

Absorption and retinol equivalence of β -carotene in humans is influenced by dietary vitamin A intake

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Abstract The effect of vitamin A supplements on metabolic behavior of an oral tracer dose of [¹⁴C] β -carotene was investigated in a longitudinal test-retest design in two adults. For the test, each subject ingested 1 nmol of [¹⁴C] β -carotene (100 nCi) in an emulsified olive oil-banana drink. Total urine and stool were collected for up to 30 days; concentration-time patterns of [¹⁴C] β -carotene, [¹⁴C]retinyl esters, and [¹⁴C]retinol were determined for 46 days. On Day 53, the subjects were placed on a daily vitamin A supplement (10,000 IU/day), and a second dose of [¹⁴C] β -carotene (retest) was given on Day 74. All ¹⁴C determinations were made using accelerator mass spectrometry. In both subjects, the vitamin A supplementation was associated with three main effects: 1) increased apparent absorption: test versus retest values rose from 57% to 74% (Subject 1) and from 52% to 75% (Subject 2); 2) an \sim 10-fold reduction in urinary excretion; and 3) a lower ratio of labeled retinyl ester/ β -carotene concentrations in the absorptive phase. The molar vitamin A value of the dose for the test was 0.62 mol (Subject 1) and 0.54 mol (Subject 2) vitamin A to 1 mol β -carotene. Respective values for the retest were 0.85 and 0.74. These results show that while less cleavage of β -carotene occurred due to vitamin A supplementation, higher absorption resulted in larger molar vitamin A values.—Lemke, S. L., S. R. Dueker, J. R. Follett, Y. Lin, C. Carkeet, B. A. Buchholz, J. S. Vogel, and A. J. Clifford. Absorption and retinol equivalence of β -carotene in humans is influenced by dietary vitamin A intake. *J. Lipid Res.* 2003. 44: 1591–1600.

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β -Carotene is a commonly consumed plant pigment (polyene) that is a biological antioxidant and a nutritional precursor of vitamin A. Within the intestine, β -carotene can be cleaved (Scheme 1) to yield two molecules of vitamin A (retinol) (1). A large percentage of consumed carotene is absorbed intact, metabolized to vitamin A and other products of uncertain vitamin A value, or excreted

(2). However, for reasons that are not well defined, β -carotene is a relatively poor source of vitamin A (3). Some of the variability of the reported vitamin A activity of β -carotene under controlled conditions can be attributed to both dose formulation and administration and the absorption and metabolism of the consumer, presumably controlled by genetic traits.

However, outside of a controlled experimental setting, a number of other factors may also affect β -carotene absorption and vitamin A value, including the type and quantity of dietary fat (4, 5), competition among coconsumed carotenoids (6), the plant or food matrix of incorporation (5), and the extent of mucosal metabolism (7). Determining the contribution of each factor to the vitamin A value is key to understanding the individual response.

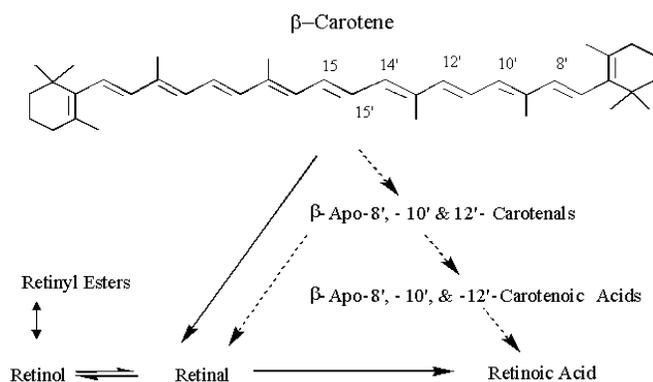
The importance of β -carotene as a primary vitamin A source in less-developed regions suggests that in these individuals, status may be an important factor (8). In Filipino children of low or marginal vitamin A stores, the bioconversion of plant carotenoids to vitamin A varied inversely with vitamin A status (9). These findings were strengthened by the use of isotope dilution methods for the assessment of body stores rather than the measurement of circulating retinol concentrations (10). A molecular basis for the observed relationship is suggested in rat studies that report increased activity of the main carotenoid cleavage enzyme, β , β -carotene 15,15'-monooxygenase, in intestinal homogenates of vitamin A-deficient animals (11–14). Moreover, it has been observed that large doses of β -carotene do not result in vitamin A toxicity (15), suggesting homeostatic control of bioconversion or saturation of the cleavage enzyme.

Using functional indicators, the earliest investigations into the vitamin A value of β -carotene estimated that between 2 μ g to 4 μ g of β -carotene were needed to have the same biological efficacy (i.e., vitamin A activity) as 1 μ g of

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Scheme 1. The proposed central cleavage (solid line) and the ex-centric cleavage (dashed line) pathways. [Reprinted from Wang et al. (50).]

retinol (16–18). In terms of molar vitamin A value, this is equivalent to 0.47 mol to 0.94 mol retinol formed from 1 mol β -carotene. More recently, stable isotope tracing has been applied to β -carotene conversion studies [for review see ref. (19)]. As an example, a study in one woman taking a pharmacological (126 mg) and physiological (6 mg) dose of deuterium-labeled β -carotene reported that vitamin A activity was highly dose dependent, resulting in molar vitamin A values of 0.034 mol and 0.5 mol retinol per mol β -carotene, respectively (20).

