

Isotopic tracer techniques for studying the bioavailability and bioefficacy of dietary carotenoids, particularly β -carotene, in humans: a review¹⁻³

Machteld van Lieshout, Clive E West, and Richard B van Breemen

ABSTRACT

Vitamin A deficiency is a serious health problem in many developing countries. Provitamin A carotenoids in fruit and vegetables are the major source of vitamin A for a large proportion of the world's population. However, the contribution of plant foods is substantial only when both the consumption and provitamin A content of such food is high and, at the same time, the bioefficacy of the provitamin A is high. With respect to provitamin A carotenoids, the term *bioefficacy* is defined as the product of the fraction of the ingested amount that is absorbed (bioavailability) and the fraction of that which is converted to retinol in the body (bioconversion). Isotopic tracer techniques can meet the need for accurate and precise estimates of the bioavailability, bioconversion, and bioefficacy of dietary carotenoids in humans. Use of such techniques will enable proper evaluation of food-based approaches to eliminating vitamin A deficiency. In addition, the putative antioxidant capacities of carotenoids can be better understood if their bioavailability is known. Here, we discuss how tracer techniques can be applied to obtain reliable and representative data. A step-by-step discussion of aspects related to these techniques is provided, including study design, choice of isotopic tracers, dosing regimen, collection of samples, chemical analysis of samples, and data analysis. *Am J Clin Nutr* 2003;77:12-28.

KEY WORDS Stable isotopes, carbon isotopes, radioisotopes, deuterium, ¹³C, ²H, bioavailability, β -carotene, carotenoids, conversion, bioefficacy, retinol, vitamin A, HPLC, extrinsic labeling, intrinsic labeling, liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry

RATIONALE FOR STUDYING BIOAVAILABILITY AND BIOEFFICACY OF DIETARY CAROTENOIDS

Provitamin A carotenoids, in particular β -carotene in fruit and vegetables, are the major source of vitamin A (retinol) for a large proportion of the world's population (1). In their 1988 guidelines on the human requirement for vitamin A, the FAO/WHO proposed that 6 μ g β -carotene in food has the same vitamin A activity as 1 μ g retinol (2). Official bodies in many countries have followed these guidelines. Recently, the Institute of Medicine (3) of the National Academy of Sciences in the United States proposed that 12 μ g β -carotene in food has the same vitamin A activity as 1 μ g retinol. Several studies in Indonesia (4) and Vietnam (5) found that as much as 21 μ g β -carotene in a mixed diet (with a ratio of vegetable to fruit of 4:1) has the same vitamin A activity as 1 μ g

retinol. More data are required on the extent of conversion of provitamin A carotenoids to retinol. However, the results of studies carried out to date suggest that alternatives to the promotion of the consumption of fruit and vegetables will be required to combat vitamin A deficiency. Data on the extent of absorption of carotenoids are also required when evaluating their putative antioxidant properties. There is a need for new techniques to quantify the absorption of carotenoids and the conversion of provitamin A carotenoids to retinol.

In this article, several terms are used that need to be defined. *Bioavailability* is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (6). *Bioconversion* is the fraction of a bioavailable nutrient (here, absorbed provitamin A carotenoids) that is converted to the active form of a nutrient (retinol). *Bioefficacy* is the fraction of an ingested nutrient (here, dietary provitamin A carotenoids) that is absorbed and converted to the active form of the nutrient (retinol) in the body (Figure 1; 7). *Functional bioefficacy* is the fraction of an ingested nutrient that performs a certain metabolic function (8), such as the ability of ingested provitamin A carotenoids to reverse or prevent abnormal dark adaptation.

Efforts to estimate or quantify the bioefficacy of dietary carotenoids in humans (Table 1) have included animal models; depletion-repletion techniques (to estimate functional bioefficacy); oral-fecal balance techniques; serum, plasma, or chylomicron responses; and the use of isotopic tracers. Much emphasis has been placed on human studies in which changes in the concentration of retinol in plasma or serum are measured in response to various dietary sources of β -carotene. Because techniques using isotopic tracers can provide the most accurate data on the bioavailability and bioefficacy of dietary carotenoids in humans, these will

¹ From the Division of Human Nutrition and Epidemiology, Wageningen University, Wageningen, Netherlands (MvL and CEW); the Department of Gastroenterology, University Medical Center, St Radboud, Nijmegen, Netherlands (CEW); and the Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago (RBvB).

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³ Reprints not available. Address correspondence to CE West, Division of Human Nutrition and Epidemiology, Wageningen University, PO Box 8129, 6700 EV Wageningen, Netherlands. E-mail: clive.west@wur.nl.

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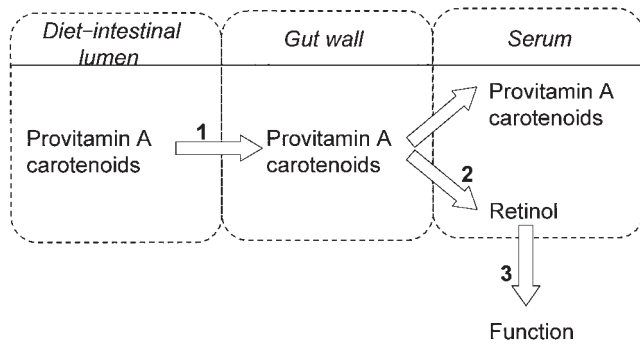


FIGURE 1. The bioavailability and bioefficacy of provitamin A carotenoids. Bioavailability = 1; bioconversion = 2, bioefficacy = 1×2 , and functional bioefficacy = 1×3 (for further explanation, *see* the text).

be discussed in detail. As outlined in Table 1, considerations in the design of such studies will be discussed.

CHOICE OF MODEL: TECHNIQUES NOT INVOLVING TRACERS

Animal models

Animal models can be very useful for studying mechanisms (9) because they allow the use of procedures that cannot be readily undertaken in humans. Such procedures include the use of radioisotopes, removal of tissues, and feeding of diets that result in deficiency. However, the results obtained from the use of animal models cannot always be extrapolated directly to humans, especially not when making quantitative estimates. With respect to carotenoid metabolism, animal models have several limitations. In their extensive reviews of the animal models used for studying carotenoid metabolism, van Vliet (10) and Lee et al (11) concluded that no animal model completely mimics human absorption and metabolism of carotenoids. For example, cats can absorb β -carotene (12) but cannot convert β -carotene, whereas rats and chickens convert >90% of absorbed β -carotene to retinol (10, 11). Ferrets, pruruminant calves, and possibly also monkeys absorb β -carotene and convert some to retinol, as do humans (10). However, ferrets are very inefficient converters of absorbed β -carotene, making them an inappropriate model for studying the bioefficacy of carotenoids (13), and the cost of housing calves and monkeys limits their use (11).

Human studies

Depletion-repletion studies and oral-fecal balance techniques

In the “Sheffield experiment” (14) conducted in the 1940s, in which male participants were conscientious objectors to military service, 16 (2 women and 14 men) subjects consumed a vitamin A-deficient diet and 7 control subjects (1 woman and 6 men) consumed the same diet but with additional supplements of retinol or β -carotene from various sources for periods ranging from 8.5 to 25 mo. Dark adaptation was used as a functional indicator of bioefficacy. In the control group, either 750 μg retinol/d ($n = 2$) or 3000 μg β -carotene/d in oil ($n = 4$) or in margarine ($n = 2$) prevented impaired dark adaptation. In the 16 subjects fed the deficient diet,

TABLE 1

Considerations in the design of studies on the bioavailability and bioefficacy of dietary carotenoids, with emphasis on isotopic tracer techniques

Consideration
Choice of model
Animals: used to study mechanisms
Humans: can provide quantitative data on
Functional bioefficacy: depletion-repletion studies
Relative bioavailability: oral-fecal balance techniques
Relative bioavailability and bioefficacy: serum, plasma, or chylomicron response after single or multiple doses of carotenoids or retinol
Absolute bioavailability and bioefficacy: isotopic tracer techniques
Study design
Defining the aim and hypothesis
Defining the study population and estimating the sample size
Choice of isotopic tracer
Isotopes
Radioactive: ^3H , ^{14}C
Stable: ^2H , ^{13}C
Method and degree of labeling
Intrinsic and uniform
Extrinsic: uniform or specific
Multiple labeling for measuring bioefficacy
Matrix of tracer
Choice of dosing regimen
Route of administration
Frequency of dosing: single or multiple
Size of dose: pharmacologic (>10 mg), physiologic (<10 mg), or low (<1 mg)
Choices related to the collection of samples
Specimen: lymph, liver, other tissues, urine, feces, serum or plasma, and chylomicrons
Number and timing: one or multiple samples collected over a short or long term
Choices related to the chemical analysis of samples
Detection methods for radioisotopes
Liquid-scintillation counter
Accelerator mass spectrometry (AMS)
Detection methods for stable isotopes
Nuclear magnetic resonance (NMR)
HPLC
Mass spectrometry (MS)
Gas chromatography (GC) coupled with MS
GC-combustion isotope ratio MS
HPLC coupled with particle beam, atmospheric pressure chemical ionization, or electron ionization liquid chromatography-MS
Method validation and quality control
Method of data analysis
Mathematical modeling: defining the required assumptions
Statistical analysis of data: choice of appropriate methods

only 3 men, who also had the lowest plasma retinol concentrations, developed impaired dark adaptation (depletion phase of the study). Either 390 μg retinol/d ($n = 1$) or 1500 μg β -carotene/d in oil ($n = 2$) was required to reverse this impaired dark adaptation (repletion phase). Other depletion-repletion studies conducted by Booher et al (15; $n = 5$) and by Wagner (16; $n = 10$) shortly before World War II reached similar conclusions, whereas in the 1970s Sauberlich et al (17; $n = 8$) concluded that, on a weight basis, only twice as much β -carotene as retinol was required to reverse impaired dark adaptation. Depletion-repletion techniques require very long

study periods (up to 2 y), however, and medical ethics committees may be reluctant to approve such studies. In addition, depletion-repletion studies yield only crude estimates of bioefficacy because only stepwise increased doses of retinol and β -carotene can be tested and compared.

In oral-fecal balance techniques, the difference between the amount of β -carotene in food consumed and that excreted in feces is assumed to represent the amount of β -carotene absorbed (18). With these techniques, gastric or bacterial degradation of unabsorbed carotenoids may contribute to overestimation of absorption. Rao and Rao (19) found indications that such bacterial degradation occurs. On the other hand, endogenously secreted carotenoids might be excreted in feces, thus leading to an underestimate of bioavailability. Thus, data on the bioavailability of carotenoids obtained with balance techniques should be interpreted with care. Early studies, carried out before carotenoids could be analyzed by HPLC, were reviewed by Hume and Krebs (14) and by Rodriguez and Irwin (20).

