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Chapter 3

EXPERIMENTAL APPROACHES TO THE STUDY OF β-CAROTENE METABOLISM: POTENTIAL OF A ^{13}C TRACER APPROACH TO MODELING β-CAROTENE KINETICS IN HUMANS

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I. INTRODUCTION

The purpose of this chapter is to briefly review the biological relevance of β-carotene (βC) to human health, previous approaches to model or study quantitative aspects of βC absorption and metabolism, and the potential merits of a ^{13}C-based stable tracer approach recently developed at Cornell and how it might be useful for βC modeling in humans. A preliminary report of this approach has been published (Parker *et al.,* 1993).

While the provitamin A activity and therapeutic usage of β-carotene in the treatment of photosensitivity disorders is well documented, a therapeutic or preventative role of βC in cancer, cardiovascular disease (CVD), or other degenerative diseases, as well as a modulator of immune function and oxidative balance, is speculative and currently an active area of investigation.

Several fundamental aspects of βC metabolism in humans are poorly understood. These include, quantitative information on the absolute absorption efficiency of βC, the magnitude of effect of factors which influence βC absorption or biotransformation of βC to vitamin A, kinetics of βC in various lipoprotein fractions, tissue sites of βC storage and biotransformation, and quantitative estimates of rates of specific tissue or total body elimination of βC. This lack of detailed knowledge of βC metabolism makes it more difficult to design and interpret appropriate clinical trials to test the role of βC in human health, predict the efficacy of dietary sources of βC in ameliorating vitamin A deficiency, and propose plausible mechanisms for reported biological effects of βC in humans and animals. It is speculated that the causative agent in many of the reported biological effects is intact βC rather than a retinoid metabolite, however the studies conducted to date cannot confirm this hypothesis. The development and application of mathematical and compartmental kinetic models, using stable isotope tracer techniques, offers the advantages of safely studying βC metabolism in humans, of providing predictive information regarding βC biokinetics, and possibly of aiding in the evaluation of the role(s) of βC in various biological responses.

A. BIOLOGICAL EFFECTS OF β-CAROTENE IN HUMANS

1. Cancer

Numerous epidemiological studies have consistently shown and continue to show that consumption of diets high in βC or high in βC-rich fruits and vegetables is correlated with a lower risk of developing some types of cancers (Ziegler, 1989; van Poppel, 1993; Gerster, 1993). Cancer clinical trials, which have been completed in exclusively high-risk subjects, indicate the βC is probably not a powerful therapeutic agent late in the process of carcinogenesis (Greenberg *et al.*, 1990, 1994; Heinonen *et al.*, 1994). Lung cancer risk may actually be increased in smokers receiving βC. Because the effects of smoking on βC metabolism are largely unknown, a mechanism for the observed increased risk is difficult to propose. If this effect is actually due to βC, a follow-up after cessation of supplementation should show a return of risk to that of the non-βC group. Information on the kinetics of total body βC elimination would be most helpful in aiding the design of such a follow-up.

However, there have been many reports showing the efficacy of supplemental βC in reversing and reducing the incidence of oral leukoplakia, a preneoplastic lesion associated with oral cancer, in high-risk subjects (Stich *et al.*, 1988; Garewal *et al.*, 1990, 1993). In addition, recent observations also suggest βC may reduce the risk of gastric cancer via an interaction with ornithine decarboxylase activity in the stomach mucosa (Bukin *et al.*, 1993, 1995). These latter findings suggest that early intervention with βC may yield greater health benefits than interventions occurring later in the progression of a disease.

2. Cardiovascular Disease

A preliminary study of Gaziano and co-workers (1990) reported a 44% reduction in all major coronary events and a 49% reduction in all major vascular events in subjects receiving 30 mg of βC every other day for 60 months compared to a placebo group. This preliminary report has created speculation that βC may have mitigating effects in CVD. However, studies investigating several possible biochemical mechanism of action for βC in reducing CVD have produced inconsistent results. Numerous small-scale clinical trials have found an increase in serum HDL–cholesterol levels following βC supplementation (Mathews-Roth and Gulbrandsen, 1974; Bencich *et al.*, 1989; Gaffney *et al.*, 1990; Manago *et al.*, 1992), while others have reported no such effect (Ringer *et al.*, 1991; Allard *et al.*, 1994). βC

has also been shown to have little protective effect on the formation of oxidized LDL, a risk factor in CVD (Princen *et al.*, 1992; Reaven *et al.*, 1993). Consequently, the role of βC as a preventative or curative agent in CVD remains speculative.

3. *Modulation of Immune Function and Antioxidant Status*

Currently, the available evidence for βC as a modulator of immune function and oxidative balance is also inconsistent. Supplemental βC has been shown to ameliorate ultraviolet light-induced suppression of cellular immune response as measured by delayed-type hypersensitivity tests in both young and older men (Fuller *et al.*, 1992; Herraiz *et al.*, 1994), alter population characteristics of immune cells by increasing the number of T-helper cells, natural killer cells, and/or cells with activation markers in healthy subjects (Alexander *et al.*, 1985; Watson *et al.*, 1994; Prabhala *et al.*, 1991), and inhibit the respiratory burst reaction in granulocytes isolated from human subjects (Clausen, 1992). However, other investigators using similar βC doses have found no effect of supplemental βC on lymphocyte subpopulations (Ringer *et al.*, 1991; van Poppel *et al.*, 1993) or neutrophil superoxide formation (Mobarhan *et al.*, 1990). While βC has been proposed to perform an antioxidant function, analogous to that of α-tocopherol, its effects on indicators of antioxidant status have little relationship to βC dose. For example, the magnitude of reduction in serum thiobarbituric acid-reactive substances (Mobarhan *et al.*, 1990) and breath pentane (Gottlieb *et al.*, 1993) was essentially equivalent between subjects receiving 120 mg/day and those receiving 15 mg/day, even though an 8-fold increase in dose and 2.6-fold greater serum βC enrichment occurred in the group receiving 120 mg/day. Lack of information on βC metabolism over these wide dose differences or the effect of repeated elevated doses on βC metabolism and biokinetics hampers interpretation of these results.

