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In Vitro Intestinal Absorption of Carotenoids Delivered as Molecular Inclusion Complexes with β-Cyclodextrin Is Not Inhibited by High-Density Lipoproteins

Elisabet Fernández-García,[†] Irene Carvajal-Lérida,[†] Francisco Rincón,[‡] José J. Ríos,[†] and Antonio Pérez-Gálvez*,[†]

[†]Department of Food Biotechnology, Instituto de la Grasa (CSIC), Avenida Padre García Tejero 4, 41012 Sevilla, Spain, and [‡]Department of Food Science and Technology, C-1 building annex, Campus of Rabanales, University of Córdoba, E-14014 Córdoba, Spain

This study analyzed the assimilation efficiency of carotenoids when they are delivered as inclusion complexes with β-cyclodextrin (CyDIC) in water. The in vitro assimilation model used was the brush border membrane vesicles (BBMV) system in which the BBMVs were incubated with CyDIC. Carotenoid suspensions in Tween were used as a reference. Regardless of the form in which the carotenoids were delivered to the BBMV preparation, a higher assimilation efficiency was observed for carotenes than for the xanthophyll lutein. At the highest donor solution concentration, supplying carotenoids in CyDIC produced a significant increase in carotenoid assimilation compared to the corresponding carotenoid suspensions in Tween. The assimilation process using CyDIC takes place by means of a dissociation process in which the carotenoids are released from the β-cyclodextrin to later be assimilated. At the highest concentration of CyDIC in the donor solution, the dissociation equilibrium will be shifted toward the free forms of the complex, thus increasing the amount of carotenoids available for assimilation. In another set of experiments, the effect of high-density lipoproteins as activity inhibitors for the receptors involved in carotenoid assimilation was analyzed. In carotenoid suspensions in Tween, with an inhibitor, a significant decrease in the assimilated quantity compared was observed with values reached without the inhibitor. Lutein presented the most significant decrease (70%). The fact that complete inhibition was not reached suggests that both simple and facilitated diffusion contributes to the assimilation process. When the donor solution composed of CyDIC and inhibitor was added, significant increases were observed in β-carotene and lycopene assimilation for all concentrations and in lutein for the highest concentration. This effect is due to the exchange between lipoprotein lipid components and CyDIC, which promotes the dissociation and liberation processes of the carotenoid, which then becomes available for assimilation.

KEYWORDS: β-Carotene; lycopene; in vitro assimilation; high-density lipoproteins; β-cyclodextrin; facilitated transport; lutein

INTRODUCTION

The passive diffusion model explaining the absorption of lipid compounds through the membrane of enterocytes, which is featured in most scientific reviews and texts on nutrient absorption, has been under intense scrutiny in the past few years. Results obtained with different experimental models, both in vitro and in vivo, offer evidence of the existence of transporters that facilitate the absorption of lipophilic compounds such as cholesterol (free or esterified), triacylglycerides, and phospholipids (*1*−*3*). Examples of the identified transporters are type B residual receptors, cluster of differentiation 36 (CD36), and Niemann−Pick C1-like 1 protein (NPC1L1).

The implication of the above-mentioned transporters in the lipid assimilation process has been demonstrated by using competitive inhibitors such as serum apolipoproteins and lipoproteins, amphipathic α-helical peptides, and synthetic drugs such as ezetimibe (*4*, *5*). The facilitated diffusion model explains the significant variability that exists among individuals with regard to lipid compound absorption efficiency has been observed by applying different assimilation models (*6*, *7*). It also explains different pathological/physiological scenarios for intestinal epithelium with deficient lipid absorption levels.

Some of the observations that have inspired the search for the true absorption mechanism for cholesterol and other lipophilic compounds have also been observed in the case of other compounds, such as carotenoids. This isoprenoid family, the presence of which in the mammalian body is exclusively due to diet, carries out a series of biological functions and actions that are of extreme interest for human health, and for this reason, events associated with assimilation of carotenoids are studied carefully. In vivo

*Corresponding author (telephone +34 954691054; fax +34 954691262; e-mail aperez@cica.es).

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clinical trials have revealed considerable variability in absorption efficiency between individuals, and nonresponders exist. The existence of ester specificity in absorption has also been demonstrated, as has competition between different compounds for absorption along with the tendency for small groups of carotenoids to accumulate in biological tissue even though eating fruits and vegetables provides a greater number of carotenoids (*8*, *9*). All of these indications have suggested the possibility of a facilitated diffusion transport mechanism that is similar to that of cholesterol.

The reconstruction of the lipid absorption process involved in the assimilation mechanism that takes place in intestinal epithelial cells has opened new channels for intentionally inhibiting cholesterol uptake. Existing alternatives to the use of vegetable sterols, which compete with cholesterol for incorporation into micelles, and polymer resins, which sequester bile salts (cholestyramine, cholestipol), now include drugs that block transporters that facilitate cholesterol assimilation (*10*). Interference at any level of the cholesterol assimilation process will affect the assimilation of other dietary lipids, such as fat-soluble vitamins and carotenoids. In fact, an increase in the consumption of fruits and vegetables when phytosterols are ingested is recommended to avoid a decrease in the plasma levels of fat-soluble vitamins and carotenoids (*11*). This recommendation is effective because interference in the micellization and assimilation processes occurs only when phytosterols are ingested, and this effect does not continue afterward (*12*). Nevertheless, treatment with drugs may have a more prolonged effect due to their metabolism and modus operandi. For example, ezetimibe is metabolized in the liver by means of phase II enzymes, which generate a glucuronidated compound that returns to the intestine and then inhibits cholesterol assimilation to a much greater degree than unmetabolized ezetimibe (*13*). If the chosen option for decreasing cholesterol absorption is treatment with drugs that block cholesterol transport in the intestinal epithelium and that decrease the expression of genes encoding the transporters involved in facilitated diffusion, it is unclear whether an increase in the dietary consumption of fruits and vegetables could compensate for the decreased plasma levels of fat-soluble vitamins and carotenoids.