Serum, plasma, or chylomicron responses

Since the 1980s, various studies, including many with limitations such as small sample sizes or the absence of a control group, have been carried out in which changes in the concentration of carotenoids and retinol in serum or plasma were measured after feeding foods or pharmanutrients (dietary supplements) containing carotenoids, retinol, or both once or more often over a fixed period (1, 21, 22). Inclusion of a group fed a diet containing synthetic β -carotene enables quantification of relative bioavailability, whereas inclusion of a group fed a diet containing retinol enables quantification of relative bioefficacy. After the consumption of foods containing β -carotene, β -carotene is released by mechanical and chemical disruption of the food matrix and is solubilized with bile salts. β -Carotene becomes incorporated in micellar particles, which cross the unstirred water layer. β -Carotene is then absorbed into the enterocytes and together with its metabolites retinol or retinyl esters incorporated into chylomicrons. Chylomicrons are transported via the lymph to the bloodstream, from which they deliver β -carotene or its metabolites to other tissues and the liver (10, 23). Therefore, a modification of the serum response technique has been to measure the response in chylomicrons, which reflects newly absorbed β -carotene and newly formed retinol (24, 25). In general, these techniques have low precision, requiring large numbers of subjects to yield reliable quantitative data.

ISOTOPIC TRACER STUDIES CONDUCTED TO DATE

The need for techniques using isotopic tracers to study the bioavailability and bioefficacy of dietary carotenoids in humans was stressed by a Task Force of the International Vitamin A Consultative Group in 1999 (1), because such techniques may provide the most reliable estimates. To date, several studies have been conducted using both radioisotope and stable-isotope techniques, and their design and results are briefly discussed below.

Radioisotope studies

In the 1960s, Goodman et al (26) and Blomstrand et al (27) measured the concentrations in lymph of single, orally administered doses of 0.4 mg or 1.3 mg [^{14}C] β -carotene in oil ($n = 2$) or 47 μg [^3H] β -carotene in oil ($n = 2$). Recoveries of β -carotene of 52%, 15%, 9%, and 17% were calculated, respectively. The value of 52% was regarded as an exception, possibly resulting from a

metabolic disorder because 90% of the radioactivity recovered in the lymph was in the β -carotene fraction. In the other 3 patients, 69–88% of the radioactivity recovered in the lymph was in the retinyl ester fraction. Three decades later, Dueker et al (28) determined the recovery of a single oral dose of 306 μg [^{14}C] β -carotene in the β -carotene and retinol fractions in the plasma, feces, and urine of one subject. In this study, 57% of the dose was recovered in the stool within 48 h postdosing. From the measurements made, it was also concluded that 3.5 μg β -carotene in oil has the same vitamin A activity as 1 μg retinol. As explained earlier, balance studies might overestimate or underestimate bioavailability, and thus bioefficacy.

Stable-isotope studies

The potential risk and increased reluctance to use radioisotopes has stimulated the use of stable isotopes. Thus, in recent years, stable isotopes and compounds labeled with stable isotopes have become increasingly available. In the 1930s, Rudolf Schönheimer and David Rittenberg conducted the first studies of the metabolism of macronutrients with the use of stable isotopes (29). Later, stable-isotope-tracer techniques were successfully developed for studying the metabolism of minerals, such as iron, magnesium, and zinc (30, 31), and of vitamins and their precursors, such as folate (32) and provitamin A carotenoids (33, 34). Because of the limited availability of organic nutrients labeled with stable isotopes and the difficulty in their quantification, few studies have been carried out so far.

Studies designed to estimate body stores of vitamin A

In healthy individuals, the liver contains ≈ 80 –90% of the total body stores of vitamin A, mostly in the form of retinyl esters. However, performing liver biopsies of humans to assess the body stores is justifiable only under certain instances, and therefore indirect techniques to estimate liver reserves of vitamin A have been developed (35, 36). These techniques include the relative-dose-response and modified-relative-dose-response methods, which provide an indication of when vitamin A liver stores are depleted (35). During the past few decades, isotope-dilution techniques have been developed to estimate body stores of vitamin A in humans (36–41).

All isotope-dilution techniques measure the degree of isotopic enrichment of retinol after a challenge dose of labeled retinyl ester. It is assumed that measurements are performed after the isotope has equilibrated with the body's vitamin A pool. Because intake of dietary β -carotene or vitamin A will continue, measurements are more likely to be made after the isotope is in a steady state with the body's vitamin A pool. A mathematical formula (37) based on estimates of the absorption and storage of retinol derived from isotope-dilution studies in rats (42) is used to interpret the data. Because most detection methods are relatively insensitive (see the section on detection methods), the first deuterated-retinol-dilution tests required large doses of labeled retinol [1.35 $\mu\text{mol/kg}$ body wt (37), 0.70 $\mu\text{mol/kg}$ (38), 0.75 $\mu\text{mol/kg}$ (39), 0.75 $\mu\text{mol/kg}$, and 0.85 $\mu\text{mol/kg}$ (40)]. However, more sensitive detection methods have enabled slightly lower doses of retinol to be used [0.61 $\mu\text{mol/kg}$ body wt (36) and 0.45 $\mu\text{mol/kg}$ (41)]. Because these techniques are developed primarily to assess body stores of retinol in retinol-depleted subjects, large doses might bias the estimates obtained. These techniques have been validated against concentrations of retinol in biopsies of liver, which is the primary storage site of retinol (37, 39).



Another technique involving the administration of 52 nmol [$^{13}\text{C}_4$]retinol to weaning rats with an average weight of 60 g at onset has been reported (43). Although the author suggested that this method is more sensitive than others, the dose administered to rats corresponds with 0.87 $\mu\text{mol/kg}$ body wt, similar to the dose used with the other methods. In fact, if related to body surface area, the dose would be relatively greater in humans than that used to date.

Studies designed to obtain qualitative information on the bioavailability and bioefficacy of carotenoids

To our knowledge, 12 studies have been published since 1990 that used compounds labeled with stable isotopes to study carotenoid bioavailability or bioefficacy in humans. Some of these investigations were reviewed previously (44, 45). Five studies provide only qualitative information, whereas 7 provide quantitative data (Table 2). In the first of the qualitative studies, which involved one man, Parker et al (46) administered a single physiologic dose of 1 mg of uniformly labeled [^{13}C]β-carotene that had been biosynthesized in green algae grown with ^{13}C as the sole carbon source. The only conclusion that can be drawn from this experiment is that β-carotene is absorbed and partially converted to retinol in the body. Yao et al (47) administered a single physiologic dose of 3 mg of uniformly labeled biosynthesized [^{13}C]lutein to each of 4 women and could measure its appearance and disappearance in plasma. You et al (48) administered a single physiologic dose of 992–994 μg uniformly labeled [^{13}C]9-*cis*-β-carotene and 6–8 μg [^{13}C]all-*trans*-β-carotene (biosynthesized by green algae) to each of 3 subjects. At least 8 mo later, each subject also consumed a single dose of 992–994 μg unlabeled 9-*cis*-β-carotene and 6–8 μg [^{13}C]all-*trans*-β-carotene. From this experiment it could be concluded that *cis*-β-carotene in part is isomerized to all-*trans*-β-carotene before or during its absorption. Burri and Park (49) fed a pharmacologic dose of 40 mg [$^2\text{H}_8$]β-carotene to each of 8 women. Although the data obtained from this study were analyzed with the use of a mathematical compartmental model designed to quantify the bioavailability of β-carotene, the only conclusion drawn by the authors was that β-carotene is converted to retinol in the body with high interindividual variation. Finally, Pawlosky et al (50) fed one woman a single physiologic dose of 5 mg [$^2\text{H}_8$]β-carotene and concluded that the appearance and disappearance in plasma of labeled β-carotene could be measured.

Studies designed to quantify the bioavailability and bioefficacy of carotenoids

Novotny et al (51) administered a single pharmacologic dose of 40 mg [$^2\text{H}_8$]β-carotene to one man. Although they estimated the bioavailability at 22%, assumptions underlying the compartmental model used limited its validity (see the section on method of data analysis). With such a study design, it is not possible to assess the bioefficacy of β-carotene.

To date, 6 studies have been conducted in which both bioavailability and bioefficacy were estimated. In an investigation involving 2 adults, Tang et al (52) administered a single dose of 3 mg [$^2\text{H}_8$]retinyl acetate and 200 g spinach grown on 30% $^2\text{H}_2\text{O}$. The spinach contained 13.8 mg uniformly labeled [^2H]β-carotene, 2.7 mg uniformly deuterated other provitamin A carotenoids, and an unstated amount of uniformly labeled carotenoids without vitamin A activity. It was concluded that the vitamin A activity of 27–72 μg β-carotene in spinach was equivalent to that of 1 μg

retinol. In another study (53), the same research group administered to one woman single doses of 126 mg (pharmacologic dose) and 6 mg (physiologic dose) [$^2\text{H}_8$]β-carotene and 9 mg [$^2\text{H}_8$]retinyl acetate on separate occasions up to 2.5 y apart. From this study, they concluded that an amount of 3.8 or 55 μg β-carotene in oil has the same vitamin A activity as 1 μg retinol after doses of 6 or 126 mg, respectively. The degree of isotopic enrichment of β-carotene in serum was not measured; thus, the bioavailability of β-carotene could not be assessed. From this study, it can be concluded that high pharmacologic doses of β-carotene are not absorbed to the same extent as are physiologic doses. Thus, estimates derived from such high doses cannot be readily extrapolated to the bioefficacy of dietary carotenoids.

Lin et al (54) administered single pharmacologic doses of 10 mg [$^2\text{H}_6$]retinyl acetate and of 20 mg [$^2\text{H}_6$]β-carotene to 11 women 1 wk apart. From the areas under the curve of the concentrations in plasma of [$^2\text{H}_3$]retinol, [$^2\text{H}_6$]retinol, and [$^2\text{H}_6$]β-carotene, and using a number of assumptions that are discussed later (see the section on mathematical modeling), the bioavailability and bioefficacy of carotene were calculated. Five of the 11 women participating in this study were designated as non-responders because they did not absorb β-carotene. In our opinion, there are 2 reasons for the low isotopic enrichment of retinol in serum in these subjects. First, the (unknown) matrix of β-carotene probably limited its absorption and thus bioconversion. Second, the enrichment being measured approached the limit of quantitation or even detection (see the section on detection methods). Be that as it may, on the basis of the results for the remaining 6 subjects, the investigators reported that the bioefficacy of dietary β-carotene was high, 74% (CV: 81%), whereas the bioavailability was only 6.1% (SE: 1.8%; range: 1.1–14.4%). A bioefficacy of 74% means that 74% of β-carotene is absorbed and converted to retinol (ie, 1.25 μg β-carotene has the same vitamin A activity as 1 μg retinol) (7). Because these results are inconsistent with each other, the assumptions underlying the model will need to be addressed (see the section on mathematical modeling).

