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Carotenoid and retinoid metabolism: insights from isotope studies☆

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Abstract

Use of isotopes as tracers has had an important role in elucidating key features of vitamin A and retinoid metabolism in animal models and humans. Their use has shown that β-carotene absorption is variable, and that the appearance of β-carotene and its metabolites in the blood by time since dosing follows characteristic patterns. Retinol formed from β-carotene shows a different pattern, as does lutein. In this article, we summarize and discuss insights and some surprises into the absorption and metabolism of vitamin A, β-carotene, and lutein that were gained with the use of isotope tracers in humans, rats, and cells as models.
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Keywords: Carotenoid; Isotope; Human; Rat; β-Carotene; Vitamin A; Lutein

Vitamin A (retinol and retinyl esters) is an essential nutrient that is necessary for normal cell growth and differentiation, immunological functions, and vision [1–5]. β-Carotene is the major source of vitamin A (retinol), as well as having antioxidant properties and immunological functions [6–8]. β-Carotene is the best known of the large carotenoid family, which also includes lutein. Lutein is concentrated in the macula, and appears to be necessary for optimal macular function and eyesight [9]. We will summarize select studies that used isotopic tracers and provided insights, and occasional surprises, into the absorption, metabolism, and excretion of retinol, retinyl esters, β-carotene, and lutein.

Vitamin A metabolism

Vitamin A (retinol and its metabolite retinoic acid) has important connections to genetics [10–14]. For example, a variety of retinoid receptors (the RXR,[1] RAR, and RARE receptors) have been identified in the nucleus, and retinoic acid as well as other retinoids is critical to the proper development of the *X–Y* axis of development. Many of its basic genetic mechanisms have been identified [10–14] and are being elucidated. Several excellent reviews on retinoid metabolism are available [14,15]. Much of what is known about the absorption and metabolism of retinoids as they might occur in vivo in humans is based on the isotope studies conducted by Sauberlich et al. [16]. The reason for this is that retinoid concentrations are highly regulated, and are stored in inaccessible human tissues such as the liver [14,15]. Furthermore, much time is needed for administered isotopes to fully equilibrate with body pools of retinoid that turn over slowly; so developing suitable protocols is not trivial [14–17].

In the past 20 years, many methods have been developed for using stable and radioactive isotope methods to trace retinoid metabolism and status in humans and rodents [17–58]. Almost all studies in rodents have used radioactive isotopes, which allow scientists to

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[1] *Abbreviations used:* AMS, accelerator mass spectrometer; AUC, area under curve; CRBP, cellular retinol binding protein, RXR, retinoid X receptor; RAR, retinoic acid receptor, and RARE, retinoic acid response element, VLDL, very low density lipoprotein.

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identify and measure metabolite concentrations [36–46]. Isotopes have been administered to rats by oral [30,31,39], intravenous [46], and gastric [47] routes.

Generally, most human study protocols have involved oral administration of retinoids and carotenoids suitably labeled with stable isotopes [48,50] whereas rat protocols have used radioactive isotopes exclusively [35–40,42–47,59]. Most labeled retinoids and carotenoids have been fed to humans as solutions (or suspensions) in oil [6,48,51,52]; although a few study protocols have involved intravenous injection of isotope labeled carotenoid/retinoid to bypass the intestine [16] or lymph cannulation of surgical patients [49,50]. All protocols, human or animal, used chemically and isotopically pure test compounds, instead of intrinsically labeled compounds in foods.

The kinetic behavior of retinol metabolism in the blood appears to be similar for humans and rats. Generally when purified retinyl acetate is fed a single large peak appears in the blood [16,30,31,39,51–53]. The peak first appears about 4–8 h post-dose for humans [16,51,52]. It may appear a little sooner in rats [31,39,53]. The peak reaches its maxima between 12 and 24 h [51,52] and is observable in the blood for many weeks after it appears. Half-lives are very long, ranging from 75 to 241 days for men fed a vitamin A deficient diet [16] and 56 to 243 days [54–57] for men and women fed vitamin A adequate diets. Kinetic profiles of retinol metabolism have been developed for the rat [36–39]. The model developed for humans, which is based on much less evidence, appears to be similar [54,57].

