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Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an in vitro digestion/ Caco-2 cell culture model

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We previously developed an in vitro model to estimate the relative bioavailability of carotenoids from a meal prepared using commercial baby foods. The general applicability of this model was tested using a stir-fried meal consisting of fresh spinach, fresh carrots, tomato paste, and vegetable oil. After in vitro digestion of the cooked meal, the aqueous fraction was separated from residual oil droplet and solids by centrifugation to quantify micellarized carotenoids. The percentages of lutein, lycopene, α-carotene, and β-carotene transferred from the meal to the micellar fraction were 29.0 ± 0.6, 3.2 ± 0.1, 14.7 ± 0.3, and 16.0 ± 0.4, respectively. Carotenoid transfer from the meal to the aqueous fraction was inhibited when bile extract was omitted from the intestinal phase of digestion. The bioavailability of the micellarized carotenoids was validated using differentiated cultures of Caco-2 human intestinal cells. All four carotenoids were accumulated in a linear manner throughout a 6-hr incubation period. Metabolic integrity was not compromised by exposure of cultures to the diluted aqueous fraction from the digested meal. The addition of 500 μmol/L α-tocopherol to test medium significantly improved the stability of the micellar carotenoids within the tissue culture environment. These results support the utility of the in vitro digestion procedure for estimating the bioavailability of carotenoids from foods and meals. (J. Nutr. Biochem. 11:574–580, 2000)

Keywords: in vitro digestion; carotenoid bioavailability; Caco-2 intestinal cells, lutein, α-carotene, β-carotene

Introduction

Carotenoids are a group of hydrophobic pigments synthesized by plants, photosynthetic algae, and bacteria that appear to confer protection against the development of some forms of cancer and cardiovascular disease.[1,2] Carotenoid bioavailability from foods or supplements is usually assessed by monitoring increases in carotenoid content of plasma or the triacylglycerol-rich fraction of plasma.[3,4] However, the wide variations in plasma carotenoid response of individuals after consuming carotenoid-rich meals suggest that the absorption of these plant pigments is affected by genetic, physiological, and metabolic factors.[4,5]

The plant matrix, the carotenoid form (synthetic vs. naturally occurring in foods), the manner of preparation of the carotenoid-rich food, and the composition of the carotenoid-rich meal are known to affect carotenoid bioavailability from foods.[6–8] For example, plasma and breast milk levels of β-carotene (BC) and retinol increased in women fed synthetic BC in a wafer, but not in women fed green leafy vegetables containing an equivalent amount of BC.[9] Moreover, the bioavailability of specific carotenoids from the same food source, for example, BC and lutein (LUT) from spinach,[10] or similar carotenoids from different matrices,[11] can differ markedly. Such observations indicate that the diversity of carotenoids in foods and the many potential food combinations used to prepare meals present a major

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challenge for predicting carotenoid bioavailability at this time.

We previously developed an in vitro method to estimate the relative bioavailability of carotenoids from various foods.[12] Commercial baby foods consisting of processed vegetables and meat were used to prepare a test meal that was subjected to in vitro digestion. The quantities of carotenoids transferred from the food matrix to the aqueous micellar fraction were determined as an estimate of the relative bioavailability of LUT, lycopene (LYC), α-carotene (AC), and BC. The bioavailability of these carotenoids in the aqueous fraction was validated by monitoring their apical uptake by differentiated cultures of the Caco-2 human intestinal epithelial cell line. The experiments reported below represent a parallel study in which the test meal was prepared from stir-fried fresh vegetables instead of the highly processed baby foods in order to evaluate the broader applicability of the in vitro digestion procedure for estimating the bioavailability of carotenoids from meals.

Materials and methods

Unless stated otherwise, all reagents and materials were purchased from Sigma Chemical Co. (St. Louis, MO USA) and Fisher Scientific Co. (Norcross, GA USA).

