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Effect of Carrot (*Daucus carota*) Microstructure on Carotene Bioaccessibilty in the Upper Gastrointestinal Tract. 1. In Vitro Simulations of Carrot Digestion

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Studies investigating carotene bioaccessibility (release from the food matrix to a solubilized form) directly from plant material during the process of digestion are scarce, mainly due to the difficulties associated with obtaining such material. Therefore, this paper examines the relationship between tissue microstructure and carotene bioaccessibility using an in vitro digestion model. Dietary oil provides a pool for the initial solubilization. Therefore, carotene partitioning into an emulsified oil phase was assessed using raw carrot tissue and carrot tissue subjected to various degrees of heating and particle size reduction and, in all cases, was found to be greatly reduced compared with juiced carrot. Carotene bioaccessibility was found to be greater from raw tissues than heated tissues of the same size. This is because heating increases the propensity for intact cells to separate, effectively encapsulating the carotene. Although the gross structure of the tissues was found to be relatively unaffected by in vitro digestion, at the cellular level some cell-wall swelling and cell death were observed, particularly close to the surfaces of the tissue. This study suggests that cell-wall rupture prior to digestion is an absolute requirement for carotene bioaccessibility in the upper intestine and that heating does not enhance carotene release from intact cells.

KEYWORDS: In vitro digestion; carrot; *Daucus carota*; bioaccessibility; carotene; cell wall; tissue viability

INTRODUCTION

Epidemiological studies have shown that an increased consumption of foods rich in carotenoids is associated with a diminished risk of cardiovascular disease and some cancers, particularly those associated with aging (*1*, *2*). However, carotenoid bioavailability is not well understood, and the wide range of values (2−90%) given in the literature reflects the usage of different meals and model systems (*3*). In nutrition studies, the term "bioaccessibility" is defined as the fraction of a nutrient that is released from a food matrix in the gastrointestinal tract. It has a different meaning from "bioavailability", which concerns the fraction actually absorbed. By its definition, bioavailability includes bioaccessibility, and both are dependent on processing of food before ingestion and structural changes of the food during digestion (*4*). Carrots are commonly consumed worldwide in both raw and cooked forms and are considered to be a major source of β-carotene in the diet. Increasing our knowledge of carotene bioaccessibility may highlight suitable preparation and processing methods for improving bioavailability and thus bioactive effects.

Carotenoids are synthesized and sequestered in plastids. In carrot, the carotenes are in a crystalline state and are located within the equivalent of the thylakoid membrane in the chromoplasts (*5*, *6*). Such structures are known as carotene bodies and provide an extremely stable environment for the carotene.

The hydrophobic nature of carotenoids prevents their diffusion directly from a plant food to the site of absorption in the small intestine. Current thinking suggests that following release from a food matrix, carotenoids dissolve within an oil emulsion before being transferred to mixed micelles in the duodenum for absorption (*7*, *8*). There is, however, a lack of information on the solubilization pathways involved in carotenoid bioavailability, including accessibility from the plant structure. Recently, studies have begun to show that the structure of food can be a controlling factor in the bioaccessibility of these compounds, resulting in a significant proportion being unavailable for absorption (*9*). Indeed, our findings in vivo indicate that raw carrot shreds arrive relatively unchanged at the terminal ileum of humans (*10*). Further work is required to address to what extent plant architecture influences carotenoid bioaccessibility, particularly as many studies continue to use pure carotenoid preparations and highly processed or homogenized foods (*11*−*16*). Although some studies have acknowledged the importance of plant tissue structure, many have used combinations of processing treatments, which make the interpretation of results difficult (*17*, *18*). As a result, the process whereby carotenoids become bioaccessible

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differs greatly between studies. It is therefore important to determine systematically the different effects of processing (e.g., heat treatment and particle size reduction) on carotenoid bioaccessibility.

Direct investigation of food digestion within the gut poses both practical and ethical problems and is at least one reason for the lack of information in this area. Therefore, validated in vitro models of digestion are vital tools for understanding the changes to food structure during digestion. Of the in vitro models designed to investigate carotenoid bioavailability, the majority have examined the uptake of carotenoids to micelles from preloaded oils (*7*, *19*−*22*) and from preloaded micelles to Caco-2 human intestinal cells (*19*, *23*). Few studies have investigated solubilization of carotenoids directly from plant tissues into dietary lipid phases, which is believed to be the first step in their bioavailability, particularly where digestion of mixed meals is concerned (*8*, *13*, *14*).

To improve our understanding of the structural characteristics of plant tissue that determine carotenoid bioaccessibility, we have investigated the solubilization of carotene from carrot tissues into simulated gastric and duodenal oil phases. Previous work from this group (*13*, *14*) investigated the movement of carotene from carrot juice into bulk oil phases. The current work seeks to expand this by investigating bioaccessibility from a range of processed carrot tissues into emulsified oil phases of physiological relevance. We have studied the effect of particle size and surface area by measuring the partition of carotene into the oil under gastric conditions from raw carrot cubes of different sizes. Different cutting and cooking protocols have also been investigated by using carrot shreds, boiled and steamed cubes, and carrot purée. This work has been accompanied by a detailed investigation, by light microscopy, of carrot microstructure pre- and post digestion. The work provides a basis for understanding the in vivo digestion of carrot described in the accompanying paper (*10*).