A survey of other quantitative efforts with stable isotopes of carotene shows doses that range from 6 mg to 40 mg (19). One concern with milligram-sized doses is that they may be saturating and exceed the average transport and cleavage capacity of the intestine. Indeed, During et al. (21) estimated that the average adult intestine can maximally cleave ~ 2.5 mg of β -carotene/day, and this value would argue against the use of milligram-sized doses, particularly when given in a single bolus. To circumvent saturation conditions when using stable isotopes, van Lieshout et al. (22) proposed a multiple dosing design in which microgram-sized doses of carbon-13-labeled β -carotene and retinyl palmitate are consumed for 3 weeks. At the end of the dose period, the relative enrichment of the two forms of isotopically labeled retinol (derived from β -carotene and preformed vitamin A) are used to estimate the relative vitamin A value of the carotene component. By this method, a molar vitamin A value for β -carotene of 0.75 was determined in Indonesian children.

Kinetic studies are facilitated by use of isotopically labeled analogs (tracer) of the natural dietary form (tracee). Whereas radioisotopes such as ^{14}C or tritium were once customary (23, 24), stable isotopes are now more common (19). The motivation for the shift, however, is mainly apprehension over the use of large radiative doses (25) and not the inducement of a superior analytic method. Recently, we (26) utilized accelerator mass spectrometry (AMS) detection of a $[^{14}\text{C}]\beta$ -carotene tracer. AMS is an isotope ratio instrument that measures $^{14}\text{C}/^{12}\text{C}$ ratios to parts per quadrillion (10^{-15}), quantifying labeled biochemicals to attomolar (10^{-18}) levels in milligram-

sized samples (25, 27). Extremely low detection limits allowed the use of low, safe radiation doses, and small sample sizes permitted a high density of sampling. Radiation exposure was comparable to that incurred naturally from cosmic rays in a single day (25). From the analysis of total excreta, dose absorption and rates of elimination were determined. Using this approach with one male subject, a molar vitamin A value of 0.53 was calculated for β -carotene. Long-term kinetics in plasma to 209 days was also possible because of the low natural abundance of ^{14}C and high sensitivity of the AMS instrument.

The aim of the present study was to determine the effect of vitamin A supplementation on the absorption and retinol equivalence of β -carotene. We studied the metabolic fate of physiologic (536 ng) oral doses of $[^{14}\text{C}]\beta$ -carotene in two female volunteers before and after a 21 day vitamin A supplementation period. Total ^{14}C was measured in plasma, urine, and stool to assess total dose absorption and excretion rates. The time course of labeled retinyl esters, retinol, and parent β -carotene were determined in plasma using liquid chromatographic separation followed by ^{14}C -AMS analysis.

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 from Sigma (St. Louis, MO). β -Carotene and *all-trans* retinol standards were obtained from Sigma. All solvents and other chemicals, unless otherwise noted, were obtained from Fisher Scientific (Santa Clara, CA). The *all-trans*- $[^{14}\text{C}]\beta$ -carotene was a gift from Hoffmann-La Roche (Basel, Switzerland). Olive oil, skim milk, sugar, bananas, and other groceries were from a local supermarket.

Dose preparation

Synthetic *all-trans*- $[^{14}\text{C}]\beta$ -carotene was purified by reversed-phase high performance liquid chromatography (RP-HPLC) as previously described (26). The purified dose was suspended in 1 ml absolute ethanol. Radiochemical purity was $>99\%$. *all-trans*- β -Carotene accounted for $>90\%$ of the dose, with the remaining being associated with *cis* isomers. Specific activity was 98.8 mCi/mmol. The dose in ethanol was added to an emulsion (shake) of the following composition: olive oil (0.5 g/kg body weight), 300 g of fresh banana, 100 ml skim milk, and 12.6 g sucrose were blended. About half of the shake was transferred to a plastic cup, the *all-trans*- $[^{14}\text{C}]\beta$ -carotene dose (in ethanol) was placed on top, and the remaining half of the shake layered atop the dose. The layers were stirred gently and consumed by the volunteer.

Subjects and experimental design

Subjects 1 and 2 were healthy women aged 41 and 43 years with a BMI of 23.6 and 26.7, respectively. Their blood lipid levels were normal. Subjects were administered a 1 year semi-food frequency questionnaire (DIETSYS-NCI Dietary Analysis System, version 3.7c). Typical daily consumption values of vitamin A and β -carotene for Subject 1 was 4,947 IU [1,484 retinol equivalents (REs)] and 1,074 μg of β -carotene. Respective intake values for Subject 2 were 3,416 IU (1,025 REs) and 1,310 μg . Subjects were

instructed to avoid foods rich in vitamin A or provitamin A carotenoids for 1 week prior to the study but to otherwise maintain their usual dietary habits. They were asked to keep a complete record of food intake for 2 weeks after dosing.

The experiment was designed to incorporate a test and retest period (Fig. 1). Subjects began complete fecal and urine collection 24 h in advance of the dose and continued complete 24 h collections until Day 16 and Day 30, respectively. On the day of dose administration, subjects were fitted with an intravenous catheter in a forearm vein. Blood was drawn into 7 ml tubes containing EDTA. A baseline blood sample was drawn (7 AM) and the dose consumed. Blood samples were drawn at 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 24, and 36 h post dosing. Blood samples were also collected between 8 AM and 8:30 AM on days 2, 3, 4, 5, 6, 9, 11, 13, 18, 25, 32, 39, and 46 in the fasted state.