In an investigation involving 3 adults, Edwards et al (25) administered a single dose of 2 mg [$^2\text{H}_4$]retinyl acetate, 6 mg β-carotene as either raw carrot or spinach, and either 20 or 1 g of added fat to each of the subjects in random order, 2–6 wk apart. The duration of sample collection was too short to measure bioavailability and bioefficacy of β-carotene in the low-fat meals. On the basis of the data from the high-fat meals, it was concluded that the vitamin A activity of 20 μg β-carotene in carrot or spinach is equivalent to that of 1 μg retinol. This corresponds to a bioefficacy of 4.7%. The bioavailability of β-carotene in carrot or spinach ranged from 3–16%.

We have conducted 2 controlled dietary studies involving the daily administration of <200 μg β-carotene and <90 μg retinol, each specifically labeled with 10 ^{13}C atoms. The degrees of isotopic enrichment of β-carotene and retinol in serum at plateau isotopic enrichment (CarRet PIE) were measured by liquid chromatography–mass spectrometry (LC-MS), and the data were analyzed by using the CarRet PIE mathematical model (7, 55, 71). In the first study (7) lasting ≤ 10 wk with 35 children, 2.4 μg (\bar{x} ; 95% CI: 2.1, 2.7) β-carotene in oil was found to have the same vitamin A activity as 1 μg retinol. In the second study (55, 71) lasting 6 wk with 77 children, 2.7 μg (\bar{x} ; 95% CI: 2.5, 2.8) β-carotene in oil had the same vitamin A activity as 1 μg retinol. If the data from both studies are pooled ($n = 111$), it is estimated that 2.6 μg β-carotene in oil (median value) has the same vitamin



TABLE 2

Studies in humans using stable-isotope tracers to study the bioavailability and bioefficacy of dietary carotenoids qualitatively or quantitatively

Reference	Intervention: a) description of subjects, b) isotope tracer ¹ and dosing regimen, c) number and timing of blood sampling ²	Analysis: a) sample preparation and measurement of isotopic enrichment, ³ b) method of data analysis	Conclusion: a) results (mean with CV), ⁴ b) comments
Qualitative studies			
Parker et al, 1993 (46)	a) 1 man, 41 y; b) Single, physiologic dose of 1 mg intrinsically, uniformly labeled [¹³ C]β-carotene in oil; c) 12 samples within 22 h	a) Plasma saponified, purified by using HPLC, (hydrogenated overnight for β-carotene), and then analyzed by GC-IRMS ⁵ (56); b) Estimation of maximal ¹³ C degree of isotopic enrichment in retinol, retinyl esters, and β-carotene in plasma	a) Maximal degree of isotopic enrichment in retinol, retinyl esters, and β-carotene in plasma with ¹³ C was 0.8%, 9.2%, and 1.2%, respectively; b) β-Carotene is converted to retinol in the body
You et al, 1996 (48)	a) 2 men and 1 woman, 23–38 y; b) Single dose of 992 or 994 μg [¹³ C]9- <i>cis</i> -β-carotene and 6–8 μg [¹³ C] <i>all-trans</i> -β-carotene followed ≥8 mo later by a single dose of 992 or 994 μg unlabeled 9- <i>cis</i> -β-carotene and 6–8 μg [¹³ C]- <i>all-trans</i> -β-carotene (each preparation was dissolved in safflower oil); c) ≤17 samples within 33 h through a catheter and then several samples by venipuncture up to 10 d	a) Plasma saponified, purified by using HPLC (hydrogenated overnight for β-carotene), and then analyzed by GC-IRMS (46, 48); b) Plasma concentration-versus-time curve of [¹³ C]retinol, [¹³ C] <i>all-trans</i> -β-carotene, and [¹³ C]9- <i>cis</i> -β-carotene in plasma	a) Substantial amounts of [¹³ C] <i>all-trans</i> -β-carotene and [¹³ C]retinol appear in plasma after ingestion of an oral dose consisting of >99% [¹³ C]9- <i>cis</i> -β-carotene, whereas only small amounts of [¹³ C]9- <i>cis</i> -β-carotene appear to be secreted in the bloodstream; b) <i>cis</i> -β-carotene in part is isomerized to <i>all-trans</i> -β-carotene before or during its absorption
Burri and Park, 1998 (49)	a) 5 adult women, 21–28 y; b) Single pharmacologic dose of 40 mg [² H ₈]β-carotene in oil; c) 5 samples within 15 h and 7 up to 21 d	a) Plasma extracted, saponified, extracted by solid phase, derivatized, and then analyzed by GC-MS ⁵ (57) for retinol and by HPLC for β-carotene (58, modified after 59); b) Compartmental model developed by Novotny et al (51)	a) A working compartmental model for β-carotene metabolism was developed, suggesting that [² H ₈]β-carotene converts to [² H ₄]retinol with high interindividual variation; b) β-Carotene is converted to retinol in the body
Yao et al, 2000 (47)	a) 4 women, 25–38 y; b) Single physiologic dose of 3 mg intrinsically, uniformly labeled [¹³ C]lutein in oil; c) 13 samples within 16 h and 7 samples up to 22 d	a) Plasma extracted, saponified, lutein purified by reversed-phase and normal-phase HPLC, hydrolyzed and hydrogenated, and then analyzed by GC-IRMS; b) Plasma concentration-versus-time curve of [¹³ C]lutein	a) Peak enrichment was measured at 16 h, enrichment still measurable at day 22; b) Appearance and disappearance of [¹³ C]lutein in plasma after a single dose can be measured
Pawlosky et al, 2000 (50)	a) 1 woman, 32 y; b) Single physiologic dose of 5 mg [² H ₈]β-carotene mixed into a breakfast drink; c) 1 sample at 8 h and 4 samples up to 29 d	a) Plasma extracted and then analyzed by particle beam LC-MS ⁵ for β-carotene; b) Plasma concentration-versus-time curve of [² H ₈]β-carotene	a) Peak enrichment was measured at 8 h, enrichment still measurable at day 29; b) Appearance and disappearance of [² H ₈]β-carotene in plasma after a single dose can be measured. Degree of isotopic enrichment of retinol in plasma was not measured
Quantitative studies			
Novotny et al, 1995 (51)	a) 1 man, 53 y; b) Single pharmacologic dose of 40 mg [² H ₈]β-carotene in oil; c) 7 samples within 12 h and 18 samples up to 113 d	a) Plasma extracted, saponified, extracted by solid phase, and then analyzed by HPLC for β-carotene (59) and by GC-MS for retinol (60); b) 5-Term and a 3-term poly-exponential equation, to describe plasma concentration-versus-time curves of [² H ₈]β-carotene and of [² H ₄]retinol, respectively ⁶	a) 22% of labeled β-carotene was absorbed: 17.8% as intact β-carotene and 4.2% as retinoid. In liver and enterocyte, 43% and 57% of β-carotene was converted to retinoid, respectively. Mean residence time of β-carotene in body was 51 d; b) Outcome is heavily affected by assumptions underlying the compartmental model ⁶
Tang et al, 2000 (53)	a) 1 woman, 47 y; b) Single doses of 126 mg (pharmacologic) and 6 mg (physiologic) [² H ₈]β-carotene in crystalline form dissolved in oil and 9 mg [² H ₈]retinyl acetate on separate occasions up to 2.5 y apart; c) 2 (and 3) samples within 6 h (and 9 h) and 6 up to 21 d after consumption of [² H ₈]β-carotene (and [² H ₈]retinyl acetate, respectively)	a) Serum extracted, purified by HPLC, derivatized, and then analyzed by electron capture negative chemical ionization GC-MS for retinol (61, 62); b) Area under serum concentration-versus-time curve of [² H ₄]retinol ⁷ compared with that of [² H ₈]retinol ⁷	a) Vitamin A activity of 3.8 and 55 μg β-carotene in oil was equivalent to that of 1 μg retinol after doses of 6 and 126 mg β-carotene, respectively; b) Degree of isotopic enrichment of β-carotene in serum was not measured

(Continued)



TABLE 2 (Continued)