MATERIALS AND METHODS

Tissue Preparation. Commercially-available washed carrots were purchased and stored at 1−2 °C in the dark for use within 2 weeks. On the day of the experiment, carrots were peeled and chopped into slices, routinely 3 mm thick. The slices were cut in half, and the inner vascular tissue was removed by hand. Only outer parenchyma tissue was used during this study. For bioaccessibility studies on carrot cubes, batches of 3 mm cubes were produced by a parallel array of double-edged razor blades 3 mm apart. Other sized cubes for surface area experiments were made by choice of appropriate thickness of slices and separation of the cutting edges. Cubes were examined microscopically for validation of size and shape. Raw grated carrot was prepared from outer parenchyma tissue using a conventional kitchen grater. Raw carrots were juiced using a Moulinex juicer (type CF1A). To remove large particles, the juice was filtered through one layer of nylon mesh of 63 μm nominal pore size (John Stanier and Co., Manchester, U.K.). A description of the juice has been presented elsewhere (*13*).

For steaming and boiling, raw carrot slices were steamed in a conventional steamer over a saucepan for 20 min or boiled in water for 20 min and then cut into cubes. Boiled carrots were mashed using a pestle and mortar until a smooth purée was formed. For the material that was to undergo digestion for bioaccessibility studies (where results are expressed as the percentage of the total carotene present), carotene was measured in the processed material. This precluded errors arising from loss of carotene from the tissue during the processing.

In Vitro Digestion. To prevent photo-oxidation of carotene, experiments were conducted whenever possible under gold fluorescent lighting (wavelength 570−580 nm). Solvents were flushed with nitrogen, and samples were prepared in amber vials and covered with a blanket of argon.

Gastric Digestion. *Emulsion Premix*. Egg L-α-phosphatidylcholine (PC), grade 1 (99% pure), was obtained from Lipid Products (Nutfield, U.K.). Solvent was removed from the PC stock solution (63.5 mM/L in chloroform/methanol) by rotary evaporation (Buchi RE 111 Rotavapor, Flawil, Switzerland). Remaining solvent was removed overnight in vacuo after the vacuum chamber had been purged with nitrogen. On the day of the experiment, dried PC was hydrated in warmed saline solution (0.15 mol/L NaCl, pH 2.5) for 30 min on an orbital shaker (200 rpm, at 37 °C) with 6−8 glass beads (3 mm diameter). The suspension of PC was sonicated in a jacketed beaker to produce single-shell liposomes using a Status Ultrasonic US200 homogenizer fitted with a titanium flat tip (12.7 mm diameter, Bandelin, Berlin, Germany) at 50% power on a 0.9 s pulse cycle for 10 min until the dispersion was transparent to the eye. The temperature was controlled by water at 1 °C circulating in the jacket. Olive oil (low acidity, Sigma, Poole, U.K.) was added to the dispersion, which was then vortexed using a benchtop Whirlimix for 1 min. This coarse emulsion was immediately passed two times through a high-pressure orifice homogenizer (Emulsiflex Type B3, Avestin Inc., Ottawa, Canada) at a pressure of 35.8 MPa to give a fine emulsion of 10% w/v olive oil stabilized by 7.1 mmol/L PC. The droplet size measured with a Coulter LS230 particle size analyzer was 3−4 μm (weight mean diameter).

Aqueous Phase. Dried PC was hydrated and sonicated as described to give a PC concentration of 7.9 mmol/L. Sufficient porcine pepsin (Sigma) was added to this bulk aqueous phase to give a final concentration of 157 U/mL of gastric fluid per sample.

Complete Gastric Mixture. A sample of 8.8 g of the aqueous phase was added to 2.2 g of carrot preparation and 2.2 g of the emulsion premix. The pH was adjusted to 2.5 with 1 mol/L HCl as necessary. A gastric lipase analogue (F-AP15, Amano Enzymes Europe Ltd., Chipping Norton, U.K.) was added to each sample to give a total concentration of 62 U/mL of gastric fluid per sample. The complete gastric mix was therefore 11.0 g of 0.15 M NaCl containing 7.8 mmol/L PC, 128 U/mL pepsin, 62 U/mL lipase, and 2% w/v olive oil with 2.2 g of carrot tissue.

All gastric digestions were carried out in the shaking incubator at 37 °C for 2 h.

Duodenal Digestion. Following gastric incubation, the pH of the samples was immediately adjusted to 6.5 with 1 mol/L NaOH and the following were added: Bis-Tris buffer, pH 6.5 (Sigma), bile salt solution containing equimolar amounts of sodium taurocholate (Sigma) and sodium glycodeoxycholate (Sigma) and $CaCl_2$ (Merck-BDH, U.K.). The bile salts and their relative proportions were chosen to mimic the hydrophobicity of bile salts in human bile (*24*). The pH was checked and adjusted to 6.5 with 1 mol/L NaOH as necessary. After the addition of porcine pancreatic lipase and colipase (Sigma), the final composition of the duodenal aqueous phase was as follows: 34 nmol/L (30 U/mL) lipase, 1160 nmol/L colipase, 7.0 mmol/L PC, 7.4 mmol/L total bile salts, 9.2 mmol/L $CaCl_2$, and 25 mmol/L Bis-Tris with a weight of 11.9 g. The duodenal digestions then proceeded for 2 h at 37 °C in the shaking incubator. Following incubation, the samples were transferred into plastic centrifuge tubes and centrifuged (17000g_{max}, 15 min, 10 °C) to separate the phases.

Lipolysis Assay. Lipolysis of the oil during the in vitro digestions was determined by titrating the released fatty acids in total digesta plus ethanol washings with 0.05 mol/L NaOH. Lipolysis was found on average to be 10% of the maximum possible under gastric conditions with a further 5% occurring during duodenal conditions. Digestion of the oil was accounted for when total carotene partitioning was calculated.