Cyclodextrins are cyclic oligomaltosaccharides obtained from starch by means of an enzymatic cycling reaction using cyclodextrin glucosyltransferase. The extraordinary ability of cyclodextrin to form inclusion complexes with hydrophobic molecules that are stored within the cyclodextrin cavity has led to a wide range of uses in areas such as agriculture, analytical chemistry, food preparation, and the pharmaceutical sector, as well as in hygiene, cosmetic, and cleaning products (*14*). The most common uses of cyclodextrins in food preparation include protection and controlled release of flavoring compounds, flavor elimination, and stabilization emulsions such as sauces, creams, butters, and mayonnaises. Within the group of cyclodextrins (α-, β-, γ-, and δ-cyclodextrin), β-cyclodextrin is the most commonly used form in pharmaceuticals and is also the most commonly studied in humans (*15*). The property of cyclodextrins that makes them relevant to pharmacology is their capacity to increase hydrophobic drug assimilation by acting as transporters to draw hydrophobic drugs close to biological membranes (skin, mucous membranes, cornea) and by increasing the availability of the complexed drug (*16*).

The purpose of this study was to use the ability of cyclodextrins to form inclusion complexes with fat-soluble compounds, which makes the fat-soluble compound more water-soluble in order to study whether the supply of common dietary carotenoids formulated as inclusion complexes with β-cyclodextrin increases carotenoid assimilation efficiency. In this study, we used in vitro brush border membrane vesicle preparations as an assimilation model. The action of β-cyclodextrin as an efficient transporter of the guest molecule (a carotenoid, in this case) to the plasma membrane could indicate that an optimal assimilation level will be reached. The efficiency of assimilation from carotenoid β-cyclodextrin inclusion complexes was also tested under conditions of blocking the receptors involved in bringing carotenoids through the plasma membrane.

MATERIALS AND METHODS

Raw Materials. Tomato, marigold, and β-carotene oleoresins were supplied by LycoRed (Beer-Sheva, Israel), Kemin Foods (Des Moines, IA), and Extractos Vegetales (La Línea de la Concepción, Cádiz, Spain), respectively.

Reagents and Solvents. Acetone, methanol, hexane, absolute ethanol, methylene chloride, and *N*,*N*-dimethylformamide, all of HPLC quality, were supplied by Teknokroma (Barcelona, Spain). Purified water was obtained using a Milli-Q purification system (Millipore, Milford, MA). β-Apo-8′-carotenal and β-cyclodextrin (98% pure) were supplied by Sigma (St. Louis, MO). Protein concentrations were determined using the disodium salt of 2,2′-biquinoline-4,4′-dicarboxylic acid provided by Fluka (St. Louis, MO) and bovine serum albumin supplied by Sigma. To measure the intestinal saccharase activity, glucose (purity ≥ 99.5), glucose oxidase from *Aspergillus niger* (type VII) with an activity of 180,200 U/g (as using glucose as a substrate), horseradish peroxidase (type II) with an activity of 150−250 U/mg (as determined using pyrogallol as a substrate), 3-dimethylaminobenzoic acid, and 3-methyl-2-benzothiazolinone were used, all of which were obtained from Sigma. Sheep serum and heparin sodium salt from porcine intestinal mucosa, grade I-A, 140 units USP/mg, were supplied by Sigma. All other reagents and solvents were of analytical quality.

Stock Solutions of Carotenoids. The separation and purification procedure used to obtain stock solutions of carotenoids (lycopene, lutein, and β-carotene) from tomato, marigold, and β-carotene oleoresins (which were subsequently used to create inclusion complexes) was as follows: 0.02 g of oleoresin was dissolved in 25 mL of ethyl ether. The dilution was placed in a separating funnel, and 10 mL of KOH in methanol (10%, w/v) was added. The saponification reaction lasted 1 h, and its purpose was to eliminate the oily matter present in the oleoresin. Once the reaction was completed, 200 mL of aqueous NaCl solution (10%, w/v) was added. The organic and aqueous phases were separated, and the aqueous phase was discarded. The organic phase was washed with distilled water until it had a neutral pH. It was then washed twice with 200 mL of aqueous Na_2SO_4 (2%, w/v). The organic phase was filtered through a solid bed of Na_2SO_4 and was then evaporated in a vacuum rotary evaporator. The residue was dissolved with an exact volume of acetone (for β-carotene and lutein) or light petroleum ether (for lycopene). The spectrophotometric measurement of the free lutein concentration in the solution was done at λ_{max} = 446 nm with an extinction coefficient of $E^{1\%}_{1\ cm}$ = 2340, and the spectrophotometric measurement of the β-carotene concentration was done at λ_{max} = 450 nm with an extinction coefficient of $E^{1\%}_{1\ cm}$ = 2620 (*17*). The lycopene concentration in the solution was measured at λ_{max} = 472 nm with $E^{1\%}_{1\ cm}$ = 3450 (*18*). Purity of stock solutions was determined by HPLC as described below.

Preparation of the Carotenoid Suspensions in Tween and Preparation of the Carotenoid/β-Cyclodextrin Inclusion Complexes. The carotenoid suspension in Tween was prepared according to the procedure described by During et al. (*19*). An aliquot of the stock carotenoid solution was mixed with 50 μL of Tween 40 suspended in acetone (20%, w/v), and the solvents were evaporated under a stream of N_2 gas. The residue was dissolved in the correct amount of buffer solution (2 mM Tris-HCl, 0.05 M D-manitol, 5 mM EGTA, pH 7.1) and shaken in a vortex mixer for 1 min. The solution was prepared on a daily basis and stored at 4 °C until use, at which point it was once again shaken in the vortex mixer for 1 min. A somewhat modified version of the procedure described by Pfitzner et al. (*20*) was used to obtain the carotenoid/β-cyclodextrin inclusion complexes. An aliquot of the stock carotenoid solution was evaporated, and the residue was redissolved in 2 mL of methylene chloride. Next, 48 mL of ethanol and β-cyclodextrin was added. The molar ratio of β-cyclodextrin to the carotenoid was maintained at 20.