Preparation of meal from fresh vegetables

All manipulations with foods were performed under subdued lighting to minimize the destruction of carotenoids. A carotenoid-rich meal was prepared from 100 g fresh spinach, 50 g fresh carrots, and 50 g tomato paste to provide primary sources of LUT, AC and BC, and LYC, respectively. The spinach and carrots were chopped finely. Seven milliliters of vegetable oil (primarily soybean oil, Hunt-Wesson, Inc., Fullerton, CA USA) were added to a temperature-regulated electric skillet that was preheated to 177°C. The vegetables were added to the skillet and fried for 4 min with constant stirring. The cooked meal was mixed with 70 mL of saline containing 150 μmol/L butylated hydroxytoluene (BHT) and homogenized to a pureed consistency with a kitchen blender (Osterizer Pulse Matic) set on "chop" for 2 min, followed by setting of "blend" for an additional 2 min to simulate mastication. Samples (2 g) of the meal were transferred to amber bottles and diluted with 34 mL of saline (150 mmol/L) containing 150 μmol/L BHT before homogenizing (Tekmar Tissumizer, Cincinnati, OH USA) for 30 sec at a setting of 50. The starting quantity of the homogenized meal subjected to the digestion procedure was increased to 6 g to provide higher concentrations of micellarized carotenoids for the investigation of carotenoid uptake by Caco-2 cells (see below).

In vitro digestion

The in vitro digestion procedure described by Miller et al.[13] was conducted with minimum modifications as reported elsewhere.[12] Briefly, the homogenized meal was acidified (pH 2) before addition of porcine pepsin to a final concentration of 1.8 mg/mL and incubated at 37°C in a shaking water bath (Precision Scientific Instruments, Model Shal. Form, Chicago, IL USA) at 95 rpm for 1 hr. The pH of the gastric digestate was then increased to 5.3 with sodium bicarbonate, and porcine bile extract and pancreatin were added to provide final quantities (concentrations) of 2.4 and 0.4 mg of bile extract and pancreatin per mL digestate, respectively, in a final reaction volume of 46 mL. The pH was elevated to 7.5 with 1 N sodium hydroxide and 10 mL aliquots of the partially digested meal were transferred to three amber glass bottles. The bottles were capped after blanketing the digesta with argon, and samples were incubated in the shaking water bath at 37°C for 2 hr.

The impact of the digestion procedure itself on the recovery of carotenoids from the starting meal was examined in pilot studies. Cooked meals were homogenized in 150 mmol/L saline containing 150 μmol/L BHT (final volume of 46 mL). Aliquots of the homogenized meal were extracted and the concentrations of carotenoids determined (see below) for comparison with those present in digestate after completion of the digestion procedure. The levels of LUT, LYC, AC, and BC were similar ($P > 0.01$) in the homogenized and the digested meals, indicating that these carotenoids were not destroyed during the digestion procedure.

Isolation of the micellar fraction

The aqueous fraction was isolated from the digesta according to Hernell et al.[14] Polyallomer ultracentrifuge tubes were filled with 6.5 mL of the digesta and centrifuged in a 50.3 Ti rotor at 167,000 × *g* at 4°C for 95 min (Beckman Model L7-65 Ultracentrifuge; Beckman Instruments, Palo Alto, CA USA). The aqueous fraction was collected from the centrifuge tubes with an 18-gauge needle attached to a 10-mL plastic syringe. The solution was filtered (cellulose acetate, 0.22 μm pore size; Gelman Sciences, Ann Arbor, MI USA) to remove microcrystalline nonmicellarized carotenoids that were not pelleted during centrifugation.[12,15]

Uptake of micellar carotenoids by intestinal cells

Stock cultures of Caco-2 cells (HTB-37, American Type Culture Collection, Rockville, MD USA) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, nonessential amino acids (10 mL/L), L-glutamine (2 mmol/L), amphotericin B (0.5 mg/mL), gentamicin (50 mg/L), sodium bicarbonate (44 mmol/L), and HEPES (15 mmol/L) in a humidified atmosphere of 95% air/5% CO_2 (v/v) at 37°C with medium changed every other day.[16] Cultures of Caco-2 at passages 23–36 were grown in 12-well dishes (Beckton Dickinson Labware, Franklin Lakes, NJ USA) and used for experiments between 11 and 14 days after reaching confluency. The differentiation of Caco-2 cells maintained in this manner is maximal at this time.[17]

Monolayers were washed two times with 1 mL Hank's balanced salts solution (HBSS) before adding 1 mL of test medium containing 0.75 mL DMEM and either 0.25 mL aqueous fraction or 0.25 mL saline (control) to triplicate wells for each test. Cultures were incubated at 37°C for either 2, 4, or 6 hr. At indicated times, medium was removed and monolayers were washed two times with HBSS containing 5 mmol/L sodium taurocholate at 22°C. Presumably, the wash with bile salt removes carotenoids adhering to cell surfaces.[12,18] Cells were scraped into 1 mL of ice-cold phosphate buffered saline containing 10% (v/v) ethanol and 45 μmol/L BHT. Samples were overlaid with argon and stored at −20°C for a maximum of 2 days.

