Long-term kinetic study of β-carotene, using accelerator mass spectrometry in an adult volunteer

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Abstract **We present a sensitive tracer method, suitable for in vivo human research, that uses β-[^{14}C]carotene coupled with accelerator mass spectrometry (AMS) detection. Using this approach, the concentration-time course of a physiological (306 μg; 200 nCi) oral dose of β-[^{14}C]carotene was determined for 209 days in plasma. Analytes included β-[^{14}C]carotene, [^{14}C]retinyl esters, [^{14}C]retinol, and several [^{14}C]retinoic acids. There was a 5.5-h lag between dosing and the appearance of ^{14}C in plasma. Labeled β-carotene and [^{14}C]retinyl esters rose and displayed several maxima with virtually identical kinetic profiles over the first 24-h period; elevated [^{14}C]retinyl ester concentrations were sustained in the plasma compartment for >21 h postdosing. The appearance of [^{14}C]retinol in plasma was also delayed 5.5 h postdosing and its concentration rose linearly for 28 h before declining. Cumulative urine and stool were collected for 17 and 10 days, respectively, and 57.4% of the dose was recovered in the stool within 48 h postdosing. The stool was the major excretion route for the absorbed dose. The turnover times ($1/k_{el}$) for β-carotene and retinol were 58 and 302 days, respectively. Area under the curve analysis of the plasma response curves suggested a molar vitamin A value of 0.53 for β-carotene, with a minimum of 62% of the absorbed β-carotene being cleaved to vitamin A. In summary, AMS is an excellent tool for defining the in vivo metabolic behavior of β-carotene and related compounds at physiological concentrations. Further, our data suggest that retinyl esters derived from β-carotene may undergo hepatic resecretion with VLDL in a process similar to that observed for β-carotene.**—Dueker, S. R., Y. Lin, B. A. Buchholz, P. D. Schneider, M. W. Lamé, H. J. Segall, J. S. Vogel, and A. J. Clifford. **Long-term kinetic study of β-carotene, using accelerator mass spectrometry in an adult volunteer.** ***J. Lipid Res.*** **2000.** 41: **1790–1800.**

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β-Carotene is a hydrocarbon polyene distributed ubiquitously throughout the plant kingdom. It is one of a large group of pigments known as carotenoids, many of which serve as metabolic precursors to vitamin A active compounds in humans and other mammals (**Scheme 1**). Of the provitamin A carotenoids, β-carotene ranks highest as a global vitamin A source and has attracted the interest of the medical community not only as a means of combating vitamin A deficiency (1, 2), but also because of its beneficial effect on photosensitivity (3). Moreover, numerous epidemiological studies have reported an inverse relationship between diets rich in β-carotene and cancer (4, 5) and these relationships led to several large chemoprevention studies. The results of the clinical trials showed β-carotene to provide no benefit, and perhaps even to be harmful (6–8). As a result, considerable controversy now surrounds the pharmacological use of β-carotene (9).

Understanding the role of β-carotene in chemoprevention and as a vitamin A source is hampered by the complexity of β-carotene metabolism and the large variance in the individual response to this compound. Although it is lipophilic and follows many of the same absorptive pathways as other dietary lipids, its bioavailability from food or as a supplement is low and variable. The source and pathological significance of this variance is not well understood and many fundamental issues of β-carotene utilization remain poorly defined: the intracellular transport of β-carotene within the enterocyte, the genetic and environmental factors that modulate the cleavage of β-carotene to vitamin A, the extent and site(s) of postabsorptive metabolism, and the mechanisms behind tissue-specific accumulation. Two excellent reviews discuss these and other issues that surround human β-carotene metabolism (10, 11).

Isotopes are needed for human kinetic studies in which a dose is monitored in the presence of endogenous concentrations of the analyte. These studies were traditionally performed with radioisotopes, although increasingly, health

Abbreviations: AMS, accelerator mass spectrometry; AUC, area under the curve; BHT, butylated hydroxytoluene; LDL, low density lipoprotein; LOQ, limit of quantification; LSC, liquid scintillation counting; RP, retinyl ester of palmitate; RO, retinyl ester of oleate; RS, retinyl ester of stearate; RP-HPLC, reversed-phase high performance liquid chromatography; TG, triacylglycerol; VLDL, very low density lipoprotein.

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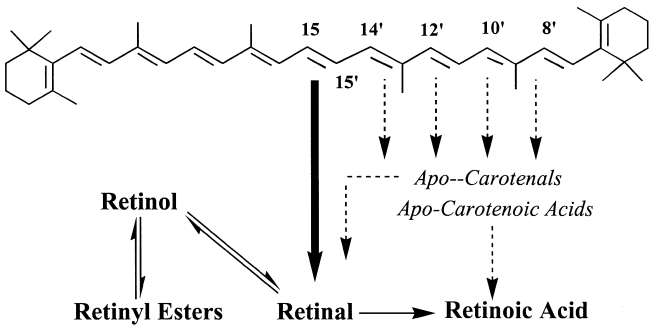

Scheme 1. Schematic of the proposed pathways of biotransformation for β-carotene that are either known or suspected to occur in humans. β-Carotene is cleaved to retinaldehyde either centrally by carotenoid 15,15′-dioxygenase (EC 1.13.11.21) or aymmetrically (excentric cleavage) first to produce a series of β-apo-carotenals. Central cleavage in the intestine is the quantitatively major pathway of conversion although cleavage in other tissues and organs is suspected. The excentric mechanism occurs in plants and microorganisms although it remains to be experimentally proven in humans. Retinol is a transport and storage form of vitamin A whereas retinoic acid is the metabolite that mediates the nonvisual function of vitamin A for growth and epithelial differentiation.

concerns associated with ionizing radiation have driven interest in stable isotope applications (12–15). We describe a new approach in bioanalytical tracing that uses accelerator mass spectrometry (AMS) and minute radiation doses of β-[^{14}C]carotene. AMS is an isotope ratio instrument that measures $^{14}C/^{12}C$ ratios to parts per quadrillion (10^{-15}), quantifying labeled biochemicals to attomolar (10^{-18}) levels in milligram-sized samples. At attomolar levels of sensitivity, the radiation exposure is reduced to negligible levels, enabling in vivo testing in healthy subjects. Further, because combustion precedes isotope ratio analysis, complex matrices such as urine and stool can be analyzed unprocessed (neat) without specific metabolite identification, facilitating the determination of bioavailability. Several excellent reviews of AMS advantages over nonisotopic and stable isotope labeling methods are available (16, 17).

We have applied AMS technology to trace the metabolic behavior of β-[^{14}C]carotene (306 μg; 200 nCi)(derived from $^{14}CO_2$ photosynthetically labeled spinach) and its metabolites in an adult male subject. The specific objectives were to establish quantitative values for bioavailability by mass balance, the vitamin A value of β-carotene, biological half-lives for β-carotene and the retinol derived from it, and probe for minor metabolites that include retinoic acids. The approach described in this report should have broad application to kinetic studies of nutrients, xenobiotics, and pharmaceuticals.

