Development of an in Vitro Digestion Method To Assess Carotenoid Bioavailability from Meals

Dean A. Garrett, Mark L. Failla,* and Robert J. Sarama†

Graduate Program in Nutrition, The University of North Carolina at Greensboro, Greensboro, North Carolina 27412

The objective of this study was to develop a model for assessing the bioavailability of carotenoids from meals using an in vitro digestion procedure. A meal was prepared using baby food carrots, spinach, and a meat, plus tomato paste. The aqueous fraction was isolated from digesta to determine the quantity of carotenoids transferred from the food to micelles. The micellarization of lutein (25−40%) exceeded ($p < 0.01$) that of α- and β-carotene (12−18%) and lycopene (<0.5%). Micellarization of carotenoids was not affected by elimination of the gastric phase of the digestive process. The absence of bile extract prevented the transfer of carotenoids from foods to micelles, whereas omission of pancreatin only reduced the micellarization of the carotenes. Differentiated cultures of Caco-2 human intestinal cells accumulated 28−46% of micellarized carotenoids from the medium after 6 h. These results support the usefulness of the in vitro digestion process as a rapid and cost-effective model for screening the bioavailability of carotenoids from meals.

Keywords: *In vitro digestion; carotenoid bioavailability; lutein; carotenes; Caco-2 cells*

INTRODUCTION

Epidemiological studies have consistently demonstrated that the consumption of diets rich in vegetables and fruits is associated with decreased risks of certain forms of cancer and cardiovascular diseases (Willet, 1994; Kohlmeier and Hastings, 1995; Zeigler, 1996). Carotenoids represent one group of phytochemicals that have received much attention as possible promoters of health since some possess antioxidant and pro-vitamin A activities (Flagg et al., 1995; Mayne, 1996). Although commonly consumed foods are likely to provide 50−100 of these plant pigments (Chug et al., 1993), past research has focused primarily on β-carotene (BC), a carotenoid that is abundant in plasma and serves as a major source of retinol.

Home gardening has been promoted as a means of supplying carotenoid-rich fruits and vegetables to meet the vitamin A requirement of populations in which there is limited availability of animal products (Bloem et al., 1996). However, the bioavailability of BC from foods is low compared to that from supplements (Brown et al., 1989; de Pee et al., 1995; Zhou et al., 1996). Previous studies also have shown that the bioavailability of carotenoids from meals is influenced by a number of factors in addition to carotenoid content, including the manner in which the foods are processed and cooked, as well as the content of lipid and fiber in the meal (Rock et al., 1992, 1998; Stahl and Sies, 1992; Erdman et al., 1993; Shiau et al., 1994).

Carotenoid bioavailability in humans generally has been assessed by monitoring the appearance of the carotenoid in plasma following the ingestion of either the purified carotenoid, a carotenoid-enriched extract of a natural source, or a carotenoid-rich meal (Micozzi et al., 1992; de Pee et al., 1995; Oshima et al., 1997). This approach is useful, although it fails to indicate the actual amounts of the ingested carotenoids that have been absorbed and metabolized (Bowen et al., 1993). Such information can be obtained from studies with the ferret and preruminant calf, since their intestinal metabolism and absorption of BC are similar to those in humans (Wang et al., 1992; Hoppe et al., 1996). However, the specialized needs of these animals limits their widespread use. The development of in vitro models for assessing carotenoid bioavailability from foods appears to provide a cost-effective alternative (Parker, 1996).

The primary objective of the present studies was to characterize the micellarization of carotenoids from a test meal using an in vitro digestion method. This technique has been used effectively for assessing the bioavailability of iron (Miller et al., 1981; Gangloff et al., 1996), phosphorus (Liu et al., 1998), amino acids (Lindberg et al., 1998), cholesterol (Fouad et al., 1991), and vitamin B6 (Ekanayake and Nelson, 1986). Baby foods (i.e., carrots, spinach, and chicken) were selected for the test meal because they are precooked and pureed to a relatively homogeneous consistency, which increases the surface area of the food available to the digestive process. These characteristics enhance the release of the carotenoid from the food matrixes during digestion (Erdman et al., 1993; Rich et al., 1998; van het Hoff et al., 1998). To validate that the carotenoids transferred to the aqueous (i.e., micellar) fraction during the in vitro digestion actually represented a measure of their bioavailability, we also examined the uptake of the micellarized carotenes and lutein (LUT) by cultures of Caco-2 human intestinal cells. This cell line spontaneously differentiates at confluency into polarized cells

* To whom correspondence should be addressed at the Graduate Program in Nutrition, University of North Carolina, P.O. Box 26170, Greensboro, NC 27412-6170. Phone: (336) 334-5313. Fax: (336) 334-4129. E-mail: mlfailla@uncg.edu.

† Winton Hill Technical Center, The Proctor & Gamble Co., Cincinnati, OH 45224.

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with enterocyte-like characteristics (Pinto et al., 1981; Hidalgo et al., 1989).

MATERIALS AND METHODS

Supplies. Gerber stage 2 commercial baby foods (spinach, carrots, chicken, ham, and beef), Wesson vegetable oil, and Kroger brand tomato paste were purchased from a local grocery. Whenever possible, the identical lot number of a specific product was purchased. Dulbecco's minimum essential medium (DMEM), fetal calf serum, L-glutamine, amphotericin B, gentamicin, nonessential amino acids, sodium bicarbonate, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pepsin, pancreatin (porcine), bile extract (porcine), and sodium taurocholate were purchased from Sigma Chemical Co. (St. Louis, MO). L-[4,5-^{3}H]leucine (specific activity 171 Ci/mmol) was purchased from Amersham Life Sciences Inc. (Arlington Heights, IL). Alamar blue dye was obtained from Alamar Biosciences, Inc. (Sacramento, CA). All other reagents and materials were purchased from Fisher Scientific Co. (Norcross, GA).