Reference	Intervention: a) description of subjects, b) isotope tracer ¹ and dosing regimen, c) number and timing of blood sampling ³	Analysis: a) sample preparation and measurement of isotopic enrichment, ³ b) method of data analysis	Conclusion: a) results (mean with CV), ⁴ b) comments
Lin et al, 2000 (54)	a) 11 women, 19–39 y; b) Single pharmacologic doses of 10 mg [² H ₆]retinyl acetate and 20 mg [² H ₆]β-carotene 1 wk apart; c) 5 samples within 20 h and 19 samples up to 28 d after consumption of [² H ₆]retinyl acetate, of these samples, 5 were within 20 h and 8 were up to 21 d after consumption of [² H ₆]β-carotene	a) Plasma extracted, saponified, extracted by solid phase, and then analyzed by GC-MS for retinol (37, 63) and by HPLC for β-carotene (modified after 63 and 64); b) Area under plasma concentration-versus-time curve of [² H ₆]retinol (from 0 to 4 d), of [² H ₃]retinol (from 0 to 4 d), and of [² H ₆]β-carotene (from 0 to 21 d); Dose-normalized ratios of the areas under the curves of [² H ₃]retinol to [² H ₆]retinol were used for calculation of vitamin A activity. Summation of the AUCs of [² H ₃]retinol and [² H ₆]β-carotene, and several assumptions were used to calculate total absorption (ie, bioavailability)	a) Vitamin A activity of 2.3 μg (CV: 140%) β-carotene in oil was equivalent to that of 1 μg retinol. Five of 11 women were designated as nonresponders. Reported absorption of β-carotene was 3.3% (range: 0–14.4%; CV: 135%); b) Bioefficacy of β-carotene in oil is 41%, whereas bioavailability is 3.3%. Obviously, these conflicting results cannot be true and the assumptions need to be addressed. CV is high
van Lieshout et al, 2001 (7)	a) 16 girls and 19 boys, 8–11 y; b) 2 doses of 80 μg [¹³ C ₁₀]β-carotene in oil and 80 μg [¹³ C ₁₀]retinyl palmitate/d over a period of ≤10 wk; c) 3 samples per child over a period of ≤10 wk ⁸	a) Serum extracted and then analyzed by APCI LC-MS ⁵ for retinol (65, 66) and for β-carotene (66); b) Bioefficacy and bioavailability of β-carotene in oil quantified by using the CarRet PIE mathematical model of β-carotene and retinol at plateau isotopic enrichment (7)	a) Plateau isotopic enrichment was reached within 21 d. 2.4 μg β-carotene in oil (between-subjects CV: 36%; within-subjects CV: 22%) has the same vitamin A activity as 1 μg retinol. The bioavailability ⁹ of β-carotene in oil was 108% (n = 33; geometric \bar{x} ; between-subjects CV: 36%; within-subjects CV: 23%); b) Bioavailability and bioefficacy of β-carotene in oil can be quantified with high precision
Tang et al, 1999 (52)	a) 1 man, 45 y, and 1 woman, 51 y; b) Single dose of 200 g spinach, grown on 30% ² H ₂ O containing 13.8 mg uniformly ² H-labeled <i>all-trans</i> -β-carotene ¹⁰ and 3.0 mg [² H ₈]retinyl acetate 3 d apart; c) Several samples up to 8 d	a) Serum extracted, purified by HPLC, derivatized, and then analyzed by electron capture negative chemical ionization GC-MS for retinol (62); b) Area under serum concentration-versus-time curve of uniformly labeled retinol compared with that of [² H ₈]retinol	a) Vitamin A activity of 27–72 μg (range; CV: 73%) β-carotene in spinach was equivalent to that of 1 μg retinol; b) Relatively high CV. Degree of isotopic enrichment of β-carotene in serum was not measured
Edwards et al, 2001 (25)	a) 1 woman and 2 men, 25–35 y; b) Single dose of 2 mg [² H ₄]retinyl acetate, 6 mg β-carotene as either raw carrot or spinach, and either 20 or 1 g of added fat; ¹¹ c) 5 samples within 8.5 h	a) Plasma triacylglycerol-rich lipoprotein fractions prepared (67), extracted (48), saponified, extracted, purified by HPLC (68), derivatized, and then analyzed by GC-MS for retinol (25); b) Baseline corrected area under serum concentration-versus-time curve of unlabeled retinyl esters compared with that of [² H ₄]retinyl esters, β-carotene, and α-carotene. Bioavailability and bioefficacy of β-carotene was estimated by assuming 80% absorption of [² H ₄]retinyl acetate	a) In the presence of 20 g fat, 20 μg β-carotene in carrot or spinach (between-subjects CV: 22%; within-subjects CV: 37%) has the same vitamin A activity as 1 μg retinol; The bioavailability ⁹ of β-carotene in carrot or spinach ranged from 3% to 16%; ¹² b) Duration of sample collection was too short to measure bioavailability and bioefficacy of β-carotene in low-fat meals. Findings on bioavailability and bioefficacy of β-carotene in vegetables confirm earlier studies (69)
van Lieshout et al, 2001 (55)	a) 38 girls and 39 boys, 7–13 y; b) 3 doses of 31 μg [¹³ C ₁₀]β-carotene in oil and 21 μg [¹³ C ₁₀]retinyl palmitate/d for 6 wk; in weeks 3–6, 2 portions of either 164 g spinach or 162 g pumpkin were consumed daily, containing 2.8 and 1.4 mg β-carotene, respectively; ¹³ c) 1 Sample at the end of each 3-wk period	a) Serum extracted and then analyzed by APCI LC-MS ⁵ for retinol (65, 66) and for β-carotene (66); b) Bioefficacy and bioavailability of β-carotene in oil quantified, and relative bioefficacy and bioavailability of β-carotene in spinach and pumpkin estimated by using CarRet PIE mathematical model of β-carotene and retinol at plateau isotopic enrichment (7, 55)	a) 2.7 μg β-carotene in oil (CV: 21%) has the same vitamin A activity as 1 μg retinol; ¹⁴ Bioavailability ⁹ of β-carotene in oil was 81% (n = 73; geometric \bar{x} ; CV: 42%); Bioavailability and bioefficacy of β-carotene in pumpkin were both 1.7 (interval: 1.3, 2.4) times those of β-carotene in spinach; b) Findings on bioavailability and bioefficacy of β-carotene confirm earlier studies (4, 5, 7)

(Continued)



TABLE 2 (Continued)

- ¹Unless otherwise specified, isotope tracers were specifically labeled.
- ²A baseline sample was collected shortly before consumption of the labeled compounds in all studies (see also footnote 8).
- ³Concentration of retinol and β -carotene in serum or plasma was measured by HPLC in all studies.
- ⁴If more than one subject was studied, values are expressed as means with CVs. The CV (SD/\bar{x}) is the biological variation within and between subjects and the variation in the chemical analysis. All studies investigated the same metabolic processes (bioavailability and bioefficacy). Therefore, it can be expected that the biological variation within and between subjects is somewhat comparable between the studies. Thus, in our opinion, the differences in CVs mentioned in this table indicate differences in the precision of the techniques used for studying bioavailability and bioefficacy and are related to the design of the studies more than to the subjects studied.
- ⁵GC-IRMS, gas chromatography–isotope ratio mass spectrometry; LC-MS, liquid chromatography–mass spectrometry; GC-MS, gas chromatography–mass spectrometry; APCI LC-MS, atmospheric pressure chemical ionization LC-MS.
- ⁶Model intestinal absorption was constrained to be inside the range of 2 SDs of $15\% \pm 4.5\%$, referring to a publication of Bowen et al (18). In the original publication of this study (70), 2.6 mg (CV: 14%) of a 15-mg dose of β -carotene was absorbed in 11 healthy men (\bar{x} age: 26 y).
- ⁷[²H₄]Retinol was the sum of [²H₃]retinol, [²H₄]retinol + [¹³C, ²H₃]retinol, and [¹³C, ²H₄]retinol. [²H₈]Retinol was the sum of [²H₆]retinol, [²H₇]retinol, [²H₈]retinol, [²H₈]retinol + [¹³C, ²H₈]retinol, and [¹³C₂, ²H₈]retinol.
- ⁸In a subgroup of 12 children, the first blood sample was collected at baseline; for the others, the first blood sample was collected on day 21.
- ⁹A 1:2 stoichiometry of the conversion of β -carotene to retinol was assumed.
- ¹⁰Spinach also contained 2.7 mg other provitamin A carotenoids (1.9 mg 9-*cis*- β -carotene, 0.6 mg α -carotene, and 0.2 mg 13-*cis*- β -carotene). In our calculations, we assumed that the vitamin A activity of other provitamin A carotenoids was one-half that of β -carotene.
- ¹¹Four different test meals were given to each of the 3 subjects, in random order, 2–6 wk apart. The meals were 1) raw carrot + 20 g fat, 2) raw spinach + 20 g fat, 3) raw carrot + 1 g fat, and 4) raw spinach + 1 g fat. Carrot meals contained 5.6 mg β -carotene and 3.1 mg α -carotene. Spinach meals contained 6.2 mg β -carotene.
- ¹²SDs reported in the original publication (25) do not take into account that each subject was measured twice. We estimated within- and between-subjects CVs for use in this table on the basis of data from a figure in the original publication. Individual data on the bioavailability of β -carotene could not be estimated from the original publication; therefore, these data are reported as a range.
- ¹³Spinach and pumpkin also contained 128 and 604 μ g other provitamin A carotenoids, respectively. In our calculations, we assumed that the vitamin A activity of other provitamin A carotenoids was one-half that of β -carotene.
- ¹⁴If data from the 2 studies are pooled, an amount of 2.6 μ g β -carotene in oil (median) has the same vitamin A activity as 1 μ g retinol, and the (median) bioavailability of β -carotene in oil was 86%.

A activity as 1 μ g retinol. Thus, the best estimate of the bioefficacy of β -carotene in oil is 36%. If the data from both studies are pooled ($n = 106$), the median bioavailability of β -carotene in oil is 86%.

The labeled β -carotene fed in the first study had a *cis-trans* ratio of 3:1. Because β -carotene occurs in foods mainly in the *all-trans* configuration, for the second study, care was taken that 90% of the labeled β -carotene was in the *all-trans* configuration. In the second study, comprising a 3-wk run-in period followed by a 3-wk treatment period, children were randomly allocated to daily receive either 164 g spinach or 162 g pumpkin, containing 2.8 and 1.4 mg β -carotene and 0.12 and 0.60 mg other provitamin A carotenoids, respectively. From this study, it can be concluded that the bioefficacy and the bioavailability of β -carotene in pumpkin are both 1.7 (interval: 1.3, 2.4) times those of β -carotene in spinach.

STUDY DESIGN

In designing intervention studies to quantify the bioavailability and bioefficacy of dietary carotenoids in humans with the use of tracer techniques, decisions need to be made at different levels (Table 1). Often, decisions made at one level will limit the decisions that can be made at other levels. Such decisions will be illustrated on the basis of the studies presented above (Table 2).

Defining the aim and hypothesis

In general terms, 3 types of studies can be envisaged in which isotopically labeled carotenoids or retinol can be used. These include qualitative studies and 2 types of quantitative studies: efficacy studies, which provide information under closely controlled conditions, and effectiveness studies, which measure the effect of

an intervention under field conditions. As discussed above, many of the initial studies were qualitative in nature and showed that β -carotene is converted to retinol in the body. More recently, attention has been directed toward measuring the extent of conversion of β -carotene to retinol within the framework of efficacy. As discussed later, mathematical models are required to obtain such data. Studies in which body pools of retinol are estimated can be used in both efficacy and effectiveness studies. Regardless of the type of study, clearly defined hypotheses should be established that can be evaluated by using appropriate statistical tests.

Defining the study population and estimating the sample size

There are 2 prior considerations in the selection of the study population. The first is to choose a population in which results are most likely to be obtained. Thus, when examining serum retinol response to feeding β -carotene or retinol, vitamin A–depleted subjects are more responsive, thus providing data that could not be obtained in a replete population. The second is to choose a population for which the results are relevant: for example, developed compared with developing countries, men compared with women compared with children. Thus, in developing countries, the question arises whether subjects should be treated to eliminate intestinal parasites. We have taken the view that untreated subjects should be studied because they are representative of the population.

The US Institute of Medicine (3) has taken the view that data from subjects in developing countries cannot be used in establishing bioefficacy or nutrient requirements because such subjects are not representative of the US population. In our opinion, reference data on bioefficacy or nutrient requirements should be based first on the most reliable data available and second on the most representative data available. Currently, the most reliable data



available for vitamin A bioefficacy were obtained in vitamin A-replete subjects in developing countries (4, 5, 7, 55, 69), and these findings are consistent with studies of smaller groups of vitamin A-replete subjects in Europe and the United States (25, 69, 72). Therefore, it seems appropriate to extrapolate the best available bioefficacy and bioavailability data for vitamin A-replete subjects to those in developed countries until more reliable data for these populations become available. To assess the validity of such extrapolations, it would be interesting to study the bioefficacy of fruit and vegetables in vitamin A-replete subjects, such as vegetarians fed a retinoid-free diet.