Among the studies aimed at determining the yield of vitamin A from β-carotene, much of the administered carotene isotope is excreted within 48 h [16,54–58]. The main excretion route is through the feces, where 20–60% of the dose of labeled carotene appears [16,54–58]. Urinary excretion accounts for another 10–30% [16,55,56]. There is very little information about other routes of excretion, though these routes clearly exist [16]. For example, retinoids are excreted in the breath; where about 10% of the dose appears [16]. The extent to which retinoids are excreted in saliva, sweat or tears is unknown even though both retinoids and retinoid transport proteins are present in tears [60].

Retinoid nutritional status assessment

Retinol status has been difficult to assess using non-isotopic methods, because its serum concentrations are tightly regulated and $\geqslant 90\%$ of its body stores are in inaccessible tissues such as liver and kidney [1–5,14,15,61]. Only small changes occur in response to changes in intake, so serum retinol concentrations provide uncertain estimates of retinol status except in severe deficiency or toxicity [2,61]. Although the modified retinol dose–response test has been used with some success in the field to estimate retinol status [62–66], isotopic methods could become the method of choice for estimating retinol status. Considerable efforts have been made recently to develop equations based on isotope dilution data that will enable liver vitamin A stores to be estimated so that it can serve as a measure of total body vitamin A stores [30,31,34,39,46,52,67–73].

Vitamin A nutritional status appears to have significant effects on retinoid metabolism [16,51,56]. People with higher retinol status appear to absorb retinol more efficiently than people with low retinol status. The amount of retinyl acetate isotope that is absorbed is highly and positively correlated to their retinol liver stores [16,51,56]. Furthermore, in a report of an individual with low retinol status who was supplemented with retinol and achieved normal status, retinol absorption improved after supplementation [51]. Dietary vitamin A intake also appears to influence retinol metabolism. Very low intakes of retinol appear to reduce (rather than increase) retinol utilization, even when retinol stores are still adequate [16]. These results appear to be counterintuitive because it would seem that individuals with lower nutritional status with respect to retinol should absorb and utilize it more efficiently. However, retinol absorption and elimination is highly variable among individuals, and some children develop vitamin A deficiency while consuming marginal amounts of vitamin A and others do not [74,75]. Body stores of vitamin A represent a balance between its absorption and elimination, processes that may not be well coordinated with one another. Therefore, some well-nourished individuals may have relatively low retinol stores because they absorb less, or excrete more, retinol than individuals with larger stores. Similarly, a marginally nourished child becomes vitamin A deficient while a comparably nourished one that absorbs and/or stores vitamin A better does not. Therefore, people with lower retinol status may well absorb retinol less well than those with higher retinol stores.

Although retinoids are key essential nutrients they are not widely dispersed among foods. So, conserving retinol in times of scarcity might be advantageous and it is possible that biologic systems have developed feedback mechanisms that conserve and thus reduce utilization of this vital nutrient in times of scarcity. This type of feedback conservation mechanism can easily explain why dietary retinoid depletion decreases retinoid utilization even when body stores of retinol are still high.

Factors influencing absorption and excretion of retinol

There are obvious limitations to the data, since so few people or animals have been fed isotopes. However, there is some evidence that gender, race, and body composition do not have a strong influence on vitamin A metabolism in humans [51,52]. There are a few reports suggesting that age may influence retinol metabolism. For example, older rats also appear to absorb more

retinol than younger ones [46]. Children suffering from respiratory disease appear to metabolize retinol faster than adults [52,76]. Furthermore, respiratory infections and fevers appear to increase retinol utilization and excretion [74,75,77,78]. The principal cause appears to be impaired mobilization from liver as a result of reduced synthesis of RBP during the acute phase response. Serum retinol may also drop as a result of irreversible loss of retinol via urine during severe infections when tubular re-absorption of RBP is impaired.