Carotenoid Analysis. Carotene concentrations in olive oil were routinely measured by diluting aliquots of the oil (20−50 μL) in 1 mL of *n*-hexane. The absorbance was measured at 450 nm using an extinction coefficient (mM/L) of 137.4 (*25*). Because the major carotenoids in carrot are α- and β-carotene (>98%) (*25*), both showing absorption at 450 nm, no distinction was made between them. The amount of carotene solubilized in oil is expressed as a percentage of the total in the tissue (as used at the start of the gastric digestion). Significant differences were assessed by Student's *t* test.

To measure total carotene content, representative processed tissue samples were roughly chopped (when necessary) and frozen with liquid nitrogen ($N_{2(l)}$). Frozen tissue was blended with further $N_{2(l)}$ in a Waring commercial blender at full power for 1 min. This was followed by homogenization in $N_{2(l)}$ with an Ultra-Turrax homogenizer (model T25) for 5 min to form a fine frozen powder dispersion. Following evaporation of the $N_{2(l)}$, the powder was stored at −20 °C prior to extraction using a modified Bligh and Dyer method (*26*). Approximately 0.2 g of carrot powder was accurately weighed into a screw-top centrifuge tube using a precooled spatula. One milliliter of methanol was added. After 15 min, with occasional mixing, 1 mL of chloroform was added and mixed vigorously.

After a further 15 min, with occasional mixing, 1 mL of ultrapure water was added and mixed well. This was followed by gentle centrifugation ($2000g_{max}$, 5 min, 20 °C) to separate the phases. The lower carotenoid-containing chloroform phase was carefully transferred into a clean dry volumetric flask. Two further 1 mL additions of chloroform were added, mixed, centrifuged, and the lower phase was collected. The combined extracts were made up to a known volume and mixed well. Carrot juice (0.2 mL) was directly extracted using the same procedure. An aliquot of the combined extracts was evaporated with nitrogen, and the carotenoids were resuspended in 1 mL of *n*-hexane for absorbance measurement at 450 nm.

Carotene concentrations in the duodenal aqueous phase were measured by HPLC. An aliquot of the aqueous phase (collected after centrifugation) was filtered through a 0.22 μm nylon filter (Nalgene) to remove debris and unsolubilized carotene. Samples were extracted by mixing 0.125 mL of the filtrate with 0.5 mL of dichloromethane (containing 0.1% BHT). The mixture was sonicated for 10 s and made up to 5 mL with acetonitrile/methanol/dichloromethane (75:20:5, v/v/v). An aliquot was centrifuged for 10 min at 12000 rpm to remove particulate material. Aliquots were placed into amber HPLC autosampler vials (Chromocol), and HPLC was conducted as described previously (*14*).

Microscopy. *Preparation of Carrot Tissue for Light Microscopy*. After in vitro digestion, approximately 20 cubes of raw, steamed, or boiled carrot were taken at random and sectioned centrally by hand with razor blades to give transverse sections ≥100 μm thick. Sections of digested grated carrot were also similarly prepared. Sections were mounted in distilled water on glass slides and covered with a coverslip. The sections, and also a sample of the puréed carrot, were examined unstained with an Olympus BX60 light microscope (Olympus, Japan) using bright field optics and photographed with a JVC 3-CCD color video camera. Images of the outer layers of the cubes and shreds were recorded using the Acquis Bio imaging program from Syncroscopy (Synoptics Ltd., Cambridge, U.K.).

Preparation of Resin-Embedded Control and Digested Carrot Tissue for Light Microscopy. Raw undigested (control) and digested (2 h gastric or 2 h gastric followed by 2 h duodenal) carrot cubes were fixed for 2 h in 3% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.2) and dehydrated in an ethanol series [10−100% (w/v)] with three changes of 100% ethanol. Tissue was infiltrated with LR White acrylic resin (Agar Scientific, Stansted, U.K.) and polymerized for 24 h at 60 °C. Sections, 1 μm thick, of the whole cross section of the cubes were cut as near as possible to the midpoint of each cube using an ultramicrotome (Ultracut E, Reichert-Jung Vienna, Austria). Sections were stained with toluidine blue (0.05% aq) and examined and photographed using the Olympus BX60 light microscope as before. This method is ideal for demonstrating changes in cell-wall morphology, but carotene and lipids are extracted by the solvents used during dehydration and embedding.

Cell Viability. Viable tissues reduce neutral solutions of 2,3,5-triphenyltetrazolium chloride (TTC), producing water-insoluble red formazan. The reduction of tetrazolium occurs at various points along the respiratory chain in mitochondria, and thus the absence of the red formazan is indicative of cell damage.

Raw carrot cubes digested in vitro were incubated in TTC solution [0.6% (w/v) in phosphate buffer (0.05 mol/L), pH 7.4] for 24 h at room temperature in the dark. The samples were cut in half with a razor blade, and the cut surface was examined using a stereomicroscope with dark field illumination. Images, with live tissue staining red, were recorded with a Nikon Coolpix 950 digital camera (Nikon, Japan).