Considering sample size, many studies conducted to date have not used sufficient subjects (Table 2) to provide data representative of any population. To a large extent, this is due to the developmental nature of the work being carried out and the costs involved. In the future, more attention will need to be paid to power. Based on the intra- and interindividual variation observed in studies conducted to date, data are available to perform power calculations.

CHOICE OF ISOTOPIC TRACER

Isotopes

Elements (atoms with the same number of protons) containing a different number of neutrons are referred to as isotopes and can be either stable or radioactive. Although for retinol and some carotenoids (but not cyclic and acyclic carotenes) it would be possible to use isotopes of oxygen, all studies to date have used isotopes of carbon or hydrogen (Table 2). Isotope effects, such as changes in the reaction rates in biological systems, are most likely to occur when using isotopes of hydrogen (73, 74) because the mass of ^2H or ^3H is double or triple that of ^1H , whereas the mass of ^{13}C or ^{14}C is only 8% or 17% higher than that of ^{12}C . Isotope effects are more likely with radioisotopes because their atomic weights are typically higher than those of their stable counterparts. One problem that does not arise when using radioisotopes in studies of carotenoid metabolism is short half-life. Both ^{14}C and ^3H have relatively long half-lives, 5760 and 12.3 y, respectively.

Other reasons dictate whether radioisotopes or stable isotopes are used in human intervention studies (30, 73). First, because methods for detecting radioactivity are very sensitive (*see* the section on detection methods), much lower doses of labeled compounds can be administered in studies using compounds labeled with radioisotopes, thus avoiding perturbation of metabolism (*see* the section on size of dose). Second, after the administration of a compound labeled with a radioisotope, the compound and its metabolites can be traced at low concentrations by measuring the radioactivity. With compounds labeled with stable isotopes, the degree of isotopic enrichment might be too low to measure precisely. Third, when using radioisotopes, whole-body measurements of radioactivity can be made. With stable isotopes, the tissue and compound in which the isotope is incorporated must be known before the tissue can be collected and the compound isolated to measure the degree of isotopic enrichment. However, for many studies in humans involving administration of radioisotopes, prior knowledge of the metabolism is also required, because not all tissues can be collected from healthy volunteers (*see* the section on specimen collection). Fourth, in the past, organic compounds labeled with stable isotopes were relatively expensive, but prices are coming down as supplies increase. Despite the

advantages of using radioisotopes, however, reluctance to use them still exists because high doses of radioisotopes may be harmful. For the future, we suggest that more discussion take place on the true risks of using radioisotopes in human nutrition research. Hence, we would like to stress that Dueker et al's (28) radioisotope study is a welcome approach after decades during which the reluctance to use radioisotopes has increased. Until there is more general acceptance of the use of radioisotopes, compounds labeled with stable isotopes will continue to be used in studies of carotenoid bioavailability and bioefficacy.

Method and degree of labeling

The method of isotopic labeling of compounds has major implications for further aspects of the study design, such as preparation and analysis of samples and subsequent analysis of data. Either intrinsic or extrinsic labeling can be used. Intrinsic labeling involves biological incorporation of isotope into compounds so that the compounds are in the same matrix as the foods consumed. Extrinsic labeling refers to the chemical incorporation of isotopes into a compound. Although much effort has been spent on intrinsically labeling vegetables such as spinach, carrots, and tomatoes, it has been difficult to produce enough vegetable to achieve sufficient enrichment of carotenoids in serum to obtain reliable data. In fact, just one abstract reporting such a study has been published to date (52).

In our opinion, the disadvantages of intrinsic labeling outweigh the advantages for studies aimed at quantifying carotenoid bioavailability and bioefficacy. First, in intrinsically labeled plants, isotopes are incorporated in all molecules instead of in only the compounds of interest. Thus, spinach or tomatoes grown on $^2\text{H}_2\text{O}$ or on $^{13}\text{CO}_2$ will also contain other labeled carotenoids (with and without provitamin A activity) and other labeled compounds, which might bias the results. Second, if the duration of exposure to the label is insufficient, the isotope will not be incorporated into all positions in the molecule to the same extent. This can lead to problems in the interpretation of analytic data and, together with the use of parent compounds labeled to an extent $< 100\%$, might lead to a low degree of isotopic enrichment of compounds of interest. As a result, the amounts of isotopic compound and plant material that need to be administered to enable adequate measurements must be increased.

A third problem is producing sufficient quantities of labeled vegetables to carry out a complete study or a series of studies. As a result of limitations in the size of the plant and the length of the growing season, it proves difficult to obtain intrinsically labeled fruit. To some extent, these problems can be overcome by not using fruit and vegetables normally consumed but by using other biological sources such as green algae. Green algae have been grown on $^{13}\text{CO}_2$ as the sole carbon source to provide labeled *all-trans*- β -carotene (46), 9-*cis*- β -carotene (48), and lutein (47). However, the algae matrix is probably different from that of most fruit and vegetables. Note that in their studies, Parker, You, and Yao et al (46–48) extracted β -carotene and lutein from the algae and dissolved the compounds in oil. Thus, intrinsically labeled β -carotene was used as an extrinsic reference dose, and the advantage of intrinsic labeling with respect to the matrix was lost. Fourth, isotope effects may be enhanced with intrinsic labeling, which results in labeling throughout the molecule. As discussed above, such an effect would be greater with hydrogen than for carbon.

Although extrinsic labeling can also be used to synthesize uniformly labeled compounds, its greatest advantage is in labeling



compounds specifically at a limited number of sites. It is necessary to decide on the number of atoms that need to be labeled in a molecule. This number should be sufficient to provide enrichment above the natural abundance (1.1% for ^{13}C and 0.016% for ^2H). Thus, for retinol and carotenoids, ≥ 3 ^{13}C atoms need to be incorporated. Because absorbed labeled β -carotene is in part converted to retinol, studies on the bioefficacy of β -carotene should use β -carotene containing ≥ 6 ^{13}C atoms located at designated sites on each half of the molecule. To be able to distinguish labeled retinol that is administered from that synthesized in vivo from β -carotene, the administered retinol should contain ≥ 3 ^{13}C atoms more than the retinol derived from the labeled β -carotene.

We carried out pilot studies using β -carotene and retinol each labeled with 6 ^{13}C atoms. However, we decided to increase the number of ^{13}C atoms in both retinol and β -carotene to 10 to improve the performance of the measurements of isotopic enrichment (65). Use of different stable isotopes, such as ^{13}C and ^2H , is of limited value for this purpose because it is necessary to create molecules differing in molecular weight. When using radioisotopically labeled β -carotene and retinol, it is possible to carry out studies using different radioisotopes, such as ^{14}C and ^3H , for each compound (26, 27).

Edwards et al (25) developed an adaptation of a triacylglycerol-rich lipoprotein fraction model that involves coadministration of 2 mg [$^2\text{H}_4$]retinyl acetate. This extrinsic reference dose of retinyl acetate controls for variation in chylomicron kinetics in vivo and for retinyl ester recovery during the preparation and analysis of the triacylglycerol-rich lipoprotein fraction and also acts as a reference with which to estimate the bioefficacy of β -carotene from food.

Apart from the number of isotopic atoms incorporated into each molecule, the positions at which isotopes are incorporated should be chosen carefully. The isotopes should be placed at chemically inert sites to minimize loss or rearrangement during metabolism, sample preparation, or analysis. This is a major concern with ^2H atoms, especially when labeled compounds are prepared by using reversible reactions (74). When using ^2H atoms for extrinsic labeling at specific positions, scrambling (ie, rearrangement of isotopes to positions in the molecule other than those in which they were initially incorporated) occurs more often than when using ^{13}C atoms (7). If scrambling occurs, adjustments in the mass spectrometry method may be required.

Isotopic purity of both intrinsically and extrinsically labeled compounds can be very high, $>95\%$ (46, 50), $>98\%$ (48), or $>99\%$ (7, 25, 47, 55, 75). Unfortunately, not all studies report the isotopic purity of the compounds used (49, 52), and some studies used compounds with a lower isotopic purity, 91% (54) or 80–81% (51, 53). High isotopic purity facilitates detection of labeled compounds by mass spectrometry and subsequent quantification of the bioavailability and bioefficacy by mathematical modeling. Low isotopic purity reduces the sensitivity and precision of mass spectrometric quantification. If multiple ions must be measured as a result, the number of variables increases, and consequently the error of the method also increases (see the section on chemical analysis of samples and the section on method of data analysis).

Matrix of tracer

When studying carotenoid bioavailability and bioefficacy, 2 types of matrices are important: oil and that of the fruit or vegetable. Oil as a matrix is important for 2 reasons: 1) it is a matrix in which carotenoids are consumed, eg, β -carotene in palm oil, and 2) it is expected that the bioavailability of carotenoids will be

maximal in this matrix. Studies on the bioavailability and bioefficacy of carotenoids in fruit and vegetables are important because controversy exists over the role of the matrix of fruit and vegetables in the bioavailability and thus bioefficacy of carotenoids. Therefore, much emphasis has been put on how bioavailability and bioefficacy in such matrices can be measured. Because intrinsically labeled vegetables have the same matrix, there has been interest in their use for studying carotenoid bioavailability and bioefficacy. As discussed above, however, there are several problems with intrinsic labeling.

In a study by Tang et al (52), [$^2\text{H}_8$]retinyl acetate and spinach containing uniformly ^2H -labeled carotenoids were administered to 2 subjects. In our recent study in Indonesia (55), we used extrinsically labeled β -carotene and retinol to quantify the bioavailability and bioefficacy of β -carotene in spinach and pumpkin. The extent of dilution of the isotopic enrichment of retinol and β -carotene was used as the basis for calculating the bioavailability and bioefficacy of β -carotene. Edwards et al (25) used extrinsically labeled retinyl acetate as an extrinsic reference to estimate the bioavailability and bioefficacy of β -carotene in carrot and spinach.

In one of the investigations by Tang et al (53), β -carotene was administered in crystalline form. The β -carotene bioefficacy of 25% reported by Tang et al was lower than that of 36% reported by us (55). One explanation for this finding might be the lower bioavailability and thus lower bioefficacy of β -carotene in large crystals than of that dissolved in oil, as shown in studies in rats (76). Thus, in future studies, more attention should be given to ensuring that β -carotene is in fact dissolved, for example, by examining the preparation microscopically.