The resulting mixture was shaken at 100 rpm in a heat bath/agitator at 37 °C for 24 h. Once the process was complete, the solution was evaporated in a vacuum rotary evaporator and dried using a stream of N_2 gas. The solid residue was homogenized and stored at −30 °C until use.

Determination of Carotenoid Concentrations in Carotenoid Suspensions in Tween and in Carotenoid/β-Cyclodextrin Inclusion Complexes. The final carotenoid concentration in a solution was measured using the following process. Sample (an encapsulated inclusion complex, 50 mg dissolved in 3 mL of water, or 1 mL carotenoid suspension in Tween) was added to 8 mL of a 1:1 (v/v) mixture of *N*,*N*-dimethylformamide and hexane. To encourage phase separation, 5 mL of aqueous NaCl solution (10%, w/v) was added. The mixture was shaken for 1 min in a vortex mixer and then centrifuged at 4000 rpm for 5 min. The organic phase was placed in a test tube, and the remaining aqueous phase was extracted once more under the same conditions. The two organic phases were combined, and the solvent was evaporated. The residue was redissolved in the proper solvent to determine the concentration at the corresponding λ_{max}.

Isolation of High-Density Lipoproteins from Sheep Serum. To obtain a fraction enriched in high-density lipoproteins (HDLs), HDLs were separated from other lipoproteins in the serum using precipitation and centrifugation (*21*). An aliquot of sheep serum (2 mL) was mixed with 0.2 mL of the working solution, which was a heparin/magnesium chloride mixture (0.6 mL of heparin solution with 40,000 units USP/mL mixed with 10 mL of 1.06 M manganese chloride solution). The mixture was shaken and left to rest for 10 min at room temperature. Finally, it was centrifuged at 1500*g* for 30 min. The supernatant contained the HDL fraction enriched with type A1 and A2 apoproteins.

Preparation of Brush Border Membrane Vesicles (BBMVs). To obtain the BBMVs, we used porcine intestinal tissue and followed the procedure described by Hauser et al. (*22*). The fresh tissue, transported to the laboratory in a buffer solution (12 mM Tris-HCl, 0.3 M D-mannitol, 5 mM EGTA, pH 7.1) and kept on ice, was perfused with an ice-cold physiological saline solution, cut into pieces weighing between 20 and 25 g, and frozen at −80 °C until use. To prepare BBMVs, the frozen tissue was cut into pieces weighing approximately 1 g each. These fragments were placed in a beaker in an ice bath, and then 60 mL of buffer solution was added to the beaker (12 mM Tris-HCl, 0.3 M D-mannitol, 5 mM EGTA, pH 7.1). This mixture was then left to rest for 30 min. After that time, the resulting suspension (which remained on ice) was shaken for 90 s with a Vibromixer (100 Hz, Graber Pfenninger, Stäfa, Switzerland) and filtered. The filtrate was diluted with 300 mL of ice water and homogenized using a liquid homogenizer (Ultra-Turrax, model T-25, IKA Labortechnik, Staufen, Germany) for 3 min. Next, solid $MgCl_2$ was added until a final concentration of 10 mM in the mixture was reached; this mixture was then homogenized for 1 min. The resulting suspension was centrifuged at 12000*g* for 5 min at 4 °C (centrifuge model Avanti J-25, Beckman Instruments Inc., Palo Alto, CA, with a rotor type JA 25.50). The pellet resulting from this centrifugation, which contained cell remains and other contaminants, was discarded. The supernatant liquid was centrifuged once again at 48000*g* for 15 min at 4 °C. This time, the supernatant liquid was discarded, and the resulting pellet was resuspended in 50 mL of buffer solution (2 mM Tris-HCl, 0.05 M D-mannitol, 5 mM EGTA, pH 7.1) and homogenized using a Potter-Elvehjem homogenizer (10 strokes). The homogenized mixture was centrifuged at 48000*g* for 15 min at 4 °C, the supernatant liquid was discarded, and the pellet (purified BBMVs) was stored at −80 °C until use.

In Vitro Assimilation Process. The prepared BBMVs were homogenized in 15 mL of buffer solution (2 mM Tris-HCl, 0.05 M D-mannitol, 5 mM EGTA, pH 7.1) with a Potter-Elvehjem homogenizer (10 strokes). Before the assimilation test was performed, a routine characterization of the product was performed. The bicinchoninic acid assay (*23*) was used to determine the protein content, and the intestinal saccharase activity was measured (*24*). One volume of BBMV preparation containing 2 mg of protein was preincubated for 5 min in a test tube placed in a 37 °C bath and agitated at 250 rpm. In this set of experiments, in which carotenoid assimilation was inhibited, an HDLs solution (50 μg of protein/mL of BBMV preparation) was added at the start of the preincubation period. After the preincubation period was complete (with or without HDLs solution), the donor solution (carotenoid suspension in Tween or carotenoid/β-cyclodextrin inclusion complex solution) was added. The test was carried out under the same conditions except that the duration was 20 min. Tests were run with three different carotenoid concentrations in the incubation medium, 0.5, 1, and 2.5 μM, with concentrations normalized for the amount of protein. After the incubation period was complete, the mixture was centrifuged at 48000*g* at 4 °C for 15 min. The supernatant liquid was discarded, and the pellet was redissolved in 1 mL of buffer solution (2 mM Tris-HCl, 0.05 M D-mannitol, 5 mM EGTA, pH 7.1) for subsequent analysis. Three measurements were taken for each carotenoid, concentration, and donor solution combination (suspension in Tween or inclusion complex solution).

Extraction and Quantification of Carotenoids Assimilated by BBMVs. The following procedure was applied for extracting and quantifying the carotenoids assimilated by BBMVs: an exact, known quantity of β-apo-8′-carotenal was added to the final BBMV suspension as internal standard for subsequent quantification. Next, 1 mL of *N*,*N*-dimethylformamide, 1 mL of ethanol, and 6 mL of a 5:1 mixture (1% BHT) of hexane and dichloromethane were added. The mixture was placed in an ultrasound bath for 5 min, shaken for 1 min in a vortex mixer, and subjected to ultrasound for an additional 5 min before being centrifuged at 4400 rpm for 5 min. The organic phase was removed, and the mixture was evaporated under a stream of N_2 gas. The dry residue was redissolved in 250 μL of acetone (HPLC quality), and the sample wa stored at −30 °C until analysis. The carotenoid measurement was taken following the HPLC procedure developed by Mínguez-Mosquera and Hornero-Méndez (*25*).