RESULTS

Carotene Partitioning. Carotene partitioning from carrot tissues into the emulsion oil phase during simulated gastric digestion, or gastric followed by duodenal digestion, is shown in **Figure 1**. Preliminary experiments showed that partitioning of carotene reached a maximum value within 2 h and did not change thereafter. Also, emulsion droplet size was found to be unchanged during this time. Considering first the gastric digestions, the raw grated shreds and raw carrot juice were found to allow the greatest amounts of carotene, 20.8 ± 3.0 and 96.8 ± 4.2%, respectively, to partition into the oil. The process of heating the tissue tended to reduce carotene partitioning. For example, the

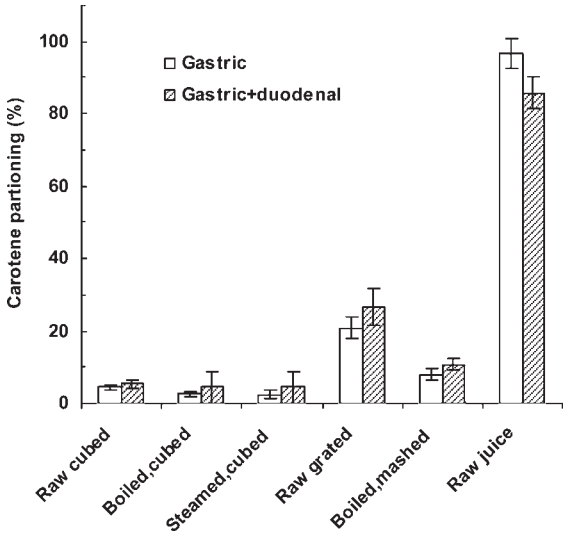

Figure 1. Carotene partitioning into gastric and duodenal emulsion phases during in vitro gastric digestion alone and gastric followed by duodenal digestion. The partitioning is expressed as a percent of the total carotene present in the raw or processed tissue before gastric digestion. Results represent the mean of 16 samples ± SD except for grated and steamed, which are the mean of 8 samples ± SD.

boiled and steamed carrot cubes released significantly less carotene, 2.6 ± 0.8 and 2.5 ± 1.0%, respectively, than the raw carrot cubes (4.5 ± 0.7%) ($P < 0.001$). Mashing the boiled carrot into a purée resulted in significantly more carotene being available for partitioning into the oil than from the boiled, cubed material ($P < 0.001$). However, when compared to the raw juiced sample, the puréed carrot released substantially less carotene into the oil.

Following the simulated gastric digestion, the digesta were incubated under duodenal conditions. This led to a further small but significant increase ($P < 0.01$) in carotene solubilization in the oil in all cases except for the juiced sample (**Figure 1**), for which carotene partitioning decreased significantly ($P < 0.001$). Enhanced partitioning under duodenal conditions could be due to further emulsification of the oil phase by surface-active agents such as bile salts. In effect, smaller droplet sizes would increase the surface area for solubilization of the carotene in the oil. In carrot juice, the observed decrease in partitioning would suggest that some of the carotene solubilized during gastric digestion had passed from the oil into the micelles of the aqueous phase. However, carotene was not detected during analysis of the aqueous phase by HPLC.

Cubes of raw carrot of various total surface areas (but of constant weight) underwent in vitro gastric digestion, and the partitioning of absolute amounts of carotene into the gastric oil phase was measured (**Figure 2**). Carotene partitioning increases with increasing surface area, but does not show direct proportionality. It is likely that there was some loss of carotene during the cutting process, a hypothesis that is supported by the negative correlation between the amount partitioned and surface area per cube, shown in the inset to **Figure 2**.

Carrot Tissue Microstructure before and after In Vitro Digestion. To determine the relationship between carrot microstructure and carotene partitioning, carrot tissue pretreated and digested as in **Figure 1** was examined by microscopy. In all digested samples, however pretreated, the gross structure and orange coloration of the carrot tissue were retained and carotene bodies were clearly visible in cells where the cell wall is intact (**Figure 3**). In sections of the digested raw carrot cubes, the cells at the surface (**Figure 3A**, between arrowheads) were cut open cleanly during the initial

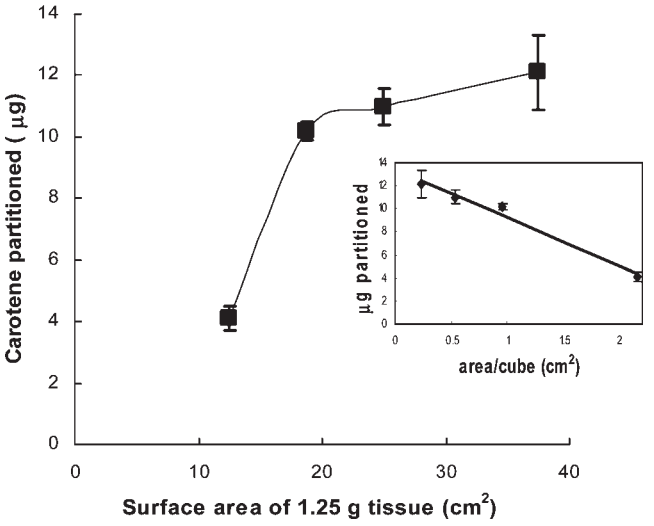

Figure 2. Effect of raw carrot surface area on carotene partitioning into a gastric emulsion following in vitro digestion. Each point represents the mean and range of results for duplicate samples. (Inset) Data replotted in terms of surface area per single cube. The line is the linear regression with correlation coefficient of −0.989.

preparation of the cubes. The cell contents, including the carotene bodies, were then free to disperse into the digestion emulsions. Intact cells below the surface retained their contents. In raw grated carrot samples, the surface of the carrot shreds (**Figure 3B**, between arrowheads) was irregular and cracked, resulting in more ruptured cells and a greater release of carotene from the surface. In contrast, in steamed carrot the cells tended to separate rather than rupture during the preparation of cubes, so that the walls of the surface cells (**Figure 3C**, between arrowheads) remained intact, effectively encapsulating the carotene during digestion. A similar observation was made for boiled carrot cubes (**Figure 3D**,**E**), but in this case the surface cells became rounded (**Figure 3E**, between arrowheads). The puréed carrot was found to contain groups of intact cells that had escaped rupture and contained carotene (**Figure 3F**), but some cell-wall breakage with carotene release is inevitable during the physical process of mashing. Even though particle size had been significantly reduced, the fact that many cells remained intact appears to have had a significant inhibitory effect on carotene accessibility. Cell rupture and cell separation in raw or cooked plant tissues during cutting are illustrated diagrammatically in **Figure 4**. Many of the intact cells in steamed or boiled carrot tissue contain spherical droplets that resemble lipid (**Figure 3F**, open arrows). These droplets may form due to disruption and coalescence of internal membranes with other lipids, but remain encapsulated if the cells are not ruptured.