CHOICE OF DOSING REGIMEN

Route of administration

Although compounds are administered by a variety of routes in pharmacologic investigations of bioavailability, in studies of carotenoid bioavailability and thus bioefficacy, most attention is directed toward the intravenous and oral routes of administration. In pharmacology, bioavailability is defined as the ratio of the area under the curve of the concentration of the compound of interest after oral administration to that after intravenous administration. However, we define bioavailability differently (see the section on rationale), which is necessary because a fraction of the ingested and absorbed provitamin A carotenoids will be converted to retinol in the body (bioconversion). As a result, serum concentrations of provitamin A carotenoids do not reflect the total response to ingested carotenoids, because part of the ingested carotenoids will be converted to retinol. Unlike the administration of hydrophilic compounds, it is impractical to administer retinol and carotenoids parenterally because of the difficulty of formulating them in a physiologic form such as chylomicrons or, in the case of retinol, bound to retinol-binding protein and transthyretin.

Frequency of dosing

Single doses of labeled compounds can be administered to provide qualitative information on the kinetics, appearance, and disappearance of labeled compounds and their metabolites in the body (25, 46–54). In all the studies of carotenoid metabolism that have used radiolabeled compounds, single



doses have been administered (26–28). There are some limitations, however, to using single doses for studying the bioefficacy of carotenoids.

First, single doses do not represent habitual intakes and thus their metabolism might differ from that of nutrients consumed over a longer period. Such differences have been found in studies on the absorption of iron and on factors affecting iron absorption. In their review of iron absorption and bioavailability, Hallberg and Hulthen (77) explain why the variation in iron absorption from the whole diet is lower than that from single meals. Almost all studies examining the effect of food components—termed effectors (22)—that modulate iron absorption have used single meals containing or not containing the effector under study. The variation in iron absorption between single meals of different compositions may be much greater than the variation in iron absorption from whole diets composed of several single meals because the latter is the mean absorption of several single meals. This does not mean that the absorption of iron from single meals per se would be falsely high or low. Similarly, single-dose studies for studying carotenoid bioavailability and bioefficacy might not result in false estimates per se, but the estimates cannot be readily extrapolated to the bioavailability and bioefficacy of carotenoids in the habitual diet. In addition, the variation in the estimates obtained using single doses might be relatively high, thus requiring a large number of subjects in a study.

Second, at some time point after the administration of a single dose of isotope, enrichment approaches baseline values. Thus, many measurements are often made when the signal-to-noise ratio is low, or, in other words, when the limit of quantitation is approached or even exceeded (*see* the section on detection methods). Third, data analysis of single-dose studies involves the use of complicated mathematical models often based on assumptions that are difficult to justify (*see* the section on method of data analysis).

In studies in which both β -carotene and retinol are administered, both should be administered at the same time (7, 25, 55). In some studies, intervals of 1 wk (54) or >6 mo (53) have been used instead of simultaneous dosing. Such dosing regimens might result in bias due to time effects.

The use of multiple doses results in a plateau of isotopic enrichment (73). Such a plateau can be obtained earlier if an initial priming dose is used. Because a plateau is reached after a prolonged period of dosing, the degree of isotopic enrichment of nutrients in serum is generally higher than that reached after a single dose, thus facilitating detection. Thus, not only can repeated measurements be made to establish the level of enrichment but individual measurements will be more reliable because they will not be near or below the limit of quantitation.

Multiple doses can be administered at different intervals. In the studies conducted by our laboratories, a dosing regimen of labeled carotenoids and retinol of 2 or 3 times per day at mealtime was chosen, because these nutrients were normally consumed at mealtime. The aim was for the label to mix with the foods ingested. Thus, at plateau isotopic enrichment, the ratio of isotopic enrichment in plasma represents the ratio of the flow of labeled and unlabeled nutrients from the gut. In our studies, plateau isotopic enrichment was reached within 21 d of the start of multiple dosing (7) and might have occurred between 8 and 21 d (55). As will be discussed later (*see* the section on mathematical modeling), plateau values can be used readily in mathematical models.

Size of dose

It is necessary to decide not only on the frequency of dosing but also on dose size. In the study by Dueker et al (28), the limit of quantification of [^{14}C] β -carotene in plasma, urine, and feces was in the attomole range (1×10^{-18} mol). This level of sensitivity enabled a low dose (306 μg) of [^{14}C] β -carotene to be administered.

Detection methods for stable isotopes are less sensitive than those for radioisotopes. Thus, the dose administered needs to be relatively high to reach measurable levels of isotopic enrichment. Four single-dose studies (Table 2; 49, 51, 53, 54) involved the administration of pharmacologic doses (>10 mg), which might be inappropriate for quantifying bioavailability and bioefficacy. The metabolism of retinol and β -carotene might be perturbed with pharmacologic doses, as was shown by Von Reinnersdorff et al (78), who administered 55 mg [$^{13}\text{C}_3$]retinyl palmitate to 11 healthy men. Although β -carotene is absorbed by passive diffusion, the absorption and metabolism of β -carotene is dose-dependent. Thus, from their own studies in rats and those of others in experimental animals and humans, Brubacher and Weiser (79) concluded that the bioefficacy of β -carotene in humans was dose-dependent when >4 mg was fed in one meal. Such an effect may explain, at least in part, the results of Tang et al (53), who administered 6 and 126 mg [$^2\text{H}_8$] β -carotene to the same woman 2.5 y apart. They reported that carotenoid bioefficacy was 25% and 1.7% with the respective doses.

To minimize the effects observed when large doses are fed (73), studies have been carried out in which amounts normally consumed in a meal have been fed (Table 2; 25, 46–48, 50, 52). However, with these physiologic doses, the problem of using relatively insensitive methods of detection of stable-isotope-labeled compounds may arise. Thus, some of the variation in the results obtained in these studies might be explained through experimental error resulting from attempting to measure isotopic enrichment beyond the limit of quantitation. These limitations were successfully overcome in studies in which multiple low doses (<200 μg β -carotene/d and <90 μg retinol/d) of stable-isotope-labeled compounds were used for a sufficiently long period to enable isotopic enrichment to reach a plateau (7, 55).

CHOICES RELATED TO THE COLLECTION OF SAMPLES

Specimen

An advantage of radioisotopes (except for very weak β -emitters such as tritium) is that whole-body measurements can be made to determine the retention of administered compounds (30), irrespective of whether the compounds have been metabolized. In addition, the route of labeled compounds through specific body tissues can be followed if such tissues can be sampled. In the radioisotope studies of Goodman et al (26) and Blomstrand et al (27), lymph was collected to measure β -carotene absorption. However, cannulation of lymph ducts in humans can be carried out only in a clinical setting. In studies in which stable isotopes have been used to assess total body stores of retinol, liver biopsies have been collected to evaluate the usefulness of plasma samples in assessing body stores (37, 42). As for lymph cannulation, liver biopsies can be taken only in a clinical setting. A technique developed by Edwards et al (25) in which newly absorbed



β -carotene and newly formed retinol are measured in triacylglycerol-rich lipoprotein fractions can also be performed only in a clinical setting.

Because retinol excretion in urine is increased in infection, isotopically labeled compounds can be used to assess the extent of such excretion. Under normal physiologic circumstances, only small amounts of retinol are excreted in feces, but the amount of β -carotene excreted can be quite large (28, 71), representing β -carotene that has not been absorbed. As discussed above, the extent to which this β -carotene has been subjected to gastric or bacterial degradation and the proportion of this β -carotene deriving from endogenously secreted β -carotene are not yet known. We measured the isotopic enrichment of β -carotene in feces during consumption of multiple low doses of [$^{13}\text{C}_{10}$] β -carotene to assess whether the bioavailability of carotenoids could be studied in this way (71). Bioefficacy cannot be assessed by using data from feces because bioconversion occurs in the body. To assess the bioavailability of β -carotene in oil using feces, complete collection of feces is necessary. With the use of data from feces it was assessed that the bioavailability of β -carotene from pumpkin ($n = 6$) was 1.8 times more efficient than from spinach ($n = 8$), which is comparable to the ratio of 1.7 observed in the same subjects with the use of data from serum. These data suggest that the bioconversion of β -carotene from both vegetables might be comparable, which was also suggested by Edwards et al (25) in their study of spinach and carrot. The collection of tissues other than serum or feces might be useful for gaining further insights into the metabolism of carotenoids.

Number and timing of collection of samples

In single-dose studies, the extent of isotopic enrichment of retinol and β -carotene in plasma is measured to enable the area under the curve of isotopic enrichment to be calculated. Because the accuracy of the estimate of bioavailability and bioefficacy depends on the frequency and timing of sampling of blood samples (80), a sufficient number of samples at representative time points (during the absorption and during the elimination phase) need to be collected. In studies by Edwards et al (25) and by Parker et al (46), samples of blood were collected up to 8.5 and 22 h after dosing, respectively, which are relatively short periods for obtaining a full picture of the appearance and disappearance of labeled compounds. Indeed, the sampling period of 8.5 h was too short in the study by Edwards et al (25). On the other hand, in another study involving one adult, Novotny et al (51) collected samples of blood for up to 113 d, which is an extremely long time. As discussed earlier, when isotopic enrichment approaches baseline values, the signal-to-noise ratio will decrease significantly, therefore dramatically increasing the analytic variation.

In multiple-dose studies, the length of time over which samples are collected is determined by the time required to reach plateau isotopic enrichment. Because a plateau is attained, fewer samples have to be collected than in single-dose studies. However, because the intraindividual variation in bioavailability and bioefficacy of dietary β -carotene is $>20\%$, collection of more than one sample would dramatically increase the power of the study or enable fewer subjects to be required (7).

CHOICES RELATED TO THE CHEMICAL ANALYSIS OF SAMPLES

Detection methods for radioisotopes

As discussed earlier, radioisotopically labeled compounds can be detected with high sensitivity (30). Another advantage is that it

is not necessary to know in which metabolite the label is incorporated. This might save time in tracking which metabolite has been formed. The activity of isotopes emitting β -radiation can be measured by a liquid-scintillation counter, whereas a crystal-scintillation counter can be used to measure isotopes emitting γ -radiation. In the study by Dueker et al (28), the activity of β -carotene in serum, feces, and urine was assessed by accelerator mass spectrometry (AMS). According to these authors, combustion of the compound of interest before analysis is an advantage because complex matrices such as feces and urine can be studied. However, with combustion, structural information is lost. In addition, sample preparation for AMS is labor intensive because it requires extraction, saponification, reversed-phase HPLC fractionation, graphitization, and combustion. Note that AMS does not require the use of radioisotopes. Instead, ions containing ^{14}C are separated from those containing ^{12}C and ^{13}C according to their mass-to-charge ratio (m/z) in the mass spectrometer. The ^{14}C isotope is preferred for isotope enrichment studies simply because it has a much lower background level than ^{13}C .