Meals were controlled for time and content on the dose administration day. Lunch was served at 5.5 h postdosing and consisted of a frozen dinner (Enchiladas, Amy's Kitchen, Petaluma, CA), a blueberry bagel with jelly, 1 apple and 1 banana, and a large chocolate chunk cookie (Pepperidge Farm). Dinner was served 10.5 h post dose and consisted of a frozen dinner (Chinese Stir Fry, Amy's Kitchen) plus the bagel and fruit taken for lunch. Each meal was selected to deliver 20 g of fat in ~600 calories; lunch contained 300 μg REs and dinner 700 μg REs.

The administration of a second [^{14}C] β -carotene dose marked the beginning of the retest period at Day 74. Three weeks prior to the start of the retest (Day 74), subjects began consuming 10,000 IU (3,000 μg REs) of vitamin A supplement daily (as retinyl palmitate, GNC, Pittsburgh, PA) and continued at that level until 2 weeks after the retest dose administration. The supplement was continued at 5,000 IU (1,500 REs) for 6 additional weeks. The supplement was not consumed on the day the retest dose was given. The same dose administration, dietary control, and sample collection was performed on the day of dosing for the retest as for the test. Additional blood samples were also collected between 8 AM and 8:30 AM on Days 2, 3, 4, 5, 6, 9, 11, 13, 18, 25, 32, 39, 46, 60, 74, 88, 102, 116, and 137 in the fasted state.

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Institutional Review Boards at Davis and Lawrence Liver-

more National Laboratories. Informed consent was obtained from each patient.

Sample preparation

Plasma was separated by centrifugation and stored at -80°C . Complete urine samples were collected in tared 3 l plastic amber urine collection containers (Fisher). The urine was prepared for ^{14}C analysis by diluting a 1 ml aliquot with 9 ml of water. A 100 μl aliquot of the dilution was transferred to a quartz combustion vial for ^{14}C determinations. Stool samples were collected in 4 ml collection bags (Fisher Scientific). Stool weights were recorded, and a volume equal to five times the sample stool mass of 1:1 2-propanol-1 M KOH was added directly to the bag. The mixture then was dispersed with a Stomacher 3500 laboratory blender (Brinkmann, Westbury, NY) for 2 min at the high setting. Samples were heated in a water bath for 2 h at 70°C and redispersed on the Stomacher for 2 min. This heating-mixing cycle was repeated once more. A 40 ml aliquot was transferred to a 50 ml polypropylene tube containing 25 g of glass beads (6 mm; Fisher Scientific). The tube was capped and shaken by wrist-action (Model 75; Burrell Scientific, Pittsburgh, PA) for 6 h at maximum speed. One milliliter of the homogenate was diluted with 9 ml 1:1 2-propanol-1 M KOH, and a 100 μl aliquot was transferred to a quartz combustion vial for AMS analysis. Total carbons for plasma, urine, and stool samples were determined by the DUMAS method (28).

Analyte determinations

Eluent fractions of the HPLC corresponding to retinol or β -carotene were collected in quartz combustion vials, and ^{14}C was measured by AMS. Because retinyl esters and β -carotene comigrated on our system, two chromatographic separations were needed. In the first analysis, free retinol was determined. In the second run, β -carotene and retinol were determined after saponification of the plasma extract; the saponification step effected the conversion of all retinyl esters to their common moiety, retinol. Final retinyl ester concentrations were determined by the difference in plasma retinol pre and post saponification. The procedure is as follows. Plasma (200 μl) was denatured with 400 μl ethanol and extracted with 3×1 ml of hexane, which was pooled. The extracts were dried under nitrogen gas, and the sam-

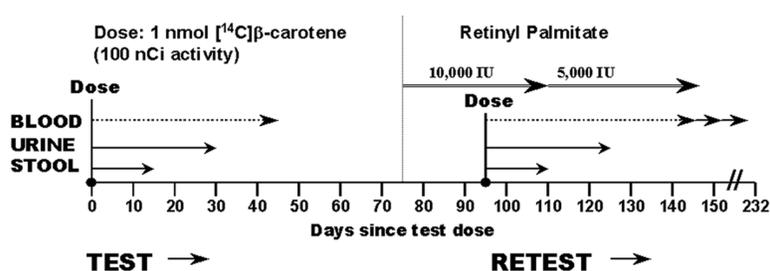


Fig. 1. Experimental design and time line. The experiment was designed to incorporate a test and retest period. In the test period, subjects began complete fecal and urine collection 24 h in advance of the dose and continued complete 24 h collections until Day 16 and Day 30, respectively. On the day of dose administration, subjects were fitted with an intravenous catheter in a forearm vein. A baseline blood sample was drawn. The subjects were then given a 1 nmol dose of [^{14}C] β -carotene in an emulsified drink. Cumulative urine was collected for 30 days and cumulative stool for 17 days. The last blood sample for the test was taken 46 days post dose. This was followed by a 7 week wash-out period. After that time, the administration of a second [^{14}C] β -carotene dose marked the beginning of the retest period. Three weeks prior to the start of the retest, subjects began consuming 10,000 IU [3,000 μg retinol equivalents (REs)] of vitamin A supplement daily and continued at that level until 2 weeks after the retest dose administration. Then the supplement was continued at 5,000 IU (1,500 REs) for 6 additional weeks. The supplement was not consumed on the day the retest dose was administered. In the retest, the same dose administration, dietary control, and sample collection were performed on the day of dosing.