Mass Spectrometry. To estimate whether the carotenoid inclusion complex was being absorbed by the BBMVs membranes, aliquots from the supernatant and the pellet were analyzed using mass spectrometry. These samples were obtained after centrifugation of the BBMV preparation, which was incubated with a carotenoid inclusion complex solution under the conditions described above. Mass spectra were recorded on a Finnigan MAT95s magnetic sector mass spectrometer (Finnigan Mat, Bremen, Germany) equipped with an ESI interface operating in negative mode. The solutions for mass spectrometric analysis were prepared by dissolving an aliquot of sample in a 75:25:0.1 (v/v/v) mixture of water/isopropyl alcohol/formic acid. The samples were introduced into the electrospray interface by an infusion pump operating with a flow rate of 20 μL/min. The ESI mass spectra, in the negative ion mode, were obtained under the following conditions: initial capillary temperature at 200 °C, sprayer voltage at 3 kV, and lens, skimmer, and octapole voltages set to get an optimal response for a reference solution of β-cyclodextrin. Nitrogen was used as the sheath gas at a pressure of 15 psi. Collision-induced dissociation within the lens transport region (CID) was promoted by applying a simultaneous 20 V capillary and lens defocusing voltage or by varying the cone voltage. An important step to consider when mass spectra of cyclodextrin complexes are obtained is to ensure that the presence of electrostatic adducts formed during the electrospray process are excluded. By increasing the voltage at the skimmer orifice, the breakage of possible clusters with cyclodextrins takes place. Our experiments did not show a substantial reduction of the presence of complex ions even we increased the cone voltages from lower to higher values or increased the CID voltages. On the other hand, the use of capillary heating to dissociate the complex could provide additional information regarding the properties of complex ions such as those between between β-cyclodextrin and lutein in the gas phase. Experiments in which the capillary temperature was increased from 150 to 300 °C were performed to produce, if possible, the breakage of possible electrostatic clusters. The experimental results showed that the complex ions remained dominant in the ESI mass spectra; therefore, the carotenoids-to-β-cyclodextrin binding energy was high enough to remain stable at high temperatures.

Statistical Analysis. Results were obtained on the basis of a mixed two-thirds level full-factorial experimental design including as factors the carotenoid type (β-carotene, lutein, and lycopene), the carotenoid concentration (0.5, 1, and 2.5 μM), the donor solution type (carotenoid suspension in Tween or β-cyclodextrin inclusion complexes), and inhibition (presence or absence of HDLs as competitive inhibitors). Results are expressed as means ± standard deviation. ANOVA and the identification of significant differences between results were performed by applying the Duncan test, and the level of statistical significance was set at $p < 0.01$. STATISTICA computer software (version 5.5 for Windows, 1999; Statsoft Inc., Tulsa, OK) was used in the statistical analysis of the results.

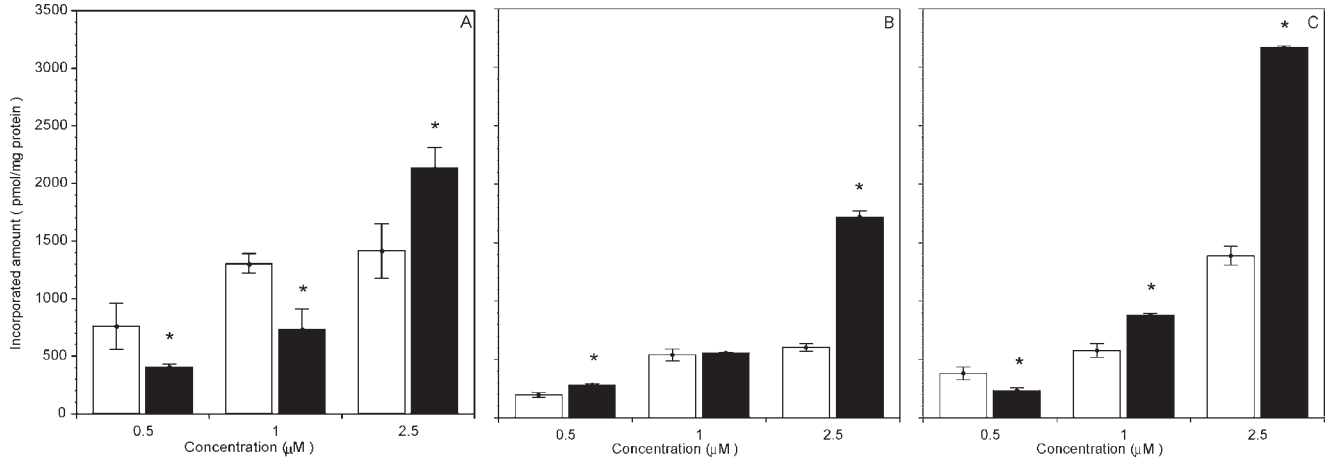

Figure 1. Assimilation of β-carotene (**A**), lutein (**B**), and lycopene (**C**) in BBMVs derived from porcine small intestine. Assimilation was measured after incubation of the three concentrations of the carotenoid suspension in Tween (white bars) or the carotenoid/β-cyclodextrin inclusion complex solution (black bars) at 37 °C for 20 min with the BBMV preparation. Assimilation is shown as the mean ± SD (n = 3); an asterisk indicates significant differences in the assimilation levels reached for the two types of donor solution, for each carotenoid and for each concentration ($p < 0.01$, Duncan test).