B. ASPECTS OF β-CAROTENE METABOLISM REQUIRING CLARIFICATION

1. *Absorption and Biotransformation*

Quantitative information on the absolute absorption efficiency of βC in humans is sparse. The effect of matrix, magnitude of dose, or coingested foods on absorption efficiency are poorly understood, if at all. Matrices such as naturally occurring complexes in plant foods, or pharmaceutical matrices such as water disperable beadlets, crystalline powders, or oil suspensions of βC, are likely to have a large impact on absorption efficiency.

Typical dietary intakes of βC range from 1 to 6 mg from foods to pharmacological supplementations of 30 to 300 mg and encompass a range over which absorption efficiency probably varies considerably.

Absorption efficiency, to the extent reflected by plasma βC levels, has been shown to be affected by several factors. βC absorption has been shown to be facilitated by coconsumption of fat and/or a meal (Dimitrov *et al.*, 1988; Prince and Frisoli, 1993). Rock and Swendseid (1992) reported lower plasma βC levels in subjects consuming 12 g of citrus pectin with 25 mg of βC compared to subjects receiving the same βC dose without pectin. Recently, Weststrate and van het Hof (1995) reported a reduction in plasma βC and lycopene concentrations in healthy subjects after consumption of a relatively high dose (12.4 g/day) and a lower dose (3 g/day) of sucrose polyester, a synthetic fat substitute, relative to controls.

In addition, there are numerous reports showing the extreme interindividual variation in plasma response following βC supplementation (Johnson and Russell, 1992; Dimitrov *et al.*, 1988; Brown *et al.*, 1989). Dietary factors, such as fat, fiber, or fat substitute content, or physiological factors, such as bile salt composition or secretion, mixed micelle formation, or general health, may have interactive and independent roles in the observed variation in plasma βC enrichment levels. The above factors and possibly other factors which are currently unknown may contribute to this variation through involvement in mechanisms which occur during absorption and intestinal conversion of βC to vitamin A. However, the relative importance of each factor to the observed variation in final plasma βC levels achieved following supplementation remains to be determined.

At present the only experimental studies concerning efficiency of absorption and intestinal bioconversion of βC to vitamin A in humans stems from two reports from the mid 1960s using radiolabeled βC. These studies were conducted in elderly hospitalized cancer patients with cannulated thoracic lymph ducts using ^{14}C- or ^{3}H-βC and reported that the amount of intact βC absorbed varied considerably (9 to 30% of dose) and that retinyl esters were the major lymphatic product of βC metabolism, representing 61 to 88% of recovered ^{3}H or ^{14}C (Goodman *et al.*, 1966; Blomstrand and Werner, 1967). However, controversy regarding postabsorptive bioconversion of βC to vitamin A and retinoic acid exists because of a lack of appropriate techniques to study such a process. While *in vitro* animal tissue studies strongly indicate central cleavage of βC to two molecules of retinal as the primary mechanism of conversion of β-carotene to vitamin A, the stoichiometry of this reaction in humans remains unconfirmed.

Zeng and co-workers (1992) employed synthetic carotenoid analogs to evaluate carotenoid metabolism in humans. The advantage of this approach is that low doses can be used because such carotenoids are not endogenous

to human plasma, unlike βC and other carotenoids of dietary origin. Ethyl-β-apo-8′-carotenoate, 4,4′-dimethoxy-β-carotene, and β-apo-8′-carotenal (100 μmol) dissolved in peanut oil were administered to healthy human subjects as part of a light breakfast. Serial venous blood samples were drawn and concentration versus time curves of the carotenoid analogs or their metabolites were obtained by HPLC. The data suggested that these carotenoids differed with respect to sites and mechanisms of biotransformation, rates of absorption and elimination, and maximum serum concentration and time to maximum concentration. Mathematical modeling was utilized to determine absorption rate by area under curve (AUC) values and elimination rate using estimated mean sojourn time. A 270-fold difference in the rate of absorption between carotenoids was reported, with ethyl-β-apo-8′-carotenoate being the fastest (1130 $\mu mol \cdot hr^{-1} \cdot liter^{-1}$), 4,4′-dimethoxy-β-carotene intermediate (159 $\mu mol \cdot hr^{-1} \cdot liter^{-1}$) and the β-apo-8′-carotenal metabolite,β-apo-8′-carotenyl palmitate, the slowest (4.2 $\mu mol \cdot hr^{-1} \cdot liter^{-1}$). Of the three analogs, only β-apo-8′-carotenal exhibited significant biotransformation to retinoid metabolites. However, the mechanism for conversion could not be determined. The large differences in plasma kinetic behavior between the carotenoid analogs employed in this study suggest structure-specific mechanisms of elimination and emphasize the need to apply stable isotope techniques to the study of uptake and metabolism of naturally occurring carotenoids.

Questions regarding the extent of postabsorptive bioconversion of βC to vitamin A persist. Animal data indicate the liver possesses this capability, but the relative importance of intestinal mucosa versus liver is unknown. Novotny and co-workers (1995) reported a compartmental model which predicted that both liver and intestinal mucosa were important sites for biotransformation of βC in the human, with 43% of total conversion occurring in the liver and 56% in the intestinal mucosa. However, the model assumed a stoichiometry of 1 mol retinol per mole βC, and the effect on the model assuming a 2:1 ratio was not discussed.

2. *Transport and Storage*

The carotenoid composition of various lipoprotein classes has been well described, as have the changes in βC concentration in such classes following large single oral doses of βC (Johnson and Russell, 1992; Traber *et al.*, 1994). However, other aspects of plasma carotenoid transport are poorly understood. These include: (1) the rates of turnover of βC in LDL and HDL, (2) the physiological significance of HDL-βC (reverse transport?), (3) the extent and rate of exchange between lipoproteins of βC in comparison with other carotenoids, and (4) the extent to which recycling of plasma βC into and out of liver, adipose, or other tissues occurs.