A possible effect of the size of the bolus dose on the kinetic behavior of vitamin A has not been adequately studied. Including fat with the dose aids absorption [14,15], but direct comparison of the effect of fat type or quantity upon retinoid absorbed has not been extensively studied. Olive oil and coconut oil both appear to aid carotenoid absorption.

In summary, results from isotopic studies show that vitamin A is usually well absorbed, and displays characteristic kinetics in most studies. A representative kinetic profile from a subject fed a bolus dose of tetradeuterated-retinyl acetate is shown in Fig. 1. Retinol (as retinyl ester) is stored almost exclusively in liver, and appears to be metabolized very slowly, with half-lives of about ≅20 weeks. The reasons for the large differences in metabolic half-life are unknown, but further studies on retinoid metabolism using isotopes may identify the reason for these differences.

β-Carotene metabolism

Much less is known about carotenoid absorption and metabolism compared to that of retinoids. Furthermore,

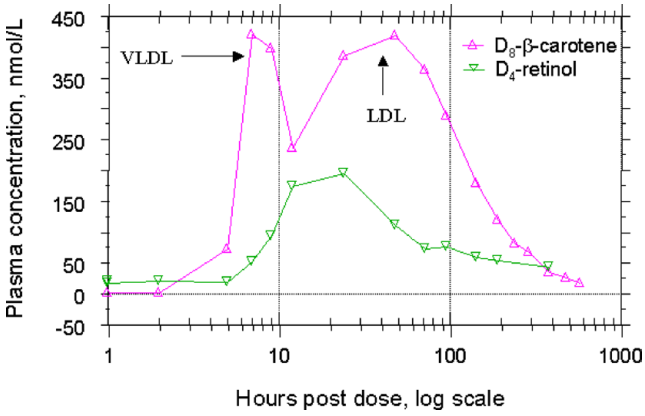

Fig. 1. Pattern of D_4-retinol in plasma from an oral dose of D_4-retinyl acetate in oil. Pattern of D_8-β-carotene in plasma from an oral dose of D_8-β-carotene in oil. Appearance, peak maximum, and disappearance from blood of a 20 mg (60 μmol) oral dose of [^{2}H]retinyl acetate (tetradeuterated or D_4 retinyl acetate) in olive oil. The figure also shows the appearance, peak maxima, and disappearance from blood of a 37 mg (68 μmol) oral dose of [^{2}H]β-carotene (octadeuterated or D_8 β-carotene) in olive oil.

almost all isotope research has centered on β-carotene and especially on the formation of vitamin A from β-carotene, almost to the exclusion of other carotenoids, and other functions.

Most carotenoid isotope studies have been conducted in the past decade; though there are a few notable exceptions (see [16,49,50,79]). Both stable and radioisotope methods have been developed [49,79–86]. Carotenoid study protocols usually involved oral feeding of chemically pure forms often labeled with isotopes [16,81–86], even though carotenoids occur naturally in plants bound to a protein as an intrinsic part of a food matrix. Therefore, care is needed in interpreting the results. Kurilich et al. [87] recently studied kale whose carotenoids were intrinsically labeled with ^{13}C. Virtually all of the carbons in the kale were labeled by growing it in the presence of $^{13}CO_2$. This novel experimental protocol has already yielded valuable data on the metabolism of β-carotene from vegetables. They found that whereas plasma [^{13}C]β-carotene peaked at 8 h and again at 24 h after dosing, [^{13}C]lutein peaked in plasma only once and at a later time, 11 h. The double peak is characteristic of β-carotene as can be seen from our work (Figs. 1, 2, 4, and 5) and those of others [16,81,82]. The [^{13}C]retinol that was derived from [^{13}C]β-carotene peaked at 24 h, presumably after being handed off from chylomicrons and very low density lipoproteins (VLDL) to retinol binding protein (RBP) in liver as can be seen in Fig. 2. Other foods that are of interest to carotenoid researchers and that might be amenable to labeling as described by Kurilich et al. [87] include carrots, sweet potatoes, or tomatoes.

