Carotenoid uptake and secretion by CaCo-2 cells: β-carotene isomer selectivity and carotenoid interactions[1]

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Abstract **In presence of oleate and taurocholate, differentiated CaCo-2 cell monolayers on membranes were able to assemble and secrete chylomicrons. Under these conditions, both cellular uptake and secretion into chylomicrons of β-carotene (β-C) were curvilinear, time-dependent (2–16 h), saturable, and concentration-dependent (apparent K_m of 7–10 μM) processes. Under linear concentration conditions at 16 h incubation, the extent of absorption of all-*trans* β-C was 11% (80% in chylomicrons), while those of 9-*cis*- and 13-*cis*-β-C were significantly lower (2–3%). The preferential uptake of the all-*trans* isomer was also shown in hepatic stellate HSC-T6 cells and in a cell-free system from rat liver (microsomes), but not in endothelial EAHY cells or U937 monocyte-macrophages. Moreover, extents of absorption of α-carotene (α-C), lutein (LUT), and lycopene (LYC) in CaCo-2 cells were 10%, 7%, and 2.5%, respectively. Marked carotenoid interactions were observed between LYC/β-C and β-C/α-C. The present results indicate that β-C conformation plays a major role in its intestinal absorption and that *cis* isomer discrimination is at the levels of cellular uptake and incorporation into chylomicrons. Moreover, the kinetics of cellular uptake and secretion of β-C, the inhibition of the intestinal absorption of one carotenoid by another, and the cellular specificity of isomer discrimination all suggest that carotenoid uptake by intestinal cells is a facilitated process.**—During, A., M. M. Hussain, D. W. Morel, and E. H. Harrison. **Carotenoid uptake and secretion by CaCo-2 cells: β-carotene isomer selectivity and carotenoid interactions. *J. Lipid Res.* 2002.** 43: **1086–1095.**

Supplementary key words *cis* isomers • α-carotene • lycopene • lutein • chylomicrons • human intestinal model

The consumption of a carotenoid-rich diet containing fruits and vegetables has been associated with a decreased risk for certain types of cancer and cardiovascular diseases (1). Approximately 600 carotenoids have been described in nature, however, only ~60 of them are present in human diet and ~20 of them in the human body. The hydrocarbon carotenoids β-C, α-C, and LYC, and the xanthophylls (or oxycarotenoids) LUT and β-cryptoxanthin are the most common carotenoids found in human diet (2) and in human blood and tissues (3). Although carotenoids are mainly found in the all-*trans* configuration of their polyisoprenoid structure, *cis* isomers can be detected in limited amounts in natural products or formed in significant amounts during food processing.

Knowledge about carotenoid absorption in humans comes mainly from extensive studies conducted with β-C and is well summarized in several reviews (4–8). Laboratory rodents do not absorb intact carotenoids and hence are not a good animal model for human carotenoid absorption and bioavailability. Ferrets, preruminant calves, and Mongolian gerbils have been used as models for studying human β-C metabolism (9–11), however, none of these are entirely satisfactory. In humans, quantitative data on β-C absorption and bioavailability have come mostly from studies involving either the intake-excretion balance approach or the plasma carotenoid response approach, in which the increase in plasma concentration after an oral load of carotenoid is measured (8). Both of these methods give only an indirect indication of intestinal absorption. Approaches using stable isotopes coupled with mass spectral analysis of carotenoids in the isolated postprandial TG-rich lipoprotein fraction of plasma give the most direct measure of carotenoid absorption (5, 8), however, they are costly and complex.

Fundamental aspects of carotenoid absorption are also largely unknown, such as quantitative information on differential uptake of carotenoids, luminal and intracellular factors regulating the process, the mechanism of intracellular transport of carotenoids and their incorporation in

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Abbreviations: α-C, α-carotene; β-C, β-carotene; CM, chylomicrons; LUT, lutein; LYC, lycopene; NEAA, non essential amino acids; OA, oleic acid; PL, phospholipids; TC, taurocholate.

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chylomicrons (CM), and the interactions among carotenoids during various steps of their intestinal absorption (4–7, 12). Given the limitations of using human subjects for investigation, a simple alternative model for studying human intestinal carotenoid absorption would be useful.

An intestinal cell culture system would be an appropriate model if the cells were able to form and secrete CM (large lipoproteins rich in TG that are required in vivo for intestinal absorption of fat-soluble nutrients such as carotenoids). The recent work of Luchoomun and Hussain (13) demonstrated that highly differentiated CaCo-2 cells cultured on membranes were able to assemble and secrete CM when they were supplemented with oleic acid (OA) and taurocholate (TC). We have used this model here to characterize the uptake and secretion of β-C by intestinal cells. In addition, we demonstrate the striking conformational discrimination in the absorption of geometric isomers of β-C and interactions between β-C and other carotenoids during their transport by CaCo-2 cells. Our results are consistent with a mechanism of facilitated uptake of carotenoids by CaCo-2 cells.

MATERIALS AND METHODS

Chemicals

All-*trans* β-C (type IV, >95% purity), OA, TC, α-C (type V, >95% purity), LYC (90–95% purity), LUT (70% purity), and other chemicals were purchased from Sigma (St. Louis, MO). [U-^{14}C]glycerol (160 mCi/mmol) and [1,2,3-^{3}H]glycerol (53.5 Ci/mmol) were obtained from Amersham (Amersham Pharmacia Biotech, NJ) and NEN Life Science Products (Boston, MA), respectively. All-*trans* β-C was purified by passage through a neutral alumina column (Type WN-3, Sigma) in hexane and then stored with *d*-α-tocopherol (0.01 molar ratio) in hexane at –20°C. The purified β-C solution contained mainly all-*trans* β-C (*cis* isomers <2%). 9-*Cis* and 13-*cis* isomers of β-C were a generous gift from Dr. A. Nagao (National Food Research Institute, Tsukuba, Japan). 9-*Cis* β-C solution in hexane contained mainly the 9-*cis* (95%) and all-*trans* (5%) isomers.13-*Cis* β-C solution in hexane contained mainly the 13-*cis* (82%) and all-*trans* (18%) isomers. LUT solution in hexane contained mainly LUT (90%) and zeaxanthin (7%) (≤3% of other compounds). α-C solution in hexane contained mainly α-C (96%) and β-C (4%). LYC solution in dichloromethane contained a mixture of all-*trans* (44–86%), 5-*cis* (6–46%), and other *cis* (8–10%) isomers of LYC. Because of variation found in the isomer composition of LYC, the sum of *trans* + *cis* LYC was used in the present work.