To investigate the effect of digestion specifically on the microstructure of the cell walls, raw digested cubes were embedded in resin, sectioned, and stained prior to light microscopy. The walls of cells at the surface of the cubes (**Figure 5B**−**D**, arrowheads) showed a marked difference in toluidine blue staining when compared to the undigested control sample (**Figure 5A**). The staining was much more diffuse after in vitro digestion, which suggests that cell-wall components such as pectins or proteins have been solubilized from these outermost cells. Following sequential gastric and duodenal digestion (**Figure 5C**,**D**), the walls of these outer cells were swollen and the middle lamella regions (the areas that join adjacent cells together) were more heavily stained, which was probably due to the movement of pectin from the cell walls into these areas. These effects were largely confined to the first three or four layers of cells at the surface of the carrot cubes where cells have been subjected to the digestion conditions for longest.

Carrot Cell Viability before and after In Vitro Digestion. Tissue viability after in vitro digestion was assessed using TTC. Following treatment with water or saline, the vast majority of cells (apart from damaged surface cells) maintained their viability (results not shown). However, following in vitro digestion, cell death was observed (i.e. cells were unable to reduce TTC into red formazan and remained orange). Carrot cubes were digested (gastric and duodenal) without oil but with and without PC. Following digestion in the presence of PC, the majority of cells remained viable (**Figure 6A**), with only cells near the surface of the cubes unable to produce red formazan. Without PC, the digested tissue was found to be orange throughout (**Figure 6B**), indicating that all cells were nonviable. That this damage was incurred in the duodenal stage of digestion was shown by examining cubes after gastric digestion alone, when cell death was largely restricted to the surface regions of the cubes and was unaffected by the presence of PC.

Digestion under duodenal conditions alone allowed the individual components to be investigated. The presence of bile salts was found to cause significant cell death and this effect was reduced in the presence of PC (results not shown). PC therefore confers some protection against cell death during in vitro duodenal digestion.

DISCUSSION

The release of carotenoids from food structure and their processing into an absorbable form have been highlighted as one of the main challenges associated with understanding and measuring carotenoid bioavailability (*3*). We have addressed this by investigating the transfer of carotene to a dietary oil. Because of the hydrophobicity of these micronutrients, this is believed to be an initial step in determining bioavailability (*7*, *8*). We chose to use olive oil as it is commonly used with both cooked and raw food.

More carotene partitioned into the oil from raw carrot preparations than from cooked tissues. This is because cells from raw vegetable tissues tend to rupture, whereas, as illustrated in **Figure 4**, cells in heated tissues tend to separate intact so that their contents remain encapsulated. For example, in raw carrots the process of juicing followed by filtration results in the majority of the cells being broken open (confirmed by microscopy), allowing unrestricted partitioning of the carotene into the oil. In contrast, heating the tissue prior to particle size reduction resulted in an increased likelihood of cell separation. Our findings are in agreement with published work in which carotenoid bioavailability was found to be greater from juiced tissue than from raw intact tissue (*27*) or from cooked puréed tissue (*22*). We suggest that heating hinders carotene release from carrot tissue as this process reduces the likelihood of cell rupture. This contradicts some previous studies in which it was postulated that cooking helps to break down cell walls, resulting in improved carotene release during digestion (*17*, *21*, *28*, *29*). However, it should be noted that many studies have underestimated the importance of particle size reduction by mechanical processing (*17*, *30*, *31*). In the few studies that have specifically investigated the effect of heating on carotenoid bioavailability, no significant differences between heated and unheated vegetables were found for carrot slurries (*32*) or cherry tomatoes (*33*). In another study, van het Hof et al. (*34*) examined the effect of heating and homogenization on carotenoid bioavailability from canned tomatoes. It was demonstrated that for β-carotene, the degree of homogenization