Preparation of Baby Food Meal. The foods for the standard meal were selected to provide sources of lutein (LUT), lycopene (LYC), α-carotene (AC), β-carotene (BC), and lipid. The meal was prepared by combining 1 g of creamed spinach, 0.7 g of carrots, 0.3 g of tomato paste, and 2 g of chicken (6% fat by weight) with 32 mL of saline containing 150 μmol/L butylated hydroxytoluene (BHT). In one set of experiments, the chicken was replaced with an equivalent amount of either ham or beef to provide a similar percentage of fat (approximately 3%) in the meal. In a parallel experiment, vegetable oil (120 μL) was substituted for meat to provide 3% fat by weight in the meal. When the uptake of micellarized carotenoids by Caco-2 cells was examined, the quantities of vegetables in the meal were increased 3-fold and the amount of chicken was kept constant (i.e., 2 g), to increase the concentration of carotenoids delivered to cultures. Ingredients were mixed by stirring with a glass rod before the meal was homogenized (Tekmar Tissumizer, Cincinnati, OH) for 30 s at a setting of 50 and microwaved (GE Spacemaker III) at a setting of high for 25 s. The meal was cooled for approximately 2 min before the heating step was repeated.

In Vitro Digestion. The standard procedure was a modification of that described by Miller et al. (1981). Briefly, the homogenized meal was cooled on ice prior to acidification (pH 2) with 1 M HCl and the addition of 2 mL of porcine pepsin (40 mg/mL in 0.1 M HCl). The homogenate was transferred to a clean amber bottle and incubated at 37 °C in a water bath (Precision Scientific Instruments; Shal. Form Model, Chicago, IL) shaking at 95 rpm for 1 h. Next, the pH of the partially digested meal was raised to 5.3 by adding 1.0−1.3 mL of 0.9 M sodium bicarbonate followed by the addition of a mixture of bile extract and pancreatin (9 mL containing 2 mg/mL pancreatin and 12 mg/mL bile extract in 100 mmol/L sodium bicarbonate solution). Final concentrations of pancreatin and bile extract in the reaction mixture were 0.4 and 2.4 mg/mL, respectively. The pH of each sample was increased to 7.5 by the addition of 1 N sodium hydroxide, 10 mL aliquots of the incompletely digested meal were transferred to three amber glass bottles, and the material was overlaid with a blanket of argon before the bottles were sealed. Samples were incubated in the shaking water bath (95 rpm) at 37 °C for 2 h to complete the intestinal phase of the in vitro digestion process.

To determine the impact of the digestion process on the recovery of carotenoids from meals, a set of standard meals was homogenized in a volume of saline equivalent to the total volume of the reaction mixture (46 mL) at the completion of the digestion process and extracted to determine the carotenoid levels. The concentration of each carotenoid in the digesta was greater than 95% of the levels present in the test meal ($p > 0.01$).

Isolation of the Aqueous/Micellar Fraction from Digesta. The aqueous fraction that contained micelles was separated from a residual oil droplet and particles of food using the method of Hernell et al. (1990). Aliquots (6.5 mL) of each digestate were transferred to ultracentrifuge tubes (Beckman Life Sciences, Brea, CA) that were placed in a 50.3 Ti rotor and centrifuged at 167000*g* at 4 °C for 95 min (Beckman L7-65 Ultracentrifuge, Beckman Instruments, Palo Alto, CA). The aqueous fraction was collected from the centrifuge tubes using an 18 gauge needle attached to a 10 mL syringe. In initial experiments, aqueous samples were transferred directly to a clean amber glass bottle. In subsequent experiments (see the Results), the aqueous fraction was passed through a filter with 0.22 μm pores (Gelman Sciences, Ann Arbor, MI) during the transfer process. Aliquots of filtrate and digesta were stored at −20 °C under a blanket of argon until analysis.

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage number 19, and stock cultures were maintained as previously described (Han et al., 1994). The complete medium contained high glucose DMEM (D7777; Sigma Chemical Co.), 10% heat-inactivated fetal bovine serum (FBS), nonessential amino acids (10 mL/L medium; M7145), L-glutamine (2 mmol/L), amphotericin B (0.5 mg/L), gentamicin (50 mg/L), HEPES (15 mmol/L), and sodium bicarbonate (44 mmol/L). Cells were grown in 12-well plastic dishes (Beckton Dickinson Labware, Franklin Lakes, NJ) in a humidified atmosphere of air/CO_2 (95:5 v/v) at 37 °C. Fresh complete medium was added every 2 days. Cultures were used for experiments between 11 and 14 days postconfluency, since previous studies have shown that Caco-2 cells exhibit maximum differentiation during this period as assessed by the activities of the marker enzymes alkaline phosphatase and sucrase (Ellwood et al., 1993).

Cellular Uptake of Micellar Carotenoids. Experiments were performed using Caco-2 cultures between the 23rd and 37th passages. Monolayers were washed twice with 1 mL of Hank's balanced salts solution (HBSS) before addition of 1 mL of the test medium containing the aqueous fraction from the in vitro digestion procedure that was diluted 1:3 (v/v) with basal DMEM. Cultures were incubated at 37 °C and harvested at indicated times. After spent medium was removed, monolayers were washed three times with HBSS containing 5 mmol/L sodium taurocholate at 22 °C and collected in 1 mL ice-cold phosphate-buffered saline containing 10% (v/v) ethanol and 45 μmol/L BHT. Samples were stored at −20 °C under a blanket of argon for a maximum of 2 days before analysis.