Sample extraction and analysis

Frozen samples of digesta, aqueous fraction, and cells were thawed and sonicated briefly. Aliquots (500 μL) were mixed with 500 μL of ethanol containing 0.5 μmol/L echinenone before extracting with 1 mL acetone three times. Acetone extracts were pooled and 1 mL of distilled water was added before re-extraction with 2 mL of hexane three times. Pooled hexane extracts were evaporated under nitrogen and reconstituted in 500 μL of mobile phase (see below). Aliquots (35 μL) were analyzed by reverse-phase high performance liquid chromatography (Hewlett-Packard, Model HP-1090, Avondale, PA USA) and carotenoids were detected at 450

nm.[19] Carotenoids were separated using a BF-C18/C4 analytical column (25 cm × 0.46 cm diameter; ES Industries, West Berlin, NJ USA) containing 5 μm particles protected by a C-18 Adsorbosphere guard column (5 μm particle size, 0.75 × 0.46 cm, Alltech, Deerfield, IL USA). Isocratic analyses were performed at a flow rate of 1.5 mL/min with a mobile phase of 65% (v/v) acetonitrile containing 13 mmol/L triethylamine, 15% (v/v) methylene chloride, and 20% (v/v) methanol containing 1.3 mmol/L ammonium acetate.[20] Results were calculated from a multilevel calibration table based on a series of external standards. Extraction efficiency generally exceeded 90% as determined by the recovery of echinenone that was added to each sample.

Cellular integrity

The potential toxicity of the diluted aqueous fraction from the digested meal on the viability and metabolic integrity of differentiated cultures of Caco-2 cells was assessed. Standard markers monitored included the morphological appearance of the monolayer, cell viability as assessed by trypan blue exclusion, protein content per well, the mean number of domes per 10 microscopic fields/well, and mitochondrial activity. Domes represent an indicator of Na, K-ATPase activity in the basolateral membrane.[21] Procedures for examining the uptake and incorporation of ^{3}H-leucine into protein and mitochondrial activity have been described in detail previously.[12,20]

Stability of micellar carotenoids

Diluted aliquots of the aqueous fraction isolated from the digestate were incubated in a cell-free tissue culture environment for 6 hr to assess the stability of the micellar carotenoids in the aqueous fraction. Some samples were supplemented with indicated concentrations of freshly prepared α-tocopherol or ascorbate to evaluate the ability of antioxidants to increase the stability of the micellarized carotenoids.

Protein assay

The quantity of cellular protein per well was determined using the bicinchoninic acid assay (Pierce, Rockford, IL USA).

Statistical analysis of data

Data represent the mean ± SEM. For each parameter tested, three separate meals were prepared for one experiment and each experiment was repeated at least once on another day with differentiated cultures at a higher passage number to provide a minimum of six independent observations. For cellular studies, the diluted aqueous fraction from each digestate was added to triplicate wells containing differentiated monolayers of Caco-2 cells to examine carotenoid uptake. The mean carotenoid accumulation from the triplicate wells was used as a single observation. Data for 6–9 aqueous fractions that were added to test cultures were pooled to determine the mean level of cellular accumulation of carotenoids from micellar medium at each time indicated. Data were analyzed using either Student's *t*-test (*Figure 1*), Student's *t*-test and repeated measures analysis (*Table 1*), one-way analysis of variance (*Figure 2* and *Table 2*), and analysis of covariance (*Figure 3*), as appropriate. Tukey-Kramer Honestly Significant Difference test was used as a post hoc comparison of statistical significance. All analyses of data were performed using the JMP statistical software program (SAS Institute, Cary, NC USA) with the alpha level set at $P < 0.01$ to determine significant differences.