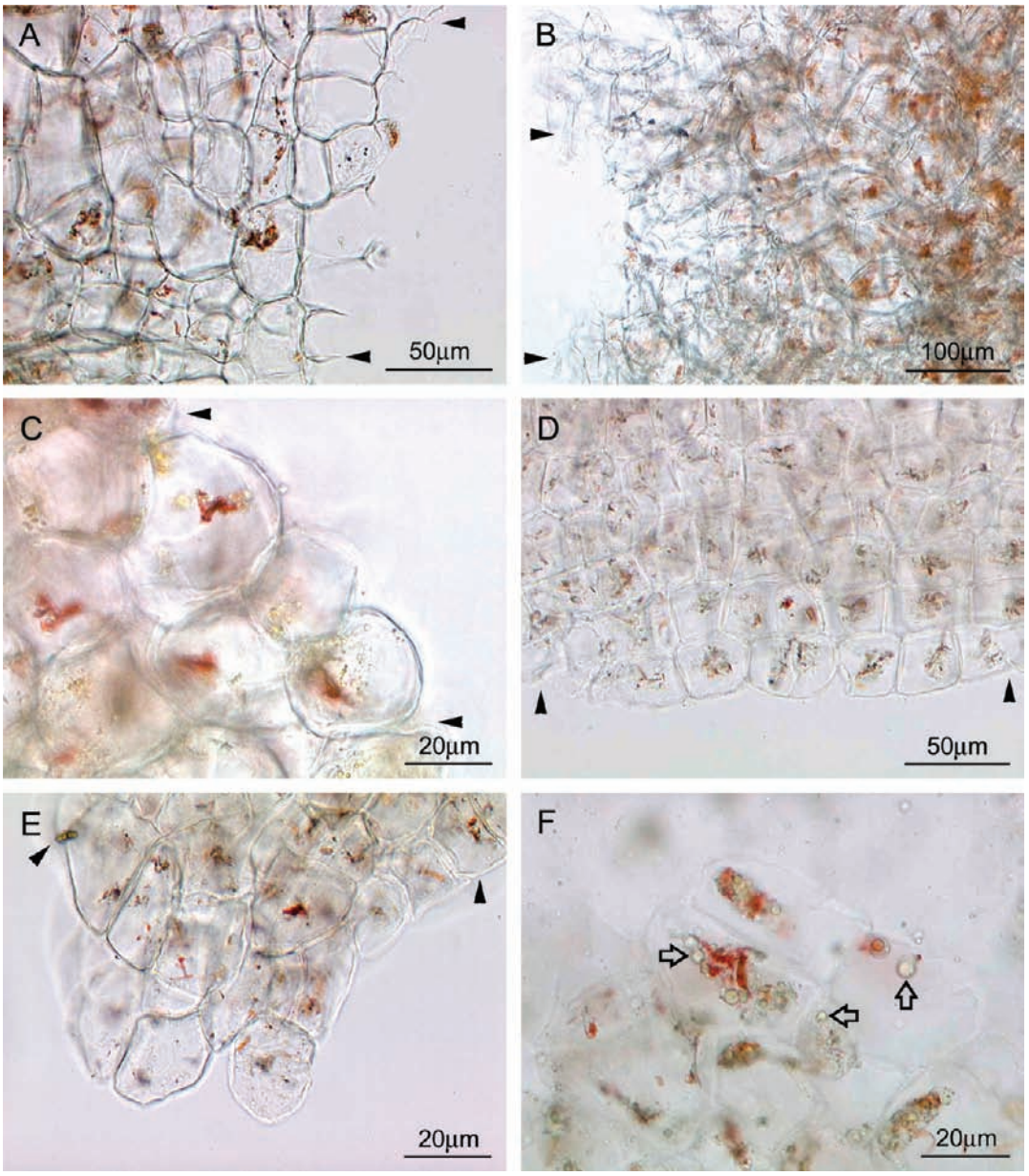

Figure 3. Unstained sections of carrot after various pretreatments and in vitro digestion (gastric followed by duodenal): (**A**) surface cells of a raw carrot cube; (**B**) surface cells of raw grated carrot; (**C**) surface cells of a steamed carrot cube; (**D**) surface cells of a boiled carrot cube; (**E**) detail of surface cells of a boiled carrot cube; (**F**) cells from puréed carrot. Surface of sample is indicated by arrowheads and yellow lipid droplets by open arrows in **F**. Carotene is present as orange/red bodies.

significantly affected carotene plasma response only when the tomatoes were not heated above the temperature at which they were canned. This suggests, at least for carotene, that particle size reduction is more important than heating for bioaccessibility. In the current study, a relatively small amount of carotene partitioning (8.0−10.8%) was observed when cooked carrots were mashed into a purée, whereas in other studies using cooked puréed carrots, 45% of the carotene partitioned into the fat phase of the stomach in vivo (*8*). Clearly, the severity of the processing conditions is of particular relevance: for example, the food blender used by Tyssandier et al. (*8*) will probably result in more cell rupture than the pestle and mortar method used in the current work.

Using cubes of different sizes, our work suggests that the area of tissue surface exposed during digestion is important in the release of carotene from raw carrot. However, the nonlinear dependence of carotene release on surface area for the same weights of tissue may indicate that some carotene is lost from the cut cells during the actual process of cutting. This hypothesis is supported by the negative correlation between surface area per cube and amount of carotene partitioned into oil (see inset to **Figure 2**). Further work is required to quantify these ideas and to conduct a comparative study on cooked tissue. However, we would predict that an increase in surface area is unlikely to increase significantly carotene partitioning from cooked material to the level observed for raw tissue. In the current study, despite having a similar surface area, the cooked carrot did not release as much carotene as the raw equivalent. Therefore, we suggest that the governing factor in carotene bioaccessibility is not surface area but the number of ruptured cells. An analogous situation has been observed for the release of nutrients from almond seeds during in vivo and in vitro digestion (*35*). Clearly, processing technique and, indeed, chewing will have a significant impact on carotene bioaccessibility.

Another reason cell rupture enhances carotene partitioning is the requirement for direct contact between the carotene-containing chromoplasts or crystals and the interface of the oil droplets (*13*). We sought to mimic in vitro the dietary emulsions likely to be produced during digestion in terms of droplet size and composition (*8*). Increasing the surface area of the emulsion had a positive effect on carotene partitioning compared to our previous work using bulk oil (*14*), when 65% of the carotene in carrot juice partitioned into the bulk oil phase during in vitro gastric digestion. In the current study in which an emulsion was used, 95% partitioning was found. However, emulsion surface area is not the only factor determining carotene partitioning into the oil phase. Tyssandier et al. (*8*) found a positive relationship between the mean diameter of lipid emulsions recovered in the stomach and the transfer efficiency of carotenoids. Therefore, they suggested that parameters related to the quality of the interface must also be significant. For example, soluble protein from vegetable tissue has been found to reduce carotene partitioning into oil (*13*, *14*). Also, smaller droplets will have smaller contact areas with the carotene-containing bodies, and with a higher internal pressure they will be less able to distort to increase the area of contact.