Cell culture

CaCo-2 cells were initially obtained from the American Type Culture Collection (Rockville, MD). The TC7 clone of the CaCo-2 cell line was a generous gift from Dr. A. Zweibaum (Unité de Recherche sur la Différentiation Cellulaire Intestinale, INSERM U178, Villejuif, France). Cells were grown on 25 or 75 cm^2 flasks (Corning Glassworks, Corning, NY) in presence of DMEM containing 25 mM glucose, 20% heat-inactivated FBS, and 1% nonessential amino acids (NEAA) (Gibco, Life Technologies Inc.). The seeding density was 20×10^3 and 12×10^3 cells/cm^2, respectively for CaCo-2 and TC7 cells with a 7-d passage frequency. Cells were incubated at 37°C in a humidified atmosphere of air/carbon dioxide (95:5, v/v) and the medium was changed every 48 h (10 or 20 ml for 25 or 75 cm^2 flasks, respectively).

For each experiment, cells were seeded at a density of 60×10^3 cells/cm^2, and grown on transwells (6-well plate, 24 mm diameter, 3 μm pore size, Corning Costar Corp., Cambridge, MA) in presence of DMEM containing 20% FBS, 1% NEAA, and 1% antibiotics. The medium was changed every 48 h for 3 weeks in order to obtain a confluent, differentiated cell monolayer. After 3 weeks, each cell monolayer was tested for its permeability (by measuring the diffusion of phenol red from the apical side to the basolateral side which was close to 0% in the presence of the intact cell monolayers used in the experiments). At the beginning of each experiment (zero-time), the apical side received 2 ml of serum-free medium (DMEM with 1% NEAA) containing TC-OA (0.5: 1.6 mM), [U-^{14}C]glycerol, or [1,2,3-^{3}H]glycerol, and β-C (see below for the mode of delivery to cells) and the basolateral side 2 ml of serum-free medium alone, followed by a incubation at 37°C. After the treatment period (usually 16 h), media from each side of the membrane were harvested; the apical medium kept at −20°C until analyses and the basolateral medium subjected to a lipoprotein fractionation (see below). The cell monolayer was washed three times with 2 ml of HBSS (Gibco) and extracted for total lipid (see below). In general, for each experimental point, the media and cell extracts of two wells were combined prior to analyses.

Method of delivery of carotenoids to cells

For delivering β-C (and other carotenoids) to cells, the Tween method previously described (14) was used with minor changes. Briefly, the required amounts of β-C or other carotenoid (up to 90 nmol) in hexane and 20 μl of Tween 40 at 20 g/100 ml in acetone were introduced into a sterilized glass tube, solvents evaporated, and the dried residue was then solubilized in 2 ml of serum-free medium and vigorously mixed. The resultant clear suspension was divided (2 × 1 ml) into two wells, each already containing 1 ml of serum-free medium with OA, TC, and labeled glycerol on the apical side. When the interaction between β-C and either α-C, LUT, or LYC was tested, the carotenoid in organic solvent was introduced in the test tube at the same time as β-C and Tween 40.

Incorporation of β-C isomers into other systems

Rat liver stellate cells HSC-T6 were obtained from Dr. William S. Blaner, Department of Medicine, Columbia University, NY, and were maintained under the cell culture conditions described recently (15). β-C (either all-*trans*, 9-*cis*, or 13-*cis* isomer) was delivered to confluent HSC-T6 cells grown on T25 flasks using the Tween method (see above). After overnight (18–20 h) incubation, β-C was extracted and analyzed from medium and cells as described below. Similar experiments were also conducted with confluent monolayers of the human endothelial cell line, EAHY, and with suspensions of the human monocyte/macrophage line U937.

Microsomes were prepared from two rat livers (Hilltop Lab Animals, Inc., Scottdale, PA) by differential centrifugation according to the procedure of Maisterrena et al. (16). Approximately 1,200 pmol of either all-*trans* β-C, 9-*cis* β-C, or 13-*cis* β-C in hexane, and 20 μl of Tween 40 at 4.5% in acetone were introduced in a Potter-Elvehjem tube and solvents immediately evaporated under nitrogen. One milliliter of the microsomal suspension (20 mg protein/ml) was rapidly added to the dry residues and homogenized on ice for 5 min. The suspension was then centrifuged at 105,000 *g* for 60 min at 4°C. The resultant pellet (microsomes) was resuspended in 1 ml of buffer.

Lipoprotein fractionation

Large CM, small CM, and VLDL were isolated from the basolateral medium by a sequential density gradient ultracentrifuga-

tion as previously described (13). Briefly, 3.5 ml of media was well mixed with 0.57 g KBr (density of 1.1 g/ml) and then overlaid with 3 ml each of 1.063 and 1.019 g/ml, and 2 ml of 1.006 g/ml density solutions using an Auto Densi-Flow (Labconco Corp., MO). The sample was subjected to three successive centrifugations at 15°C (SW41 rotor, Beckman Instruments Inc., CA): *a*) at 40,000 rpm for 33 min, then *b*) at 40,000 rpm for 3 h 28 min, and finally *c*) at 40,000 rpm for 17 h (17). After each centrifugation, 1 ml of the top was collected corresponding to large CM (Sf > 400), small CM (Sf 60–400), and VLDL (d < 1.006 g/ml, Sf 20–60) fractions, respectively, and replenished with 1 ml of 1.006 g/ml density solution prior to the next centrifugation.

Lipid extraction

From the cell monolayer, total lipids (including carotenoids) were extracted twice with 2 ml of 2-propanol-dichloromethane (2:1, v/v) at room temperature for 30 min and once with 2 ml of 2-propanol-dichloromethane (2:1, v/v) at 4°C overnight in dark (18). The three extracts were combined, solvents evaporated, and lipids redissolved in 1 ml of methanol-dichloromethane (84:16, v/v) and kept at −20°C for TG and carotenoid analyses. From apical media (before and after treatment), basolateral media, and the different lipoprotein fractions (0.1 to 0.5 ml aliquot), lipids were extracted using the method of Folch et al. (19). The lipid extract in chloroform-methanol (2:1, v/v) was used for TG analysis.