ple was resuspended in 200 μ l 1:1 CH₃CN/2-propanol. An aliquot (20 μ l) was loaded onto a liquid chromatograph and the eluent corresponding to the retinol peak (2.7–3.1 min; 325 nm) was collected into a quartz combustion tube. The remaining lipid extract was dried under nitrogen and digested in 220 μ l of 5% KOH in methanol (220 μ l with 0.7% pyrogallol) for 1 h at 70°C under nitrogen. Water (200 μ l) was added, and the sample extracted with 3 \times 1 ml volumes of hexane. The extracts were pooled, dried under nitrogen, and the sample resuspended as before. A 20 μ l aliquot was separated by HPLC. Total retinol (2.7–3.1 min; 325 nm) and β -carotene (8.9–9.4 min; 450 nm) eluent fractions were collected into quartz combustion tubes. The HPLC apparatus was an Agilent 1100 chromatograph with a variable wavelength detector (Palo Alto, CA) fitted with an Agilent Zorbax Eclipse XDB-C18 column (3.5 μ m, 3.0 \times 150 mm) and an Eclipse XDB-C8 guard cartridge. The mobile phase conditions were: 0–1 min 100% Solvent A (70:30 CH₃CN-CH₃OH + 0.02% ammonium acetate), followed by 1–4 min linear ramp to 65% Solvent B (1:1 CH₃CN-MeCl₂ + 0.02% ammonium acetate) at 0.5 ml/min; hold until 10 min.

Recovery experiments showed that retinol was 90% recovered for both pre- and postsaponification extractions; therefore, the cumulative retinol recovery was 81%. Retinyl ester extraction recovery (using retinyl palmitate as the test compound) was 90% in the first extraction, and 87% of that was recovered after saponification, for a cumulative recovery of 78%. β -Carotene recovery for the whole procedure was a minimum of 92%. [¹⁴C]retinyl ester concentrations were calculated by adjusting for saponification losses relative to [¹⁴C]retinol and then subtracting the presaponification [¹⁴C]retinol from the postsaponification [¹⁴C]retinol collection.

AMS analysis

¹⁴C determinations were made at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory

(27). HPLC metabolite fractions and urine and stool dilutions in quartz tubes were supplemented with 50 μ l of tributyrin (40 mg/ml) in methanol, taken to dryness under vacuum (RC 10.10 rotary concentrator system; Jouan Inc., Winchester, VA), and converted to graphite (29). Final metabolite concentrations were calculated by relating the ¹⁴C signal to the known specific activity of the molecule. The [¹⁴C]retinoids derived from β -carotene cleavage were assigned a specific activity of 49.4 mCi/mmol (one-half that of its [¹⁴C] β -carotene precursor).

Data are expressed as amol ¹⁴C metabolite/ml plasma or a percentage of administered or absorbed dose for urine and stool.

AUC and half-life calculations

AUC values were determined using a linear interpolation of straight line connection between the data points with tabulation of the integral of each interpolation from 0 to time t using Origin (Microcal; Northampton, MA). Rate constants and half-lives ($t_{1/2}$) were estimated by linear regression analysis of semi-log plots of the concentration time plots. Urinary and stool output rates were determined from linear fits of the plots after equilibration had been reached.

RESULTS

Mean plasma retinol and β -carotene concentrations are summarized in **Table 1**. Mean differences in β -carotene concentrations between test and retest periods were significant ($P < 0.05$). Retinol concentrations did not differ significantly. A summary of fecal and urinary excretion data is displayed in **Fig. 2** and Table 1. Subject 1 absorbed 57% of the test dose, although fecal excretion extended over 6 days. Fecal elimination shortened to 4 days under

TABLE 1. Vitamin A value and kinetic parameters after administration of 1.0 nmol oral dose [¹⁴C] β -carotene

	Subject 1		Subject 2	
	Test	Retest	Test	Retest
Plasma retinol ^a	1.53 (0.09)	1.58 (0.16)	1.29 (0.08)	1.38 (0.07)
Plasma β -carotene ^a	0.28 (0.07)	0.43 (0.05) ^b	0.33 (0.03)	0.42 (0.03) ^b
Absorbed dose (%) ^c	57	74	52	75
Cumulative dose excreted in stool (%) ^d	47	28	51	28
Absorbed dose excreted in stool (%)	10	2	3	3
Absorbed dose excreted in urine (%) ^e	35	4	29	5
Absorbed dose excreted in urine from 0–72 h (%)	31	3	26	3
Urinary excretion rate 5–30 days (% absorbed dose/d)	0.102	0.028	0.082	0.054
Absorbed dose stool excretion rate (% absorbed dose/d)	0.046	0.032	0.033	0.046
Urinary/stool excretion rate ratio	2.21	0.88	2.44	1.18
AUC _{0–5} ^f				
¹⁴ C	22,636	24,955	89,505	132,920
Retinyl esters	3,976	15,018	22,850	63,207
β -Carotene	1,666	6,787	8,378	32,740
Retinol	743	1,054	1,885	6,606
Retinyl esters/ β -carotene	2.39	2.21	2.73	1.93
% Recovery in components (SUM/ ¹⁴ C)	28	92	37	77
Vitamin A value	0.62	0.85	0.54	0.74
Half-life ($t_{1/2}$) retinol (d)		140		243
Half-life ($t_{1/2}$) β -carotene (d)		20		35
Turnover time β -carotene (d)		29		51

^a Mean (\pm SD), sampling of time points at 0, 0.27, 0.61, 1.0, 1.5, 2.0, 4.0, and 6.0 days postdosing.

^b Significantly different at $P < 0.05$.

^c First 5 days for Subject 1; 2.5 days for Subject 2.

^d Percent of total dose over 17 days of stool collection.

^e Urine collections occurred over 30 days.

^f mol \cdot h/ml.