Evaluation of the Potential Cytotoxicity of the Aqueous Fraction of Digested Meals. Prior to examining the uptake of micellar carotenoids derived from the in vitro digestion of carotenoid-rich meals, we evaluated the potential cytotoxicity of the diluted aqueous fraction on cultures of Caco-2 cells. Gross morphological appearance of the monolayer, cell viability as assessed by trypan blue exclusion, and the number of domes per microscopic field (an indicator of basolateral Na, K-ATPase activity; see Hidalgo et al. (1989)) were similar in differentiated cultures incubated for 6 h in either saline diluted 1:3 (v/v) with basal DMEM or the aqueous fraction separated from digesta and diluted 1:3 (v/v) with basal DMEM. The following biochemical activities were also monitored.

Uptake and Incorporation of [^{3}H]Leucine by Caco-2 Cells. Test media were removed from cultures after 6 h, and the monolayers were washed twice with DMEM before addition of 1 mL of DMEM containing 1 μCi [^{3}H]leucine to each well. After incubation at 37 °C for 30 min, the medium was removed and the monolayers were washed twice with cold HBSS. Cellular material was scraped into 1 mL of phosphate-buffered saline containing 10% ethanol and 45 μmol/L BHT. The cells were homogenized for 5−10 s (Sonic Dismembrator, Fisher Scientific Co.), and an aliquot (400 μL) was mixed with ice-cold trichloroacetic acid (TCA; 306 mmol/L) and vortexed. Samples were incubated at 0 °C for 15 min before centrifugation at 16000*g* for 10 min to pellet-precipitated [^{3}H]proteins. The protein pellet was washed with TCA (306 mmol/L) and solubilized in 0.5 mL of 1 mol/L sodium hydroxide. Aliquots (200 μL) were added to 3 mL of Ultima Gold scintillation fluid (Packard Instrument Co., Meriden, CT) and analyzed by liquid scintillation spectrophotometry (Beckman LS 6000 SE; Beck-

man Instruments, Fullerton, CA) to quantify [^{3}H]protein. Aliquots of the cell sonicate were also analyzed to determine total ^{3}H.

Mitochondrial Activity. The reduction of the Alamar blue dye by cultured cells provides an indicator of mitochondrial activity (Ahmed et al., 1994). Monolayers exposed to either control or micelle-containing medium for 6 h were washed before the addition of 1 mL of phenol red-free DMEM containing 10% Alamar blue dye. Cultures were returned to the incubator, and aliquots (200 μL) were removed every 15 min and replaced with a similar volume of fresh medium. The absorbance of the test samples was corrected by subtracting A_{520} (nm) of an aliquot of the medium from cell-free wells from A_{520} (nm) of the medium removed from wells with cells after identical lengths of time.

Protein Assay. The protein content in the cells was determined by the bicinchoninic acid method (Pierce, Rockford, IL).

Carotenoid Extraction and Analysis. The preparation of meals and processing of all samples were performed under yellow lighting to minimize the photodecomposition of the carotenoids. Frozen samples were thawed, and cell homogenates were sonicated (Sonic Dismembrator, Fisher Scientific Co.) at a setting of 3 for 10 s on ice. Aliquots (500 μL) of the samples were combined with 500 μL of ethanol containing 0.5 μmol/L echinenone (recovery standard) and extracted three times with 1 mL of acetone. A 1 mL sample of distilled water was added to the pooled acetone extracts before the samples were re-extracted into 2 mL of hexane three times. The pooled hexane extract was evaporated under a stream of nitrogen. Dried samples were reconstituted in 500 μL of mobile phase and analyzed by reversed-phase high-performance liquid chromatography (HPLC; Hewlett-Packard, Model HP-1090, Avondale, PA) using a UV/vis detector with wavelength set at 450 nm. Carotenoids were separated using a BF-C18/C4 phase analytical column with 5 μm particles (25 cm × 0.46 cm; ES Industries, West Berlin, NJ) that was protected by a C-18 Adsorbosphere guard column with 5-μm particles (0.75 × 0.46 cm; Alltech, Deerfield, IL). Analytes were eluted from the column with a mixture 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 (mobile phase) at a flow rate of 1.5 mL/min (Martin et al., 1996). Results were calculated from a multilevel calibration table generated using a series of external standards, after correction for extraction efficiency based on the recovery of echinenone.

Statistical Analysis of Data. Data represent the mean ± SEM. For studies characterizing the in vitro digestion procedure, three separate meals were prepared and treated as indicated for each experiment. Experiments were repeated at least once to provide a minimum of six independent observations for each factor examined. For cellular studies, the diluted aqueous fraction from each digestate was added to three replicate wells with (carotenoid accumulation and cellular integrity) or without (micellar carotenoid stability) differentiated monolayers of Caco-2 cells. The mean response of three wells was treated as a single observation, and 6−9 such experiments were conducted to generate the mean ± SEM. The data were analyzed using student's *t*-test (Figures 1 and 2; Table 1), one-way analysis of variance (ANOVA; Figures 3 and 4), analysis of covariance (Figures 5 and 6), and repeated measures analysis and student's *t*-test as appropriate (Table 2). The Tukey−Kramer honestly significant difference test (HSD) was used as a post-hoc comparison of statistical significance. All statistical analyses were performed using the JMP statistical software program (SAS Institute, Cary, NC). Statistical significance was set at an α level of $p < 0.01$.