The absorption and bioavailability of β-carotene, and the amount of vitamin A derived from it (its retinol equivalence) in humans and rats [81,82,85,86,88,89] are variable. Part of the variability reported in the literature is a result of the demanding methodology necessary for these measurements. Retinyl esters formed from β-carotene appear in serum at about the same time as β-carotene itself appears, and these retinyl esters were virtually impossible to identify and measure prior to the availability of sensitive radioisotope tracers. Furthermore, the retinol equivalence of β-carotene are sometimes estimated from the integrated areas under the concentration curves (AUCs) of β-carotene, retinyl ester, and retinol in serial blood. AUC models are handicapped because β-carotene, retinyl ester, and retinol can undergo further metabolism, can leave blood for tissues, and can be irreversibly lost via urine, outcomes that cannot be measured easily by this method [88]. However, even when these difficult methodological issues are solved, variability remains high [55,56]. Reasons for the variability might include environment, food matrix effects, genetic factors, and/or pathologic conditions [89–91].

Tracer studies can facilitate the understanding of human metabolism. Accelerator mass spectrometry

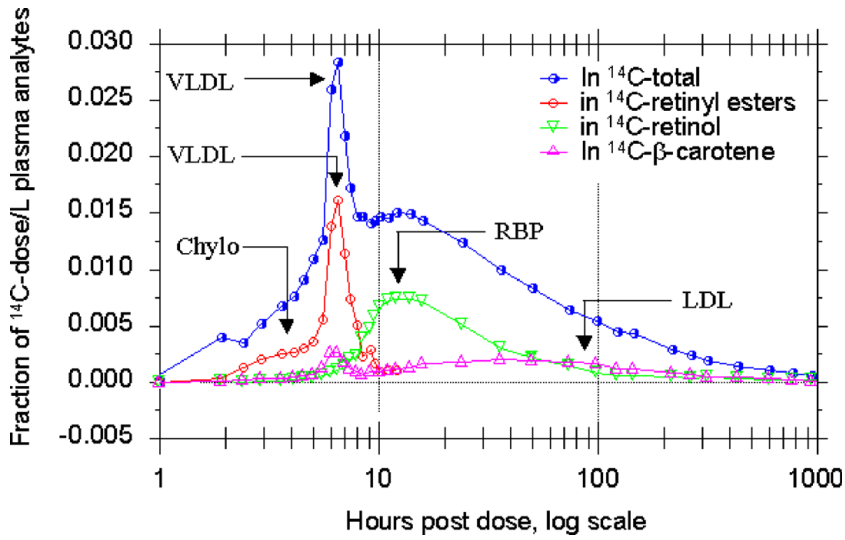

Fig. 2. Patterns of ^{14}C in plasma following a small (1 nmol) oral dose of [^{14}C]β-carotene in an oil-based banana smoothie to an adult woman (*subject 02*). The ^{14}C in plasma that is associated with the labeled retinyl esters, retinol, and β-carotene fractions accounts for about one-half of the total radioactivity. The remainder is associated with yet-unidentified carotenoid and retinoid metabolites, possibly epoxides, apo-carotenals, and retinoic acids.

(AMS) is an isotope ratio instrument that measures the ratios of long lived radioisotopes ($^{14}C/^{12}C$) in isolated chemical fractions for biological and chemical research to attomol levels (10^{-18}) in milligram-sized samples. The detection sensitivity and small sample size requirements of AMS make it ideal for human nutrition studies [55,56].

