trans* α- and β-carotene > xanthophylls > lycopene in the basolateral compartment. The quantity of β-carotene secreted by Caco-2 cells was within the range reported in lymph from human subjects administered a dose of radioactive β-carotene (Blomstrand and Werner, 1967; Goodman et al. 1966). Apical uptake of all-*trans* β-carotene from Tween 40 micelles and its subsequent secretion across the basolateral membrane were significantly greater than the uptake and secretion of 9-*cis* and 13-*cis* β-carotene (During et al. 2002). It was further found that apolar carotenoids appeared to inhibit uptake and absorption of one another in the cell model. Finally, secretion of lutein in chylomicra by Caco-2 cells was similar when the carotenoid was presented to the apical surface of Caco-2 cells in either mixed micelles (Chitchumroonchokchai et al. 2004), i.e., the physiological vehicle, or in Tween 40 micelles (During et al. 2002).

VII | Physiological relevance of *in vitro* models for assessing the bioavailability of provitamin A carotenoids in humans

Ultimately, studies of free-living human subjects are required to determine the efficiencies of the absorption and conversion of provitamin A carotenoids from foods prepared in various manners and ingested as components of traditional meals. However, the difficulty and expense of conducting well-controlled human studies precludes systematic investigation of the effects of plant genotype, meal preparation method, and host factors on provitamin A bioavailability for local populations.

[8] A synthetic nonionic detergent (polyoxyethylenesorbitol monolaureate) that solublizes fat-soluble molecules.

In the Introduction we proposed that *in vitro* biochemical and cellular methods represent cost-effective surrogates for an initial screening of relative bioavailability of provitamin A carotenoids. Support for this hypothesis is provided by direct comparison of the observations from *in vivo* and *in vitro* studies discussed in Sections 4 and 6, respectively, as summarized in Table 4. Human studies have consistently shown that bioavailability is increased when the plant matrix is destroyed by cooking and by other forms of processing, and when carotenoids solubilized in oil are ingested instead of natural foods. These factors also

TABLE 4

COMPARISON OF RESULTS FOR GASTROINTESTINAL METABOLISM AND BIOAVAILABILITY OF CAROTENOIDS USING *IN VIVO* AND *IN VITRO* APPROACHES

Human studies and animal models	*In vitro* models
Digestion, source and processing	
Carotenoids are stable during gastric and small intestinal phases	High recovery and limited isomerization during simulated digestion
Micellarization of lutein > apolar carotenoids	Micellarization of lutein > β-carotene > lycopene
↓ absorption if pancreatic or biliary insufficiency	↓ micellarization when pancreatin or bile lowered or deleted
↑ absorption from cooked and mashed foods	↑ micellarization after cooking
↑ absorption from oil vs. foods	↑ micellarization from oil vs. food
↑ absorption of *trans* vs. *cis* β-carotene	↓ micellarization of *trans* vs. *cis* β-carotene ↑ uptake and secretion of *trans* β-carotene vs. *cis* β-carotene by Caco-2 cells
Effects of dietary components	
Absorption of lycopene saturated at supra-physiologic doses	Saturable uptake of β-carotene by Caco-2 cells
lyso-phosphatidylcholine ↑ absorption of β-carotene and lutein	*lyso*-phosphatidylcholine ↑ micellarization and uptake by Caco-2 cells
Phytosterols/stanols ↓ β-carotene absorption	β-sitosterol ↓ β-carotene uptake by Caco-2 cells
Antagonistic and agonistic effects between carotenoids affect absorption	Antagonistic effects between carotenoids affect micellarization, uptake and secretion by Caco-2 cells
Intestinal cell metabolism	
Partial conversion of β-carotene to retinoids	Conversion of β-carotene to retinyl esters by Caco-2 cells
Secretion of carotenoids and retinyl esters in chylomicra	Caco-2 cells secrete carotenoids and retinyl esters in chylomicra

increase the micellarization of carotenoids during simulated digestion. In addition, impaired secretion of digestive enzymes, bicarbonate, and bile in response to a meal can decrease absorption of fat-soluble compounds, just as reduced concentrations of pancreatin and bile extract decrease micellarization of carotenoids during simulation of the small intestinal phase of digestion. Collectively, these results indicate that the relative bioavailability of provitamin A carotenoids from staple food crops can be probed by monitoring the extent of micellarization of provitamin A carotenoids during simulated digestion.

Studies using Caco-2 cells have shown that both apical uptake and basolateral secretion of all-*trans* β-carotene are more efficient than those of *cis* isomers of the carotenoid, and that other dietary compounds can affect these transport processes. These observations parallel findings in human subjects and animals and lend support to the use of the Caco-2 cell model to confirm the accessibility of carotenoids that are micellarized during simulated digestion. Conditions required for induction of BCO1 expression in Caco-2 cells and the secretion of chylomicra have been defined. Determination of BCO1 mRNA, protein, or enzyme activity may be considered as a means of assessing the possible influences of food preparation methods and meal composition on the absorption and conversion of provitamin A carotenoids. There is, however, a need to validate the *in vitro* approaches by directly comparing the relative bioavailabilities of provitamin A carotenoids from the same test meal fed to human subjects and subjected to simulated digestion coupled with the Caco-2 cell model.

The *in vitro* approach should ultimately prove useful for estimating the bioavailability of provitamin A from many cultivars with the aim of selecting a subset of cultivars that merit further examination in animal models, most likely in gerbils or perhaps ferrets. The results of the animal studies in turn should provide the basis for selecting the most promising cultivars of staple crops for investigation of provitamin A bioavailability in human subjects in local settings. Thus it is important to identify mechanisms that integrate *in vitro* and *in vivo* studies to decrease vitamin A deficiency in developing countries.

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