RESULTS

Characterization of the in Vitro Digestion of Carotenoid-Rich Meal. The concentrations of lutein (LUT), lycopene (LYC), α-carotene (AC), and β-carotene (BC) present in the total digestate were 0.83 ± 0.01, 1.95 ± 0.03, 0.93 ± 0.01, and 1.96 ± 0.02 μg/mL, respectively. All four carotenoids were detected in the aqueous fraction following in vitro digestion, although the efficiency of their transfer from the foods differed. The relative level of LUT in the aqueous fraction (26.0 ± 0.4%) exceeded that of the carotenes (16.0 ± 0.5%) and LYC (5.1 ± 0.2%) (Figure 1). To ensure that the carotenoids in the aqueous fraction were actually in micelles, the aqueous fraction was passed through a filter with 0.22 μm pores (Wang et al., 1994). Filtration did not alter the quantities of LUT, AC, and BC ($p < 0.01$), whereas the quantity of LYC in the filtrate was decreased to less than 0.5% that in the digesta (Figure 1). Aqueous fractions were filtered in all subsequent experiments. Results will not be presented for LYC in light of the very low micellarization of the carotenoid in the test meal.

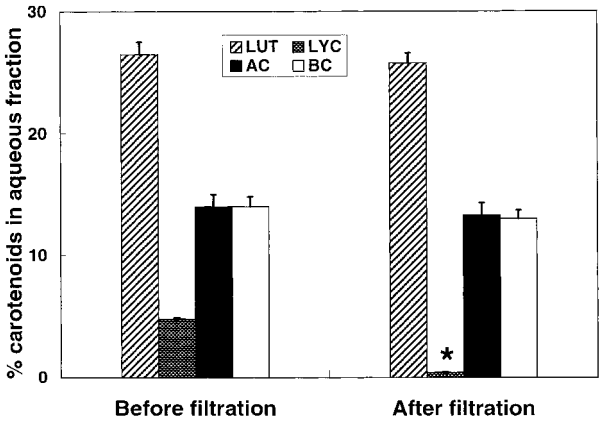

Figure 1. Effect of filtration on the carotenoid content of the aqueous fraction after in vitro digestion of the test meal. The aqueous fraction was collected after centrifugation of the digestate and extracted either before or after passage through a filter with 0.22 μm pores. The quantities of carotenoids present in extracts from the total digestate and its aqueous fraction were determined by HPLC as described in the Materials and Methods. Values were expressed as the percentage of the total carotenoid (digestate) present in the aqueous fraction. Data represent the mean ± SEM from three separate experiments with three replicate samples per experiment ($N = 9$). An asterisk over the error bar denotes that the lycopene content differed significantly ($p < 0.01$) in pre- and postfiltration samples. The levels of lutein and carotenes in pre- and postfiltered samples were similar.

Next, we examined the contributions of the gastric and intestinal phases of the in vitro digestion procedure to the transfer of carotenoids from the test meal to the aqueous fraction. Carotenoids were not detected in the filtered aqueous fraction when the digestive materials, i.e., HCl, pepsin, bicarbonate, bile extract, and pancreatin, were deleted from the mixture (data not shown). When the digestion process was limited to the gastric phase (i.e., HCl and pepsin for 1 h), insignificant levels (<1%) of carotenoids were present in the neutralized aqueous fraction (data not shown). When digestion of the test meal was initiated at the intestinal phase (i.e., pancreatin and bile extract added to homogenized meals at neutral pH), the percentages of LUT, AC, and BC transferred to the aqueous fraction did not differ significantly ($p > 0.01$) from that in samples subjected to the complete digestion process (Figure 2).

The central role of bile extract in the micellarization of hydrophobic compounds such as carotenoids is well documented (Erdman et al., 1993; Parker, 1996). The contribution of bile extract on the transfer of carotenoids

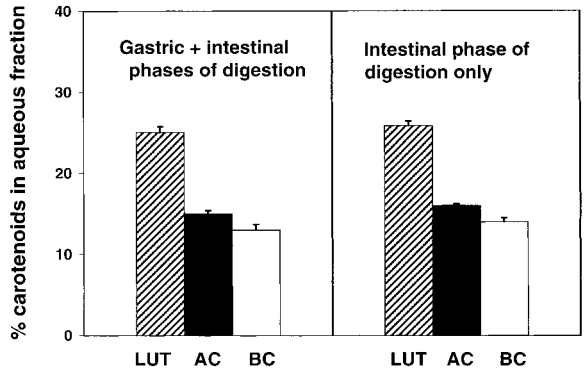

Figure 2. Contributions of the gastric and intestinal phases of the in vitro digestion process to the transfer of carotenoids from the test meal to the aqueous fraction. Digestion of the homogenized meal was initiated at either the gastric or the intestinal phase. When the impact of the gastric phase only was assessed, acidic samples were neutralized with $NaHCO_3$ prior to centrifugation and extraction. The amount of each carotenoid recovered in the aqueous fraction was expressed as a percentage of the respective carotenoid in the digesta. Data are the mean ± SEM from two separate experiments with three replicates per experiment ($N = 6$).