Lipid analysis

At zero-time, [U-^{14}C]glycerol or [1,2,3- ^{3}H]glycerol was added to the apical medium of cells in order to label and quantify the newly formed TG and phospholipids (PL) pools. Total lipid extracted from cells, media, and lipoprotein fractions were separated by TLC using pre-coated plates of silica gel 60 (0.5 mm thick, Merck, Germany) and chloroform-methanol-water (65:25:4, v/v/v) as the mobile phase. Lipid bands corresponding to TG and PL (including [U-^{14}C]TG and PL or [1,2,3-^{3}H]TG and PL) were first visualized with iodine vapor, scraped from the plate, dissolved in 5 ml of scintillation cocktail liquid, and finally counted in a scintillation counter.

Extraction and analysis of carotenoids

Carotenoids were extracted from apical and basolateral media (at zero-time and after treatment), using a procedure established for serum (18). Thus, 100 μl of medium and 300 μl of 2-propanol-dichloromethane (2:1, v/v) were placed in a tube and vortexed for 1 min, followed by a centrifugation for 1 min using an IEC microcentrifuge (International Equipment Company, MA). The resultant supernatant was directly applied to the HPLC system. Carotenoids were extracted from lipoprotein fractions (300–500 μl) (1 volume), once with 2-propanol-dichloromethane (2:1, v/v) (3 volumes) and twice with hexane (3 volumes). The three extracts were combined, dried, redissolved in 300 μl of methanol-dichloromethane (84:16, v/v), and applied to the HPLC system. For cells, an aliquot of the total lipid extract in methanol-dichloromethane (84:16, v/v) obtained previously (see above) was directly analyzed by HPLC.

Carotenoids were analyzed using a Waters HPLC system equipped with a Model 717 plus autosampler, a Model 996 photo diode array detector, and a Millenium^{32} chromatography manager (Waters T system, Milford, MA). Carotenoids were eluted on a TSK gel ODS 120-A C18 reverse phase column, 4.6 × 250 mm (Tosohaas, Montgomeryville, PA), with methanol-dichloromethane (84:16, v/v) as the mobile phase (flow rate of 0.9 ml/min). Retention times of LUT, α-C, all-*trans* β-C, 9-*cis* β-C, 13-*cis* β-C, and LYC (*trans* + *cis*) were 5.0, 14.9, 15.9, 17.2, 17.6, and 18.2–19.0 min, respectively. Each carotenoid was quantified from its peak area by comparison with a standard reference curve established with different amounts of the respective standard carotenoid (from 0.5 to 500 pmol) in methanol-dichloromethane (84:16, v/v) at 450 nm.

ApoB measurement

ApoB levels in basolateral media and lipoprotein fractions were analyzed using a sandwich enzyme-linked immunosorbent assay described previously (13, 20).

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis of results was assessed by one way ANOVA coupled with the Fisher's test. Relationships between two variables were examined by simple or logarithmic regression analyses. The choice of the regression (simple vs. logarithmic) was determined by the squared value of the regression coefficient (R^2); the regression given the highest R^2 value was chosen. All statistical analyses were performed using the Statview, version 5.0 (SAS Institute, Cary, NC). A value of $P < 0.05$ was considered significant.

RESULTS

Lipid and apoB compositions of lipoproteins secreted by CaCo-2 cells under standard experimental conditions

The standard conditions were defined as follows: 3 week-differentiated CaCo-2 cell monolayers cultured on 3 μm-pore size transwells (Corning Costar) were incubated

TABLE 1. Distribution of apoB and newly synthesized TG and PL in cells and in the different lipoprotein fractions under standard conditions

			Distribution in Lipoprotein Fractions[c]			
	Total in Cells	Total Secreted	Large CM Sf > 400	Small CM Sf 60-400	VLDL d < 1.006	Rest d > 1.01
	^{3}H-pmol	*^{3}H-pmol*		*% of total secreted*		
TG[a]	10727 ± 2147	4340 ± 1226	52.1 ± 7.8	36.4 ± 5.9	6.7 ± 2.8	4.8 ± 2.9
PL[a]	2662 ± 746	762 ± 189	11.4 ± 4.9	10.9 ± 5.3	4.2 ± 4.0	73.5 ± 9.3
	ng	*ng*		*% of total secreted*		
ApoB[b]	n.a.	2523 ± 166	10.0	22.2	18.4	49.4

[a] Data for TG and PL represent means ± SD, n = 6 to 8 independent experiments.
[b] Data for apoB represent means of triplicate assays from one experiment.
[c] The recoveries of TG, PL, and apoB in total lipoprotein fractions versus total secreted in the basolateral medium were 82.1 ± 8.6% (n = 6), 69.4 ± 33.3% (n = 6), and 65.4%, respectively.
n.a. = not analyzed.

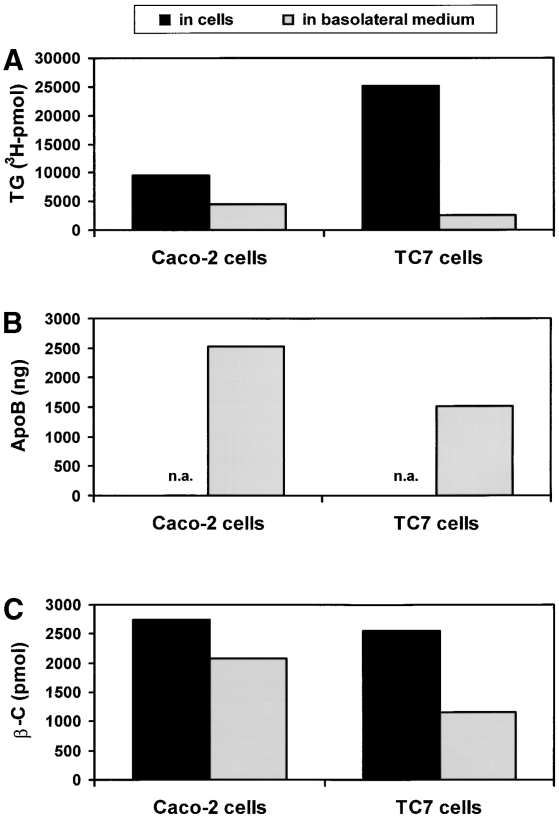

Fig. 1. Comparison of CaCo-2 and TC7 cells for their ability to produce and secrete newly synthesized triglycerides (TGs) (graph A), to secrete apolipoprotein B (apoB) (graph B), and to take up and secrete β-carotene (β-C) (graph C). Differentiated CaCo-2 and TC7 cell monolayers were used. The apical side received serum-free medium containing TC-OA at 0.5:1.6 mM plus [^{3}H]glycerol at 45 μM (~1.5 μCi/ml, 33.3 μCi/μmol), Tween 40 at 0.1%, and all-*trans* β-C at 5.2 μM. The basolateral side received serum-free medium. After 16 h incubation, basolateral media of two wells (4–5 ml) were combined and subjected to a lipoprotein fractionation. Lipids were extracted from the medium, lipoprotein fractions, and cells. Data for TG and β-C are averages of two independent treatments and data for apoB are means of triplicate assays from one treatment. (n.a. = not analyzed.)