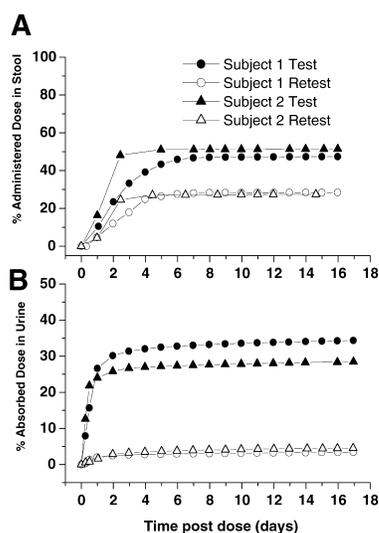


Fig. 2. The cumulative stool (A) and urine (B) recovery of ^{14}C expressed as a percentage of administered (stool) and absorbed (urine) dose following oral ingestion of [^{14}C]β-carotene. The point of discontinuity in the cumulative rates for stool was used to determine dose absorption. Vitamin A supplementation (retest) was associated with higher dose absorption and reduced urinary excretion of ^{14}C .

retest with an increased absorption to 74%. Subject 2 displayed a more rapid excretion of the nonabsorbed [^{14}C]β-carotene. In the test period, 48% was lost (52% absorbed) in two collections, while in the retest period, 25% was lost (75% absorbed) in two collections. ^{14}C signal was present in the 0–6 h urine collection in both subjects and in both test periods. The largest single output occurred within 48 h in all cases. Urinary excretion dominated fecal excretion after the absorption phase, contrary to a previous report in a male subject (26). The rate of urinary ^{14}C excretion was 2.2 and 2.4 times that of stool for Subjects 1 and 2, respectively, in the test period. Output of the absorbed dose

via urine over the first 30 days post dosing was much smaller in the retest period than in the test period in both subjects. Specifically, absorbed dose excreted in the urine of Subject 1 was 31% during the test and 3% during the retest; Subject 2 excreted 26% during the test and 3% during the retest. After dose equilibration at Day 5, the rate of urine loss was low for both subjects and in both periods; as a result, differences in total excretion were largely attributable to the early time points.

Plasma β-carotene and retinyl ester concentration before and after supplementation for the first 50 h are shown in Fig. 3. In test absorptive period (0–10 h), analyte concentrations rose to a first maximum centered at 8 h for both subjects, passing through a small shoulder at the leading edge of main peak. A similar structure for both subjects and both analytes was observed in the retest with the obvious addition of a well-resolved first peak (shoulder peak in the test). Concentrations for the two analytes within both subjects moved in parallel, suggesting a single transporter for both analytes. Concentrations of circulating β-carotene were persistent and equivalent at ~2,000–4,000 attomolar for both subjects in the distributive period (10–24 h) of both the test and retest. Subject 2 doubled the circulating β-carotene in the retest phase over the test phase, however. Subject 1 displayed a strong resurgence in retinyl ester concentration that was muted after supplementation and decayed away in both instances between 15 h and 50 h. Subject 2 displayed a very attenuated rise in retinyl esters in the retest that was not present in the test.

The integrated ratio of retinyl ester/β-carotene from 0 h to 5 h (AUC_{0-5}) estimates the vitamin A value of β-carotene (Table 1). AUCs tabulated over longer periods could be influenced by postintestinal actions and therefore are not viewed as valid estimators of intestinal cleavage. The derived ratios were 2.39 and 2.73 for Subjects 1 and 2, respectively, during the test period. Retest values were 2.21 and 1.93 for Subjects 1 and 2, respectively. Assuming cen-

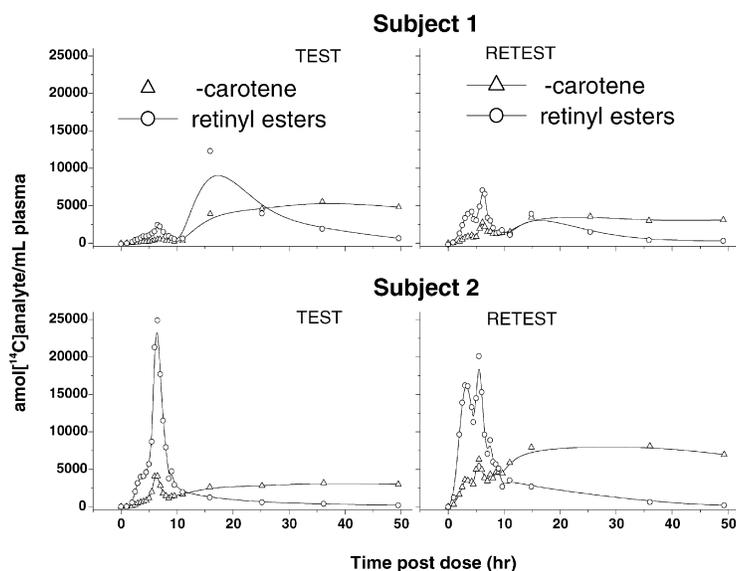


Fig. 3. Time course plots from 0–50 h for labeled β-carotene and retinyl esters isolated from plasma. Hexane extracts of denatured plasma were fractionated by HPLC and followed by accelerator mass spectrometry (AMS) measurement of ^{14}C concentrations. Molar values for the metabolites were calculated 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.

tral cleavage of β -carotene and adjusting for dose absorption from the stool information, the conversion ratio (vitamin A value) for the test was 0.62 and 0.54 mol vitamin A to 1 mol β -carotene for Subjects 1 and 2, rising to 0.85 and 0.74 mol vitamin A to 1 mol β -carotene in the retest. (A value of 1, interpreted as meaning that 1 mol of ingested β -carotene, is equivalent to 1 mol of preformed vitamin A.) Another way to examine the bioconversion is to plot the concentration ratio of retinyl ester/ β -carotene at discrete time points (Fig. 4). The data displayed an overall depletion of retinyl esters relative to β -carotene concentrations with time, except for apparent influxes coincident with the times of second and third meals (indicated by arrows). Ratios in the test period were consistently higher than retest ratios for both subjects.