MATERIALS AND METHODS

Chemicals

All chemicals were checked for ^{14}C content by AMS prior to use. Tributyrin (glycerol tributyrate) was purchased from ICN Pharmaceuticals (Costa Mesa, CA) and dissolved in methanol purchased from Sigma (St. Louis, MO). All solvents and other chemicals unless otherwise noted were obtained from Fisher Scientific (Santa Clara, CA). $^{14}CO_2$ was purchased as bicarbonate from American Radiochemicals (St. Louis, MO). Standards of 13-*cis*- and 13-*cis*-4-oxo-retinoic acid were gifts from Hoffman-La Roche (Nutley, NJ). All-*trans*-retinoic acid and β-carotene were obtained from Sigma.

Radiation safety/human subjects

The Radiation Safety and Humans Subjects Committees of the University of California at Davis and Lawrence Livermore National Laboratories approved all protocols. Informed written consent was obtained from the volunteer under the guidelines established by the Human Subject Review Committee.

Biosynthesis and purification of β-[^{14}C]carotene

β-[^{14}C]carotene was prepared by growing spinach (*Spinacia oleracea*) in an atmospherically sealed chamber pulsed with $^{14}CO_2$ (18, 19). Spinach plants that were 30 days of age were placed in the chamber and allowed to equilibrate for 1 day prior to the administration of a total of 50 mCi of $^{14}CO_2$ provided at the rate of 10 mCi/day for 5 days. Exposure was initiated by adding 10 mCi as a solution of $NaH^{14}CO_3$ to excess 18 N H_2SO_4. After the final $^{14}CO_2$ exposure, the plants were maintained for 72 h in the box prior to harvesting. The aerial parts of the plant were harvested and the β-[^{14}C]carotene was extracted (20). Final radiometric and chemical purity was >98% by analytical reversed-phase high performance liquid chromatography (RP-HPLC) at 220 and 450 nm with analysis of 1-min eluent fractions by liquid scintillation counting (LSC). The specific activity of the extracted β-carotene was determined by absorption spectroscopy (ε = 140,655 at 453 nm) (21) and LSC to be 0.35 Ci/mol.

Dose formulation

β-[^{14}C]carotene was suspended in ethanol and an aliquot containing 200 nCi of activity (0.57 μmol; 306 μg) was added to 1 g of olive oil (Star, Extra Virgin) in a 5-ml Teflon centrifugation tube. The bulk of the ethanol was removed under a stream of argon and the remainder by placing the open tube under vacuum for 8 h. The β-carotene solution in oil was then quantitatively transferred to a gelatin capsule that was stored in a screw-cap glass vial under argon at −80°C until dosing.

Dosimetry

A highest radiation exposure model (22) was created on the following assumptions. The half-life of ^{14}C used was 5370 years with a quality factor of 1. The model assumed that the dose of β-[^{14}C]carotene would be 100% absorbed as retinol, with 80% of it being deposited in liver and the remaining 20% uniformly distributed among other tissues. A biologic half-life of 154 days (23) was used for retinol. The total effective dose equivalent was determined to be 28 μSv.

Subject, diet, and specimen collection

The subject was a 35-year-old, nonsmoking male in good health with a body mass index of 22.2 kg/m^2. The subject's usual nutrient intake was assessed from a diet diary kept for 1 week prior to dosing and indicated an average daily intake of 4.4 mg of β-carotene, 4.6 mg of provitamin A carotenoids, and 874.4 μg retinol for a total of 1,614 retinol equivalents. Intake of other nutrients was within recommended ranges. The morning preceding dosing the subject began complete 24-h urine and stool collections. Later that day he was fitted with an intravenous catheter in a forearm vein. A baseline blood sample (7 ml) was drawn on the following morning into an ethylenediaminetetraacetic acid-containing tube. The patency of the catheter was maintained by

flushing it with 2 ml of saline followed by 1 ml of heparin solution. The line was primed by removal of 2 ml of blood (discard) immediately before sampling. After the baseline draw the subject consumed half of an apple fritter pastry that was immediately followed by the dose in a gelatin capsule (25 g of total fat; 24 g from the fritter plus 1 g of olive oil from the dose). Apple juice was provided to facilitate swallowing of the capsule. Blood samples were drawn 0.50, 1.08, 1.75, 2.58, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, 9.00, 9.50, 10.00, 11.00, 12.08, 13.00, 14.00, 15.75, 21.25, 25.17, 28.00, 32.00, and 35.75 h postdosing, and between 8:00 and 8:30 AM on days 2, 3, 4, 5, 6, 7, 8, 10, 13, 14, 16, 20, 28, 42, 49, 58, 77, 99, 128, 147, and 209 in the fasted state.

A snack of half an apple fritter pastry (24 g of fat) was eaten 4 h postdosing, and a lunch (two Gorditas from Taco Bell®; 28 g of fat total) at 5 h and a late dinner at 15 h (turkey pot pie and whole apple) were consumed (21 g of fat). All foods were low or devoid of β-carotene. Fluid intake for the first 48 h was restricted to water and apple juice. Complete 6-h urine collections (four specimens) were made during the first 24-h period. Complete 24-h urine samples from day 2 through day 17 were made in 3-liter amber containers (Fisher Scientific) containing 1 g of ascorbic acid. Complete 24-h stool collections were made through day 10 in 3-liter sterile laboratory blender bags (65-μm-thick polyethylene; Stomacher, available through Fisher Scientific). As far as possible, urine samples were stored at 4°C during collections.

Specimen preparation

Plasma was separated by centrifugation and transferred into Cryo-Lok vials (4 ml) and stored at −80°C until processing. A 40-ml aliquot was taken from each urine container and stored at −80°C until analysis. For the stool, 0.5 to 1 liter of 0.5 M KOH was added to the collection bag and the mixture was dispersed with a Stomacher laboratory blender (model 3500; Fisher Scientific). Samples were blended for 2 min (high setting) and aliquots were removed for analysis. Urine and stool collections made over the first 4 days postdosing were screened for radioactivity by LSC for 5 h. Samples containing >5 dpm/100 μl were diluted 10-fold with 0.5 M KOH to reduce the level of ^{14}C for AMS determinations. Only the 24- to 48-h stool collection required dilution.

AMS sample preparation and measurement

The ^{14}C determinations were made at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratories (Livermore, CA) (16, 24). Aliquots of neat plasma (30 μl), urine (100 μl), and stool (150 μl) sample were analyzed neat (without fractionation or admixture). Plasma carotenoids and retinoids were also separated by HPLC and the fractions were mixed prior to AMS ^{14}C determination with a tributyrin carrier (in methanol) containing 1 mg of carbon that was low in ^{14}C (25, 26). Sample preparation was done in disposable plastic or glassware at the University of California at Davis and transported to Livermore for graphitization and ^{14}C measurement (27). Excess ^{14}C concentrations over natural abundance were converted to β-carotene equivalents, using the specific activity of the β-carotene and carbon content.

AMS measurement times were typically <3 min/sample to a counting precision of ≤3%. The assay precision was determined from the amount of scatter in the results obtained from the analysis of individually prepared replicates (N ≥ 3) of plasma, urine, and stool and averaged about 4% for each specimen. A limit of quantification (LOQ) for neat analyses was defined as two times the standard deviation of the assay imprecision associated with predose samples. For HPLC fractions this was established by analysis of baseline fractions. The LOQs were 18, 32, and 17 amol of β-[^{14}C]carotene for plasma, urine, and stool, respectively. The LOQ for collected HPLC fractions was 3 amol of β-[^{14}C]carotene (26).