Table 1. Primary Effects of the Factors Concentration, Donor Solution Type, and Inhibition on the in Vitro Intestinal Assimilation of β-Carotene, Lutein, and Lycopene in Brush Border Membrane Vesicle Preparations

factor[a]	β-carotene	lutein	lycopene
concentration	2.085	784	2.087
donor solution type	1.370	579	1.502
Inhibition	720	−256	398

[a] Concentration levels: 0.5, 1, and 2.5 μM. Donor solution type: carotenoid suspensions in Tween or carotenoid inclusion complexes with β-cyclodextin. Inhibition: presence or absence of solution of HDLs.

RESULTS AND DISCUSSION

The in vitro absorption model based on the BBMV system has been successfully used to evaluate the assimilation of different fat-soluble compounds, including carotenoids (*26*), as well as to determine the existence of a facilitated diffusion mechanism. This research has been facilitated by the use of different inhibitors that block the activity of the proteins that intervene in the facilitated diffusion mechanism (*27*).

Figure 1 shows the carotenoid content, expressed in picomoles per milligram of protein, that was incorporated into the BBMV plasma membrane after incubation for 20 min at 37 °C for β-carotene, lutein, and lycopene from the carotenoid suspension in Tween or from the aqueous carotenoid CyDIC solution, depending on the initial carotenoid concentration (0.5, 1, or 2.5 μM). In some cases, a large standard deviation from the mean absorption value was observed due to data dispersion. This fact may point to variations in the composition and density of the transporters that are available in different BBMV preparations, as indicated previously (*2*) and as occurs in vivo (*28*).

The comparison of transport efficiency among carotenoids indicates that carotenes assimilate better than the xanthophyll lutein. The amount of assimilated lutein was equal to 26 and 52% of the amounts of assimilated β-carotene and lycopene, respectively, at the 0.5 μM concentration. **Table 1** lists the coefficients calculated on the basis of the linear model, which correlates the response (assimilation) with the primary factors concentration and carotenoid type. It can be observed that the coefficients for β-carotene and lycopene were very similar to one another and were different from the lutein coefficients for the two listed factors. This fact may be interpreted in two ways. The first hypothesis is that the three carotenoids have the same assimilation mechanism and thus that the different levels of assimilation efficiency are the result of the different chemical characteristics of the three compounds. A prior study has shown that lutein is less accessible than the carotenes when they are incorporated into individual carotenoid solutions (*29*). This is in agreement with the results of this study. The presence of hydroxyl groups on the end rings gives the lutein molecule a certain polarity that could make its incorporation into the plasma membrane more difficult. This hypothesis is supported by a similar effect for β-carotene and lycopene regarding the donor solution type factor. However, with regard to the different effects of inhibition (the addition of HDLs as competitive inhibitors) for the two similar carotenoids (720 for β-carotene and 398 for lycopene), a second hypothesis may be suggested. If the mechanism of facilitated diffusion applies, transporters may have a greater affinity for compounds of a more hydrophobic nature. Although it has been shown that residual receptors perform their activity without important structural discrimination, with regard to facilitating the assimilation of free and esterified cholesterol as well as triacylglycerides, Werder et al. (*2*) suggest that the SR-BI receptor may first facilitate the assimilation of more hydrophobic lipids. This second interpretation will acquire greater importance when the assimilation results in the presence of an inhibitor are analyzed.

When BBMV preparations were incubated with different concentrations of the carotenoid suspension in Tween (**Figure 1**), in the case of lycopene, an increase in the amount absorbed as the initial concentration increases was observed, whereas the absorbed amounts of β-carotene and lutein at concentrations of 1 and 2.5 μM were not different at the two concentrations for either compound (with p values of 0.381 and 0.607 for β-carotene and lutein, respectively), although they were significantly greater than those reached with a concentration of 0.5 μM. At the above-mentioned concentration levels it is possible that the transport saturation level of these carotenoids was reached and surpassed under the established experimental conditions. In a study by Moore et al. (*26*), researchers observed linear (nonsaturated) β-carotene transport up to a concentration of 6 μM. Although this study was carried out following the same experimental model, in vitro BBMVs, the vesicles used by Moore were isolated from rat intestinal mucosa, not porcine mucosa as used in this study, which could have led to these differences. Our results are more comparable to those of studies by During et al. (*19*) and Reboul et al. (*30*) in which concentrations of 2 and 5.56 μM

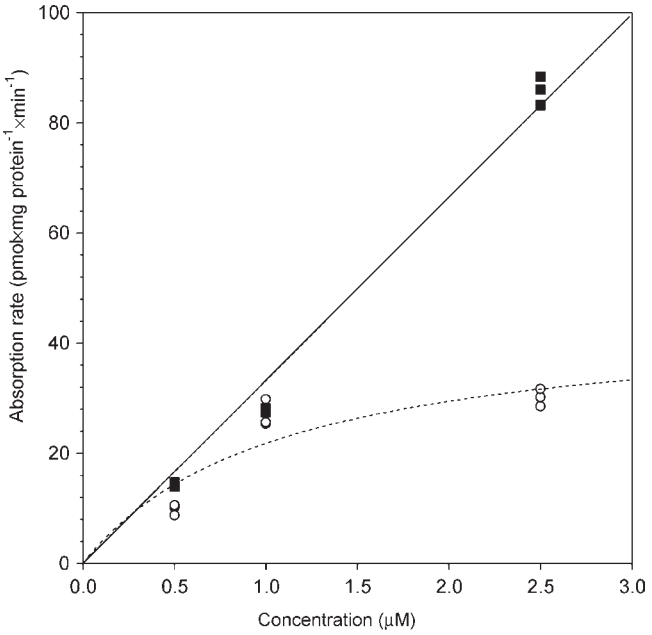

Figure 2. Lutein assimilation in BBMVs derived from porcine small intestine as a function of the initial concentration (0.5, 1, or 2.5 μM) in the donor solution of lutein suspension in Tween (circles) or lutein/β-cyclodextrin inclusion complex solution (boxes).

established in the donor solution reached transport saturation for β-carotene and lutein, respectively.