with serum-free medium containing TC-OA at 0.5:1.6 mM and [^{3}H]glycerol at 45 μM. Under these conditions, after 16 h incubation, one third of newly synthesized TG and one fifth of newly synthesized PL were secreted in the basolateral medium (**Table 1**). The two combined CM fractions contained 88% of total secreted labeled TG and 22% of total secreted labeled PL. Finally, of the total amount of apoB secreted in the basolateral medium, 32% was associated with the two CM fractions.

Comparison of CaCo-2 and TC7 cells for their ability to secrete both CM and β-C

After 3 weeks of differentiation on transwells (same starting date), both CaCo-2 and TC7 cell monolayers were incubated under standard conditions in presence of 5.2 μM of all-*trans* β-C. The total amount of labeled TG (cells + basolateral medium) synthesized by TC7 cells was 2-fold higher than for CaCo-2 cells. Although TC7 cells synthesized larger amounts of TG, those TG remained preferentially in cells and were poorly secreted (1.8-fold less TG in the basolateral medium of TC7 cells compared with CaCo-2 cells) (**Fig. 1A**). Of total labeled TG secreted, ~90% was recovered in the two CM fractions for both types of cells (data not shown). The amount of apoB secreted by TC7 cells was 1.7-fold less than that of CaCo-2 cells (Fig. 1B). Thus, the TC7 clone was less efficient in the production and secretion of CM than the original parent cell line, CaCo-2. As a probable consequence, the amount of β-C found in the basolateral medium of TC7 cells was 1.8-fold lower than that of CaCo-2 cells (Fig. 1C).

Effect of the initial β-C concentration and incubation time on β-C uptake and secretion into lipoproteins by CaCo-2 cells

Differentiated CaCo-2 cell monolayers were incubated with different concentrations of β-C (from 0.12 to 22 μM) for 16 h under the standard conditions described above. Both the amounts of β-C in CaCo-2 cells and secreted in the basolateral medium increased linearly with increasing initial β-C concentration (up to 6 μM) and then plateaued for initial β-C concentration higher than 10 μM (**Fig. 2A**, **B**). The best fitting curves were obtained by logarithmic regression analyses (R^2 = 0.931 for Fig. 2A, R^2 = 0.770 for Fig. 2B) indicating that both β-C accumulation in cells and β-C secretion were two saturable processes. In the linear concentration conditions, the extent of absorption of β-C was 11.0 ± 2.5% (Fig. 2B) and the amount of β-C found in the two combined CM fractions was 78 ± 11% of the total β-C secreted (Fig. 2C) (mean ± SD, n = 12 points). The exact distribution of β-C in the basolateral medium was 45.0 ± 9.9%, 33.5 ± 8.0%, and 9.8 ± 2.7%, respectively, in large CM, small CM, and VLDL fractions (mean ± SD, n = 12 points, data not shown). The two variables of Fig. 2C (percent of β-C in CM vs. initial β-C concentration) were independent (by simple regression: R^2 = 0.057, P = 0.4549). On another hand, the amount of β-C secreted in CM was directly associated with β-C concentration present at the apical side of CaCo-2 cells (for 0.2 to 5.99 μM) (by simple regression: R^2 = 0.825, $P <$ 0.0001, and n = 12 points), however, the amount of β-C in CM was not measured for initial β-C concentration higher than 6 μM. **Figure 3** indicates that the cellular content of β-C, its secretion into the basolateral medium, and incorporation in CM were increased in a curvilinear way with incubation time up to 16 h. Thus, although the data shown in Fig. 2 do not represent true initial rates, we estimated apparent V_{max} and K_m values for the rates of cellular uptake and secretion from the data presented in Fig. 2. We found V_{max} = 6,500 pmol β-C/16h and K_m = 10 μM for cellular accumulation of β-C, and V_{max} = 3,500 pmol β-C/16h and K_m = 7 μM for secretion of β-C from CaCo-2 cells.

Effect of β-C isomerization on β-C uptake and secretion by CaCo-2 cells

Differentiated CaCo-2 cells were incubated with ~1 μM of total β-C with different amounts of *cis* isomers (2% *cis*,