βC supplementation studies in humans have shown sustained elevated levels for several days to weeks following βC cessation (Dimitrov *et al.*, 1988; Johnson and Russell, 1992). These observations suggest considerable recycling of βC between extrahepatic tissues and plasma. Reports of βC concentration in human tissues indicate that adipose tissue is a major storage site (Dagadu, 1967; Parker, 1988). Parker (1988) reported an average adipose βC concentration of 0.62 μg/g in adults. This concentration would yield a total body adipose βC content of about 11 mg for a 75-kg adult of 24% body fat. Also, an average plasma concentration of 0.3 μ*M* would yield an average total plasma pool size of roughly 0.5 mg. Thus oral doses of 15 to 100 mg, commonly employed in the study of βC metabolism, would clearly perturb steady-state kinetics in these two body pools.

Using smaller oral doses of carotenoid analogs, Zeng *et al.* (1992) reported a 50-fold difference in serum concentration between the carotenoid analogs 4,4′-dimethoxy-β-carotene and ethyl-β-apo-8′-carotenoate and the metabolite of β-apo-8′-carotenal, β-apo-8′-carotenyl palmitate. It was also shown that the time post dosing to reach this maximum concentration range between 5.5 and 27.1 hr. This variation suggests marked differences in transport or volume of distribution between carotenoids of different structures.

3. *Elimination*

Few estimates of the rates of elimination of carotenoids from the bloodstream in the human have been published. Zeng and associates (1992) estimated mean sojourn time of the three carotenoid analogs using the simulation, analysis, and modeling (SAAM) computer program to be 144 hr for 4,4′-dimethoxy-β-carotene, 209 hr for ethyl-β-apo-8′-carotenoate and for the β-apo-8′-carotenal metabolites, 124 hr for β-apo-8′-carotenol and 43 hr for β-apo-8′-carotenyl palmitate. These values, with the exception of that for β-apo-8′-carotenyl palmitate, are much longer than the mean residence time of 51 hr estimated for d_8-β-carotene by Novotny *et al.* (1995) using SAAM. To date, the report of Notovny *et al.* (1995) represents the only attempt to quantitatively estimate kinetic parameters of βC metabolism such as mean transit time, mean residence time, fractional catabolic rate, or rate of total body elimination. Moreover, the effects of physiological conditions such as pregnancy, lactation, malnutrition, infection, inflammation, or chronic illness on βC kinetics have yet to be explored.

C. LIMITATIONS OF ANIMAL MODELS IN THE STUDY β-CAROTENE METABOLISM

Animals used for studies of βC metabolism include the rat, mouse, hamster, guinea pig, chicken, ferret, preruminant calf, monkey, and baboon.

There are substantial and well-characterized differences between species in βC metabolism. Rodents, including the rat, mouse, and guinea pig, exhibit highly efficient intestinal conversion of βC to vitamin A with the consequence that litttle βC is absorbed intact (Thompson *et al.*, 1950; Huang and Goodman, 1965). Moreover, VLDL and HDLs are the major lipoproteins involved in the transport of lipids in most rodents, whereas in the human LDL is the predominant lipid carrier, especially of βC. Studies with ferrets reveal a very unique handling of vitamin A by this animal such that serum retinyl ester levels are greatly elevated relative to the human even in the fasting state (Ribaya-Mercado *et al.*, 1992; Wang *et al.*, 1992). It was clearly shown in the βC radiotracer studies that humans are moderate absorbers of intact βC and bioconvert βC primarily to retinyl esters without elevated serum levels of retinyl esters (Goodman *et al.*, 1966; Blomstrand and Werner, 1967). In addition, Gugger *et al.* (1992) reported the complete elimination of βC from ferret plasma within 76 hr of a single oral dose of 10 mg βC/kg body wt. This result is at variance with many human studies which show elevated serum βC for several weeks following a single oral dose (Novotny *et al.*, 1995; van Vliet *et al.*, 1995). Rabbits are also "white fat" species, in that carotenoid deposition in adipose tissue is well below that observed in humans and insufficient to result in the typical yellow hue of human fat. At the present time an animal model which parallels the human with respect to known aspects of βC absorption, metabolism, transport, and tissue incorporation has not been developed. Consequently, extrapolation of data derived from animal models to humans is fraught with uncertainity and limitations.

Animal models do offer the advantage of the capability to obtain data on solid tissue kinetics of βC assimilation and elimination. For obvious reasons this cannot be done in humans, in which under most circumstances only plasma, blood cells, and adipose tissue can be repeatedly sampled from the same subject.

D. USE OF NONTRACER VERSUS TRACER METHODS FOR MODELING PURPOSES IN HUMANS

1. Nontracer Methods

Studies involving unlabeled βC depend on detection and measurement of concentration changes of βC in plasma or plasma fractions such as chylomicrons. While chylomicron response may be useful for the study of issues related to βC absorption, this fraction is not useful for examination of postabsorptive events. A carefully designed study by van Vliet *et al.* (1995) compared the utility of a chylomicron (TGR) fraction with that of

plasma to follow the absorption of a single oral dose of βC. The TGR fraction exhibited a well-defined peak in βC concentration at 5 hr postdose, coinciding with the absorption of triglyceride, while plasma βC concentration did not change significantly over this period. The dose was 15 mg βC, of which 11% was estimated to be absorbed, using several assumptions. Thus, the effective (absorbed) dose in this model may have been about 1.6 mg. The insensitivity of plasma βC to single doses of βC was further illustrated by the results of Johnson and Russell (1992). These authors reported that 7 of 11 subjects showed no increase in plasma βC concentration following a single oral dose of 120 mg βC. In contrast to the findings of van Vliet *et al.* (1995), who reported chylomicron βC responses in all subjects studied, these 7 subjects exhibited no significant βC enrichment in the chylomicron fraction, suggesting impairment of absorption or efficient conversion to retinyl esters in these subjects.