Recent studies using accelerator mass spectrometry

A distinct benefit of an AMS approach over more conventional isotope methods is the ability to measure very low levels of total ^{14}C, and thus to track minor metabolites for long periods [92,93]. Recently we [56] described the effect of vitamin A nutritional status (adjusted with vitamin A supplementation) on the β-carotene metabolism of two healthy adult women using AMS. We fed small (0.5–1.0 nmol) doses of [^{14}C]β-carotene that were emulsified with olive oil (0.5 g/kg body weight), 300 g of fresh banana, 100 mL skim milk, and 12.6 g of sucrose in a banana 'milk-shake.' Each individual was given the isotope tracer twice. The women ate their normal diet before their first isotope dose and for 46 days afterward while their blood samples were collected. Seven weeks after this first dose, the women began taking a vitamin A supplement that supplied 3000 RE (10,000 IU) retinyl palmitate per day. The women consumed this vitamin A supplement for 21 days, and then they were given their second isotope dose. They continued to consume vitamin supplements containing 3000 RE vitamin A per day for 14 days after the second dose, and then their vitamin A supplement was decreased to 1500 RE (5000 IU) retinyl palmitate per day for the remainder of the study. Concentrations of [^{14}C]β-carotene, [^{14}C]retinyl esters, and [^{14}C]retinol in plasma were measured during 46 days after the first dose and 56 days after the second. Feces were collected for two weeks and urine for one month after each dose. The AMS detection limits for plasma, urine, and feces were 0.4, 0.04, and 0.12 fmol [^{14}C]β-carotene/mL, respectively, based on double the uncertainty in the background. These respective concentrations correspond to absolute detection limits of 0.008, 0.004, and 0.006 fmol for [^{14}C]β-carotene. The pattern of ^{14}C in plasma is shown in Fig. 2. It shows the total radioactivity as well as that which occurs as intact [^{14}C]β-carotene, [^{14}C]retinol, and [^{14}C]retinyl esters. The sum of the radioactivity in intact [^{14}C]β-carotene, [^{14}C]retinol, and [^{14}C]retinyl esters accounted for ≅70% of the total radioactivity. Therefore, there are additional and as yet unidentified ^{14}C-labeled carotenoid/retinoid metabolites that we are trying to isolate, identify, and quantify by taking advantage of the exquisite sensitivity AMS.

The [^{14}C]retinyl esters (derived from the [^{14}C]β-carotene dose) appeared in plasma as two peaks, one at ≅4 h the other at ≅6.5 h after dosing. We hypothesize that the first [^{14}C]retinyl ester peak (that appeared at ≅4 h) was associated with chylomicrons while the second [^{14}C]retinyl ester peak that appeared at ≅6.5 was associated with a mix of chylomicrons and very low density lipoproteins [56]. The integrated area under the curve (AUC) for both [^{14}C]retinyl ester peaks can serve as a relative measure of the yield of vitamin A from β-carotene [81,82].

The [^{14}C]β-carotene appeared in plasma also as two peaks, one at ≅6.5 h the other at ≅50 h. We hypothesize that the first[^{14}C]β-carotene peak (that appeared at ≅6.5 h) was associated with a mix of chylomicrons and

very low density lipoproteins (VLDLs) while the second [^{14}C]β-carotene peak that appeared at ≅50 h was associated with low density lipoproteins (LDLs). The integrated area under the curve (AUC) for both [^{14}C]β-carotene peaks can serve as a relative measure of the bioavailability of the [^{14}C]β-carotene. The ratio of the AUC for both [^{14}C]retinyl ester peaks to the AUC for both [^{14}C]β-carotene peaks can also serve as a relative measure of the yield of vitamin A from β-carotene.

Loss of [^{14}C]β-carotene and its metabolites after an oral dose of [^{14}C]β-carotene is shown in Fig. 3. Almost 40% of the administered [^{14}C]β-carotene appeared to be excreted rapidly in the feces, apparently unabsorbed. The remainder is apparently absorbed, and subsequently excreted more slowly in the urine and stool. Because the exquisite sensitivity of AMS enabled the loss of ^{14}C in feces to be quantified, the AUCs described above can be normalized for the amount of [^{14}C]β-carotene absorbed.

Using AUCs and irreversible losses of ^{14}C in feces and urine as described in the paragraphs just above, we [56] determined the effect of vitamin A nutritional status on the bioavailability of [^{14}C]β-carotene and yield of [^{14}C]vitamin A from it. To our surprise, the yield was 0.54 mol [^{14}C]vitamin A from 1 mol of [^{14}C]β-carotene before supplementation, and 0.74 mol [^{14}C]vitamin A after it, indicating that vitamin A supplementation resulted in more vitamin A being formed from β-carotene. We also found that while there was less cleavage of [^{14}C]β-carotene to vitamin A upon supplementation, it was more than compensated for by a higher absorption of [^{14}C]β-carotene [56]. Although our dataset included only two subjects, our yields of vitamin A from β-carotene matched those of other investigators [7,8,71,83–85,94] except for one [95] that reported higher values.