Detection methods for stable isotopes

Nuclear magnetic resonance and HPLC

The sensitivity of detection methods to assess isotopic enrichment is generally lower than that of methods to assess radioactivity (30), and there is considerable variation in sensitivity between the various methods. Nuclear magnetic resonance (NMR) is useful for measuring the degree of isotopic enrichment of macronutrients. However, serum concentrations of micronutrients such as β -carotene and retinol are very low. Unless plasma samples are of the order of 1 L, NMR is not a useful technique for measuring isotopic enrichment of micronutrients.

In 2 studies (Table 2; 49, 51), hexane extraction, saponification, and solid-phase extraction of plasma were followed by HPLC with an ultraviolet light detector to separate [$^2\text{H}_8$] β -carotene from unlabeled β -carotene in such a way that adjacent peaks were resolved sufficiently for accurate measurements of peak area to be obtained (baseline separation). The development of this method was described by Dueker et al (59). In another paper (74), they noted that [$^2\text{H}_6$] β -carotene could not be baseline separated from β -carotene. Lin et al (54) used a computer program to integrate these peaks and to simulate baseline separation. Because [$^2\text{H}_8$] β -carotene can be baseline separated from unlabeled β -carotene, the rate of metabolism of these compounds would differ in biological systems such as humans. The shorter retention time of [$^2\text{H}_8$] β -carotene in a reversed-phase HPLC system suggests that it is more polar than unlabeled β -carotene, probably because the ^2H atoms are located to a large extent on methyl side chains of the β -carotene molecule (74). This could influence the rate of incorporation of β -carotene into mixed micelles and subsequently absorption from the gut. It is known that polarity affects the absorption of carotenoids. For example, lutein is more polar than β -carotene and has been estimated to be absorbed 5 times more efficiently (72).

Mass spectrometry

Compared with the NMR instruments discussed above, mass spectrometers are many orders of magnitude more sensitive. The limits of quantification are in the attomole range (1×10^{-18} mol) for accelerator mass spectrometers and in the femtomole range



(1×10^{-15} mol) for organic gas chromatography–mass spectrometry (GC-MS) and LC-MS instruments. Organic mass spectrometers are also highly selective detectors that can be used to distinguish carotenoids and retinoids on the basis of their molecular weight and fragmentation patterns. Furthermore, tandem mass spectrometry combines mass selectivity with fragmentation pattern selectivity, resulting in an even more selective detector of organic ions. These features allow mass spectrometers to be more selective and specific than ultraviolet (UV), visible (VIS), or infrared spectrophotometers. In addition, mass spectrometers are more sensitive than absorbance-based detectors. For example, van Breemen (81, 82) compared visible absorbance detection, electrospray, and atmospheric pressure chemical ionization (APCI) mass spectrometry during LC-UV/VIS-MS for the measurement of β -carotene and α -carotene and showed that electrospray and APCI mass spectrometry were up to 100-fold more sensitive than absorbance detection. Because mass spectrometers separate ions of molecules on the basis of m/z , this analytic instrument is ideal for the sensitive and selective detection and quantification of isotopically labeled carotenoids and retinoids. Furthermore, stable-isotope labeling is preferred over the potentially more hazardous radiolabeling.

Mass spectrometers designed for the analysis of organic molecules are widely available and permit the determination of molecular weights as well as fragmentation patterns. These include GC-MS, LC-MS, MS-MS, and LC-MS-MS instruments. Some specialized instruments used for the measurement of isotopic enrichment include AMS instruments, which are expensive and available at only a few facilities, and isotope ratio mass spectrometry (IRMS) instruments, which are more widely available than AMS instruments but still less common and more expensive than organic mass spectrometers. Both of these types of specialized mass spectrometers require highly purified samples and involve the combustion of organic compounds into carbon dioxide and water, thus losing structural information. The abundance of isotopically labeled carbon, oxygen, or hydrogen in these products may be measured at high precision. AMS instruments require the most careful and demanding sample preparation of any mass spectrometer and are intolerant of highly isotopically enriched samples, which tend to contaminate the laboratory and the instrument. IRMS may be used for a wider range of isotopic enrichment and unlike AMS can be interfaced to a GC system for online sample purification.

Because online GC-IRMS is more convenient than purifying a compound before IRMS, most studies involving isotopic enrichment of carotenoids and retinoids have used the former approach. Another advantage of GC-IRMS is that it is not necessary to know in advance which GC peak will contain the labeled compound. However, the selectivity of this approach is provided solely by GC, because all structural information is lost during combustion. Even though GC provides some separation and selectivity for GC-IRMS, the high temperatures during GC induce isomerization and extensive band broadening of carotenoids such as β -carotene, which results in a loss of sensitivity (59). Because carotenoids and retinoids do not separate efficiently during GC, HPLC is usually used to purify the peak of interest before GC-IRMS. Note that LC cannot be coupled to IRMS because the combustion products from the mobile phase would interfere with the analysis.

Despite the limitations of GC-IRMS, this technique has been applied to the measurement of the isotopic enrichment of carotenoids and retinoids. For example, Parker et al (46) developed

a labor-intensive and time-consuming method for carotenoids in which the *all-trans*- β -carotene fraction was saponified, purified by using HPLC, hydrogenated overnight to perhydro- β -carotene, and then analyzed by GC-IRMS. This method was used by You et al (48) to measure the degree of isotopic enrichment of 9-*cis*- β -carotene. In addition, this method was modified by Yao et al (47) by the addition of a hydrogenolysis step and was used to measure the degree of isotopic enrichment of lutein.

In a variation of the GC-IRMS approach for retinol analysis, Tang et al (61, 62) replaced the IRMS with an organic mass spectrometer equipped with electron capture negative ion chemical ionization. Although this type of mass spectrometer is much more widely available than the IRMS instrument, sample preparation was still complex and laborious, consisting of liquid-liquid extraction of the serum samples, followed by HPLC purification and then derivatization of retinol to form the trimethylsilyl ether. Finally, derivatized retinol and its octadeuterated analogue were monitored during GC-MS as an abundant fragment ion formed by elimination of the trimethylsilyl group. This method permitted the detection of 0.01% [$^2\text{H}_8$]trimethylsilyl-retinol in the presence of unlabeled trimethylsilyl-retinol. In addition to the low throughput and labor-intensive sample preparation, this approach increases the possibility of deuterium exchange and loss during ionization and fragmentation in the chemical ionization source of the mass spectrometer.

In a similar study of retinol, Furr et al (37) developed a protocol in which retinol and retinyl esters were extracted from serum samples by using hexane, purified by semipreparative HPLC, saponified, recombined, purified again by using another HPLC system, and then analyzed by GC-MS. Handelman et al (60) reported a more selective method in which a derivatization step was added. Edwards et al (25) developed another labor-intensive technique that required preparation of plasma triacylglycerol-rich lipoprotein fractions (67), hexane extraction (48), saponification, hexane extraction, HPLC purification of carotenoids and retinol (68), derivatization of retinol to form trimethylsilyl ether, and then analysis by GC-MS for retinol (25).

Dueker et al (59) developed a procedure to measure the isotopic enrichment of β -carotene in serum that avoided GC-MS and eliminated derivatization. However, serum had to be extracted twice, first by using an organic solvent and then by solid phase extraction. Then, 2 HPLC purification steps, one reversed-phase and the second normal phase, were necessary to remove interfering compounds before offline MS-MS analysis with positive ion electron impact ionization.

A logical simplification of the procedure used by Dueker et al (59) would be to carry out HPLC separation online with mass spectrometric detection and quantification, and ≥ 2 research groups have developed such LC-MS solutions. For example, we used reversed-phase HPLC with online APCI for the analysis of the degree of isotopic enrichment in β -carotene and in its metabolite isotopically labeled retinol (66). Also, Pawlosky et al (50) used HPLC with a particle beam mass spectrometric interface and negative ion electron capture ionization for the measurement of the degree of isotopic enrichment of β -carotene. Both of these methods used solvent extraction of serum without any derivatization followed by online LC-MS quantification. Furthermore, these LC-MS approaches used widely available organic mass spectrometers interfaced directly to the HPLC system, avoided the thermal degradation problems of gas chromatography, and prevented the loss of structural information and selectivity that would occur with



sample combustion during GC-IRMS. Unlike the method of Dueker et al (59), only a single extraction step and a single HPLC step were necessary, mass spectrometric quantitation was carried out online during HPLC, and ^{13}C labeling instead of ^2H labeling was used so that problems associated with loss or scrambling of the label were minimized.

In addition to APCI and particle beam LC-MS, we have evaluated electrospray LC-MS for the quantitative analysis of isotopically enriched β -carotene and retinol (65). Although electrospray LC-MS is highly sensitive for the detection of both β -carotene (83) and retinol (84), with detection limits similar to APCI and particle beam (≤ 1 pmol), we found that the dynamic range of this technique was insufficient for studies involving trace levels of isotope enrichment (84). For example, in studies of [$^{13}\text{C}_5$]retinol and [$^{13}\text{C}_{10}$]retinol in human serum, labeled and unlabeled retinol coeluted during reversed-phase HPLC, and the high proportion of unlabeled retinol suppressed the ionization of the labeled form. Furthermore, the electrospray standard curves for retinol (65) and β -carotene (66) were nonlinear.

In contrast with electrospray, APCI produces a linear detector response over ≥ 4 orders of magnitude for both retinol and β -carotene, and there was no evidence of ion suppression when measuring trace amounts of labeled compounds in the presence of a large excess of unlabeled material (66). For comparison, particle beam with negative ion electron capture detection was reported to show a linear detector response for β -carotene over a range of only 2 orders of magnitude. Thus far, the use of particle beam LC-MS has not been reported for the analysis of studies of isotopically enriched retinol in human serum. Thus, its application to the study of the bioconversion of β -carotene to retinol remains untested.

In summary, among the methods that have been reported for the determination of the bioavailability, bioconversion, and bioefficacy of β -carotene, organic mass spectrometry offers higher selectivity and greater availability than either AMS or IRMS. Additional advantages of organic mass spectrometry include faster, simpler, and less expensive sample preparation. Comparing the organic mass spectrometry techniques currently available, the online chromatographic techniques of GC-MS and LC-MS offer the greatest convenience, sensitivity, and speed. In particular, LC-MS stands out as the most practical technique because it eliminates the need for sample derivatization, avoids the possibility of thermal degradation of the sample, and requires only a single serum extraction step before analysis. By minimizing sample manipulation, the probability of introducing experimental error is also minimized. In addition, the efficiency and speed of sample preparation and subsequent LC-MS analysis are ideal for large numbers of samples from representative groups. Further refinements and improvements in this approach should involve the incorporation of MS-MS, ie, LC-MS-MS and LC-MS-MS to improve the selectivity of this APCI-based method. The use of more selective LC-MS-MS methods should also enable multiple carotenoids and their *cis* and *trans* isomers to be monitored during a single analysis.