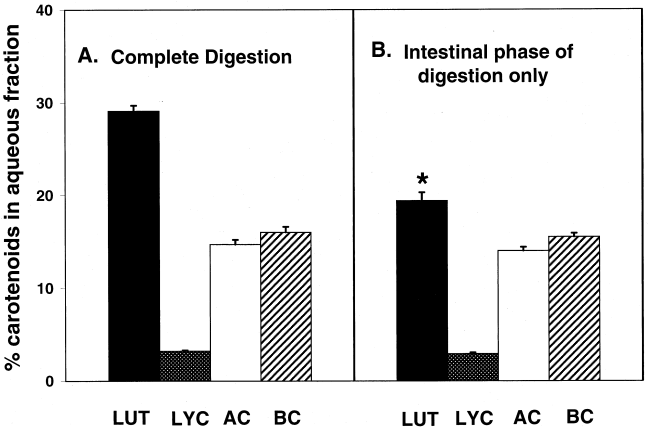

Figure 1 Relative contribution of gastric plus intestinal phase (complete digestion process) versus only the intestinal phase of the in vitro digestion process on the transfer of carotenoids from test meal to the aqueous fraction. Digestion of the homogenized meal was initiated at either the gastric (A) or intestinal (B) phase. The procedure for the complete digestion process (gastric and intestinal phases) is described in the Materials and methods section. When digestion was limited to the intestinal phase, the sample was acidified and pepsin added, but the mixture was neutralized immediately with $NaHCO_3$ followed by addition of a mixture of bile-pancreatin extract. All samples were incubated with shaking at 37°C for 2 hr for the intestinal phase before isolating the aqueous fraction by centrifugation. The quantity of each carotenoid present in the filtered aqueous fraction was expressed as a percentage of the respective carotenoid in the digesta. Data are the mean ± SEM from 9 replicate meals. The presence of an asterisk (*) above the error bar indicates that the mean level of lutein (LUT) differed significantly ($P < 0.01$) in the aqueous fraction after either intestinal or gastric plus intestinal digestion processes. The concentrations of lycopene (LYC) and carotenes in the micellar fraction were similar ($P > 0.01$) for the two treatments. AC, α-carotene; BC, β-carotene.

Table 1 Characterization of Caco-2 cultures treated with control (DMEM + 25% saline) and test medium (DMEM + 25% micellar fraction from digested meal) for 6 hr*

Characteristic	DMEM + 25% saline	DMEM + 25% micellar
Protein content per well (mg)	1.27 ± 0.03	1.31 ± 0.02
Cell viability	95.5 ± 0.9	95.1 ± 1.1
Domes per microscopic field†	2.1 ± 0.15	2.2 ± 0.14
^{3}H-LEU uptake (dpm/30 min/mg protein)	20811 ± 621[a]	18043 ± 360[b]
^{3}H-LEU incorporated into protein (% acid ppt ^{3}H)	9.4 ± 0.3[a]	13.3 ± 0.4[b]
Mitochondrial activity‡ (A_{520}/30 min/well)	0.20 ± 0.003	0.21 ± 0.004

*Listed characteristics were assessed as described in the Materials and methods section after 6 hr of exposure of cultures to control (75% DMEM + 25% saline) or test (75% DMEM + 25% aqueous fraction from digested meal) medium. The results represent the mean ± SEM from either two or three experiments using triplicate cultures for each assay. Different letters as superscript within a row indicate that there is a statistically significant difference ($P < 0.01$) between means.
†Domes are indicative of basolateral Na, K-ATPase activity in polarized cells (Hidalgo et al.[21]). Cultures were examined at 100 × magnification.
‡Mitochondrial activity was monitored by the rate of reduction of Alamar Blue dye.
DMEM–Dulbecco's modified Eagle's medium. LEU–leucine.