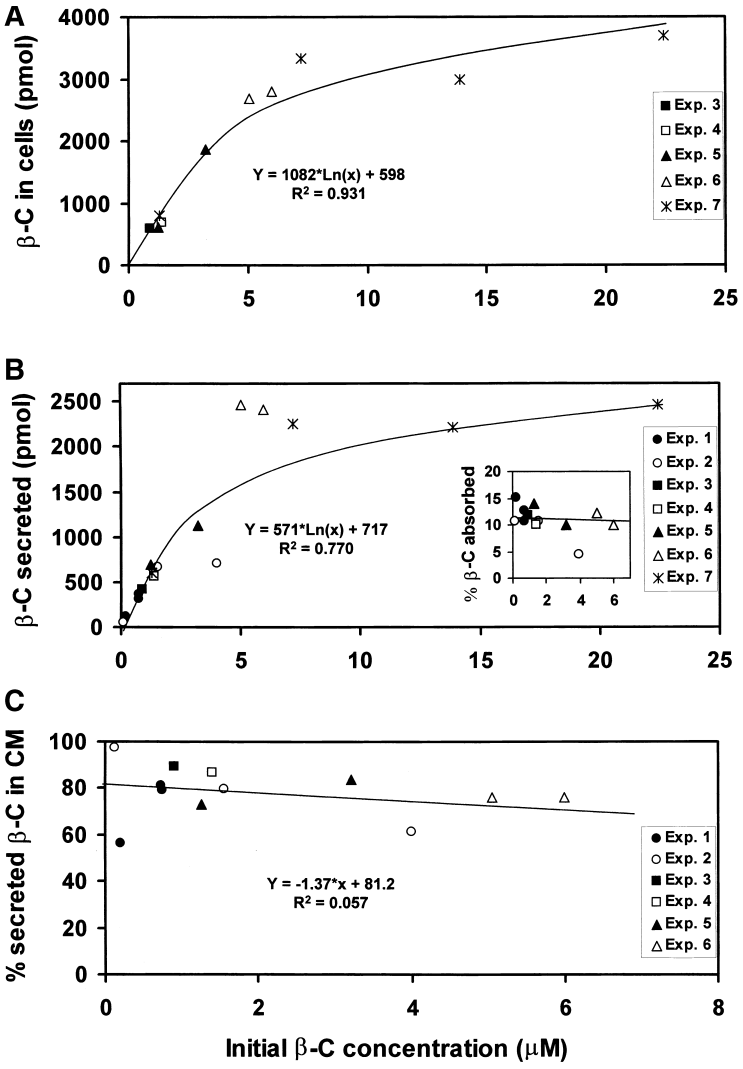

Fig. 2. Effects of the initial β-C concentration (0.12 to 22 μM) on β-C cellular content (graph A), β-C secretion in the basolateral medium (graph B), and β-C incorporation into large and small chylomicrons (CM) (graph C). The inset in graph B shows the extent of absorption of β-C for the range of initial β-C concentration between 0.12 and 6 μM. Differentiated CaCo-2 cell monolayers were used. The apical side received serum-free medium containing TC-OA at 0.5:1.6 mM, [^{3}H]glycerol at 45 μM (1.4 μCi/ml, 40 μCi/μmol), and different amounts of β-C (2 wells/ treatment). The basolateral side received serum-free medium. After 16 h incubation, basolateral media of two wells were combined and subjected to a lipoprotein fractionation. β-C was then extracted from cells, total medium, and the different lipoprotein fractions and analyzed by HPLC. Data in graphs A, B, and C are individual values obtained from seven independent experiments.

33% 9-*cis*, 99% 9-*cis*, or 79% 13-*cis*). The amounts of β-C in cells and in basolateral medium were reduced with increasing degree of 9-*cis* isomerization of β-C initially added at the apical side of cells (**Table 2**). For instance, when 9-*cis* β-C (99% *cis*, 1% all-*trans*) was used, total β-C amounts in cells and in basolateral medium were decreased by 4.7- and 15-fold, respectively, compared with all-*trans* β-C (98% all-*trans*, 2% *cis*). Similarly, a high percentage of 13-*cis* configuration of β-C (79% *cis*, 21% all-*trans*) reduced β-C cellular content by 5.5-fold and β-C secretion by 5.2-fold. Finally, in the apical medium after incubation at 37°C for 16 h, we observed that all-*trans* β-C was partly isomerized into *cis* β-C, while the opposite was not true.

Study of incorporation of β-carotene isomers by other systems

Rat stellate cells (HSC-T6) were incubated with either all-*trans*, 9-*cis*, or 13-*cis* β-C at a final concentration of ~1 μM for 20 h. Around 3.5% of the initial all-*trans* β-C amount added to the cell culture medium was incorporated into HSC-T6 cells, while less than 1% of *cis* isomers were taken up by these cells ($P < 0.0001$ in comparison with all-*trans* β-C) (**Fig. 4A**). In a cell free system, rat liver microsomes were enriched with either all-*trans*, 9-*cis*, or 13-*cis* β-C as described above. After homogenization for 5 min, microsomes contained 28%, 12%, and 11% of the initial β-C amount, respectively, for all-*trans*, 9-*cis*, or 13-*cis* β-C (Fig. 4B). Thus, *cis* isomers of β-C were significantly ($P < 0.02$) less incorporated in rat liver microsomes than the *trans* isomer. In contrast to the results with intestinal and liver cells, no isomer discrimination was observed with endothelial cells or with monocyte/macrophages. Overnight incubation of EAHY cells with 1 μM all-*trans* β-C resulted in 37 ± 8% (n = 5) uptake versus 32 ± 13% for the 9-*cis* isomer. Similarly, for U937 cells the amounts of uptake were 27 ± 12% (n = 4) for the all-*trans* isomer and 25 ± 12% (n = 6) for the 9-*cis*.

Comparison of the extent of absorption of individual carotenoids

Differentiated CaCo-2 cells were incubated with all-*trans* β-C, 9-*cis* β-C, 13-*cis* β-C, α-C, LUT, or LYC at an initial concentration of 1 μM for 16 h. As shown previously (Table 2), extents of absorption of the *cis* isomers of β-C were significantly lower (2% and 3%, respectively, for 9-*cis* and 13-*cis* β-C), compared with that of all-*trans* β-C (~11%) ($P < 0.0001$)

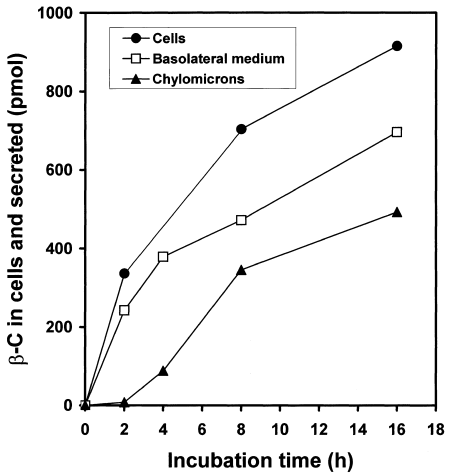

Fig. 3. Effects of incubation time (2 h to 16 h) on β-C cellular content (solid circle), β-carotene secretion in the basolateral medium (open square), and β-carotene incorporation into large and small CM (solid triangle). Differentiated CaCo-2 cell monolayers were used. The apical side received serum-free medium containing TC-OA at 0.5:1.6 mM, [^{3}H]glycerol at 45 μM (1.4 μCi/ml, 40 μCi/μmol), and β-C at 1 μM. The basolateral side received serum-free medium. After the desired time of incubation, basolateral media of two wells were combined and subjected to a lipoprotein fractionation. β-C was then extracted from cells, total medium, and the different lipoprotein fractions, and analyzed by HPLC. Data are individual values obtained from one experiment.

(**Fig. 5**). The extent of absorption of α-C was not significantly different from that of all-*trans* β-C, while extents of absorption of LUT and LYC were significantly lower 7% ($P = 0.0004$) and 2.5% ($P < 0.0001$), respectively.