Method validation and quality control

As outlined by Rodriguez-Amaya (85), the acquisition of reliable analytic data requires representative samples, validated analytic methods, quality assurance, adequately trained personnel, and ancillary support staff and facilities. The question is not only how good the method is, but also how well it is being used in the laboratory. The main performance variables that should be taken

into account in assessing any analytic method are as follows: accuracy, or closeness of the measured value with the true value; precision, a measure of the repeatability and reproducibility; specificity, the ability of a method to measure the compound of interest exclusively; the limit of detection, the lowest concentration of a compound that the analytic process can differentiate reliably from background concentrations (generally defined as 3 times the SD of the concentration measured in a substrate blank); the limit of quantification, the lowest concentration of a compound that can be measured with a stated degree of confidence (generally defined as 10 times the SD of the substrate blank); linearity, the range of concentrations over which the method has been shown to give a linear response; and the scope of the method, or the number of different substrates to which the method can be successfully applied (86).

Quality control consists of obtaining data of the appropriate fitness for their intended use. The quality of data are affected (in random order) by man, material, manipulation (in the sense of interpretation), machine, and method. The quality of data can be monitored by an effective quality-control program to prevent, identify, and correct errors. It is outside the scope of this article to explain this in much detail, but we stress the importance of using in-house control samples, of participating in interlaboratory trials, and of using certified reference materials for this purpose (85).

METHOD OF DATA ANALYSIS

Mathematical modeling

Modeling can be defined as the creation of a simplified representation of the world (87). When building models, Ockham's razor should be applied liberally. Ockham's razor states that the simplest explanation of an entity (here: β -carotene metabolism) is preferred (88). So, the number of pools and variables should be kept to the minimum required to reflect the behavior observed. For effective modeling, decisions should be made about which features of the real world will be included in the model and to what extent they need to be simplified. These decisions are based on the required function of the model, as well as on constraints imposed by our limited understanding of the world (87).

For studying carotenoid bioavailability and bioefficacy, 3 types of mathematical models have been described: compartmental models, area under the curve, and models based on plateau isotopic enrichment reached by multiple dosing (CarRet PIE). Novotny et al (51) developed one model with 5 compartments and one model with 3 compartments to estimate the dynamics of β -carotene metabolism, such as the absorption of β -carotene and the conversion of β -carotene to retinol, from data on plasma concentration-versus-time curves of labeled β -carotene and its metabolite labeled retinol. The compartmental models were based on several assumptions, including an assumption about the outcome parameter. Thus, to describe the absorption of dietary β -carotene, an estimate of the absorption of dietary β -carotene was included in the compartmental model. The need for such an assumption decreases the reliability of the final results. In Novotny et al's compartmental model, intestinal absorption was constrained to be inside the range of 2 SDs of $15 \pm 4.5\%$ on the basis of data presented by Bowen et al (18). In the original publication of this study (70), 2.6 mg (CV: 14%) of a 15-mg dose of β -carotene was absorbed in 11 healthy men with a mean age of 26 y. In a study of 4 females, Burri and Park (49) used the same



compartmental model as Novotny et al to conclude that β -carotene converts to retinol with a high interindividual variability. In the study by Lin et al (54), the fractional absorption of [$^2\text{H}_6$] β -carotene was calculated by using several assumptions. The assumptions used by Lin et al (54) seem difficult to justify considering the conflicting results of the study on bioavailability (6%) and bioefficacy (74%) of β -carotene. They calculated the fractional absorption of [$^2\text{H}_6$]carotene by using formulas developed by van Vliet et al (24) and data presented by Novotny et al (51), resulting in assumptions such as a plasma β -carotene half-life of 864 h derived from a mean sojourn time divided by 1.4.

The other single-dose studies (Table 2; 25, 46–48, 50, 52–54) plotted plasma or chylomicron concentrations of labeled compounds versus time. Those studies in which labeled retinol was also administered (52–54) can calculate and compare areas under the curves of plasma concentrations of labeled retinol derived from administered labeled retinol and of labeled retinol derived from administered labeled β -carotene. In the technique developed by Edwards et al (25), areas under the curves of chylomicron concentrations of labeled retinol derived from administered labeled retinol and of unlabeled retinol derived from administered β -carotene in spinach or carrot can be calculated and compared. If the isotopic purity of labeled compounds is low, the isotopic enrichment measured at various masses has to be summed, thus increasing the variation of the estimate of isotopic enrichment and decreasing the precision of the estimates of the area under the curves. A limitation of methods based on area under the curve is that these methods are influenced by further metabolism of carotenoids, such as clearance from serum and excretion of carotenoids and retinol (23), making such methods inappropriate for studying the bioavailability and bioefficacy of carotenoids quantitatively. As already discussed by van Vliet (10), single-dose studies cannot be used to study the effect of individual SLAMENGGHI factors on bioavailability and bioefficacy if those factors also affect the clearance of carotenoids or retinol from serum or plasma. SLAMENGGHI is a mnemonic for factors that affect the bioavailability and bioefficacy of carotenoids in humans. The factors are as follows: S, the species of carotenoid; L, molecular linkage; A, the amount of carotenoids consumed in a meal (*see* the section on size of dose); M, the matrix in which the carotenoid is incorporated (*see* the section on matrix of tracer); E, effectors of absorption (*see* the section on frequency of dosing); N, the nutrient status of the host; G, genetic factors; H, host-related factors; and I, mathematical interactions (21, 22).

The above-mentioned limitations of single-dose studies can be overcome by multiple dosing, during which plateau isotopic enrichment is reached, because such a regimen diminishes the variation in the estimates of bioefficacy and bioavailability. At the plateau level, the isotopic enrichment in plasma represents the ratio of the flow of labeled and unlabeled nutrients from the gut. If the duration and frequency of consumption of labeled and unlabeled compounds are sufficient, which can be tested in a design similar to that of our first study (7), it can be expected that this steady state also reflects a steady state at the slow-turnover tissue level and takes into account extrahepatic uptake and cleavage of absorbed β -carotene.

In our studies in which multiple doses of stable isotopes were used, data analysis was conducted by using the CarRet PIE mathematical model based on isotopic enrichment in serum of β -carotene with [$^{13}\text{C}_{10}$] β -carotene and of retinol with [$^{13}\text{C}_5$]retinol and with [$^{13}\text{C}_{10}$]retinol at plateau isotopic enrich-

ment (7, 55, 71). Although the appropriate studies have not been carried out, it would be possible to quantify the bioefficacy of β -carotene in fruit and vegetables provided that the intake of unlabeled retinol from food is sufficient to observe a sufficiently large dilution of the isotopic enrichment during the vegetable period compared with the control period. This would enable absolute quantification of the bioavailability and bioefficacy of β -carotene in fruit and vegetables and other categories of food. The narrow CI around the mean bioavailability and bioefficacy of β -carotene in oil indicates that the CarRet PIE model results in estimates with a high precision. For the quantification of the bioavailability of β -carotene, this model requires an assumption about the stoichiometry of cleavage of β -carotene into retinol. Like Edwards et al (25), we used the most efficient stoichiometry possible, ie, 1 mol β -carotene yields 2 mol retinol, for our estimates of bioavailability, thus obtaining estimates of the minimum absorption. The use of a less efficient stoichiometry, eg, a 1:1 molar equivalent, would result in a higher estimate of the bioavailability. In addition, it was assumed that labeled and unlabeled β -carotene fully mix and that both compounds are metabolized in the same way but not necessarily to the same extent.

Statistical analysis of data

In making estimates of bioavailability and bioefficacy, it is necessary to provide a measure of precision related to the biological and analytic variation. Unfortunately, many of the studies carried out to date have been limited to few subjects and sometimes only one subject, rendering such studies difficult to evaluate. As mentioned in the section on defining the aim and hypothesis, the appropriate statistical tests to evaluate the results of the study should be applied.

CONCLUSIONS


Several isotope techniques have been developed to study the bioavailability and bioefficacy of dietary carotenoids in humans. From these studies, several conclusions can be derived. First, in studies using isotope techniques it has been shown that β -carotene is converted to retinol in humans (7, 25, 46, 48, 49, 51–55). Second, the bioavailability of β -carotene and of other carotenoids with or without provitamin A activity—such as 9-*cis*- β -carotene (48) and lutein (47), respectively—can be studied with the use of isotope techniques. Third, quantitative data on carotenoid bioavailability and bioefficacy obtained by using isotope techniques are currently limited. In fact, reliable estimates of the bioavailability and bioefficacy of β -carotene in oil have been derived only from our 2 studies (7, 55). It can be concluded from our second study in Indonesia that the absorption and conversion of β -carotene from pumpkin are both 1.7 times more efficient than those of β -carotene from spinach (55, 71). At this point in time, no reliable estimates derived from isotope techniques on the absolute bioavailability and bioefficacy of carotenoids in fruit and vegetables are available. It is envisaged that increased understanding of carotenoid metabolism will enable further development of the design and mathematical models required for obtaining such absolute data.

Fortunately, many of the technologic problems affecting earlier isotope studies have been overcome by the increased sensitivity of GC-MS and LC-MS methods. Of these 2 methods, the LC-MS methods have the additional advantage that they do not require labor-intensive sample preparation. This will be essential in the



analysis of large numbers of samples arising from studies with adequate power. LC-MS with APCI has emerged as the most effective and convenient technique for studying the bioavailability, bioconversion, and bioefficacy of provitamin A carotenoids.

In our opinion, the use of mathematical models requiring assumptions that are difficult to justify should be reconsidered. In addition, single-dose studies have led to the analysis of data from measurements approaching the limit of detection. In contrast with the case in studies of mineral metabolism, it will be difficult to overcome the problems hindering the intravenous administration of β -carotene and retinol because of their lipophilic nature.

In conclusion, considerable progress has been made in the past decade to enable carotenoid bioavailability and bioefficacy to be studied with the use of isotope techniques. This approach should now be used to quantify the bioavailability and bioefficacy of other provitamin A carotenoids and to quantify the extent to which individual SLAMENGI factors affect the bioavailability and bioefficacy of carotenoids under various conditions. In addition, some of the principles developed can be applied in studies of the bioavailability and bioefficacy of other micronutrients such as folic acid. 

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