Carbon content/other assays

Total carbon was determined as described previously (28). The mean total carbon content of the urine samples was as follows: 0.56 ± 0.18% carbon (mass/mass) with a range from 0.21 to 1.01%; stool, 1.85 ± 0.71% with a range from 0.81 to 2.76%; and plasma, 3.75 ± 0.30% with a range from 3.53 to 3.96% (mean ± SD). Total plasma triacylglycerol (TG) was determined with an enzymatic reagent kit (triglyceride/GPO-Trinder; Sigma). Plasma concentrations for β-carotene and retinol were determined by RP-HPLC with internal standards (29, 30).

Metabolite fractionation

The approach for isolating β-carotene, retinyl esters, retinol, and retinoic acids involved two stages: first, segregation of plasma analytes into neutral (β-carotene, retinyl esters, retinol) and acid-extractable (retinoic acids) classes; and, second, separation of analytes within these two fraction by RP-HPLC followed by AMS analysis of collected eluent fractions. Retinyl esters (retinyl palmitate, oleate, and stearate) were determined as retinol after saponification. Metabolites were separated as follows: A 200-μl aliquot of plasma was deproteinated by addition of 200 μl of 0.025 N KOH in ethanol with vigorous mixing. The metabolites were then extracted with three 0.5-ml volumes of hexane (neutral fraction), which were pooled. The aqueous phase was adjusted to pH 3 with 200 μl of 0.6 N HCl and the acidic metabolites were extracted with three 0.5-ml volumes of 90:10 hexane – ethyl acetate (acidic fraction), which were pooled. The solvent from the acidic fraction was removed under a stream of argon and the residue was resuspended in 50 μl of CH_3CN and analyzed directly by AMS, or the analytes were fractionated by RP-HPLC (see HPLC conditions below).

The neutral fraction was dried under argon in a glass amber vial (containing 1 μg of butylated hydroxytoluene, BHT) and resuspended in 50 μl of 2-propanol, and the analytes were separated by RP-HPLC. Retention windows for retinol and retinyl esters/β-carotene were individually collected in amber glass vials. The retinol fraction was analyzed directly by AMS. For select samples, complete 1-min or 30-sec serial HPLC collections were analyzed by AMS (see below for conditions). Retinyl palmitate and oleate coeluted on our system at the front of the β-carotene peak while retinyl stearate eluted on the backside of the peak. A 3-min eluent fraction (9–12 min) containing the three esters and β-carotene was collected, concentrated to dryness under argon, and then saponifed in 5% KOH in methanol (220 μl with 0.7% pyrrogallol) for 1 h at 70°C in a capped vial evacuated with argon. To the alkaline solution was added an equal volume of water, and retinol plus β-carotene were extracted with three 0.5-ml volumes of hexane, which were pooled. The pooled hexane extracts were concentrated to dryness under a stream of argon in a glass amber vial (with 1 μg of BHT) and the sample was resuspended in 50 μl of 2-propanol. A 20-μl aliquot was then separated on an isocratic HPLC system and eluent fractions corresponding to retinol and β-carotene were collected separately and analyzed by AMS. For time points occurring after 99 h, collected β-carotene/retinyl ester fractions were treated as only β-carotene and analyzed by AMS without saponification.

HPLC conditions for neutral and acidic fractions

An isocratic RP-HPLC apparatus was used for plasma sample analyses. The HPLC apparatus consisted of a Hewlett-Packard (Palo Alto, CA) 1100 chromatograph with a variable wavelength detector fitted with a Hewlett-Packard Eclipse XDB-C18 column

(3.0 × 150 mm, 3.5-μm particle size) and an Eclipse XDB-C18 guard cartridge. Separation of neutral fraction metabolites was performed with an isocratic mobile phase of 49:21:30 acetonitrile–methanol–2-propanol (with 0.1% ammonium acetate) at a flow rate of 0.55 ml/min. The eluent was monitored at 325 nm for retinol and 450 nm for β-carotene with a wavelength switch from 325 to 450 nm at 4.8 min. The eluent output corresponding to the analytes was collected directly into a quartz combustion tube and processed for AMS analysis (26, 27). The eluent collection window for each analyte was approximately 10 sec wider (at the front and end of the absorption peak) than the peak to ensure quantitative recovery of the analyte.

Separation of acidic fraction metabolites was performed with an isocratic mobile phase consisting of 88:12 methanol–0.1% ammonium acetate in water. The eluent was monitored at 351 nm. Samples were cochromatographed with authentic standards of retinoic acids to establish identity and evaluate chromatographic performance. Gradient HPLC conditions that ramped into 100% methanol from the initial conditions were performed on select samples to probe for highly retained metabolites.

Data analysis

Plasma peak concentrations (C_{max}) and times were determined directly from the data points. Rate constants (k) for the distributive and elimination phases were estimated by linear regression analysis of semilog plots of the data with the slope of the line equal to k. Compound turnover time was defined as the inverse of the elimination rate constant (k_{el}) or $1/k_{el}$. Half-lives ($t_{1/2}$) were calculated as ln 2/k. A discontinuity in the rate of change was used to define the transition between the two plasma phases. Area under the curve (AUC) values for the β-carotene and retinyl ester in the first absorptive period (5.5–10 h) were obtained from integration of a guassian fit, using the Levenberg-Marquardt algorithm (Origin 6.0; Microcal, Northampton, MA). Other AUCs were determined by the trapezoidal approximation. Statistical analyses were performed with Origin and, as a visual aid, many of the plots were fitted with a cubic B-spline.

RESULTS

Photosynthetic labeling

The photosynthetic labeling of spinach with $^{14}CO_2$ yielded 525 g (wet) stem and leaf. A 50-g sample provided 1.8 mg of β-[^{14}C]carotene. The specific activity of the extracted β-carotene was 0.35 Ci/mol. Radio- and chemical purity were greater than 98% although the all-*trans*-β-carotene contained ~6% *cis* stereoisomeric forms as estimated by RP-HPLC. As *cis* isomers of all-*trans*-β-carotene can arise as artifacts from the processing, no attempt was made to separate the all-*trans* form further.

Bioavailability (mass balance)

Plots of the cumulative loss of β-[^{14}C]carotene in urine and stool are presented in **Fig. 1**. The cumulative stool collections from 0 to 48 h contained 57.4% of the administered dose (two collections); therefore, 42.6% of the dose was bioavailable (0.243 μmol of β-[^{14}C]carotene was absorbed). For the ensuing 192-h period (8 days) an amount equivalent to 7.8% of the administered dose was eliminated in the stool. For the urine, only minor amounts of ^{14}C appeared in the 0- to 6-h sample, consistent with the time lag observed in the plasma response (5.5-h lag). The fraction of the administered dose excreted from 0 to 96 h was 0.05, 0.08, 0.18, 0.29, 0.34, 0.19, and 0.07% of the administered dose for time points 0–6, 6–12, 12–18, 18–24, 24–48, 48–72, and 72–96 h, respectively. When normalized to a 24-h period, the largest single dose output occurred from 0 to 24 h with diminishing output thereafter. After 4 days (96 h) the output stabilized and assumed a linear form through day 17, the final day of cumulative collection.