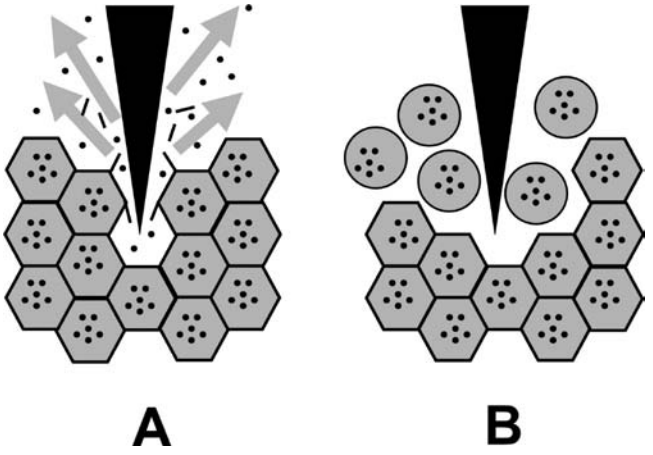

Figure 4. Diagram representing (**A**) cell rupture typical of fresh raw fruits and vegetables [cell contents such as carotene crystals (shown as black circles) are released as shown by the gray arrows] and (**B**) cell separation found in cooked vegetables and mealy fruits (cells remain intact and lipid-soluble contents remain encapsulated), after Brett and Waldron (*40*).

Within the duodenum, lipid droplets received from the stomach coexist with micellar phases. Carotene may continue to partition into the droplets or solubilize directly from the plant tissue into the micelles (*15*). With the exception of the juiced sample, carotene continued to partition into the oil phase to a small extent in duodenal conditions. A decrease in partitioning is consistent with the increased negative charge on the oil droplets due to the bile salts (*13*). A micellar pathway along which the carotene is transferred from the tissue to the oil is a further possibility. However, Rich et al. (*15*) found this pathway to be virtually absent in the presence of PC. A significant decrease in carotene was found in the duodenal emulsion phase following digestion of carrot juice. This could be due to transfer of the carotene from the oil droplets to micelles. Borel et al. (*7*) have shown this is possible when lipolysis is taking place, as in the present work. However, negligible carotene levels were detected in the micellar phase for all samples (results not shown), and this may reflect the low level of lipolysis in the simulated duodenal digestions. The cause for the decrease in carotene levels in the oil at this stage is unclear. It is possible that carotene did solubilize to some extent in the micellar phase but was undetected.

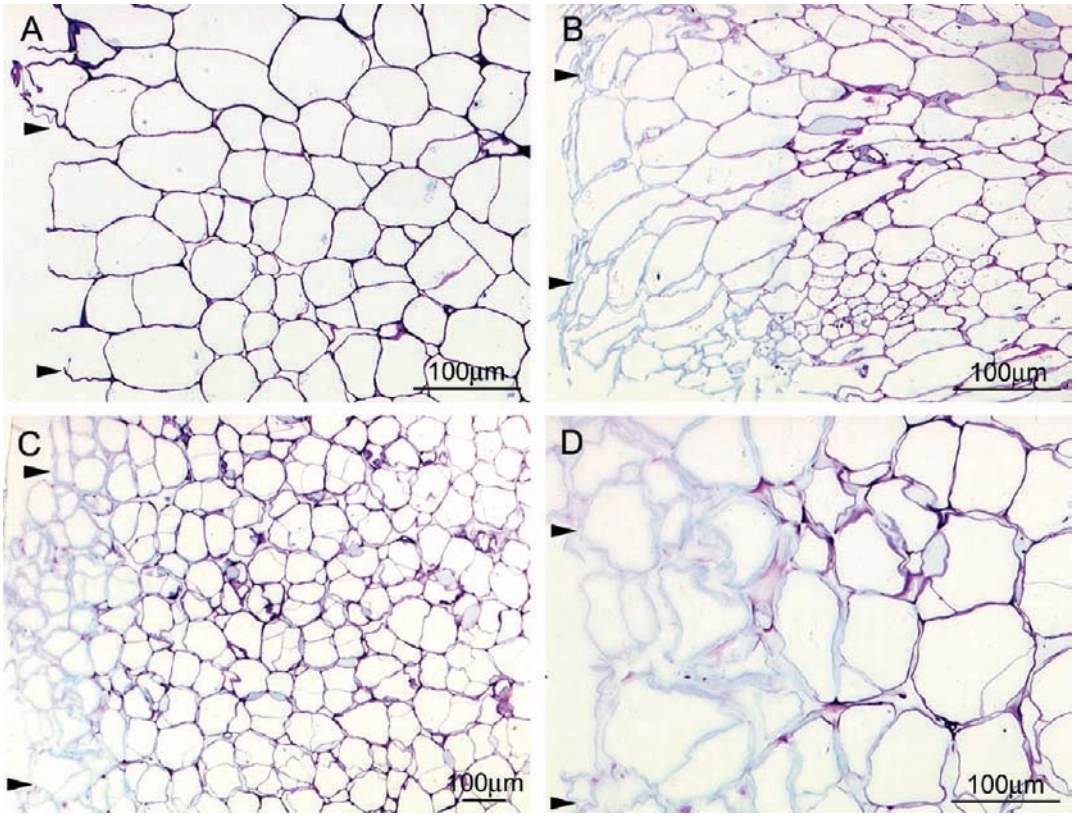

Figure 5. Effect of in vitro digestion on the cell walls of raw carrot cubes: (**A**) undigested carrot control; (**B**) 2 h gastric digestion; (**C**) 2 h gastric digestion followed by 2 h duodenal digestion; (**D**) as (**C**), showing the surface region at higher magnification. The outer surface is indicated by arrowheads. Samples were embedded in LR White resin, and 1 μm thick sections were stained with toluidine blue.