While effective doses of 1 to 2 mg seem small, the total body pool of βC can be estimated at 15 to 20 mg, with a total plasma pool of about 0.5 to 1 mg. In comparison with these pool sizes, a 2-mg effective dose is relatively large. In addition, the average daily effective dose of βC from food sources is likely to be less than 1 mg, since matrix effects probably impair bioavailability of βC to a larger extent than with supplements such as that used by van Vliet *et al.* (1995). The minimum effective dose required to yield a significant TGR fraction response, taking into account the error associated with measurement, has not been determined, but is not likely to be much less than 1 mg. Consequently, use of unlabeled βC, even coupled with use of the TGR fraction, is probably not well suited to study βC uptake and metabolism at effective doses typically derived from dietary sources.

2. *Tracer Methods*

A tracer method by definition is one which utilizes a trace or minute amount of labeled material, a sensitive and precise detection device, an administration protocol which ensures physiological and metabolic processing similar to that of the tracee, and does not perturb the mass or underlying kinetics of the tracee (Green and Green, 1990; Wolfe, 1992). An ideal tracer is chemically and physically identical to the tracee, but distinguishable from the tracee via substitution of one or more atoms within its structure. The location of the substituted atom(s) should minimize atom exchange and physiological discrimination of the tracer from the tracee.

βC tracers are potentially useful in the study of many aspects of βC metabolism including absorption, biotransformation to retinoids, transport, storage, and elimination. There are four methods of labeling βC,

two radioactive forms (^{3}H and ^{14}C) and two stable isotope forms (^{2}H and ^{13}C). Goodman *et al.* (1966) employed ^{3}H-βC and Blomstrand and Werner (1967) utilized both ^{3}H- and ^{14}C-βC to follow the steady-state kinetics of βC over a 22-hr time period. These two radiotracer studies utilized approximately 0.05 mg of ^{3}H-βC and 0.4 to 1.3 mg of ^{14}C-βC (Goodman *et al.*, 1966; Blomstrand and Werner, 1967). The effective dose, assuming 30% of dose was absorbed, for ^{3}H-βC is about 15 μg and about 390 μg for ^{14}C-βC. Both values are upper end estimates and are well below the total body βC pool size (16 to 20 mg), average plasma pool (0.5 mg), and adipose storage site (11 mg). The use of radioactive isotopes also involves health risk to the subjects and possible isotopic effects with ^{3}H have been reported (Bell *et al.*, 1986; Argound *et al.*, 1987). Stable isotopes, on the other hand, have no known physiological or health risks when given in small doses (amounts that do not perturb mass of tracee) and have been shown not to induce isotopic effects on chemical reaction. Under acidic conditions, deuterium is known to exhibit very large dissociation constants, which must be taken into consideration when using this stable isotope.

Two approaches using stable tracers to study βC metabolism in humans have been reported (Parker *et al.*, 1993; Dueker *et al.*, 1994). At Cornell, we have developed a ^{13}C-based stable tracer approach utilizing biosynthetic βC completely substituted with ^{13}C (per-labeled βC) and isotope ratio mass spectrometry. This approach is described below. Dueker *et al.* (1994) have used synthetic d_8-βC and conventional tandem mass spectrometry, and a compartmental model based on data derived from a single subject has been proposed (Novotny *et al.*, 1995). These data were obtained following a single dose of 40 mg βC, of which the model predicted 22% (about 9 mg) was absorbed. While this effective dose may represent up to one-half the estimated total body pool of βC, Novotny *et al.* (1995) argued that steady state kinetics prevailed throughout the period of observation.

A stable tracer method which couples the use of βC highly enriched in ^{13}C with high-precision isotope ratio mass spectrometry offers several advantages over approaches requiring conventional mass spectrometry. The most salient feature is the ability to use low doses representative of true tracer conditions (e.g., 0.015 to 1.0 mg in the case of [^{13}C]βC). Because small changes in carbon isotope ratio can be detected with high precision of measurement, long-term plasma kinetics (including terminal half-life estimates) can be obtained even for analytes with slow rates of elimination (e.g., βC). These features are clearly advantageous for the collection of data for mathematical modeling.

II. METHODS

A. DOSE PREPARATION AND ADMINISTRATION

Unicellular green algae was grown in a closed growth chamber with $^{13}CO_2$ as the sole carbon source by Martek, Inc. (Columbia, MD). This resulted in the biosynthesis of per-labeled (>98% ^{13}C) [^{13}C]β-carotene, as determined by electron ionization mass spectrometry and by isotope ratio mass spectrometry of the corresponding perhydro-β-carotene analog following serial dilution with unlabeled βC. All-*trans*-[^{13}C]βC was purified from a crude hexane extract of algal lipids by repeated crystallization from petroleum ether. The purified [^{13}C]βC used in subsequent experiments consisted of 95 to 97% all-*trans*-βC with α-carotene making up most of the remaining 5 to 3 %. One milligram of [^{13}C]βC was completely dissolved in 1 g of high oleic acid safflower oil (HOASO; Stepan Co., Maywood, NJ) and emulsified into 70 ml of non-vitamin-fortified skim milk, 30 g mashed banana, and an additional 19 g of HOASO using a hand held homogenizer. The banana was added for emulsion stability and taste.

Male subjects ranging in age from 27 to 41 years were placed on a low-carotenoid diet 48 hr prior to the [^{13}C]βC dose to allow for the clearance of gastric and intestinal carotenoids. On the morning of the dose, subjects were fitted with an indwelling catheter with a three-way stopcock in a forearm vein. A baseline blood sample was taken, followed by the consumption of the [^{13}C]βC-containing banana milk drink. A standardized light breakfast (one-half bagel) was provided with an additional 100 ml of non-vitamin-fortified skim milk in order to rinse both the glass which contained the dose and the subject's mouth. The low-carotenoid diet was continued 24 hr post dose. A standard lunch and evening meal were consumed 3 and 9 hr post dose, respectively. Subsequent blood samples were taken at the times shown in Figs. 2–5.