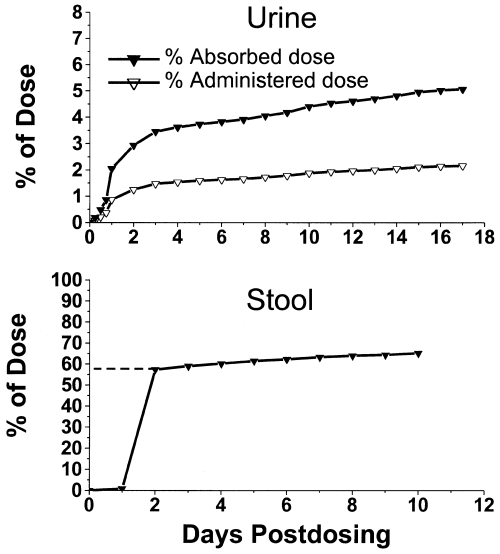

Fig. 1. The cumulative urine (top) and fecal recovery (bottom) of ^{14}C expressed as a percentage of dose after oral ingestion of β-[^{14}C]carotene. A total of 57.4% of the administered dose was recovered in the stool in 48 h, indicating 42.6% absorption. After equilibration at day 4, the fecal rate of excretion continued to be approximately 9-fold greater than the rate of excretion in the urine.

The cumulative loss of label in urine and stool from days 4 through 10 was 0.98 and 8.69% of the absorbed dose (0.055 μmol), respectively. The average daily loss in the urine and stool was 1.61% of the absorbed dose or 0.0092 μmol (4.9 ng of β-[^{14}C]carotene per day). In relative terms, the excretion of label by the fecal route was ≈9-fold greater than by the urinary route.

Time course of total ^{14}C in plasma

The concentration-time profile of ^{14}C in plasma for 5 days (top) and 209 days (bottom) postdosing is shown in **Fig. 2**. The y axis units are expressed as femtomoles of ^{14}C per milligram of total carbon (C) because the response represents a contribution from β-[^{14}C]carotene and all its labeled metabolites. Carbon-14 nuclide concentrations above background were not detectable until 5.5 h postdosing. The initial peak began to rise at 5.5 h, reached a maximum at 8 h, and declined to a nadir at 10 h. This peak was highly symmetrical. After 10 h the ^{14}C concentration rose again to a second maximum at 12 h and then continued to rise to a plateau with its highest point at 21 h. The third maximum was followed by a monoexponential descent after 36 h that settled to a terminal elimination slope around day 50.

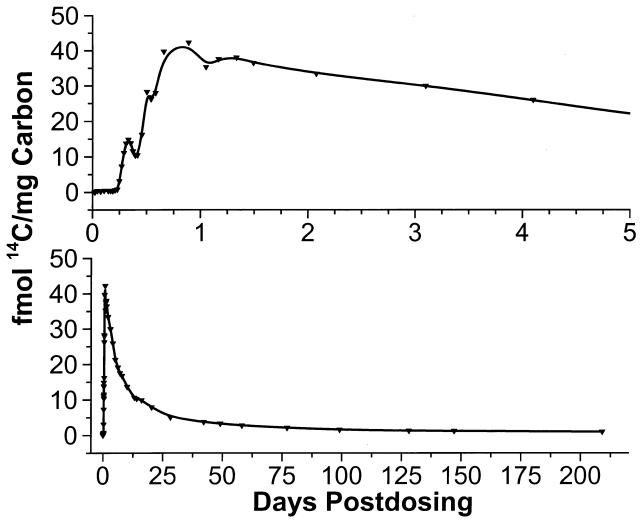

Fig. 2. Time course of ^{14}C in plasma for 5 days (top) and 209 days (bottom) postdosing (cubic B-spline fits to the data points). A high density of sampling was taken during the absorptive period and 30-μl aliquots were graphitized and analyzed by AMS without fractionation or admixture. The time of first appearance of ^{14}C was at 5.5 h, with multiple peaks at later times. Neat analysis does not include metabolite speciation and signal represents total ^{14}C.

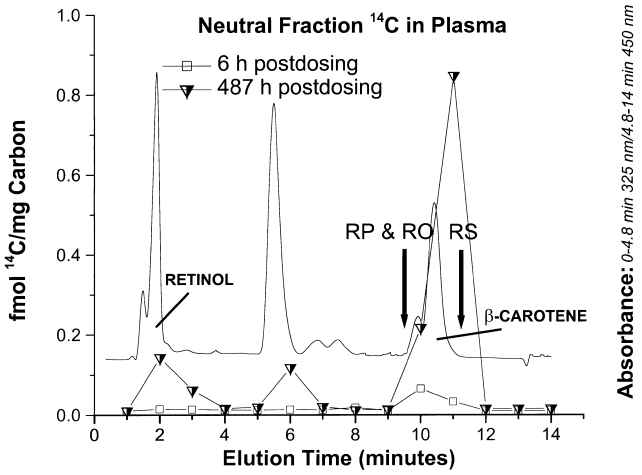

Fig. 3. Representative radiochromatogram of the neutral fraction plasma extracts without saponification of time points at 6 h (squares) and 487 h (triangles) postdosing. The HPLC eluent is collected at 1-min intervals and analyzed for ^{14}C by AMS. The elution times for retinol and β-carotene are 2.0 and 11–12 min, respectively. The elution times of retinyl palmitate (RP), oleate (RO), and stearate (RS) are marked with arrows. For plasma analyzed until 99 h postdosing, retinyl esters and β-carotene were collected in a single 3-min (9–12 min) fraction, which was then saponified to generate retinol from collected retinyl esters. β-Carotene and retinol were then separated by HPLC and the level of ^{14}C was quantified by AMS. Samples after 99 h were not expected to contain significant concentrations of labeled retinyl esters and were analyzed by a single HPLC separation. The 6-h time point shows significant ^{14}C in the β-carotene/retinyl ester retention window at 10 and 11 min and trace ^{14}C in the retinol fraction. The 487-h time point shows elevated ^{14}C signal at the 2-, 6-, and 9- to 12-min fractions. The identity of the compound appearing at 6 min is unknown

Short-term plasma kinetics

The plasma drawn 6 and 487 h postdosing was subjected to HPLC separation and the full elution series was collected at 1-min intervals and analyzed for ^{14}C (**Fig. 3**). In our system retinol elutes early, at 2.0 min, and β-carotene elutes later, between 10 and 11 min. The elution times of retinyl esters of palmitate (RP), oleate (RO), and stearate (RS) are marked with arrows. Plasma from the 6-h time point shows a small amount of ^{14}C signal in the β-carotene/retinyl ester retention window at 10–11 min and undetectable signal in other fractions. The 487-h time point shows elevated ^{14}C signal in the 2-min fraction (retinol), 6-min fraction (unknown), and in the 10 to 11-min fraction (β-carotene/retinyl ester). The identity of the compound at 6 min is unknown.