Study of potential interactions between β-C and either α-C, LUT, or LYC during their intestinal absorption by CaCo-2 cells

First, individual effects of three carotenoids (α-C, LYC, and LUT) on β-C absorption were examined (**Fig. 6A**, **B**). CaCo-2 cells were incubated with all-*trans* β-C at a fixed concentration (1 μM) and either α-C, LYC, or LUT at increasing concentrations of 1, 2, and 5 μM. α-C and LUT did not show any effect on secretion or cellular uptake of β-C. The most marked effect on β-C absorption was observed with LYC. Indeed, the extent of β-C absorption was reduced by 1.4-fold ($P < 0.05$) and 4.9-fold ($P < 0.0001$), respectively, for β-C-LYC ratios of 1:1 and 1:5 compared with β-C alone (Fig. 6A). Similar and significant reductions in the cellular uptake of β-C were observed with β-C-LYC ratio of 1:1 and 1:5 (1.6-fold, $P < 0.005$, and 4.7-fold, $P < 0.0001$, respectively) (Fig. 6B). Effect of β-C on the intestinal absorption of the other three carotenoids (α-C, LYC, and LUT) was then investigated (Fig. 6C, D). In this second experiment, CaCo-2 cells were incubated with one of the three carotenoids (α-C, LYC, or LUT) at a fixed concentration (1 μM) and all-*trans* β-C at increasing concentrations of 1, 2, and 5 μM. β-C did not interfere with the intestinal absorption of LUT, but showed a marked effect on α-C absorption. Indeed, in presence of β-C, the extent of α-C secretion was significantly reduced (by 1.4- and 2.2-fold, respectively, for the α-C-β-C ratio of 1:1 and 1:5, $P < 0.05$) (Fig. 6C). The cellular uptake of α-C was also decreased with increasing β-C concentration, but $P > 0.05$ (Fig. 6D). Finally, β-C also had an effect on LYC absorption with a reduction of both secretion and cellular uptake of lycopene by 2.3- and 1.7-fold, respectively, in presence of the LYC-β-C ratio of 1:5 compared with LYC alone (but $P > 0.05$). Although the extents of absorption of the four carotenoids (β-C, α-C, LUT, and LYC) were very different (ranging from 2.5% to 11%) (Fig. 5), the present data indicate similar extents of cellular uptake for these four carotenoids (ranging from 15% and 18%) (Fig. 6B, D).

DISCUSSION

The first purpose of this study was to establish an in vitro cell culture system mimicking the in vivo intestinal absorption of carotenoids, which involves several crucial steps: release of carotenoids from the food matrix, solubilization of carotenoids into mixed micelles formed in the lumen, cellular uptake of carotenoids by intestinal mucosal cells, and secretion of carotenoids and their metabolites into the lymphatic circulation (4–7). In the present study, β-C and other carotenoids were presented to CaCo-2 cells in aqueous solution with Tween 40 micelles. This mode of delivery, used previously for cell culture (14),

TABLE 2. Effect of β-C isomerization on its cellular uptake and secretion by CaCo-2 cells under standard conditions

Treatments	All-*trans*	All-*trans*/9-*cis*	9-*cis*	13-*cis*
	total pmol of β-C[a]			
Initial conditions at 0 h				
Apical medium	4,577 (2% *cis*)[b]	4,220 (33% *cis*)	4,082 (99% *cis*)	4,454 (79% *cis*)
Basolateral medium	~0	~0	~0	~0
After 16 h incubation				
Apical medium	2,251 (12% *cis*)	2,320 (45% *cis*)	2,333 (97% *cis*)	2,777 (79% *cis*)
Cells	629 (4% *cis*)	491 (11% *cis*)	134 (85% *cis*)	114 (13% *cis*)
Basolateral medium	345 (0% *cis*)	283 (0% *cis*)	23 (0% *cis*)	66 (13% *cis*)

[a] Data are expressed as total pmol of β-C (*trans* + *cis* isomers) and represent means of two independent experiments.

[b] Percent of total *cis* isomers of β-C present in the analyzed sample.

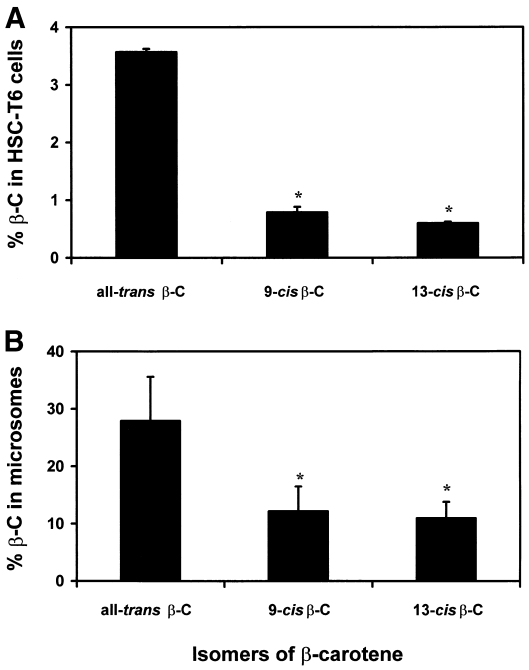

Fig. 4. Incorporation of β-C isomers by rat stellate cells HSC-T6 (graph A) and by rat liver microsomes (graph B). In graph A, confluent HSC-T6 cells cultured on T25 flasks were incubated with either all-*trans*-, 9-*cis*-, or 13-*cis*-β-C at 1 μM for 20 h. After incubation, β-C was extracted from cells and total medium and analyzed by HPLC. In graph B, one ml of a microsomal suspension (20 mg protein/ml) obtained from rat livers was homogenized in presence of 1,200 pmol of either all-*trans* β-C, 9-*cis* β-C, or 13-*cis* β-C, and Tween 40 at 0.09%. Data in graph A are means ± SD of triplicate assays (three flasks of HSC-T6 cells per β-C isomer tested) with $*P < 0.0001$ compared with all-*trans* β-C. Data in graph B are means ± SD of triplicate assays and $*P < 0.02$ compared with all-*trans* β-C.