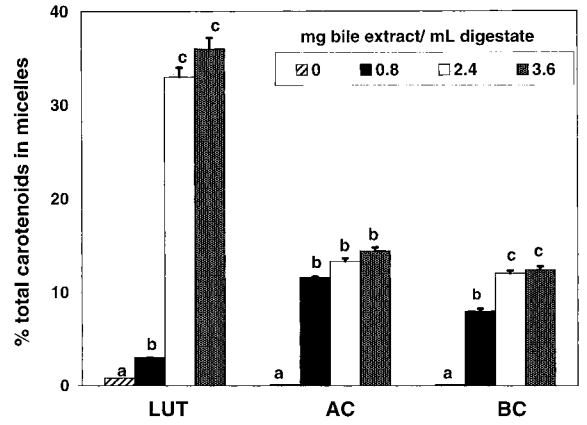

Figure 3. Effect of bile salt content on the transfer of carotenoids to the aqueous phase during the in vitro digestion of the test meal. The test meal was digested as described in the Materials and Methods except that the concentration of bile extract in the reaction mixture was adjusted to 0, 0.8, 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 filtrate from the aqueous fraction after centrifugation was quantified by HPLC and expressed as a percentage of that present in the digesta. Data are the mean ± SEM from three experiments with three replicate samples for each experiment ($N = 9$). Different letters above the error bars denote that mean levels of the indicated carotenoid differ significantly ($p < 0.01$) in response to the amount of bile extract in the digestate.

from the baby food meal to the aqueous fraction during in vitro digestion was investigated by titrating the quantity of bile extract added to the mixture during the intestinal phase from 0 to 3.6 mg/mL. The concentration of bile extract added to the standard mixture was 2.4 mg/mL. Omission of bile extract during the intestinal phase of digestion resulted in the absence of detectable levels of carotenes and the transfer of less than 1% of LUT from the meal to the aqueous fraction by the end of the digestion procedure (Figure 3). The quantities of the three carotenoids in the aqueous phase were significantly increased ($p < 0.01$) when 0.8 mg/mL bile extract was present during the intestinal phase of digestion, although the magnitude of the increase was greater for the carotenes than for LUT. Further elevation of the amount of bile extract in the digestate to 2.4 mg/mL was associated with a marked increase in the level of LUT in the aqueous fraction ($p < 0.01$), whereas the quantities of AC and BC levels in the aqueous phase increased only slightly. The concentrations of the carotenoids in the aqueous fraction were similar ($p > 0.01$) when the quantity of bile extract in the reaction mixture was either 2.4 or 3.6 mg/mL.

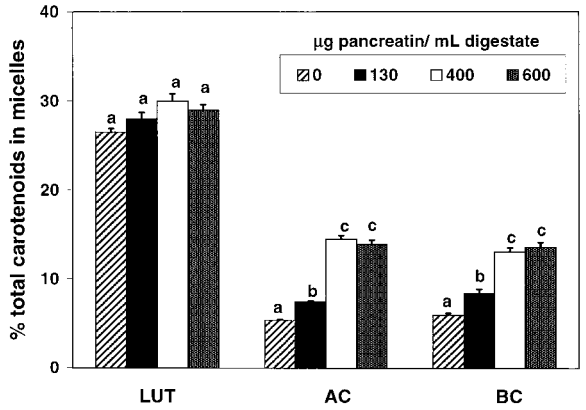

Figure 4. Effect of pancreatin content on the micellarization of carotenoids during in vitro digestion. The quantity of pancreatin present in the digestion mixture was adjusted to 0, 0.13, 0.40, or 0.60 mg/mL; the pancreatin content in the standard procedure was 0.4 mg/mL. Carotenoids were extracted from the digestate and the filtered aqueous fraction following centrifugation and analyzed as described in the Materials and Methods. The data represent the mean ± SEM from three experiments with three replicate samples per experiment ($N = 9$). Different letters over the error bars for each carotenoid denote that the means differed significantly ($p < 0.01$) in response to pancreatin content.

The amount of pancreatin added to the reaction mixture for the intestinal phase was varied from 0 to 0.6 mg/mL to assess the role of pancreatic enzymes on the in vitro transfer of the carotenoids from the test meal to the aqueous fraction. The concentration of pancreatin in the standard digestion mixture was 0.4 mg/mL. The levels of AC and BC transferred to the aqueous fraction decreased by approximately 50% when pancreatin was not included in the intestinal phase of the digestion procedure (Figure 4). The amounts of the carotenes present in the aqueous fraction increased significantly ($p < 0.01$) when the pancreatin content was elevated to 0.13 mg/mL. Elevating the pancreatin level to either 0.4 or 0.6 mg/mL further increased the percentage of carotenes transferred to the aqueous fraction ($p < 0.01$). Surprisingly, the absence of pancreatin in the digestion mixture did not significantly ($p > 0.01$) alter the percentage of LUT recovered in the aqueous fraction (Figure 4).

To examine the possible effect of the lipid source on carotenoid micellarization, four meals containing the mixture of baby food vegetables and chicken, beef, ham, or vegetable oil as the lipid source were digested. The fat content of each test meal was similar, i.e., 3 wt %. The percentage of LUT micellarized was significantly ($p < 0.01$) higher than that of the carotenes regardless of the lipid source (Table 1). Replacing chicken with similar quantities of either ham or beef increased the amount of LUT transferred to the aqueous fraction by 30−40% ($p < 0.01$). Substitution of meat with vegetable oil also markedly increased the micellarization of LUT ($p < 0.01$). In general, the levels of carotenes (Table 1) and LYC (data not shown) in the aqueous fraction were not markedly affected by the source of fat.