B. PLASMA COLLECTION, STORAGE, AND EXTRACTION

Blood collected at each time point was allowed to remain on ice for 15 min prior to centrifugation at 1800*g*. Plasma was transferred to 5-ml cryogenic vials and stored at −80°C until analyzed. Plasma lipids were extracted from duplicate 2.2-g plasma aliquots for isotope ratio analysis or from 0.25-g aliquots for HPLC quantification of retinol and βC after addition of internal standard (retinyl acetate). Plasma aliquots were deproteinized with 1 vol of ethanol and lipids extracted with 3 vol of hexane (Optima Grade, Fisher Scientific, Rochester, NY).

C. PLASMA ANALYTE QUANTIFICATION

The method of Thurnham *et al.* (1988) was modified for the quantification of plasma βC and retinol. Plasma extracts were dissolved in 40 μl of dimethylforamide and vortexed and then 210 μl of acetonitrile/methanol/chloroform (47/47/6, v/v/v) was added. Reconstituted samples were vortexed and sonicated for 40 sec prior to being transferred to autosampler vials and sealed under nitrogen. The HPLC system consisted of a photodiode array detector (Waters 996, Millipore Corp., Milford, MA) with Millennium software, a Waters 717 plus autosampler, and a Hewlett–Packard Model 1050 pump. Analytes of interest were separated using acetonitrile/methanol/chloroform (47/47/6, v/v/v), with 0.05 *M* of ammonium acetate and 1% triethylamine at a flow rate of 1.2 ml/min and a 4.6 × 15-cm Spherisorb ODS-2 column (LKB Instruments Ltd., Surrey, UK) maintained at 26°C using a column heater (Timberline Instruments Ltd., Boulder, CO). This analysis does not discriminate between ^{13}C-enriched and nonenriched analytes, but rather measures the total concentration of each isotopomer. The retention times of retinol, retinyl acetate (internal standard), and β-carotene were 2.1, 2.6, and 16.9 min, respectively. Plasma concentrations of retinol and βC were calculated using a standard curve for each analyte and an internal standard to correct for volume recovery.

D. SAMPLE PREPARATION FOR GAS CHROMATOGRAPHY–COMBUSTION–ISOTOPE RATIO MASS SPECTROMETRY (GC-C-IRMS)

Unesterified retinol was separated from retinyl esters and βC using reverse-phase semipreparative HPLC (Vydac TP201 column, 10 mm × 25 cm, Separations Group, Hesperia, CA) using methanol/dichloromethane (76/24, v/v) as the mobile phase at a flow rate of 1.2 ml/min. Eluant was monitored at 325 nm and two fractions were collected: (1) retinol, retention time of 9.7 min, and (2) retinyl esters plus carotenes, collection interval was 16.5 to 18 min. The retinyl ester–carotene fraction was saponified with absolute ethanol and saturated aqueous potassium hydroxide at 45°C for 25 min and the resulting retinol and βC were extracted with hexane. Retinyl ester–retinol and βC were separated by liquid–liquid partitioning using hexane and dimethylformamide (DMF) (2/5, v/v). The βC partitioned into hexane (>95%) and the retinol into DMF (>95%). Further purification of all-*trans*-βC from other isomers was performed using analytical reverse-phase HPLC (Vydac TP201, 4.6 mm × 15 cm, Separations Group) and methanol/dichloromethane (95/5, v/v) at 0.9 ml/min. The purified all-*trans*-

βC fraction was dissolved in DCM and hydrogenated to its thermally stable perhydro-βC analog using platinum oxide (Aldrich Chemical Co., Milwaukee, WI) under hydrogen gas overnight at 65°C. The hydrogenated βC samples were filtered to remove the platinum oxide, redissolved in hexane, and subjected to isotope ratio analysis as described below.

E. GC-C-IRMS ANALYSIS

Methodologic issues concerning use of GC-C-IRMS have been discussed by Goodman and Brenna (1992). The carbon isotopic composition of perhydro-βC was determined using a 5880A Hewlett–Packard GC interfaced to a Finnigan MAT 252 high-precision GIRMS via a ceramic combustion furnace maintained at 850°C. Perhydro-βC was injected onto a DB-1 capillary column (0.32 μm i.d. × 15 m, J&W Scientific, Folsom, CA) using cool on-column injection. The linear velocity of the carrier gas (helium) was 20 cm/sec and the GC programmed from 60 to 265°C at 25°C/min, from 265 to 300°C at 10°C/min, and held at 300°C for 4 min and then increased to 325°C at 30°C/min and held for 10 min to ensure elution of any remaining compounds. Perhydro-βC eluted at about 13 min (approximately 303°C). The column eluant was continuously and quantitatively combusted to CO_2, which was swept into the ionization chamber. In this particular application the masses 44 ($^{12}CO_2$) and 45 ($^{13}CO_2$) are of most interest and were continuously monitored. The mass 46, used to calibrate for ^{17}O contamination in the mass 45 channel, has negligible effect on $^{13}C/^{12}C$ carbon isotope ratios for the experimental data that is presented below; however, it was also continuously monitored. The computer-generated delta units ($\delta^{13}C$), reflecting the $^{13}C/^{12}C$ isotope ratio of the sample relative to an international carbonate standard (PeeDee Belemnite, PDB), were converted to atom percentage ^{13}C using the

$$\text{Atom \% } ^{13}C = \frac{(100 \times R45) \times (\delta^{13}C/1000 + 1)}{1 + (R45) \times (\delta^{13}C/1000 + 1)} \quad (1)$$

R45 represents the ratio of the signal intensities in the *m/z* 45 channel to the *m/z* 44 channel for the PDB CO_2 standard and is defined as 0.0112372. The atom percentage excess (APE) ^{13}C in the perhydro-βC peak was obtained by subtracting the baseline atom percentage ^{13}C from that of all subsequent time points. Thus, APE represents the proportion of plasma βC which is labeled at any point in time and takes into account natural abundance^{13}C or ^{13}C enrichment persisting from previous doses of [^{13}C]βC in a given subject.