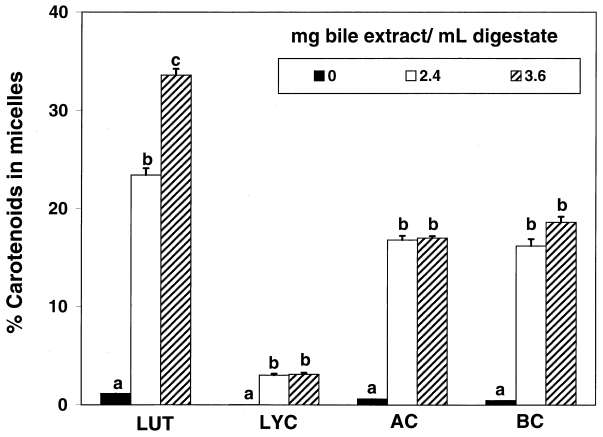

Figure 2 Bile extract is required for the transfer of carotenoids to the aqueous phase during in vitro digestion of the vegetable meal. The meal was digested as described in the Materials and methods section, except that the concentration of bile extract in the reaction mixture during the intestinal phase was adjusted to either 0, 2.4, or 3.6 mg/mL; the concentration of bile extract in the standard procedure was 2.4 mg/mL. The amount of carotenoids present in the filtered aqueous fraction after centrifugation are expressed as a percentage of that present in the digesta. Data are the mean ± SEM for a total of 9 separate meals at each level of bile tested. Different letters above the error bars denote that mean levels of the indicated carotenoid differs significantly ($P < 0.01$) in response to the level of bile extract added to the digestion mixture. LUT, lutein; LYC, lycopene; AC, α-carotene; BC, β-carotene.

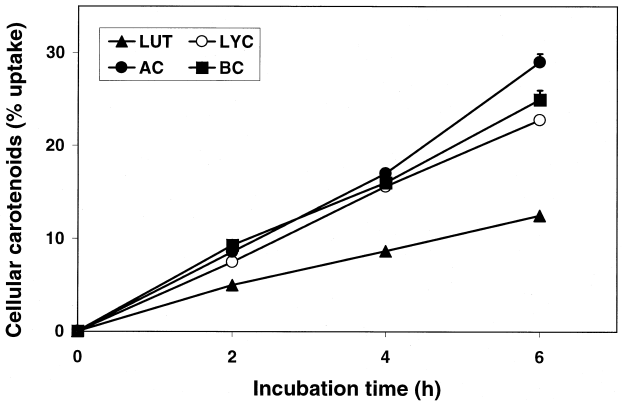

Figure 3 Uptake of micellar carotenoids by differentiated cultures of Caco-2 cells. Cultures were incubated in Dulbecco's modified Eagle's medium containing 25% (v:v) freshly isolated aqueous fraction from digested meal. Cellular levels of carotenoids were determined as described in the Materials and methods section after 2–6-hr incubation. Data represent the mean ± SEM for 9 samples. The diluted aqueous fraction from each meal was added to triplicate wells to determine average carotenoid uptake for each sample at timed intervals. The percentage of medium lycopene (LYC) and carotenes accumulated by monolayers of Caco-2 cells was greater ($P < 0.01$) than that of lutein (LUT). AC, α-carotene; BC, β-carotene.

Results

Characterization of the transfer of carotenoids from test meal to aqueous fraction

The standard meal consisted of fresh spinach, fresh carrots, and tomato paste as sources of LUT, the carotenes (AC and BC), and LYC, respectively. These vegetables were stir-fried using vegetable oil as the lipid source. The concentrations of LUT, LYC, AC, and BC in the digesta at the completion of the in vitro procedure were 2.4 ± 0.05, 6.8 ± 0.2, 0.88 ± 0.04, and 1.87 ± 0.03 μg/mL, respectively. Measurable quantities of all four carotenoids were present in the filtered (0.22 μm pore size) aqueous fraction of the digestate. However, the efficiency of transfer of the carotenoids from the foods to micelles differed. The results presented in *Figure 1A* show that the percentage of LUT transferred from the meal to the aqueous fraction (29.0 ± 0.6%) exceeded that of LYC (3.2 ± 0.1%), AC (14.7 ± 0.3%), and BC (16.0 ± 0.4%).

The quantity of LUT in the aqueous fraction of the digestate was significantly ($P < 0.01$) lower when the digestion procedure was initiated at the intestinal phase (i.e., diluted homogenized meal was not exposed to 10 mM HCl and pepsin for 1 hr at 37°C; *Figure 1B*) than when samples were subjected to the complete digestion process (*Figure 1A*). In contrast, the amounts of micellarized LYC and the carotenes were similar ($P > 0.01$) for samples subjected to either the standard (gastric and intestinal phases) or only the intestinal phase of the digestion process. Carotenoids were not detected in the filtered aqueous fraction when the digestive factors, that is, HCl, pepsin, bicarbonate, bile extract, and pancreatin, were omitted from the reaction mixture (data not shown).