This saturation effect was not seen in the absorption experiments that were carried out with aqueous inclusion complex solutions. Taking lutein as a representative example, the presence or absence of saturation in absorption can be seen in detail in **Figure 2**. This figure represents the absorption rate [expressed in pmol × (mg of protein × min)$^{-1}$] relative to the rate found for the initial lutein concentration for experiments performed with carotenoid suspensions in Tween and in aqueous carotenoid CyDIC. In the case of incubating BBMVs with a lutein suspension in Tween, the absorption rate approximated a hyperbolic curve (R^2_{adj} = 0.8780), whereas the absorption rate followed a linear pattern when the absorption experiment was performed with an aqueous solution of the inclusion complex (R^2_{adj} = 0.9834). A similar fashion was observed for β-carotene, whereas lycopene assimilation data presented only a linear trend.

The saturation of the absorption rate is one of the first signals favoring the argument for the existence of a facilitated diffusion mechanism, as indicated earlier (*9*, *19*, *30*). In this case, based on the saturation concentration, subsequent increases in the concentration of the donor solution did not cause significant increases in the absorption rate, due to the saturation of membrane transporters that facilitate carotenoid assimilation. When the assimilation process occurs from the aqueous carotenoids CyDIC solution, the linearity between the absorption rate and the concentration could indicate that assimilation is taking place by means of simple diffusion. However, it must be considered that a saturation effect exists when assimilation occurs from inclusion complex solutions, but this occurs when concentrations are higher than the maximum used in the present study, meaning that the possibility that the assimilation process may also occur by facilitated diffusion cannot be ruled out. The experiment only allowed observation of the linear response region. There is a strong possibility that simultaneous assimilation processes (simple and facilitated diffusion) are inherent to the in vitro model used to estimate assimilation, making nonspecific absorption possible. This will be explained later in the study.

Regardless of the mechanism of assimilation, a relevant factor must be considered for carotenoid assimilation from aqueous carotenoid CyDIC solutions. In the range of experimental concentrations used in this study, the process can be explained by Fick's law. The inclusion of carotenoids in cyclodextrin leads to a considerable increase in the water solubility of the carotenoid and thus, in its partition coefficient P, which itself implies an increased diffusion rate. However, the carotenoid assimilation process using inclusion complexes does not take place all at once, as the inclusion complex is not completely absorbed; rather, the carotenoid molecule is first released from the complex and is subsequently assimilated. **Figure 3** shows the mass spectra for the aqueous lutein inclusion complex solution (**Figure 3A**) and for the lysate from a BBMV preparation that was previously incubated with an aqueous solution of lutein CyIC and then separated from that solution by centrifugation after incubation (**Figure 3B**). In **Figure 3A**, two pronounced fragment ions are observed; one peak at m/z 1701, corresponding to the deprotonated ion form of the inclusion complex with one molecule of lutein [β-CD + lutein − H]$^-$, and the second peak at m/z 2269, which could correspond to a complex ion of β-cyclodextrin including two lutein molecules [β-CD + 2 × lutein − H]$^-$. In **Figure 3B** the main peak at m/z 567corresponds to the deprotonated ion form of lutein [lutein − H]$^-$.

The fact that cyclodextrin (whether in free form or within inclusion complexes) is not absorbed has already been documented previously (*31*). The process of liberating the complex-bound molecule in cyclodextrin creates an equilibrium between the inclusion complex and the free forms of the molecules that constitute the complex, which in this case are cyclodextrin and a carotenoid. The dissociation rate depends on factors that include the energy of the bond between the cyclodextrin and the guest molecule and the initial concentration of the complex in solution (*32*). At equal concentrations, a higher energy bond makes dissociation difficult and slows the process. The significance of this factor cannot be seen from the experiments in this study, because the dissociation process is part of the assimilation process, which is affected by other factors, including the ability to diffuse through the membrane in the case of assimilation by simple diffusion or the individual affinity of each transporter for each carotenoid type in the case of assimilation by facilitated diffusion.

The observed effect was that the absorption efficiency of the donor solution increased when the concentration of the donor solution increased. At the lowest concentration (0.5 μM), the efficiency of absorption from the carotenoid suspension in Tween was significantly higher than the level reached with the corresponding solutions of carotenoid CyDIC, except for the case of lutein ($p < 0.01$), as can be seen in **Figure 1**. For the 1.0 μM concentration level a heterogeneous behavior was still observed. Therefore, the efficiency of assimilation from the carotenoid suspension in Tween was still higher for β-carotene, and this relationship continued to hold at lower concentrations ($p < 0.01$). For lutein, significant differences between the two values were not observed ($p = 0.845$). However, this tendency was reversed for lycopene. In this case, the assimilation efficiency was significantly higher when the inclusion complex solution was used ($p < 0.01$). Finally, at the 2.5 μM concentration, better absorption efficiency was reached when assimilation was performed from the carotenoid inclusion complex solution, regardless of its type (carotene or xanthophyll). The increases in the absorbed quantity were 50.6, 185, and 128% for β-carotene, lutein, and lycopene, respectively.

The particular nature of the equilibrium between the complexed state and the corresponding free forms is that, a priori, the