Table 1. Effect of Lipid Source on the Micellarization of Carotenoids during in Vitro Digestion of Meals[a]

lipid source	LUT[b]	AC[b]	BC[b]
chicken	27 ± 0.5[a]	15 ± 0.5[b]	13 ± 0.7[a]
ham	39 ± 0.9[c]	11 ± 0.3[a]	13 ± 0.3[a]
beef	35 ± 0.6[b]	12 ± 0.2[a]	13 ± 0.2[a]
vegetable oil	61 ± 1.0[d]	11 ± 0.4[a]	16 ± 0.4[b]

[a] Meals were prepared with chicken, ham, beef, or vegetable oil as a source of lipid and digested as described in the Materials and Methods. The fat content of all meals ranged from 2.5% to 3.0% by weight. Carotenoids in the digestate and the filtered aqueous fraction were extracted and quantified as described in the Materials and Methods. The data represent the mean ± SEM from three independent experiments with three replicate test meals digested samples per experiment ($N = 9$). The percentage of LUT transferred to the micellar fraction was significantly ($p < 0.01$) greater than those of AC and BC regardless of the lipid source for the meal. The presence of different letters as superscripts within a column indicates that the mean percentages of the indicated carotenoid present in the micellar fractions differ significantly ($p < 0.01$). [b] Percent transferred to the micellar fraction.

Uptake of Micellar Carotenoids by Caco-2 Cells. Prior to conducting experiments to assess Caco-2 uptake of micellarized carotenoids derived from the in vitro digestion of meals, we examined the possible cytotoxicity of the aqueous fraction on the Caco-2 monolayer. These studies did not reveal any differences in gross morphological appearance, cell viability, the number of domes per microscopic field, or protein content per well in cultures incubated in either DMEM with 25% saline or DMEM containing 25% aqueous fraction from meals (Table 2). Additional experiments examined the possible influence of the micellar fraction on the uptake and incorporation of the radiolabeled leucine. Exposure to the diluted micellar fraction did not alter [^{3}H]leucine uptake, and actually increased ($p < 0.01$) the incorporation of [^{3}H]leucine into protein above that of cultures incubated in the control medium (Table 2). Moreover, mitochondrial activity was similar in control and test cultures as assessed by the reduction of Alamar blue dye (Table 2).

We also examined the stability of carotenoids in the aqueous fractions in a cell-free, tissue culture environment. In general, the carotenoids derived from the digestion of meals prepared from baby foods were stable (Figure 5). After 6 h of incubation, 92.0 ± 1.3% and 86.0 ± 3.0% of the LUT and BC were recovered from the medium, respectively. Recovery of AC was similar to that of BC after 2 and 4 h of incubation, but declined significantly ($p < 0.01$) to 65.0 ± 2.4% of the initial level after 6 h.

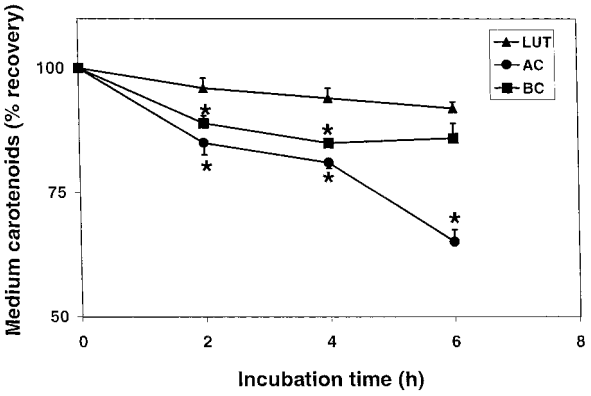

Figure 5. Stability of micellar carotenoids generated by in vitro digestion of the meal in a cell-free tissue culture environment. The aqueous fraction was diluted with 3 volumes of basal DMEM and incubated in cell-free tissue culture dishes incubated in a humidified chamber with 95% air/5% carbon dioxide at 37 °C. The medium was collected and extracted as described in the Materials and Methods to quantify the amount of each carotenoid present after 0, 2, 4, and 6 h. Data are the mean ± SEM of two separate experiments with three replicates for each experiment. An asterisk above or below the error bars indicates that the percentage of carotenoid recovered differs significantly ($p < 0.01$) from that in the test medium prior to incubation in a cell-free environment (i.e., at 0 h).

Next, the uptake of micellar carotenoids present in the aqueous fraction from digested meals by differentiated monolayers of Caco-2 cells was investigated. The levels of LUT, AC, and BC in the test medium were 300 ± 5, 20 ± 0.6, and 120 ± 4 nmol/L, respectively. The control medium did not contain detectable levels of the carotenoids (<1 nmol/L). Monolayers accumulated carotenoids in a linear manner from the medium for up to 6 h. The cellular content of LUT, AC, and BC after 6 h was 76.0 ± 2.3, 9.2 ± 0.3, and 33.0 ± 0.5 pmol/mg protein, respectively. Since the concentrations of the respective carotenoids in the test medium differed, results presented in Figure 6 represent the percentage of medium carotenoid present within Caco-2 cells at the indicated times. The percentage of medium AC that was present in cells after 6 h was significantly greater ($p < 0.01$) than for LUT and BC, respectively.