Figure 4 shows the concentration-time profile for β-[^{14}C]carotene (top), [^{14}C]retinyl esters (middle), and [^{14}C]retinol (bottom) from 0 to 99 h postdosing. Consistent with the neat analysis was a lag in the time of appearance of 5.5 h for both retinyl esters and β-carotene, which at 8 h postdosing rose to a first maximum of 1,488 and 602 fmol of analyte per ml of plasma, respectively. This was followed by a decrease to a nadir at 10 h. This initial peak was highly symmetrical for both analytes. After the nadir at 10 h the concentration of both labeled retinyl esters and β-carotene rose to a plateau spanning between 14 and 21.3 h, with a C_{max} at the 21.3-h time point of 1,826 and 1,737 fmol/ml for [^{14}C]retinyl esters and β-[^{14}C]carotene, respectively. The second maximum for retinyl esters was close in magnitude to the first maximum, whereas the second maximum for β-carotene was approximately 3-fold higher. Elevated [^{14}C]retinyl ester concentrations were maintained from 5.5 to 21 h postdosing. The time course of plasma TG concentration (milligrams of TG per deciliter) and [^{14}C]retinyl esters (femtomoles per milliliter) is presented in **Fig. 5**. The TG concentration began to rise at 1 h from fasting levels and reached a broad maximum near 5.5 h.

[^{14}C]retinol was first detectable at 5.5 h, similar to retinyl esters and β-carotene, and displayed a small, symmetrical peak (5.5–10 h) with a maximum of 412 fmol/ml at 8 h. After a low point at 9 h, its concentration increased linearly to a C_{max} of 3,389 fmol/ml at 28 h postdosing (Fig. 4). The substantial disappearance of [^{14}C]retinyl esters from plasma by 28 h postdosing also marked the transition point from increasing to declining [^{14}C]retinol concentrations.

The AUCs for labeled β-carotene, retinyl esters, and retinol in the first absorptive period (5.5–10 h) were 1,759, 4,741, and 1,141 area units (fmol·h/ml), respectively. Accordingly, the molar ratio of retinyl ester to carotene was 2.7, that is, 2.7 mol of retinyl ester was present for each mole of β-carotene. The molar ratio of (retinyl esters plus retinol) to β-carotene was 3.3 in this same period whereas the molar ratio of retinyl esters to retinol was 4.2.

Long-term plasma kinetics

Retinol and β-carotene. Semilog plots of β-[^{14}C]carotene and [^{14}C]retinol concentrations from 7 to 209 days

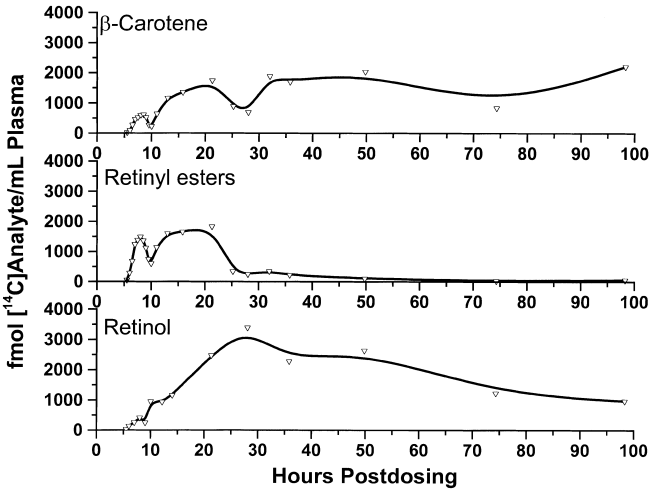

Fig. 4. Time course plots from 0 to 99 h for labeled β-carotene (top), retinyl esters (middle), and retinol (bottom) isolated from plasma (cubic B-spline fits to the data points). Hexane extracts of denatured plasma were fractionated by HPLC and followed by AMS measurement of ^{14}C concentrations. Molar values for the metabolites were calculated from the known specific activity of the original β-carotene dose. The multiple peaking apparent in the β-carotene and retinyl ester plots is highly parallel, suggesting similar metabolism in the postprandial period.

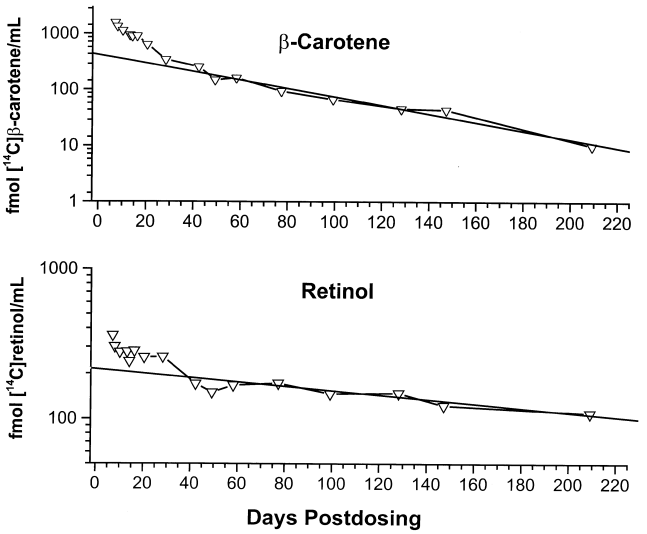

Fig. 6. Semilog plots of labeled retinol (top) and β-carotene (bottom) from 7 to 209 days, showing the fitted regression lines (straight lines) to the terminal decay portion of the plots. The discontinuity in the rate of slope change occurred at approximately 40 and 77 days for β-carotene and retinol, respectively. For β-carotene half-lives of 13 and 40 days were calculated, corresponding to a distribution and terminal decay slope, respectively. For retinol, values of 41 and 209 days were obtained for these same parameters. On the basis of the terminal decay slope the turnover times for β-carotene and retinol were 58 and 302 days, respectively.

are shown in **Fig. 6**. The disappearance pattern was biphasic for β-carotene (Fig. 6, top) and retinol (Fig. 6, bottom), although the retinol decay was more dynamic in the first 77-day period. The discontinuity in the rate of slope change occurred at approximately 40 and 77 days for β-carotene and retinol, respectively. The fitted regression lines for the terminal decay slope (k_{el}) appear in the plots. The estimated regression equations were as follows: β-carotene, $y = 6.03028 - 0.01732(x)$, $R = 0.98382$; retinol, $y = 5.36627 - 0.00332(x)$, $R = 0.94969$. For β-carotene distribution and terminal decay $t_{1/2}$ values of 13 and 40 days were obtained. For retinol, values of 41 and 209 days were obtained for these same parameters. On the basis of the k_{el} rate constant, the turnover times for β-carotene and retinol were 58 and 302 days, respectively.

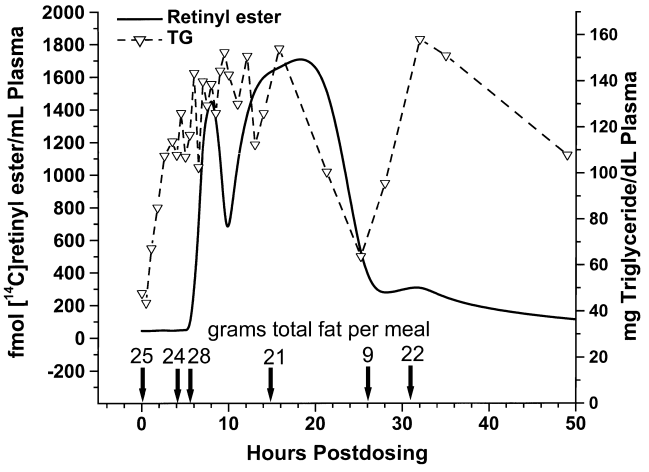

Fig. 5. Plot showing the concentration-time course of [^{14}C]retinyl esters (solid line, cubic B-spline fit to the data points) and total plasma triglyceride (dashed line) for 50 h postdosing. Numbers above the x axis refer to the time and total mass of dietary fat consumed with meals for the first 31 h postdosing.