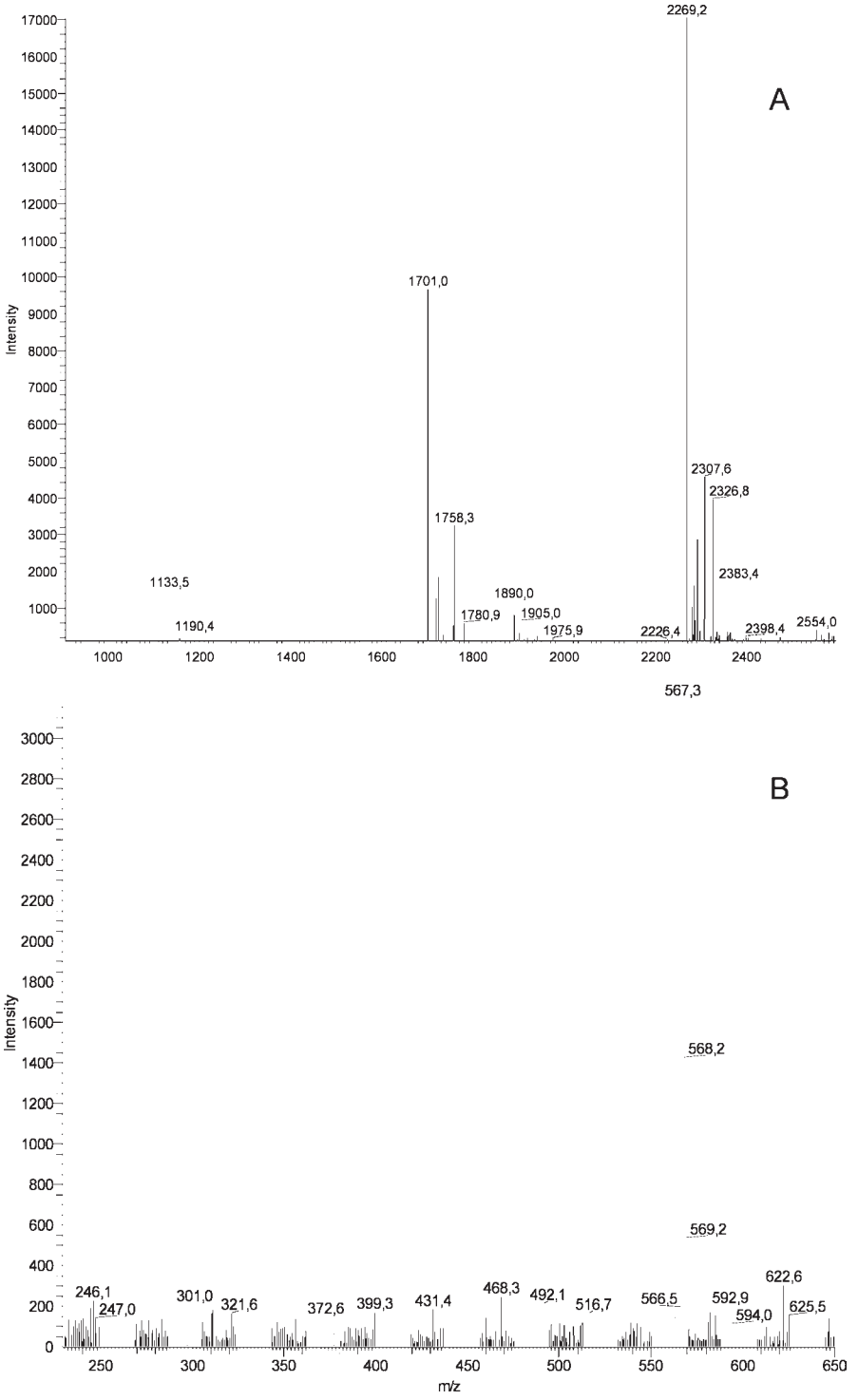

Figure 3. Mass spectrum of an aliquot of aqueous lutein inclusion complex solution (**A**) and the lysate from a BBMV preparation that was previously incubated in an aqueous solution of lutein β-cyclodextrin inclusion complexes and separated from that solution by centrifugation after incubation (**B**).

maximum concentration of the guest compound (a carotenoid, in this case) is limited by its solubility. With this condition in mind, for concentrations of 0.5 and 1 μM, the dissociation process did not reach a favorable optimum. For this reason, inclusion complexes showed lower assimilation efficiency than that reached using carotenoid suspensions in Tween. At the highest concentration, the dissociation equilibrium will be shifted toward the free forms, thus increasing the concentration of the guest compound available for assimilation. It is even possible for the concentration of the free compound to exceed the maximum set by its solubility (*33*), which further heightens the transport efficiency. This double effect explains the significant increases observed when using the highest concentration of carotenoid inclusion complexes in the donor solution.

Figure 4 shows the carotenoid content, expressed in picomoles per milligram of protein, that was incorporated into the BBMV plasma membrane after 20 min of incubation at 37 °C for β-carotene, lutein, and lycopene, from either the carotenoid suspension in Tween or the aqueous carotenoid CyDIC solutions, depending on the initial concentration of the carotenoids (0.5, 1, or 2.5 μM), when the BBMV preparation had been previously incubated with a high-density lipoprotein solution.

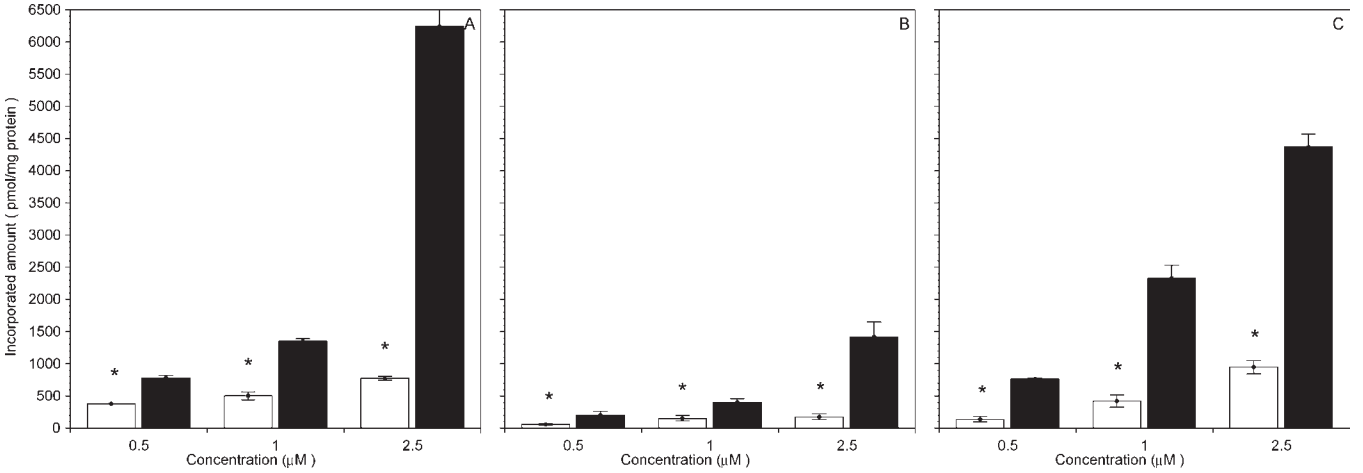

Figure 4. Assimilation of β-carotene (**A**), lutein (**B**), and lycopene (**C**) in BBMVs derived from porcine small intestine. Assimilation was measured after incubation of the three concentration levels of the carotenoid suspension in Tween (white bars) or the carotenoid/β-cyclodextrin inclusion complex solution (black bars) at 37 °C for 20 min with the BBMV preparation, which was preincubated with a high-density lipoprotein solution (50 μg/mL) for 5 min. Assimilation is shown as the mean ± SD (n = 3); an asterisk indicates significant differences in the assimilation levels reached for the two types of donor solution, for each carotenoid and for each concentration (p < 0.01, Duncan test).