The time courses for the appearance and disappearance of [14 C]retinol in plasma over 9 days following dosing is presented in Fig. 5. In all cases, the concentration rose to a single peak (albeit broad for Subject 1 retest), with a maximum concentration of ~ 24 h for Subject 1, and much earlier at ~ 12 h for Subject 2. The absence of multiple peaking is consistent with the controlled release of retinol with retinol binding protein (RBP) following hepatic uptake of retinyl esters.

Figure 6 shows the fraction of the total plasma 14 C concentration that is comprised of the sum of retinol, retinyl esters, and β -carotene as functions of subject, vitamin supplementation, and time. Integrated AUCs have the effect of dampening fluctuations due to meals and other effects, facilitating observation of general trends. The three assayed components accounted for only 60–70% of the isotopic label throughout the distribution and elimination phases of both subjects, with and without vitamin A sup-

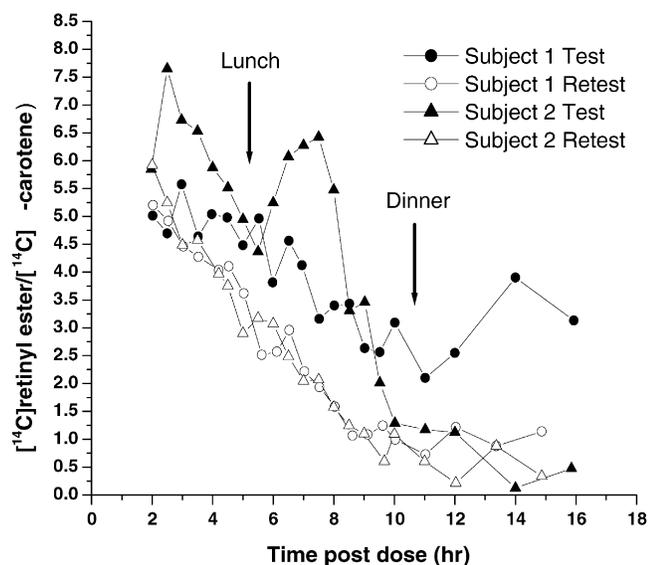


Fig. 4. Concentration ratio of retinyl ester/ β -carotene at discrete time points. In general, the data displayed a negative slope in response to depletion of retinyl ester concentrations relative to β -carotene concentrations. Solid arrows indicate timing for second and third meals. Ratios in the test period were consistently higher than retest ratios for both subjects for 10 h.

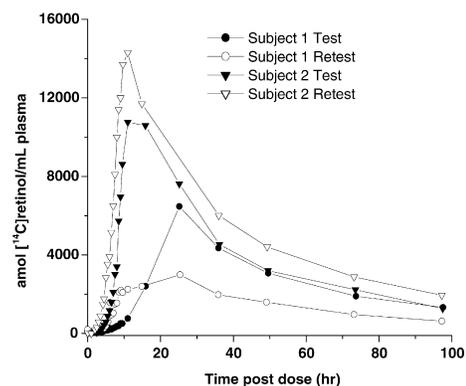


Fig. 5. Time course line plots of [14 C]retinol in plasma from 0–100 h. Unlike β -carotene and retinyl esters, retinol concentrations rose to a single peak.

plementation. A slight downward trend was apparent in Subject 1. In the absorptive phase, the main effect of supplementation was improve recovery of the components; recoveries prior to supplementation were surprisingly low, ranging from near zero (Subject 2) to 30% (Subject 1) in the first 6 h; similar retest values ranged were $\geq 90\%$ and $\geq 75\%$ in Subject 1 and 2, respectively.

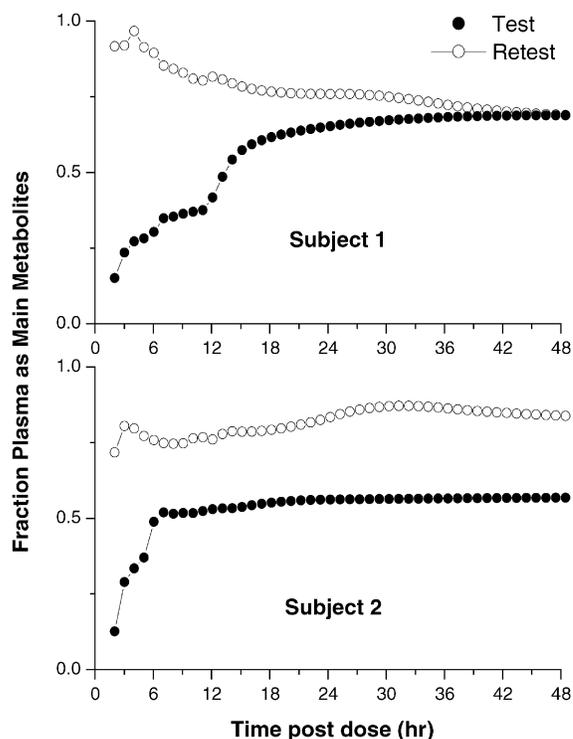


Fig. 6. The fraction of the total plasma 14 C activity accounted for in the main analytes, retinol, retinyl esters, and β -carotene as a function of subject, vitamin supplementation, and time. The three assayed components accounted for only 60–70% of the isotopic label throughout the distribution and elimination phases of both subjects, with and without vitamin supplementation. In the absorptive phase, the main effect of supplementation was increased recovery of total 14 C in main analytes; recoveries prior to supplementation were surprisingly low.