The obligatory requirement for bile salts in the solubilization and subsequent absorption of carotenoids is well established.[22] As expected, negligible amounts of carotenoids were detected in the aqueous fraction when bile extract was omitted during the intestinal phase of digestion (*Figure 2*). Addition of the standard quantity of bile extract (2.4 mg/mL) significantly ($P < 0.01$) increased the levels of all four carotenoids present in the aqueous phase. Elevation

Table 2 Stability of micellar carotenoids generated by in vitro digestion of fresh vegetable meal in a cell-free tissue culture environment for 6 hr

	% Carotenoid recovered			
		Vit E		Vit C
	Control	100 μmol/L	500 μmol/L	100 μmol/L
LUT	81 ± 1.2[b]	90 ± 0.6[a,b]	96 ± 0.9[a]	77 ± 1.5[b]
LYC	49 ± 3.2[b]	52 ± 0.5[b]	76 ± 1.3[a,b]	47 ± 2.0[b]
AC	57 ± 2.5[b]	56 ± 0.9[b]	95 ± 3.0[a]	54 ± 1.7[b]
BC	52 ± 2.3[b]	61 ± 0.9[a,b]	95 ± 1.0[a]	54 ± 2.0[b]

*The aqueous fraction from the in vitro digested meal was diluted 4-fold with basal DMEM and supplemented with either 0, 100, or 500 μmol/L α-tocopherol (Vit E), or with 100 μmol/L ascorbic acid (Vit C). Aliquots were added to cell-free tissue culture dishes and incubated in a humidified chamber with 95% air/5% carbon dioxide at 37°C for 6 hr. The medium was collected and extracted as described in the Materials and methods section to quantify the amount of each carotenoid present at 0 and 6 hr. Data are the mean ± SEM for aqueous fraction prepared from 6 test meals.

[a]The percentage of carotenoid recovered from wells after 6-hr incubation differed significantly ($P < 0.01$) from Control (i.e., no vitamin E or vitamin C supplement).

[b]The percentage of carotenoid recovered was significantly ($P < 0.01$) lower than that added to wells at 0 hr.

DMEM–Dulbecco's modified Eagle's medium. LUT–lutein. LYC–lycopene. AC–α-carotene. BC–β-carotene.

of the level of bile extract to 3.6 mg/mL slightly, but significantly ($P < 0.01$), increased the micellarization of LUT, but not the hydrocarbon carotenoids.

Cellular uptake of micellarized carotenoids generated by in vitro digestion

The apical uptake of carotenoids by differentiated cultures of Caco-2 cells from test medium was monitored to validate the bioavailability of carotenoids present in the aqueous fraction of the digested stir-fried meal. Cultures were incubated for as long as 6 hr in medium consisting of one volume of the filtered aqueous fraction and three volumes of DMEM. The starting concentrations of LUT, LYC, AC, and BC in the diluted aqueous fraction were 0.42 ± 0.01, 0.28 ± 0.008, 0.11 ± 0.004, and 0.16 ± 0.006 μmol/L, respectively. Cellular accumulation of all four carotenoids increased linearly with length of exposure and the amount of cellular LUT (57 ± 1.2 pmol/mg protein) exceeded ($P < 0.01$) that of LYC (48 ± 1.2 pmol/mg protein), AC (30 ± 0.9 pmol/mg protein), and BC (37 ± 0.8 pmol/mg protein) after 6 hr. However, the percentage of medium LYC and carotenes accumulated by the cells was significantly ($P < 0.01$) greater than that of LUT after 2, 4, and 6 hr of incubation (*Figure 3*). The greater accumulation of LYC and the carotenes than of LUT suggests differences in uptake, metabolism, and/or efflux of the hydrocarbon carotenoids and LUT.[12]

Metabolic activities of Caco-2 cells were examined to assess possible toxicity of the aqueous fraction of the digesta. Data in *Table 1* indicate that exposure of monolayers for as long as 6 hr to the diluted aqueous fraction derived from the in vitro digestate did not alter cell viability, average number of domes per microscopic field, protein content per well, or mitochondrial activity, as assessed by reduction of Alamar Blue dye.[23] Cellular acquisition of ^{3}H-leucine from medium was decreased by 10% ($P < 0.01$), whereas the incorporation of ^{3}H into protein was slightly, but significantly ($P < 0.01$), higher in cultures exposed to diluted aqueous fraction than in cultures incubated in control medium. Together, these results suggest that metabolic integrity was not compromised by exposure to the diluted aqueous fraction of the digestate.