Detection of retinoic acids at attomolar levels

Figure 7 shows the total retinoic acids concentration-time course for 209 days (Fig. 7, top). The time course profile was similar in appearance to that of retinol but on a smaller scale and with more dynamic concentration fluctuations. The k_{el} rate constant was determined to be 0.003 day^{-1}, corresponding to a $t_{1/2}$ of 231 days and a turnover time of 333 days. A linear regression fit of the concentration data from 5.5 to 25 h is shown in Fig. 7 (bottom). The estimate line had a slope of 5.2 with units of femtomoles of retinoic acids per hour. The HPLC separation of acidic fraction metabolites from plasma drawn 99 h postdosing is shown in **Fig. 8**. Thirty-second fractions were collected and analyzed for ^{14}C and expressed as femtomolar retinoic acid equivalents. The sample was cochromatographed with unlabeled 13-*cis*-4-oxo-, 13-*cis*-, and all-*trans* retinoic acids, which displayed retention times of 3.1, 8.2, and 8.6 min, respectively. Additional time points at 0, 8, 48, 72, and 480 h were also analyzed (data not shown). In the 99-h time point, a value of 11.4 fmol of ^{14}C per milligram of carbon was observed in the 2.5- to 3-min fraction, which corresponded (by elution time) to 13-*cis*-4-oxo-retinoic acid; a value of 1.8 fmol of ^{14}C per milligram of carbon was detected in the 3.5- to 4-min collection al-

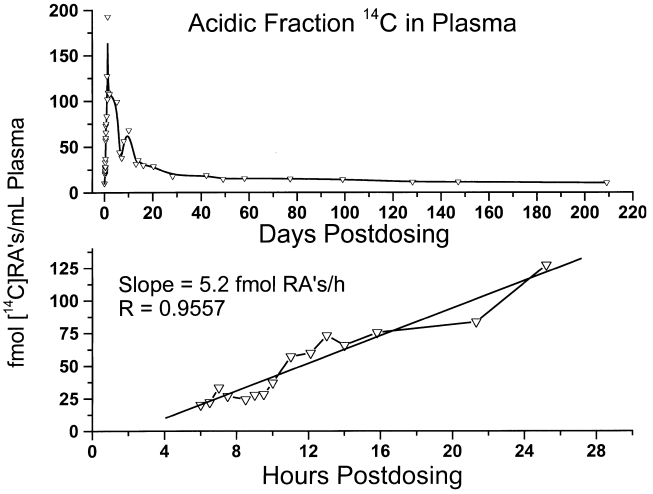

Fig. 7. Time course of acidic fraction ^{14}C for 209 days postdosing (top) (cubic B-spline fits to the data points). Excess ^{14}C concentrations over natural abundance were converted to [^{14}C]retinoic acid equivalents, using the specific activity of the retinoic acid derived from β-[^{14}C]carotene and total carbon content. The k_{el} rate constant was determined to be 0.003 day^{-1}, which corresponds to a $t_{1/2}$ of 231 days and a turnover time of 333 days. A linear fit of the [^{14}C]retinoic acids concentrations for the first 25 h postdosing is shown in the bottom panel. Total [^{14}C]retinoic acids rose at an average rate of 5.2 fmol of [^{14}C]retinoic acids per h and likely reflect vitamin A catabolism.

though the identity of this compound is not known. 13-*cis*-4-oxo-Retinoic acid was consistently detectable in fractions other than the 0 time point, whereas lesser concentrations of ^{14}C were observed in the 13-*cis*- and all-*trans*-retinoic acid fractions in the 480-h time point.

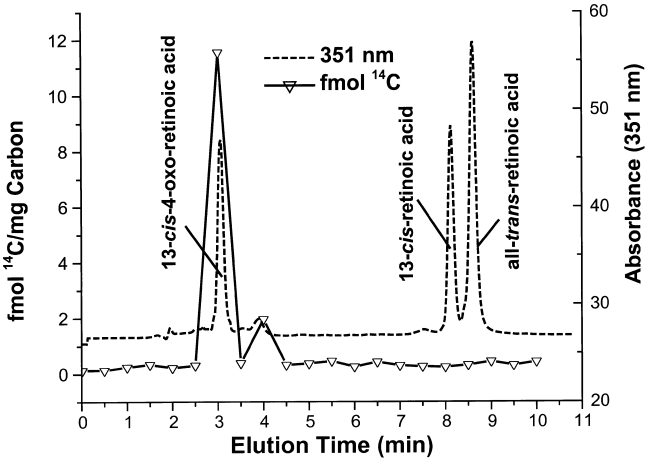

Fig. 8. Radiochromatogram (triangles) and associated absorbance at 351 nm (dashed line) of acidic fraction metabolites isolated from plasma 99 h postdosing. Extracts were supplemented with unlabeled compounds to facilitate identification. Thirty-second eluent fractions were collected and analyzed for ^{14}C by AMS. Eluent collections from 2.5–3 and 3.5–4 min were enriched in ^{14}C. The first peak at 3 min corresponded by retention time to 13-*cis*-4-oxo-retinoic acid, whereas the identify of the peak at 4 min is unknown.

Endogenous levels

The β-carotene dose was less than one-tenth the average daily intake of β-carotene for the American diet, and there was no discernable change in total plasma retinol or β-carotene concentrations for the initial 24 h postdosing, suggesting steady state conditions were maintained. Over the full course of the study, these values did not change significantly. As confirmation, the means of sample groups (N = 3) at the start, middle, and end of the study were tested for significance by one-way analysis of variance: group 1 (time 0, 7, 14, and 20 days); group 2 (time 28, 42, 49, and 58 days), and group 3 (99, 128, 147, and 209 days). The following values (micromoles per liter) were obtained for retinol and expressed as means (±SD): group 1, 1.403 (±0.00209); group 2, 1.492 (±0.00392); group 3, 1.420 (±0.0046). The means were not significantly different (P = 0.102). For β-carotene the following values (micromoles per liter) were obtained: group 1, 0.465 (±0.0079); group 2, 0.432 (±0.00337); group 3, 0.4625 (±0.00269). The means were not significantly different (P = 0.71695).

DISCUSSION

At this juncture, two radioisotope studies of cancer patients, performed in the 1960s, form much of the basis for bioavailability estimates of β-carotene in humans. In these studies β-[^{14}C]carotene doses from 6 to 52 μCi were administered to lymph-cannulated patients with reported absorption values of 8–17% and one extreme at 52% (31, 32). Our value of 42.6% β-carotene bioavailability is consistent with the prevailing concept (33, 34) that β-carotene absorption is inefficient, even under favorable absorptive conditions. The fecal output of ^{14}C was biphasic (Fig. 1) with the initial spike from 0 to 48 h arising from unabsorbed β-carotene. The slow output of label in the stool after 48 h is attributed to biliary excretion of metabolites and the normal sloughing of intestinal cells that had accumulated β-carotene and/or its metabolites. As expected, urine was not a major excretory route for intact β-carotene (due to aqueous insolubility) per se, although derived vitamin A and vitamin A catabolites are excreted in urine (35).