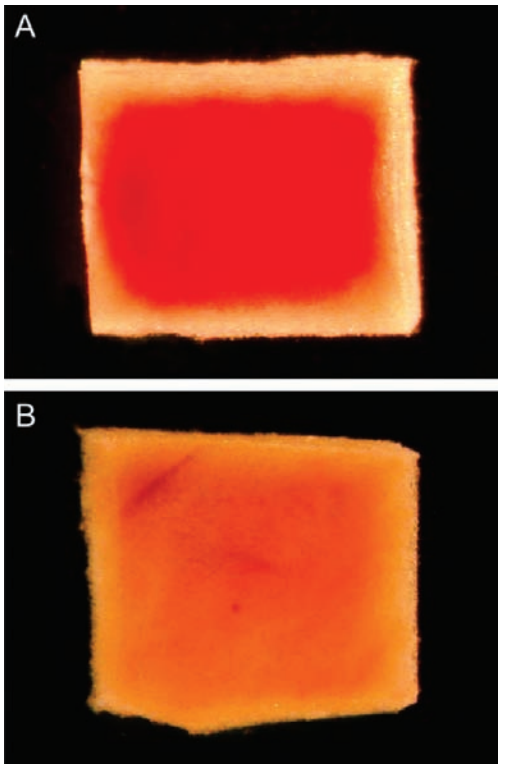

Figure 6. Effect of in vitro digestion on carrot cell viability: (**A**) with and (**B**) without phospholipid. Raw 3 mm carrot cubes were digested (2 h gastric followed by 2 h duodenal). Digested cubes were incubated in 2,3,5-triphenyltetrazolium chloride for 24 h and cut in half, and the cut surface was photographed.

We estimate from the data of Maser et al. (*36*) that the bile salt plus lecithin concentrations we used would give mixed micelles. The shape and composition of the micelles are critical for efficient uptake, and it is feasible that carotene may have solubilized into mal-shaped micelles that were subsequently removed by filtration during preparation for HPLC. It is quite possible that the decrease in carotene solubilization into the duodenal oil phase we observed for carrot juice may have occurred in all systems in this investigation. However, any such decrease could have been masked by a concomitant slow release of carotene into the oil from the large carotene pool present in the carrot tissue. It is also important to consider possible carotene loss due to oxidation. Carotene in chromoplasts within tissue is very stable, but once carotene is solubilized it is susceptible to oxidation, and under the low oxygen partial pressures pertaining to the present work, it can act as a chain-breaking antioxidant, reacting with a lipid peroxyl radical to give bleached carotene products.

Although the food matrix is widely believed to be an important factor in carotenoid bioavailability, it has been little examined following digestion. We found that the gross structure of the tissues was relatively unaffected by in vitro digestion, with material remaining intact and orange in color, as we have found in vivo (*10*). Cells that had escaped rupture remained intact and encapsulated carotene, whether raw or cooked. However, on closer inspection the microstructure of the cell walls did undergo some interesting changes. For example, in the majority of cases, the walls in the first few layers of cells at the surface of the digested raw carrot cubes appeared swollen, and staining with toluidine blue was reduced. This phenomenon is often observed in cooked vegetable tissue or fruits that have undergone ripening and is associated with movement of water into voids left in the cellulose−hemicellulose network by solubilized pectin. Indeed, Redgwell et al. (*37*) found a correlation between swelling and the degree of pectin solubilization, suggesting that swelling occurs as a result of changes to the viscoelastic properties of the cell wall. Studies on carrots have shown that the softening of the texture of the tissue on cooking is associated with loss of high molecular weight pectic polysaccharides (*38*). The mechanism for pectin solubilization from the raw carrot cell walls during in vitro digestion is not clear. However, acid hydrolysis of glycosidic linkages in the gastric environment is one possibility in conjunction with changes in physical constraints that result from turgor loss. The displacement of Ca^{2+} cross-links by H^{+} will also destabilize pectin. It is significant that cell-wall swelling occurred primarily in the simulated gastric phase of digestion, and there was little further swelling in the near-neutral conditions of the duodenal phase.

Viability assessment of raw carrot following in vitro digestion without PC revealed that cell death had occurred throughout the tissue. This could be due to acid denaturation of membrane proteins and the detergent action of the bile salts, resulting in cell lysis. The presence of PC in the in vitro digesta served to protect the cells from death. This protective effect has been observed before (*39*) and can be attributed to the lowered intermicellar concentration of monomeric bile salts available for partitioning into membranes with consequent damage. Heating is known to destroy carrot organelle membranes (*14*), so in the heated tissue the chromoplast and cell membranes cannot provide a barrier to carotene release. Therefore, it is significant that the remaining cell-wall barrier, even when damaged (by heating or by digestion), can prevent carotene release. It acts as a hydrophilic barrier both to the uptake of solubilizing bile salts and mixed micelles and to the export of the solubilized carotene.

The current results have confirmed our in vivo findings (*10*) that cell rupture is an absolute requirement for carotene release from carrot tissue and that carotene remaining within intact cell walls is inaccessible during upper gut digestion. Cell rupture prior to digestion, such as that caused by mastication or food processing, will thus determine carotene bioaccessibility. We recognize that the systems used here are models and that further work is required, for example, investigating carotenoid uptake by physiologically-relevant micelles formed when there has been substantial lipolysis and comparing cut with masticated carrot. In general, studies have shown low uptake of carotene into micelles in vitro, and this pathway could be a greater limiting factor in carotenoid bioavailability than the food matrix (*4*). Nevertheless, this work highlights the fact that, to understand nutrient bioaccessibility, the physical structure of food and its changes during digestion must be considered.

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