Stability of micellar carotenoids in tissue culture environment

The stability of the micellarized carotenoids generated by in vitro digestion of the stir-fried meal was examined in the tissue culture environment. One volume of the filtered aqueous fraction was diluted with three volumes of DMEM, aliquots were added to cell-free wells, and the dishes were incubated at 37°C in a humidified chamber with an atmosphere of 95% air:5% CO_2. The recovery of LUT after 6 hr was 81 ± 1.2% of the initial level in medium (*Table 2*). In contrast, the concentrations of LYC and carotenes decreased approximately 50% by 6 hr. This marked decrease in the levels of the hydrocarbon carotenoids was relatively linear with losses of 12–19% and 32–41% detected after 2 and 4 hr, respectively. Such losses likely occurred because of spontaneous oxidation and/or the presence of pro-oxidant compounds in the aqueous fraction from the meal. These possibilities were tested by the addition of α-tocopherol or ascorbic acid to medium. Addition of 100 μmol/L α-tocopherol to medium significantly ($P < 0.01$) increased the recovery of LUT and BC to 90 ± 0.6% and 61 ± 0.9% of the starting concentration, respectively, but failed to significantly increase ($P > 0.01$) the levels of LYC and AC after 6 hr. In contrast, there was minimal loss of carotenoids during the 6-hr incubation when test medium was supplemented with 500 μmol/L α-tocopherol. The addition of 100 μmol/L ascorbic acid to test medium failed to improve ($P > 0.01$) the recovery of the micellar carotenoids after 6 hr.

Discussion

We previously developed an in vitro method to estimate the relative bioavailability of carotenoids from foods and meals.[12] The commercial baby foods used in the initial studies were prepared by cooking the pureed food at 145°C for 35–45 min (personal communication, H.J. Heinz & Co., Pittsburgh, PA USA). In those studies, the large surface area of the cooked and pureed foods may have enhanced the extent of micellarization of the carotenoids in the meal.[8,24] Here, we sought to test the general applicability of the in vitro digestion model as a rapid procedure for estimating the bioavailability of carotenoids by preparing a meal with minimally processed vegetables. Fresh spinach and carrots were combined with tomato paste and stir-fried in vegetable oil at 177°C for 4 min.

Because micellarization of hydrophobic species facilitates their transfer to the brush border surface of enterocytes for uptake and subsequent absorption,[25] we chose the degree of micellarization during the digestion process as an indicator of carotenoid bioavailability. The efficiency of transfer of individual carotenoids of interest from the stir-fried vegetable matrix to the aqueous or micellar fraction in response to in vitro digestion varied; that is, LUT was more readily micellarized than the carotenes and LYC. This differential pattern of micellarization of the carotenoids in our in vitro studies is similar to our previous study in which a baby food meal was subjected to in vitro digestion.[12] Likewise, Castenmiller et al.[10] recently reported that LUT bioavailability was significantly greater than that of the carotenes when subjects were fed a meal consisting of either blanched, whole, or processed spinach. Consideration of the relative hydrophobicity of the various carotenoids, their location and chemical form in plants, and their transfer between the oil droplet and micelles offer some insights about the relative bioavailability of these plant pigments in foods. LUT is usually located in chloroplasts, whereas the carotenes and LYC are found in chromoplasts.[26,27] Carotenes in carrot root are associated with proteins surrounded by a thick membranous sheet, and LYC is present in the tomato as crystalloids that develop within or along thylakoid bodies.[28] Others have suggested that the location and speciation of BC and LYC in the plant matrix likely limits their transfer to the oil droplet that forms during the gastric phase of digestion.[8,24]

Micellarization of LYC from the meal containing tomato paste in response to in vitro digestion was very low in this and our previous studies.[12] Approximately 3–4% of LYC in

the stir-fried vegetable meal was transferred to the aqueous fraction, whereas less than 1% of LYC in the baby food meal with tomato paste was micellarized during in vitro digestion.[12] Others also have reported low bioavailability of LYC from tomato juice fed to human subjects,[29,30] although processing[31] and cooking in the presence of oil[30] enhanced the bioavailability of the hydrocarbon carotenoid. Cooking tomato products likely improves the bioavailability of LYC by solubilizing a portion of the aggregated LYC crystals in oil droplets.[32] Our data suggest that limited cooking and, perhaps the use of vegetable oil instead of animal fat, enhanced the micellarization of tomato paste LYC from the stir-fried vegetable meal compared to brief warming of the pureed baby food meal containing tomato paste.[12]