For the retest-test ratios for the percent ^{14}C recovered in the urine, **Fig. 7** illustrates the effect vitamin A supplementation had on the urinary excretion of label. No effect would yield values centered near unity. However, retest ratios were lower in both subjects, consistent with decreased output of ^{14}C during supplementation. The values rose in both subjects with time; however, their appearances were different. Values rose for ~ 24 h and then remained largely unchanged in Subject 1. In Subject 2, values rose gradually for the duration of the plot.

DISCUSSION

β -Carotene is thought to diffuse from mixed micelles in the small intestine through the unstirred water layer and into the enterocyte before entering the lymph with chylomicrons. The passive nature of this process does not suggest that vitamin A supplementation would result in uptake regulation of β -carotene. In this study, however, we observed that supplemental retinyl palmitate (retest) was associated with a decrease in the recovery of the ^{14}C label in the first several stool collections (Fig. 2; Table 1). We do not know the basis of the effect; however, vitamin A is important in controlling epithelial health and gut integrity (30–32), and changes in the gut in response to supplementation in our subjects may provide one explanation for the observed effects. A second explanation has to do with biliary excretion of the ^{14}C label. Using our balance technique, an enhancement in the biliary excretion of absorbed β -carotene and its metabolites could give the appearance of decreased absorption by increasing the output of ^{14}C in the stool. In the case of lipophilic compounds, biliary excretion is largely a result of a compound being metabolized in the gut wall prior to transportation to the liver via the hepatic portal vein (33). Elimination is then distributed between the bile and the urine. Our urine data indicate that a substantial portion of the dose was eliminated in the urine in the unsupplemented subjects (Fig. 2); therefore, it is reasonable to assume that a fraction of the dose was cleared in the bile as well (34). Regardless of which theory is correct, it is clear that sup-

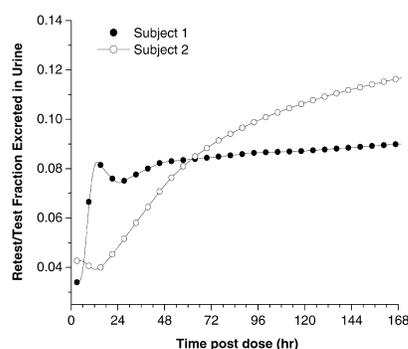


Fig. 7. Ratios of absorbed dose collected in the urine for retest over test period for 168 h post dose administration. The effect of supplementation was to depress the excretion of ^{14}C at the early time points.

plementation altered the bioavailability of the carotene dose, although the balance between uptake and elimination cannot be fully explained on the basis of stool data. An understanding of the identity of the urinary and stool products may help resolve this issue. Further studies are needed to better understand the mechanism governing this effect.

The primary objective of the current study was to investigate the effect of dietary vitamin A on the vitamin A value of β -carotene. In this limited sample set, we have seen that supplementation was associated with a higher molar vitamin A value. In accord with the higher dose absorption, the absolute AUC_{0-5} for both β -carotene and retinyl esters increased for both subjects. We focused on retinyl esters and β -carotene because plasma retinol is not of intestinal origin. The fraction of the dose converted to retinyl esters, however, was lower in both subjects, suggesting that a smaller percentage of absorbed β -carotene molecules were actually cleaved in the retest. As vitamin A value is the product of absorption and the fraction of absorbed β -carotene that is cleaved to vitamin A, the larger absorption in the retest is responsible for the higher molar vitamin A value in these studies. Determined molar ratios ranged from 0.54 to 0.85, which are comparable to values obtained by methods that used functional indicators as end points as well as some stable isotope methods (19). Intracellularly, the activity of the principal known carotenoid cleavage enzyme β , β -carotene 15,15'-monooxygenase may serve as a key regulatory point (12, 35, 36) in the bioconversion. In the rat, vitamin A deficiency can increase intestinal cleavage activity, while excess vitamin A or β -carotene is inhibitory (36). Our results suggest absorption can be more important than cleavage capacity in determining β -carotene's vitamin A value.

We determined the metabolic behavior of ^{14}C as well as the major analytes [^{14}C] β -carotene, [^{14}C]retinyl esters, and [^{14}C]retinol in plasma. The concentration profile for labeled retinyl esters and β -carotene (Fig. 3) showed two maxima in the absorptive phase for both subjects, with a larger and more resolved first maxima in the retest; retest β -carotene and retinyl ester AUC_{0-5} were, on average, 3- to 4-fold greater in magnitude than corresponding test period AUC_{0-5} (Table 1). In contrast, ^{14}C AUC_{0-5} were essentially unchanged in Subject 1, and were $\sim 50\%$ larger in Subject 2. This discrepancy between the ^{14}C recovered in the analytes versus total ^{14}C activity suggests that substantial label resides with other metabolites. The plots in Fig. 6 illustrate the magnitude of this effect over time. Values below 1 indicate the degree to which metabolites other than the main components constitute the incoming dose. Prior to supplementation, only a small fraction of the label was recovered as the main component in the early absorptive period. The effect lessened with time, and at later periods, $\sim 50\text{--}70\%$ of the label is present in plasma as the main metabolites. The patterns were markedly different in response to supplementation; supplementation increased the recovery of the label in the main components. This may suggest a sparing effect of vitamin A supplementation on the β -carotene and vitamin A utilization.