III. RESULTS AND DISCUSSION

A. EXAMPLES OF [^{13}C]βC-GC-C-IRMS DATA

The following figures include selected data to illustrate the types of kinetic curves that can be generated using this approach and which may subsequently be subjected to empirical or compartmental modeling.

The qualitative changes in ^{13}C enrichment in plasma βC (APE) over the initial 50 hr and over the entire course of data collection, after dosing with 1 mg ^{13}C-labeled βC, are shown in Figs. 1 and 2. The reproducibility of APE measurement and the purification procedure is illustrated by the fact that error bars, representing isotope ratio measurement of two independent plasma samples, are usually within the figure symbols. Two general features common to all ^{13}C enrichment versus time curves are the peak in enrichment at 5 hr, and a second, broader peak between 24 and 48 hr. These two peaks of ^{13}C appearance and elimination represent the movement of labeled βC into and out of the lipoproteins that are known to be involved in the transport of βC during absorption and distribution. Earlier studies using large doses of unlabeled βC and lipoprotein separation by ultracentrifuga-

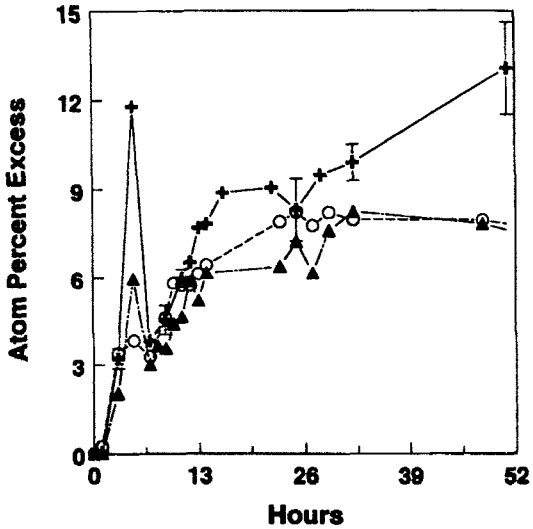

FIG. 1. A comparison of the short-term plasma kinetic patterns of atom % excess (APE) ^{13}C in plasma βC fractions versus time, from 0 to 51 hr, in three subjects following an oral dose of 1 mg [^{13}C]βC. Each symbol represents the following: subject 1 (+), subject 2 (▲), subject 3 (o). Each point represents the mean ± SD of two independent determinations.

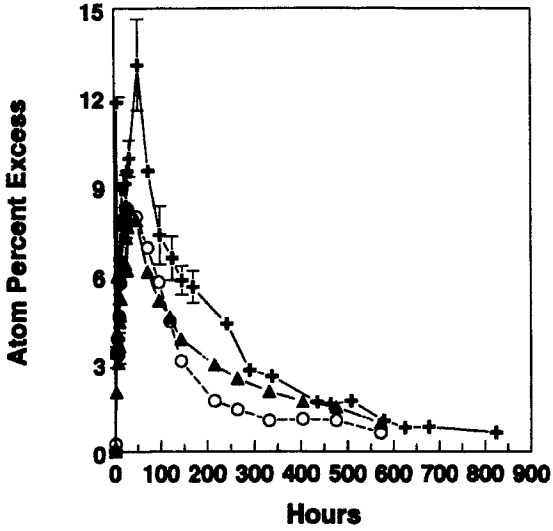

FIG. 2. A comparison of the long-term plasma kinetic patterns of atom % excess (APE) ^{13}C in plasma βC fractions versus time in three subjects following an oral dose of 1 mg [^{13}C]βC. Each symbol represents the following: subject 1 (+), subject 2 (▲), subject 3 (o). Each point represent the mean ± SD of two independent determinations.

tion indicate that the 5-hr peak represents the secretion and clearance of chylomicron-associated [^{13}C]βC (Krinsky *et al.*, 1958; Cornwell *et al.*, 1962; Johnson and Russell, 1992; van Vliet *et al.*, 1995). β-Carotene appears to be absorbed exclusively via this route. This 5-hr peak also coincides exactly with the peak in [^{13}C]retinyl esters (data not shown) or with unlabeled retinyl ester in the case of dosing with unlabeled βC (van Vliet *et al.*, 1995). Retinyl esters are a commonly used marker for chylomicrons.

The second broad peak between 24 and 48 hr represents labeled βC secreted by the liver associated with very low density lipoproteins (VLDL) and very likely encompasses the period during which these VLDL particles undergo lipolysis to IDL and LDL. As this lipolytic process occurs relatively rapidly, the broad and extented nature of this second peak probably reflects recycling of labeled βC into and out of the liver, i.e., hepatic reprocessing of VLDL or LDL particles. This phenomenon may be investigated further using modeling approaches and is reflected in the compartmental model recently proposed by Novotny *et al.* (1995).

The "absorption" peak (5 hr) and "distribution" peak (24–48 hr) are incompletely separated, indicating that hepatic secretion occurs shortly after hepatic uptake of chylomicron remnants containing labeled βC. This

overlap of absorption and distribution processes has also been evident in studies using high doses of unlabeled βC. In subjects absorbing only small amounts of βC (labeled or unlabeled), the 5-hr peak may represent only a shoulder on the leading side of the distribution peak. In such instances, use of absorption phase AUC or similar approaches to estimate absorption efficiency would be of limited use. This further underscores the need for development of mathematical models that would be more independent of observation of a distinct absorption peak.