The localization of the carotenoid within the oil droplet formed within the gastrointestinal tract is another factor that may influence its transfer to the micelle. Hydrocarbon carotenoids are buried within the core of the oil droplet, whereas the polar xanthophylls such as LUT are located near the surface.[33] Zeaxanthin in phospholipid-triglyceride emulsions was found to readily move to the aqueous phase, whereas such transfer of the hydrocarbon carotenoid BC from the emulsion to the aqueous fraction required lipase activity.[33] Thus, the location of LUT at the surface of the oil droplet likely facilitated its greater micellarization compared to the carotenes and LYC during digestion.

We evaluated the impact of gastric digestion on the micellarization of carotenoids from the stir-fried vegetable meal. A greater percentage of LUT was transferred to the aqueous fraction when digestion was initiated at the gastric phase than when digestion was limited to the intestinal phase (*Figure 1*). In contrast, the gastric process did not contribute to the amount of LYC, AC, or BC present in micelles at the completion of the intestinal phase of digestion for either the stir-fried meal or the baby food meal.[12] This observation is in line with reports that the levels of plasma retinol and hepatic carotenes were not altered by omeprazole-mediated inhibition of gastric secretion in rats fed carrot extract.[34] We homogenized the cooked meal to a puree prior to initiating in vitro digestion to ensure representative sampling for analyses. Particle size of foods has been shown to affect carotenoid bioavailability in humans.[35] The dimensions of particles of ingested fresh vegetables in the stomach are likely to be larger than those in our in vitro study. Thus, gastric digestion in vivo is expected to contribute to the disruption of the larger particles of foods, thereby increasing release and coalescence of dietary fats into oil droplets. In turn, pancreatic lipases hydrolyze ester linkages facilitating the formation of mixed micelles.[25]

The Caco-2 human colonic epithelial cell line was used to assess the actual availability of carotenoids present in the aqueous fraction of the in vitro digestate. The morphological and biochemical similarities of differentiated Caco-2 cells to mature enterocytes has resulted in the widespread use of this cell line for the investigation of the apical uptake and metabolism of numerous nutrients and drugs.[21,36] The aqueous fraction of the digestate was diluted in tissue culture medium before addition to differentiated cultures of Caco-2 cells. Monolayers of differentiated Caco-2 cells accumulated carotenoids in proportion to both the carotenoid content of the medium and the duration of exposure (*Figure 3*), which demonstrated the availability of the micellarized carotenoids from the digestate. These results are similar to our previous observations with Caco-2 cells exposed to micelles prepared in vitro in the presence of crystalline BC and LUT,[20] and the aqueous fraction generated from in vitro digestion of commercial baby food carrots and spinach.[12] Studies are now needed to examine the transfer of intracellular carotenoids into chylomicra for transport across the basolateral membrane.

Deleterious effects of the diluted aqueous fraction from the digestate on the morphological or biochemical integrity of the Caco-2 cell were not observed (*Table 1*). However, the hydrocarbon carotenoids in micelles generated from in vitro digestion of the cooked fresh vegetables were less stable in the tissue culture environment (*Table 2*) than previously observed with the aqueous fraction from the baby food meal.[12] Wei et al.[37] also reported that BC solubilized in tetrahydrofuran is unstable in tissue culture medium. Because tissue culture media such as DMEM are devoid of antioxidants,[38] we assumed that such losses occurred by spontaneous oxidation or the presence of pro-oxidants from foods in the aqueous fraction. The addition of 500 μmol/L α-tocopherol, but not ascorbate, to medium significantly improved the stability of all four carotenoids. It is possible that LYC itself may have protected the other carotenoids against oxidative modification because it demonstrates the highest antioxidant activity of all carotenoids in organic solvents.[39]

The results from this study further support the usefulness of the in vitro digestion procedure as a simple model for screening the relative bioavailability of the numerous carotenoids in various plant foods. The in vitro digestion system may be particularly useful for providing insights about appropriate traditional foods for improving the vitamin A status in populations that rely on fruits and vegetables as their primary source of this vitamin. Similarly, judicious use of the in vitro digestion model and the Caco-2 cell line should provide new insights about the uptake and transfer of dietary carotenoids and their metabolites across the intestinal epithelium.

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