DISCUSSION

Here we report the development of a relatively simple and cost-effective in vitro procedure to estimate the bioavailability of carotenoids from a meal. A standard meal was formulated using highly processed baby food vegetables and chicken. The baby foods were selected

Table 2. Characterization of Caco-2 Cultures Treated with Control (DMEM + 25% Saline) and Test (DMEM + 25% Micellar Fraction from in Vitro Digested Meal) Material for 6 h[a]

characteristic	DMEM + 25% saline	DMEM + 25% micellar fraction from digested meal
cell viability	95.0 ± 1.3	94.7 ± 0.8
protein content per well (mg)	1.31 ± 0.03	1.28 ± 0.01
no. of domes per microscopic field[b]	2.2 ± 0.14	2.1 ± 0.15
[^{3}H]LEU uptake (dpm/30 min/mg of protein)	20811 ± 621	21324 ± 846
[^{3}H]LEU incorporated into protein (% acid ppt ^{3}H)	9.4 ± 0.3[a]	12.6 ± 0.4[b]
mitochondrial activity[c] (ΔA_{520}/30 min/well)	0.21 ± 0.005	0.23 ± 0.009

[a] Listed characteristics were assessed as described in the Materials and Methods after exposure of cultures to control (75% DMEM plus 25% saline) or test (75% DMEM plus 25% micellar fraction from digested meal) medium for 6 h. The results represent the mean ± SEM from either two or three experiments for each indicator. Different letters as superscripts within a row indicate that the means differ statistically ($p < 0.01$). [b] Domes are indicative of basolateral Na,K-ATPase activity in polarized cells (Hidalgo et al., 1989). Cultures were examined at 100× magnification. [c] Mitochondrial activity was monitored by the rate of reduction of Alamar blue as described in the Materials and Methods.

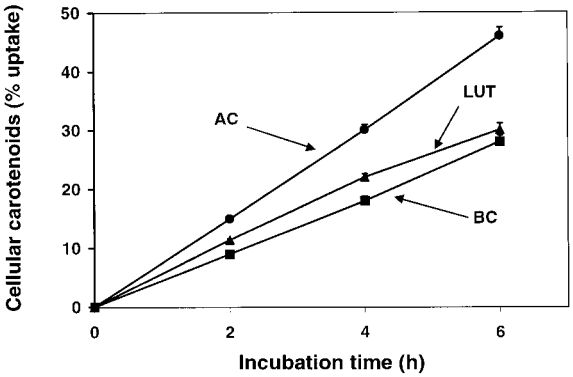

Figure 6. Uptake of micellar carotenoids by Caco-2 cultures. Cultures were incubated in DMEM with the aqueous fraction from the digested meal (3:1 v/v) for indicated periods. Cellular levels of carotenoids were determined as described in the Materials and Methods. Data represent the mean ± SEM of three experiments with three replicate samples per experiment ($N = 9$). The uptake of AC was greater ($p < 0.01$) than those of LUT and BC. LUT and BC did not differ significantly ($p > 0.01$).

for this initial series of experiments since they were precooked and pureed, thereby providing a large surface area to facilitate the digestion and micellarization process (Erdman et al., 1993). Spinach, carrots, and tomato paste were the primary sources of LUT, AC, and LYC, respectively. The meat was added as a source of fat for the formation of the oil droplet, a likely reservoir for lipophilic phytochemicals before transfer to mixed micelles (Parker, 1996; Furr and Clark, 1998). Prior to the in vitro digestion procedure being initiated, the meal was heated briefly in a microwave since this represents a typical activity for preparing these foods for infants. Carotenoids do not appear to be destroyed by microwaving foods (Chen and Han, 1990). Our initial results showed that the percentages of the carotenoids of interest transferred from the food to the aqueous fraction of the digestate varied. LUT was more readily solubilized than the carotenes and LYC. Since all manipulations of the starting meal were identical, this differential transfer of these carotenoids is likely due to the greater hydrophilicity of oxycarotenoids than hydrocarbon carotenoids and differences in their subcellular locations and molecular interactions in plant foods. The preferential micellarization of LUT in the in vitro system is in line with several previous observations. Castenmiller et al. (1999) reported that the bioavailability of LUT was greater than that of the carotenes in a human feeding trial using spinach as the carotenoid-rich food. It is interesting to note that LUT and zeaxanthin are located in chloroplasts complexed with chlorophyll and are readily released by polar solvents and detergents (Kirk, 1978). In contrast, the carotenes in carrot root are encased in a thick, membranous sheet comprised of large proteins, making their transfer to the oil droplet less likely than that of LUT (Erdman et al., 1993). The location of the carotenoid within the oil droplet that forms during digestion is affected by its polarity and probably represents another factor that influences transfer to the mixed micelle. Hydrocarbon carotenoids are buried in the core of the oil droplet, whereas oxycarotenoids such as LUT reside near the surface (Borel et al., 1996). This difference likely facilitates the transfer of LUT into micelles. It is important to note that the residual oil droplet was negligible at the end of the digestion process in our in vitro system.

Micellarization of LYC in our model was extremely poor. Several investigators have reported that plasma levels of LYC were not altered after ingestion of a single meal of tomato juice (Brown et al., 1989; Stahl and Sies, 1992). It is interesting that plasma LYC content was increased after ingestion of a single meal of tomato juice that had been boiled with 1% corn oil for 1 h (Stahl and Sies, 1992). Although heating the baby food meal containing tomato paste in a microwave failed to increase the transfer of LYC to micelles in our study, we have found that cooking (stir-fried at 177 °C for 4 min) the tomato paste with fresh vegetables and oil increased the micellarization of LYC during in vitro digestion 8-fold (manuscript in preparation).