B. UTILITY AND POTENTIAL ADVANTAGES OF THE [^{13}C]βC TRACER APPROACH

In general, relatively large single oral doses are needed to produce measurable increases in plasma βC concentration, during either absorption or distribution phases (Dimitrov *et al.*, 1987). A single dose of 1 mg would not be expected to result in such peaks. In fact, no such peaks in plasma total βC corresponding with absorption or distribution were observed in these studies, as illustrated by the plots shown in Fig. 3. The lack of an absorption peak at doses of 1 mg or below probably reflects both the

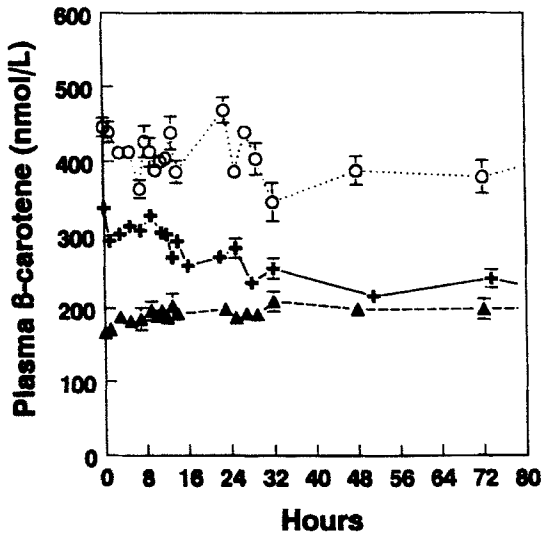

FIG. 3. Total plasma β-carotene (labeled plus unlabeled βC) concentration, from 0 to 75 hr, in three subjects following an oral dose of 1 mg [^{13}C]β-carotene. Each symbol represents the following: subject 1 (+), subject 2 (▲), subject 3 (o). Each point represents the mean ± SD of two independent determinations.

duration of chylomicron secretion of lymph into plasma (2 to 3 hr) and the rapid rate of removal of chylomicrons from plasma by the liver. The half-life of chylomicrons in the human has been estimated to be roughly 11 min (Berr *et al.,* 1985; Cortner *et al.,* 1987; Redgrave *et al.,* 1993). The plots in Fig. 3 also indicate that the changes in ^{13}C enrichment in plasma lipoproteins seen with 1-mg doses occur under steady-state conditions. That is, labeled βC is replacing unlabeled βC in the absence of a measurable change in total βC concentration. Such conditions are desirable from a modeling perspective, as discussed further below. Conventional mass spectrometry generally requires use of larger βC doses, as reported by Novotny *et al.,* 1995, in order to observe details of absorption kinetics.

^{13}C-Labeled βC is clearly evident in plasma by 3 hr after a dose of 1 mg [^{13}C]βC, illustrating the enhanced sensitivity of GC-C-IRMS to detect and measure βC early in the process of absorption. In contrast, no measurable increase in concentration of labeled βC was observed prior to 5 hr post dosing with 40 mg of d_8-βC (Novotny *et al.,* 1995).

The approach described here is clearly suitable for obtaining data on the long-term kinetics of plasma βC elimination in humans as well. Figures 2 (linear plot) and 4 (log plot) illustrate data obtained over 800 hr following a dose of 1 mg [^{13}C] βC. Even at such prolonged times, changes in carbon isotope ratio in the plasma βC pool can be observed when measuring at 100-hr intervals. Such capability is needed in order estimate the terminal half-life of plasma components with slow turnover rates, such as βC or other lipids transported in the core of LDL particles. In general, an observation period spanning at least five half-lives is desirable for such estimations. In the examples shown here, βC elimination appears roughly biphasic over the period from 48 hr through the end of data collection, with a relatively rapid phase (through approximately 150 hr) followed by a slower phase. More substantive analyses will be required to yield a more detailed description of these kinetics, and such efforts are underway. Published reports of the rate of disappearance of plasma βC following its removal from the diet also suggest a biphasic elimination curve (e.g., Rock *et al.,* 1992), but no efforts have been made to model such data, which suffer from the drawback of changing pool size.

In our model, subjects are allowed to resume self-selected diets after 36 hr postdose. This may result in changes in plasma βC concentrations as a function of occasional large fluctuations in dietary intake of several milligrams. Since APE represents the proportion of plasma βC molecules that are labeled with ^{13}C, addition of unlabeled βC to the plasma pool from dietary sources will decrease APE even though the concentration of labeled βC remains unaffected. In such instances, the data can be expressed as the plasma concentration of labeled βC (e.g., nmol/liter) by multiplying APE

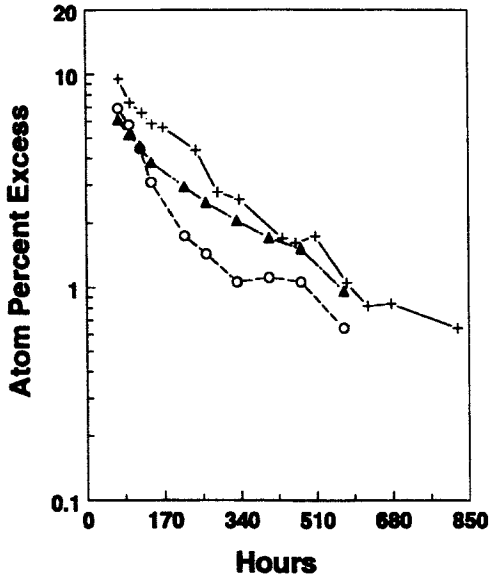

FIG. 4. Presentation of the terminal elimination kinetics of atom % excess (APE) ^{13}C in plasma βC fractions versus time in three subjects following an oral dose of 1 mg [^{13}C]βC as semi-log plots. Each symbol represents the following: subject 1 (+), subject 2 (▲), subject 3 (o). Each point represents the mean ± SD of two independent determinations.

by the plasma total βC concentration (labeled plus unlabeled, as determined by HPLC) and correcting for the extent of ^{13}C labeling in the βC dose. A comparison of [^{13}C]βC data expressed as APE with that expressed as plasma concentration (nmol [^{13}C]βC/liter) is shown in Fig. 5.