When the donor solution was composed of a carotenoid suspension in Tween and was in the presence of an inhibitor, a significant drop in the assimilated quantity was observed compared to the same concentration and carotenoid type in a Tween suspension but without an inhibitor. The assimilation efficiency was still higher for carotenes than for the xanthophyll lutein. In fact, it was with lutein that the sharpest assimilation decrease was observed (a 70% drop in the presence of an inhibitor for all three concentrations). As with the tests performed without the inhibitor, a curved pattern for β-carotene and lutein assimilation (as in **Figure 2**) and a linear pattern for lycopene were observed. **Table 1** presents the linear effects (p < 0.05) of factors affecting the considered response (carotenoid assimilation). For β-carotene and lycopene, the coefficients were positive, whereas the lutein coefficient was negative. This fact indicates that the assimilation of carotenes involves different receptors from those involved in the assimilation of the xanthophyll lutein. In any case, the presence of the inhibitor did not completely block the carotenoid assimilation process, given that the decrease never reached 100%. This supports the idea of two coexisting assimilation mechanisms, facilitated diffusion and simple diffusion, the latter of which does not require the help of protein transporters and is therefore not affected by the presence of inhibitors of these transporters. Coexistence of both assimilation mechanisms has been found for the assimilation of dephytylated chlorophyll derivatives (*34*). It is also true that with the type and the amount of inhibitor used in this trial, a lower inhibition capacity was achieved without managing to completely block all transport compared with other inhibitors and/or quantities (*35*).

Several studies have used HDLs as efficient transport inhibitors for cholesterol and other lipids, pinpointing the mechanism of inhibition at the specific bond between the lipoprotein and the receptor, which is prevented from transporting lipids from other donor particles (*2*, *36*). In the present study, it was also shown that HDLs are effective carotenoid transport inhibitors. This fact has various physiological repercussions. The first is the contribution made by intestinal epithelial cells as a source of lipoproteins/apoproteins (*35*) that are capable of limiting the transport of lipid compounds such as carotenoids; this applies to the entire human population. The specific differences lie in the ability of each individual to regulate the synthesis and function of those receptors implicated in lipid assimilation. Therefore, genetic differences or differences in the expression of receptors involved in lipid transport would explain the nonresponder phenomenon observed in in vivo studies of carotenoid assimilation. These groups of individuals do not register a significant increase in the plasma levels of any of the ingested carotenoids (*7*, *37*). Another situation that differs from the one described above, but which also affects assimilation efficiency, occurs during inflammatory processes that affect the intestinal epithelium, such as inflammatory intestinal disease. In patients affected by an inflammatory process, plasma vitamin levels are significantly lower than those measured in healthy individuals (*38*). In addition to considering that patients are under oxidative stress and that the wearing effect on antioxidants would be intensified by this condition, it should also be considered whether the inflammatory process affects the function of receptors involved in the uptake of lipid antioxidants.

In fact, the carotenoid inclusion complex formulation was used in this study not only to test its effect on the efficiency of carotenoid assimilation in an uninhibited transport situation but also to test it with intentional limits on the assimilation capacity of transporters involved in the process. The results are quite noteworthy. The process of carotenoid assimilation from inclusion complexes reached its maximum efficiency under "inhibited" conditions, as shown by the data in **Figure 4**. When the level of assimilation efficiency was compared between the presence and the absence of an inhibitor using a donor solution composed of carotenoid CyDIC, significant increases were observed for all concentrations in the cases of β-carotene and lycopene in the presence of an inhibitor. For example, with the 1 μM concentration of carotenoid CyDIC increases of 85.6 and 165% were observed for β-carotene and lycopene, respectively. With a donor solution composed of lutein CyDIC, there was a significant increase found only with the highest concentration when transport was inhibited; similar results were not found for cases in which transport was not inhibited.

Barring the previously mentioned exception of lutein inclusion complexes (at low- and middle-range concentrations), an increase in assimilation efficiency was observed in the presence of the transport inhibitor, which in fact was not expected when this study was designed. In this case, the interaction between the inclusion complex and the high-density lipoprotein components used as transport inhibitors had a beneficial effect on the efficiency of assimilation. It has been shown that many lipid

and protein compounds compete with drugs to be included in the cyclodextrin cavity and can even release a previously complexed guest compound, showing a preference for compounds with cyclodextrin. Tokumura et al. (*39*) used this competition phenomenon as a strategy for increasing the bioaccessibility of a drug complexed with β-cyclodextrin. By incorporating phenylalanine as a competitive agent with a drug CyDIC donor solution, the bioaccessibility of the drug increased. In the present study, the exchange occurred among lipoprotein lipid compounds and the carotenoid/β-cyclodextrin inclusion complex; this exchange probably involved cholesterol, as it associates very well with β-cyclodextrin (*40*). The inhibitor role that HDLs from intestinal epithelial cells play in carotenoid assimilation, as indicated previously, will have a synergic effect on the assimilation of carotenoids from carotneoid CyDIC.

The experimental design of in vivo studies that allow verification of the assimilation efficiency from carotenoids CyDIC formulation with the co-ingestion of compounds that compete for complexation will be useful for determining the assimilation efficiency of carotenoids and other lipophilic bioactive compounds. This is of special interest for re-establishing plasma antioxidant levels in individuals whose transport conditions are compromised due to a pathological or physiological cause.

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