Presystemic reactions within the intestinal mucosal wall dictate the early metabolite profile. Although there is limited human information on this subject, cytosolic extracts of human enterocytes and intact Caco-2 cells can metabolize retinol to retinoic acids and oxygenated end products of retinoic acid metabolism (7). Studies in the ferret have reported “substantial” conversion of β -carotene to polar end products that include retinoic acids and asymmetric cleavage products, i.e., apo-carotenoids (37, 38). Similar metabolism in our subjects might explain the poor recoveries of label in the main metabolites. It can be supposed that supplemental vitamin A in the retest satisfied immediate vitamin A needs in the enterocytes, therefore directing metabolism toward storage products (retinyl esters). We previously identified 13-*cis*-4-oxo retinoic acid as a blood metabolite of [14 C] β -carotene (26). Moreover, RP-HPLC analysis of plasma (data not shown) revealed one unknown component that has the chromatographic character of the neutral oxidation product of β -carotene (epoxide?).

The poor plasma recoveries of 14 C in the main metabolites coincided with large losses of the absorbed dose in the first 0–6 h urine: 0–6 h test urines contained 8% and 14% of the absorbed dose in Subjects 1 and 2, respectively. Conversely, higher plasma recoveries after supplementation coincided with marked reductions in 14 C in the urine (Table 1; Fig. 7). Thus, intestinal metabolism (test) would appear to generate products that are poorly retained and readily eliminated into the urine. Indeed, β -carotene, retinyl esters, and retinol are not found in normal urine; many of the metabolites identified to date are chain-shortened, oxidized, conjugated products (39, 40). Vitamin A supplementation was also associated with a reduction in the long-term output of label in urine, suggestive of modifications in postabsorptive turnover.

The plot of retinyl ester/ β -carotene concentrations in the absorption phase displayed a negative slope, consistent with differential postabsorptive handling of the compounds. The descent in the ratio was marked by several rises, most visibly in the test period, which were coincident with the meals. Fielding et al. (41) also demonstrated the importance of the second meal effect. They fed a high-fat breakfast and lunch of differing fat constituents and showed that 50 min after lunch, there was a rise in triglycerides that matched the content of breakfast. Lunch fats did not appear in the plasma until 2 h after lunch. Therefore, it is probable that in our study, fat from the breakfast and lunch meals was present to shuttle more [14 C] β -carotene doses into the lymph and ultimately into the circulation. van Vliet, Schreurs, and van den Berg (42) observed similar rises in subjects following a 15 mg oral dose of β -carotene. They attributed their initial drop in the ratio to saturation in the cleavage enzyme and the rise to mean either more efficient transport and chylomicron incorporation, or the establishment of new equilibrium with the enzyme. However, a saturation explanation would not seem tenable, given the small (and presumably nonsaturating) size of our doses.

The high degree of parallelism between the [14 C]reti-

nyl esters and [14 C] β -carotene in our studies suggests that a tightly linked or single mechanism controls the incorporation of β -carotene and the retinyl esters derived from it into nascent chylomicrons (43). This finding is at variance with ferret intestinal perfusion studies, which found β -carotene was released more slowly than its retinoid metabolites in the lymphatics (44). Subject 1 displayed a second interesting phenomenon, which we had originally observed in a single male subject (26); that is, the latent rise in retinyl esters that peaked at 18 h. The secondary meal effect has been examined above. A second consideration for the rise in Subject 1 (and to a lesser extent in both subjects and both test periods) is resecretion of retinyl esters from the liver with hepatic lipoproteins. The occurrence of such a pathway is deemed important since some the anticancer effects of vitamin A have been attributed to circulating retinyl esters (45). Indeed, ~5–10% of circulating vitamin A in fasting plasma is in the form of retinyl esters (46), and while some of the retinyl esters may be associated with traces of chylomicra not removed by the liver, the magnitude of the rise in our experiments would suggest a new input. Dogs, ferrets, and cats are reported to transport retinyl esters in plasma with lipoproteins (47, 48), and our data suggest that humans may have this capacity as well. An analysis of the apoB protein forms (intestinal vs. hepatic) associated with latent rises would help resolve this issue.

The time course of retinol in the plasma was very different for both subjects from retinyl esters and β -carotene, consistent with the controlled release of retinol with RBP following hepatic uptake of retinyl esters. There was no evidence for the absorption of free retinol from the intestine in the form of an early concentration peak, contrary to observations by us (26). It is noteworthy that the late maxima in Subject 1 (relative to Subject 2) were preceded by the large (test) and moderate (retest) retinyl esters rises past 10 h, consistent with a precursor-product relationship. The long-term kinetics of β -carotene and retinol were also calculated in the retest and were found to be 140 days and 243 days for retinol and 20 days and 35 days for β -carotene. This is generally consistent with an earlier study utilizing [14 C]retinyl acetate that reported mean $t_{1/2}$ s of 154 days with a range of 75 days to 241 days (18). β -Carotene half-lives have been reported as less than 12 days (49) and 40 days (26). The scarcity of data on elimination of these bioactive compounds necessitates the collection of larger data pools from diverse cross sections of the population.

In conclusion, we have shown that vitamin A supplementation at levels moderately above recommended daily allowance has effects on β -carotene metabolism that are readily detected when using AMS detection. Notable effects included a reduction in the postprandial ratio of retinyl esters to β -carotene with a concomitant increase in total dose absorption. Increased dose absorption resulted in a higher overall vitamin A value for β -carotene in response to supplementation, which was accentuated by attenuation in early metabolism of the dose to urinary end products. ■

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