We designed a series of studies to characterize the effects of various factors on the micellarization of carotenoids in the in vitro model. These include the impact of the gastric phase of digestion, the contributions of bile extract and pancreatin, and the lipid source on carotenoid micellarization. First, we examined the contribution of the digestive phases in the subsequent micellarization of carotenoids from the test meal. Surprisingly, the micellarization of LUT and carotenes was similar when the meal was subjected to either only the intestinal phase of digestion or the standard procedure that subjects the test meal to both the gastric and intestinal phases of digestion. This finding is similar to the report by Grolier et al. (1998) who found that treatment with omeprazole, an inhibitor of the gastric H^+-pump, did not alter plasma retinol and hepatic levels of BC and AC in rats fed a diet with carrot extract for several weeks. In contrast, Tang et al. (1996) reported that inhibition of gastric acid secretion by omeprazole reduced the serum concentrations of BC in a group of volunteers fed a single meal supplemented with water miscible BC. It also has been reported that an acidic environment favors the in vitro transfer of BC from carrot juice to vegetable oil (Rich et al., 1998). Differences in the source of BC (crystalline vs food source) and method of preparation of test food (i.e., cooked and pureed vs raw) likely contribute to the above discrepancies. It is interesting to note that although achlorohydria is relatively common in the elderly (Saltzman et al., 1994; Tang et al., 1996), several teams have reported that carotenoid absorption is not necessarily decreased with advanced age (Maiani et al., 1989; Brady et al., 1996).

The combination of bile salts and pancreatic enzymes is essential for the efficient micellarization of lipophilic species (Hofmann and Borgstrom, 1962; Monsbach et al., 1980). As expected, we found that the transfer of carotenoids to the aqueous fraction was inhibited when bile extract was omitted from the digestion mixture. The standard amount of bile extract in the in vitro assay (2.4 mg/mL) is similar to levels present in the small intestine in the fasted state, i.e., 4−6 mmol/L (Charman et al., 1997). Since elevation of the level of bile extract to 3.6 mg/mL did not enhance carotenoid micellarization in our model, the concentration of bile extract routinely used does not appear to be limiting under the defined conditions. The release of components incorporated into the triglyceride emulsion formed in the stomach for transfer to micelles also requires the activity of pancreatic colipase-dependent lipase (Tso, 1994). The similar degree of LUT micellarization in the absence and

presence of pancreatin was unexpected. However, this observation may be related to the very small particle size of the pureed foods and the low lipid content of the meal. Together, these characteristics may have facilitated transfer of carotenoids from the food matrix to micelles.

Dietary fat appears to be necessary for the efficient solubilization of lipophilic compounds. Lipids provide a sink for fat-soluble phytochemicals, drugs, and vitamins in the stomach, stimulate the release of bile salts from the gall bladder, and expand the size of the bile salt micelle (Tso, 1994; Furr and Clark, 1997). Hollander and Ruble (1978) demonstrated an increase in BC absorption in rats when fatty acids were added to a micellar perfusate containing BC. The importance of lipid in the absorption of dietary BC also has been noted in human studies. For example, Prince and Frisoli (1993) found that ingestion of water-miscible BC in the absence of fat was not associated with a detectable change in serum BC, but serum levels of BC increased 2-fold when lipid was administered with the carotenoid. Also, it has been reported that serum BC concentration increased in proportion to the lipid content of the meals (Shiau et al., 1994). We compared the effect of varying the lipid source on carotenoid micellarization. Ham, beef, or vegetable oil was substituted for chicken with the total lipid content of the meal remaining relatively constant at 3%. The change in lipid source increased LUT, but not carotene, micellarization. Further studies are needed to determine the possible influences of dietary lipid composition and perhaps meat protein on LUT micellarization.

The Caco-2 human colonic carcinoma cell line was chosen as a model system to validate the bioavailability of the carotenoids present in the aqueous or micellar fraction after completion of in vitro digestion of the meal. The Caco-2 cell exhibits many morphological and biochemical similarities with enterocytes, thereby lending itself to studies of apical uptake and metabolism of nutrients and drugs (Pinto et al., 1983; Hidalgo et al., 1989). We have reported elsewhere that differentiated cultures of Caco-2 are capable of accumulating BC and LUT from mixed micelles (Garrett et al., 1999). Here, the potential cytotoxicity of the soluble components from the digestion mixture, e.g., proteolytic and lipolytic enzymes, was a concern. Glahn et al. (1996) and Gangloff et al. (1996) have assessed iron bioavailability by evaluating iron uptake by Caco-2 cultures after the in vitro digestion of meat and dairy products. Gangloff et al. (1996) reported that the digestate itself was toxic to cells. Inactivation of the proteolytic enzymes by heat or addition of antiproteases failed to protect cell integrity. This suggested that components in the foods were toxic. These researchers successfully used a dialysis bag to protect cells from high molecular weight toxic compounds while providing access to bioavailable iron. We simply diluted the aqueous fraction with tissue culture medium to assess carotenoid uptake by cells. Pilot studies showed that micellar carotenoids were stable for 4−6 h in the tissue culture environment. Also, cells retained their morphological and biochemical integrity when exposed to the diluted micellar fraction and accumulated carotenoids in a linear manner. The ability to expose cultures of Caco-2 to the diluted aqueous fraction without any apparent toxicity may have resulted from the removal of undigested food particles and the residual oil droplet from digesta.

In conclusion, the in vitro digestion system appears to provide a useful alternative to animal and human models for rapidly screening carotenoid bioavailability from foods and meals. The ability to carefully control the in vitro environment should be useful for systematically investigating the impact of various processing methods and dietary factors on the bioavailability of carotenoids from specific foods. The application of micellarized carotenoids to Caco-2 cultures provides an additional tool for studying the intestinal metabolism of carotenoids and the transfer of these compounds and their metabolites across the basolateral membrane of the enterocyte. Finally, the coupled in vitro digestion and Caco-2 models seem to be applicable for the examination of the bioavailability of phytochemicals in addition to carotenoids.

ABBREVIATIONS USED

AC, α-carotene; BC, β-carotene; LUT, lutein; LYC, lycopene; BHT, butylated hydroxytoluene; DMEM, Dulbecco's modified eagles medium; FBS, fetal bovine serum; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography.

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