The plasma concentration-time course of β-[^{14}C]carotene, and those of its cleavage products retinyl esters and retinol, were interpreted within the context of the present understanding of β-carotene and lipoprotein metabolism. Briefly, in the enterocyte, β-carotene and its cleavage products are incorporated into chylomicrons (35, 36), which are released into circulation, rapidly degraded to remnants, and cleared by hepatic and other tissue (37). In the liver retinyl esters are released and undergo a cycle of hydrolysis and reesterification before storage or hydrolysis to retinol and secretion from the liver bound to retinol-binding protein. Retinyl esters are not thought to be resecreted by the normal liver (37), although β-carotene undergoes hepatic resecretion with very low density lipoproteins (VLDL). These VLDL are either cleared or delipidated to low density lipoprotein (LDL) (10, 38, 39).

The concentration time-courses of β-[^{14}C]carotene and

[^{14}C]retinyl ester in the initial 24-h period postdosing were qualitatively similar in profile (Fig. 4). The first rise peaked at 8 h for both compounds and displayed a high degree of symmetry (gaussian). The peak symmetry is a product of the rapidity with which chylomicron remnants are cleared after the influx of labeled chylomicrons from the first meal: Rapid removal limits the potential for multiple recirculations or accumulation—events that would skew the peak to the right. We consider this assumption reasonable because chylomicron remnant half-lives are short (about 5 min) (40) and their components do not appear to exchange to longer-lived lipoproteins in plasma to any appreciable degree (41, 42). There was not an observable lag between the appearance of β-[^{14}C]carotene and that of the retinyl esters derived from it, suggesting a tight metabolic linkage at the intestinal levels between β-carotene absorption and bioconversion. This parallelism was observed despite the fact that, whereas vitamin A transport is mediated by cellular retinol-binding protein type 2, proteins that mediate the flow of intact β-carotene across the cytoplasm have not been identified (43, 44).

After a nadir at 10 h, plasma concentrations of retinyl esters and β-carotene increased again in parallel, sustaining a broad plateau between 14 and 21 h. Whereas the second rise in β-carotene was anticipated (hepatic resecretion with VLDL), the second rise in retinyl esters was not. Two hypotheses can be proposed to explain the second rise in retinyl esters: *i*) the secretion of retinyl esters with VLDL after hepatic uptake of chylomicron remnants, and *ii*) multiple inputs of β-carotene from the intestine associated with subsequent meal(s).

As regards the first hypothesis, there are several lines of evidence that suggest retinyl ester secretion with hepatic VLDL: *i*) The observed parallelism between retinyl esters and β-carotene concentration-time course for 24 h postdosing suggests similar metabolism; this is significant because β-carotene undergoes hepatic resecretion with VLDL (45); *ii*) experiments in dogs have documented retinyl ester secretion from the liver (46); and *iii*) approximately 5–10% of the total vitamin A in fasting plasma is present in the form of retinyl esters and several studies have shown that retinyl esters can travel with hepatically derived VLDL and LDL (42, 47–50). The second hypothesis of multiple inputs of β-carotene from the intestine due to a subsequent meal(s) and/or irregular secretion of lipid has been previously suggested (11, 34). This phenomenon has been attributed to the relatively poor solubility of β-carotene in triglyceride (51, 52), which leads to significant deposition of β-carotene within the enterocyte membrane. The β-carotene is then released and incorporated into newly synthesized chylomicrons with subsequent fat intake (10).

We do not have a unique explanation of the breadth of the secondary peak of retinyl esters, but multiple processes may be occurring that include multiple inputs of retinyl esters with chylomicron from subsequent meals, transfer among lipoprotein classes either within the plasma or via resecretions, and delayed clearance of chylomicron and other TRL (hepatic or intestinal) (53–57). In our study, multiple fat-containing meals were consumed after dosing of moderately high fat content and the subject demonstrated a sustained postprandial triglyceridemia for 22 h postdosing, indicating that the conditions for delayed TRL removal were present (58). In support of this contention is also the observation that the decline in TG levels around 18 h was closely followed by the decline in [^{14}C]retinyl ester concentrations (see Fig. 5).

[^{14}C]retinyl esters and β-[^{14}C]carotene concentrations sharply declined in concentration between 21 and 25 h and the disappearance of [^{14}C]retinyl esters after the second maximum was biexponential: from 21 to 36 h a steep descent followed by a gradual decline to low concentrations after 50 h. This gradual disappearance has been attributed to the clearance of a small fraction of retinyl esters associated with LDL (59, 60), with contributions from the recycling of retinyl ester from extrahepatic tissue. The β-[^{14}C]carotene concentrations dropped coincidently with the sharp drop in retinyl esters, suggesting a common lipoprotein carrier for both compounds. It then reached a nadir at 28 h and then rose again to reach a broad maximum between 32 and 168 h postdosing before a consistent decline in plasma concentration. The late rises in β-[^{14}C]carotene is attributed to recycling and accumulation in LDL and high density lipoprotein with longer residence times. The rate of disappearance of plasma carotenoids appears slower than the rate of turnover of lipoprotein, consistent with the concept of exchange into and out of other tissues (11).

Unesterifed [^{14}C]retinol concentrations began to rise at 5.5 h. The timing and shape of the initial rise were similar to those of retinyl esters and β-carotene, indicating intestinally derived retinol associated with chylomicrons. Integrated areas from 5.5 to 10 h indicated that retinol is a small but significant metabolite of intestinal β-carotene metabolism: 19% of the lymph-transported vitamin A was in the form of retinol. Previous studies reported values between 2 and 10% (31, 32). Our value may be inflated by the appearance of [^{14}C]retinol associated with retinol-binding protein. Clearly this form of vitamin A begins to dominate the labeled retinol pool after ~10 h postdosing and minor contributions from this form are possible at earlier time points. After the initial absorption peak between 8.5 and 10 h, retinol concentrations rose linearly to peak at 28 h. The substantial disappearance of retinyl esters from plasma between 21 and 25 h closely preceded the transition from increasing to declining retinol concentrations, suggesting the influx of retinyl ester is driving retinol release into circulation (a precursor-product relationship). This is consistent with the hypothesis that dietary and recirculating plasma vitamin A is the first option for maintenance of vitamin A homeostasis as described in the "last in/first out" model (59–61).

We observed that postprandial plasma concentrations of β-[^{14}C]carotene and [^{14}C]retinyl esters follow a distinctly different pattern than postprandial TG concentrations in the initial hours after the first meal and dose. In the present study, TG concentrations began to rise 1 h postdosing and reached a plateau phase at 5.5 h postdos-

ing, 30 min before the first appearance of ^{14}C (Fig. 5). The large disparity between the timing of the lipid and β-carotene/retinyl ester response suggests that β-carotene and vitamin A do not follow the same absorption pattern as other dietary lipids. However, it seems unlikely that the early rise (30 min) in plasma TG is due to the meal TG consumed at the time of dosing. In this regard, it has been proposed that the cephalic phase of digestion is accompanied by the emptying of lacteals containing secreted chylomicons from a previous meal (62–64).