The stable tracer approach described above offers several advantages over other tracer techniques employed for the study of βC metabolism in humans. First, the health risks involved with the use of radioactive tracer methods are completely avoided. The radiotracer studies conducted in the mid 1960s (Goodman *et al,* 1966; Blomstrand and Werner, 1967) both involved hospitalized patients, many with serious illnesses such as cancer. In such cases, the added risk associated with small doses of tritium or ^{14}C are small in comparison with existing deleterious conditions. However, in otherwise healthy persons, such an argument cannot be made. It is often assumed that the sensitivity associated with radiotracer techniques far exceeds that of stable tracer approaches. While in some cases, depending on the specific instrumental analyses employed, that may be true. In this case, however, the doses of βC needed to obtain high-quality data are of the same order as those of radiotracer methods. The dose used to generate the

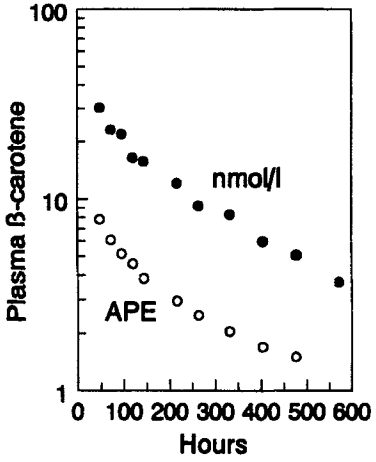

FIG. 5. A comparison of the terminal elimination kinetics of plasma βC expressed as atom % excess (APE; open circles) or as nmoles per liter (filled circles) following a single oral dose of 1 mg [^{13}C]βC. Symbols represents the mean values for subject 3.

data shown here (1 mg) is of the same order (or less) than those of the ^{14}C studies of Blomstrand and Werner (1967). In fact, doses of ^{13}C-βC considerably less than 1 mg may be employed with the GC-C-IRMS method used here, depending of the extent of ^{13}C enrichment of the dose. This is due both to the high precision of measurement of carbon isotope ratio offered by high-precision isotope ratio mass spectrometers and to the relatively low total body pool of βC estimated to be on the order of 15–25 mg in most individuals (Parker, 1988). The detection limits and precision of GC-C-IRMS are orders of magnitude improved compared to conventional quadrupole mass spectrometry (Brenna, 1994). Fractions of tracer/tracee below 0.002 generally cannot be accurately measured by conventional MS, while ratios of 0.00001 can be reliably quantified by high-precision IRMS (Goodman and Brenna, 1992; Brenna, 1994). Consequently, approaches involving conventional MS generally require higher doses of labeled βC. For example, the δ_8-βC method of Dueker *et al.* (1994), using tandem MS/MS, employed a dose of 40 mg labeled βC, of which 9 mg was estimated to have been absorbed (Novotny *et al.*, 1995). The precision (and consequently sensitivity) of the GC-C-IRMS approach is manifested in the ability to measure small changes in isotope ratio in βC over long periods of time, as discussed above.

In addition to the advantages of safety, sensitivity, and precision of measurement, the current approach is unlikely to be complicated by chemical exchange or isotope effects on the metabolism or disposition of βC in

biological systems. Deuterium is susceptible to exchange with hydrogen, depending on the acidity of the hydrogen atom involved, although the likelihood of such exchange occurring with βC is small. On the other hand, octadeuterated βC can be baseline-resolved from unlabeled βC by reverse-phase HPLC (Deuker *et al.*, 1994), suggesting that interaction with lipophilic environments (e.g., cell membranes or hydrophobic regions of enzymes) may be affected by deuteration. In contrast, we have been unable to resolve per-^{13}C-labeled βC from natural abundance βC by HPLC, suggesting that the risk of isotope effects *in vivo* are very small.

C. ADDITIONAL CONSIDERATIONS OF THE [^{13}C]βC APPROACH

The GC-C-IRMS approach employed here cannot be used without caution. The isotope ratio of eluting GC peaks can be substantially reduced by coeluting or partially resolved substances of different isotope ratio. Recently, Goodman and Brenna (1994) have published techniques using exponentially modified Gaussian and Haarhoff-VanderLinde functions to integrate overlapping fatty acid methyl ester peaks possessing different isotope ratios. Second, the relationship between delta$^{13}C_{PDB}$ and fraction ^{13}C (^{13}C as proportion of total carbon) is not linear over the entire range of ^{13}C enrichment. Consequently, the actual relationship must be determined using appropriate calibration curves to account for any nonlinearity within the range of ^{13}C enrichment encountered. Last, measurement precision is limited by analyte concentation in sample. Therefore, analytes present in low concentration in plasma may require the collection of larger blood samples. Alternatively, curve-fitting algorithms may produce satisfactory precision and accuracy for samples of low signal intensity (Goodman and Brenna, 1995).

IV. CONCLUSIONS

Stable tracer approaches to the study of βC metabolism in humans offer several advantages over the use of unlabeled or radioactive βC. β-Carotene highly enriched with ^{13}C, coupled with the use of gas chromatography–combustion–high precision isotope ratio mass spectrometry, constitutes a safe and sensitive approach which requires only very small doses of βC typical of (or less than) daily dietary intake and which do not perturb endogenous pool sizes. Subtle changes in isotope ratio can be measured over long periods of time with a low risk of isotope effects, permitting estimation of terminal half-lives of βC or its metabolites in plasma. Conse-

quently, it appears that such an approach will be useful in obtaining data needed to model the kinetics of plasma βC and its retinoid metabolites in humans, providing insights into aspects of absorption and metabolism of βC which have been difficult to address through direct experimental means.

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