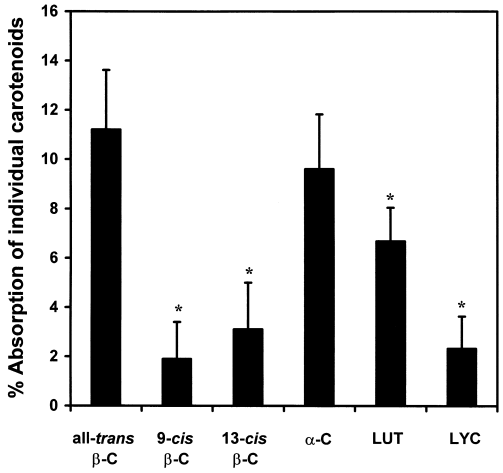

Fig. 5. Study of the extent of absorption of various individual carotenoids. Differentiated CaCo-2 cell monolayers were used. The apical side received serum-free medium containing TC-OA at 0.5:1.6 mM, cold glycerol at 45 μM, Tween 40 at 0.1%, and either all-*trans* β-C, 9-*cis* β-C, 13-*cis* β-C, α-C, LUT, or LYC at an initial concentration of 1 μM (2 wells/ treatment). The basolateral side received serum-free medium. After 16 h incubation, carotenoids were extracted from the basolateral media and analyzed by HPLC. Data are means ± SD obtained from three or more independent experiments. $*P < 0.0005$ compared with the extent of all-*trans* β-C absorption. Recoveries (16 h vs. 0 h) of all-*trans* β-C, 9-*cis* β-C, 13-*cis* β-C, α-C, LUT, and LYC were 72 ± 4, 64 ± 5, 68 ± 3, 68 ± 5, 67 ± 6, and 62 ± 8, respectively.

gave the best results in term of solubility, recovery and secretion of β-C, compared with β-C solubilization in TC-OA. Because the main focus of this study was to define an in vitro system for studying intestinal absorption of carotenoids, we did not attempt here to reproduce physiochemical conditions in the intestinal lumen involving mixed lipid micelles containing bile salts, fatty acids, monoglycerides, and PL. However, our cell culture model could be used in conjunction with an in vitro digestion procedure (21) in which carotenoids are transferred from the food to bile salt micelles to assess the bioavailability of carotenoids from foods in vitro.

One obligate step for lipophilic compounds such as carotenoids to cross the intestinal epithelial cells is their incorporation into CM. Under normal cell culture conditions, CaCo-2 cells are unable to form CM. However, using conditions defined previously (13), we found that differentiated CaCo-2 cells cultured on membranes and supplemented with TC-OA were able to secrete small and large CM. Since the TC7 clone of the CaCo-2 cell line was reported to exhibit β-C cleavage activity and thus might be a better model for carotenoid metabolism/absorption than the parent CaCo-2 cell line (14), we also examined the ability of TC7 cells to synthesize CM. Compared with CaCo-2 cells, TC7 cells exhibited (2-fold) more TG in cells and (2-fold) less TG in basolateral medium, presumably due to their lower capacity to assemble CM. As a consequence, the amount of β-C secreted by TC7 cells was twice lower than that by CaCo-2 cells. Because CaCo-2 cells were more efficient than TC7 cells in term of both CM formation and β-C transport and because β-C cleavage might complicate studies on β-C absorption per se, the parent CaCo-2 cells were used for further studies to investigate the intestinal absorption of β-C and other carotenoids.

In contrast to previous in vivo models, the present in vitro cell culture system gives us the possibility to dissociate the different steps involved in the intestinal absorption of carotenoids. Indeed, we demonstrated that CaCo-2 cells were able to take up β-C and to incorporate it into CM and secrete it at the basolateral side. These two steps (cellular uptake and secretion in CM) were curvilinear, time-dependent, saturable, and concentration-dependent processes. Saturation occurs at β-C concentrations higher than the physiological range (equivalent to a daily intake of 100 mg or more). Indeed, the β-C concentration of 1 μM (or 400 pmol/cm^2 of CaCo-2 cells; the concentration commonly used in this study) was estimated to be close to the physiological level of β-C found in the gut (200 pmol/cm^2 of surface of absorption) after a normal daily β-C intake of 5 mg (assuming that the absorption process of β-C takes place mainly in the upper half of the small intestine (length = 3.5 m, diameter = 4 cm) and villi increase the surface of absorption by a factor 10). Thus, in contrast to earlier studies (22, 23) proposing a passive diffusion process, our data

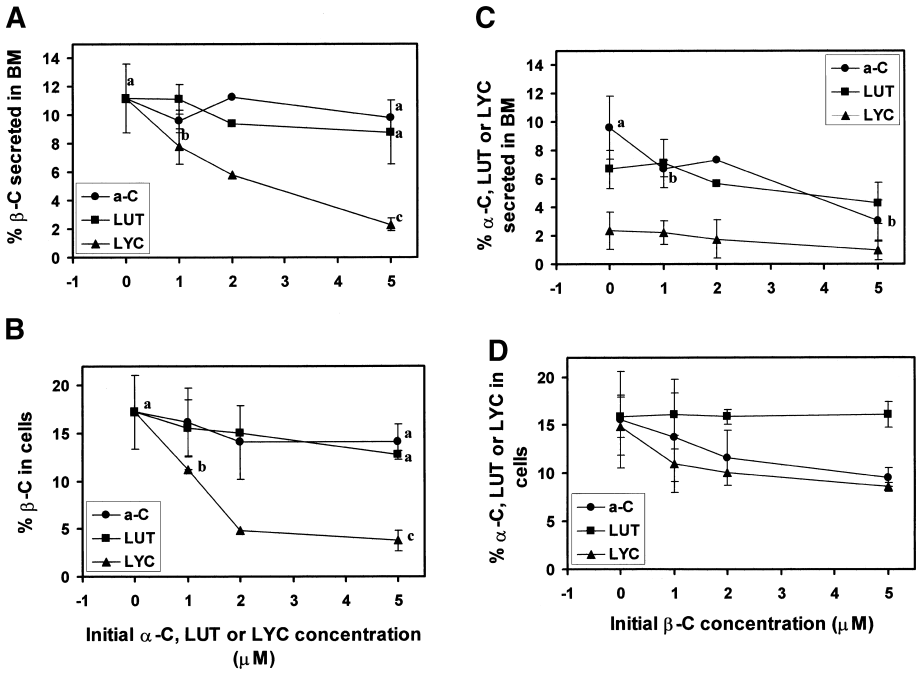

Fig. 6. Potential carotenoid interactions: effect of either α-C (solid circle), LUT (solid square), and LYC (solid triangle) on β-C cellular uptake and secretion into the basolateral medium (graphs A and B) and effect of β-C on cellular uptake and secretion into the basolateral medium of either α-C (solid circle), LUT (solid square), or LYC (solid triangle) (graphs C and D). Differentiated CaCo-2 cell monolayers were used. The apical side received serum-free medium containing TC-OA at 0.5:1.6 mM, cold glycerol at 45 μM, Tween 40 at 0.1%, and one or two carotenoids (either all-*trans* β-C, α-C, LUT, or LYC) (two wells/ treatment). In graphs A and B, 1 μM of β-C was incubated with varying concentrations (from 0 to 5 μM) of either α-C, LUT, or LYC. In graphs C and D, 1 μM of either α-C, LUT, or LYC was incubated with different concentrations of β-C (from 0 to 5 μM). After 16 h incubation, carotenoids were extracted and analyzed by HPLC. Data with error bars are means ± SD obtained from three or more independent experiments. Effects of a considered carotenoid at the different concentrations (1, 2, and 5 μM) were compared with the initial conditions in the absence of the carotenoid (0 μM). (a, b): $P < 0.05$ and (a, c): $P < 0.0001$.

suggest that intestinal transport of β-C might be facilitated by the participation of a specific epithelial transporter.