Long-term kinetic information is needed to accurately calculate final elimination rates of biological molecules with slow turnover rates. Green and Green (61) suggested that sampling times from plasma retinol should be at least 200 days to permit accurate calculation of a final elimination rate. This time period ensures equilibration of the tracer with slowly exchanging endogenous pools. Our data showed the disappearance of retinol from plasma was biexponential after day 7 and the terminal slope phase was not apparent until approximately 77 days postdosing. A $t_{1/2}$ of 209 days was determined. Alternatively, the turnover time, or the time required for the total mass or pool of tracer to be replaced by newly appearing substrate, was 302 days. Our $t_{1/2}$ compares well with those derived from a depletion/repletion study of eight healthy volunteers after ingestion or infusion of [^{14}C]retinyl acetate. These authors reported a mean $t_{1/2}$ of 154 days with a range from 75 to 241 days (23). This study was conducted with preformed vitamin A whereas our results are based on β-carotene as the vitamin A source. Accordingly, some postabsorptive (peripheral) conversion of β-carotene to vitamin A may occur, which would be expected to result in an apparent lengthening of the observed plasma half-life of retinol. This concept of postintestinal bioconversion is supported by detection of carotenoid cleavage enzyme (15,15′-dioxygenase) activity in the liver, corpus luteum, and kidney (1), and predicted in a compartmental model generated from octadeuterated β-carotene kinetic data (13).

There are limited data on the biological half-life for β-carotene. Unlike vitamin A, which is principally stored in the liver (50–80%), β-carotene is mainly distributed in the fat depots (1) and is not subject to any known metabolic regulation. Accordingly, pharmacologically sized dose (nonisotopic methods) result in elevated plasma responses and it is the concentration decay after such treatments that serves as much of the basis for reported biological half-lives for β-carotene. Rock et al. (65) reported half-lives of β-carotene of less than 12 days. The high background plasma concentration of β-carotene limits the sensitivity of nonisotopic methods. The apparent distribution half-life of 13 days obtained in our study agrees with this value, although we found the terminal decay slope to indicate an elimination half-life of 40 days and a turnover time of 58 days.

We report a molar vitamin A value of 0.53 for β-carotene in our subject. This value was arrived at by using the following logic and assumptions. The AUC analysis of the first postprandial peak indicated that 3.3 mol of retinyl ester plus retinol (vitamin A) appeared per mole of β-carotene. This calculation is based on the assumptions of central cleavage and similar kinetic time courses for the three analytes. If central cleavage is assumed (see Scheme 1), 1 mol of β-carotene will yield 2 mol of vitamin A, and, accordingly, 3.3 mol of vitamin A is equivalent to 1.65 mol (3.3/2) of β-carotene. By this reasoning, the ratio of metabolized to unchanged β-carotene is 1.65 to 1, or can be expressed as 62% [(1.65/2.65) × 100] of the β-carotene that was absorbed underwent cleavage to vitamin A. If a less efficient stoiciometery for the bioconversion is assumed, for example a 1-to-1 molar value, then using the same reasoning as above, 77% [(3.3/4.3) × 100] of the β-carotene absorbed is predicted to have undergone cleavage to vitamin A and other products. What is clear is that, by our estimates, a minimum of 62% of the absorbed β-carotene was cleaved to vitamin A and by this reasoning the vitamin A value of β-carotene dose was 0.53. [The calculation is as follows: Assuming central cleavage, 1 mol of β-carotene yields 2 mol of vitamin A. If 62% of the absorbed β-carotene were cleaved to vitamin A, 1 mol of β-carotene would yield 1.24 mol of vitamin A. Thus, the vitamin A value of the absorbed dose is 1.24. Yet, the dose was 42.6% absorbed. So, the actual vitamin A value is (1.24) (0.426) or 0.53.]

A vitamin A value of 0.53 is interpreted as meaning that 1 mol of ingested β-carotene is equivalent to 0.53 moles of vitamin A. This compares with the study by Sauberlich et al. (23), who reported that the amount of crystalline β-carotene necessary to meet the vitamin A requirement of adult men was approximately twice that of retinol (≅0.5 molar vitamin A value for β-carotene) when given under optimal absorptive conditions.

As discussed in the previous section, the proposed delayed secretion of β-carotene and retinyl esters that resulted in multiple concentration peaks with later meals complicate our vitamin A value calculation. Our value was calculated by AUC analysis of the first postprandial β-carotene/retinyl esters/retinol plasma curves from 5.5 to 10 h. We chose not to integrate the entire 0- to 24-h period because the source of the second rise in retinyl esters cannot be confidently ascribed to secondary intestinal inputs. For this reason we based our calculation on the 5.5- to 10-h region and assumed that the molar ratio of β-carotene to retinyl ester would be reproduced in later absorptive events if they occurred.

We performed a preliminary analysis of acidic metabolites to assess the potential of our approach for quantifying low level retinoic acid metabolites derived from β-carotene. Retinoic acid is an endogenous component of human blood and four forms are known to occur in human plasma: all-*trans*-retinoic acids, all-*trans*-retinoyl β-glucuronide, 13-*cis*-retinoic acid, and 13-*cis*-retinoyl β-glucuronide, which are present in about equivalent concentrations of 1–1.3 ng/ml (66, 67), as well as 4-oxo derivatives of these compounds. We were able to detect a metabolite that migrates as 13-*cis*-4-oxo-retinoic acid on an RP-HPLC system as shown in a representative HPLC trace (Fig. 8). A second, smaller activity peak appearing at 4 min did not migrate with any of our standards. This activity peak may cor-

respond to all-*trans*-4-oxo-retinoic acid, but we did not have that standard. This sample in Fig. 8 was taken 99 h postdosing and a similar metabolite profile was observed at 74 h. A later time point (487 h) showed some incorporation of label into a peak that migrates with 13-*cis*- and all-*trans*-retinoic acid (data not shown). A more thorough analysis of several more time points is needed to firmly establish the identity and relative proportions of the acidic vitamin A compounds. A linear fit of the [^{14}C]retinoic acids concentrations from 5.5 to 24 h displayed a slope of 5.2 fmol of retinoic acids formed per hour and presumably reflects vitamin A catabolism (Fig. 7).

In conclusion, several of the challenges confronting in vivo β-carotene investigations are overcome through application of the described methodology: Physiological doses are easily traced for long durations and dose bioavailability is obtained by mass balance of the 0- to 48-h stool samples; with attomolar levels of sensitivity, conversion of β-carotene into minor metabolites such as retinoic acids is possible under physiological conditions and affords an opportunity to better define the ability of β-carotene to deliver bioactive retinoids to tissues and organs. From application to larger sample set, the underlying basis for the variability associated with β-carotene metabolism can be more thoroughly examined. AMS satisfies both the analytical and ethical requirements for tracer applications in human subjects. The small blood volumes required per analysis enable studies of children, from whom only fingerprick volumes (100–200 μl) might be obtainable. Given limited sample volumes, the ratio of β-carotene to retinyl esters at a single time point in the initial postprandial (6-h time point) could serve as a means of estimating bioconversion efficiency. The influence of factors such as vitamin A status and diet matrix on bioconversion could also be investigated.

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