The idea of a specific transport in intestine is also supported by our observations showing the preferential transport of all-*trans* β-C versus either 9-*cis* or 13-*cis* isomers of β-C through CaCo-2 cell monolayers. A preferential accumulation in plasma and in postprandial lipoproteins of all-*trans* β-C versus 9-*cis* β-C was also observed in human (24–26) and ferret (27), but those in vivo studies could not provide any information about the mechanism of that selective transport. In the present study, we found that discrimination between β-C isomers occurred at the level of cellular uptake in CaCo-2 cells and HSC-T6 cells derived from liver, but not in endothelial cells or monocyte macrophages. That cellular specificity for β-C isomers is consistent with the hypothesis of a specific epithelial transporter(s). Moreover, by using the present in vitro model, the results indicate that there is a specific intracellular mechanism leading to the preferential incorporation into CM of the all-trans β-C versus its *cis* isomers.

A human study (28) reported a significant accumulation of all-*trans* β-C in plasma of subjects who ingested only 9-*cis* β-C, suggesting that 9-*cis* β-C was isomerized into all-*trans* β-C during its intestinal absorption. In contrast, we did not observe any increase of all-*trans* β-C in cells or in the basolateral medium when 9-*cis* β-C was presented to CaCo-2 cells. Therefore, the in vivo isomerization of 9-*cis* β-C suggested previously (28) could take place in the gastrointestinal lumen before cellular uptake, probably under the action of enzymes related to gut microflora since the spontaneous isomerization of 9-*cis* β-C to all-*trans* β-C is not thermodynamically favored (29).

In addition to providing new information concerning the mechanism of intestinal transport of carotenoids, the present in vitro cell culture system seems like a good model for the in vivo human intestinal absorption of carotenoids. Under linear concentration conditions at 16 h incubation and under cell culture conditions mimicking the in vivo postprandial state, 11% of all-*trans* β-C passed through CaCo-2 cell monolayers. In agreement, the human intestinal absorption of β-C was also 11% using the approach of carotenoid and retinyl ester responses in the TG-rich lipoprotein plasma fraction (30) and ranged between 9–17% using the lymph-cannulation approach (31, 32). Finally, from total β-C secreted by CaCo-2 cells, 80% was associated with the two CM fractions (45%, 34%, and 10%, respectively, in large CM, small CM, and VLDL), indicating the importance of CM assembly for β-C secretion as was shown previously for retinyl ester secretion (33).

By using the present in vitro model, we found a differ-

ential absorption among the carotenoids tested with all-*trans* β-C (11%) > α-C (10%) > LUT (7%) > LYC (2.5%). In agreement with our data, several studies using either mesenteric lymph duct cannulated rats (34), preruminant calves (35), or human subjects (36, 37) converge to indicate that LYC is poorly absorbed. In contrast to our data and a previous study (37), Kostic et al. (38) reported that LUT absorption was 2-fold higher than β-C absorption. However, in this study (38), it was assumed that β-C metabolism in the intestinal mucosa was a minor part of its transfer into plasma, contributing to lower values of β-C absorption compared with the nonprovitamin A carotenoid, LUT. Indeed, there is evidence that at least 35% (up to 75%) of the absorbed β-C is converted to retinyl esters (30–32, 37). Interestingly, the present data show that, for the four individual carotenoids tested (all-*trans* β-C, α-C, LYC, and LUT), the extent of secretion varied over a wider range (2.5–11%) than the extents of cellular uptake (15–18%). This observation indicates that the structure of the carotenoid might play a major role in its ability to be incorporated into CM.

During the last decade, it has been suggested that carotenoids compete for their absorption and metabolism, but there is discrepancy between the different findings "both in magnitude and in direction of the interactions observed" (12). For instance, in rats, liver vitamin A storage (used as a measure of β-C absorption) was enhanced by a small dose of LUT, but reduced by a larger dose of LUT (39). In humans, β-C reduced the apparent LUT absorption (38, 40, 41), while LUT had either no effect (38) or reduced the apparent β-C absorption (40, 41). In the present study, neither LUT nor β-C affected significantly the transport of each other through CaCo-2 cell monolayers. The inhibitory effect of LUT on β-C response observed in vivo could be attributed at least partly to the fact that LUT inhibits β-C cleavage enzyme (42). In addition, β-C improved the apparent LYC absorption (36), while LYC had no significant effect on the apparent β-C absorption in human (36, 41) and in ferret (43). In contrast, our data show a mutual negative interaction between LYC and β-C during their passage through CaCo-2 cell monolayers. The discrepancy between our data and the previous in vivo data concerning carotenoid interactions (36, 38–41) might be due to the fact that most of the in vivo studies, by measuring plasma carotenoid response (called here "apparent carotenoid absorption"), assume that carotenoids have similar rates of clearance from plasma and of recirculation between tissues and plasma. Thus, carotenoid metabolism in the intestinal mucosa is only a part of what is being measured in these studies. In the present in vitro cell culture system, the main carotenoid interactions occur only between nonpolar carotenoids (β-C/α-C and β-C/LYC), suggesting that these hydrocarbon carotenoids exhibiting similar structural characteristics could follow similar pathways for their cellular uptake and/or incorporation into CM. These mutual interactions are also consistent with the idea of a facilitated uptake process.

In summary, we describe here an in vitro system mimicking the in vivo intestinal absorption of β-C and present data consistent with the suggestion that β-C uptake by the intestine occurs via a facilitated process. This in vitro model should prove useful for obtaining detailed, quantitative information about intestinal absorption of carotenoids other than β-C and for investigating fundamental mechanisms of intestinal carotenoid absorption.

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