The higher yield that accompanied vitamin A supplementation seems unexpected at first. However, that may be because we tend to focus on the relevance of β-carotene for human health, to the exclusion of its other functions [96]. Thus, we typically think of β-carotene as being a precursor for retinol only, whereas β-carotene has many functions, even the potential to be derived from retinoids. β-Carotene is critical to normal embryonic development, growth, and reproduction of birds and fish, irrespective of its ability to form vitamin A [97–100]. Furthermore, it is well known that β-carotene conversion to retinol can be very poor [2,15,17,48,86,101], which would be surprising if its role as a precursor for vitamin A was of central importance. Therefore, a less restrictive view of retinoid and carotenoid metabolism suggests that retinoid status can influence carotenoid metabolism, that carotenoid status can influence retinoid metabolism, and that carotenoid status can even influence carotenoid metabolism as we [100] have recently shown. Carotenoid depleted chicks have markedly diminished incorporation of yolk- and diet-derived carotenoids into tissue carotenoids [100].

Studies have shown that β-carotene is absorbed better when it is provided with fat or in a fatty meal [7,15,61,101], but little specific information is known about this relationship. Patterns of ^{14}C in plasma from an oral dose of [^{14}C]β-carotene provided to the same subject in a bolus dose with oil, and without oil, are shown in Fig. 4. In the absence of fat, the first [^{14}C]retinyl ester peak at ≅3.5 h after dosing (which we associate with chylomicrons) is quite small, while the second [^{14}C]retinyl ester peak at ≅6.5 h after dosing (which we associate with a mix of chylomicrons and VLDL) is unaffected. The appearance of the [^{14}C]β-carotene plasma peak is also smaller in the absence of fat. Bile fat may be adequate for uptake of carotene that is cleaved to vitamin A in the enterocyte, but insufficient for maximum plasma carotene levels. Thus, it appears

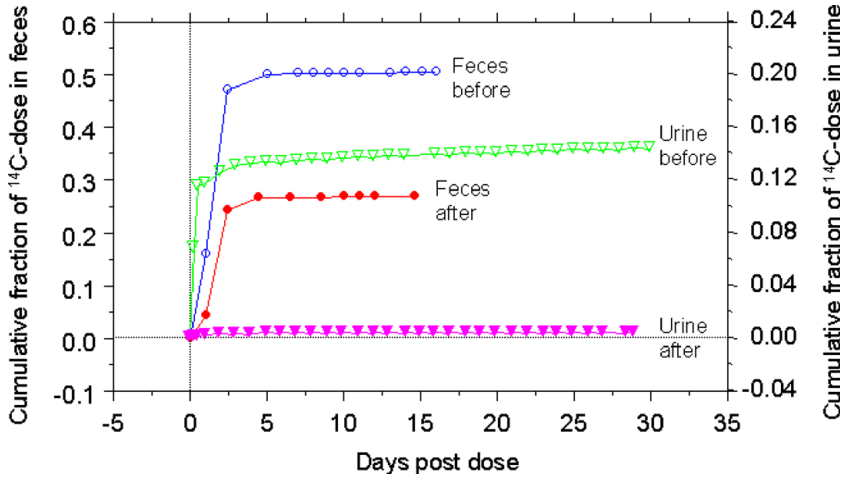

Fig. 3. Loss of ^{14}C in feces and urine following a small (1 nmol) oral dose of [^{14}C]β-carotene in an oil-based banana cocktail to an adult woman (*subject 02*) before and after retinol supplementation. Almost 40% of the administered [^{14}C]β-carotene appears to be excreted in the stool, apparently unabsorbed. The remainder is absorbed, and then excreted more slowly in the urine and stool.

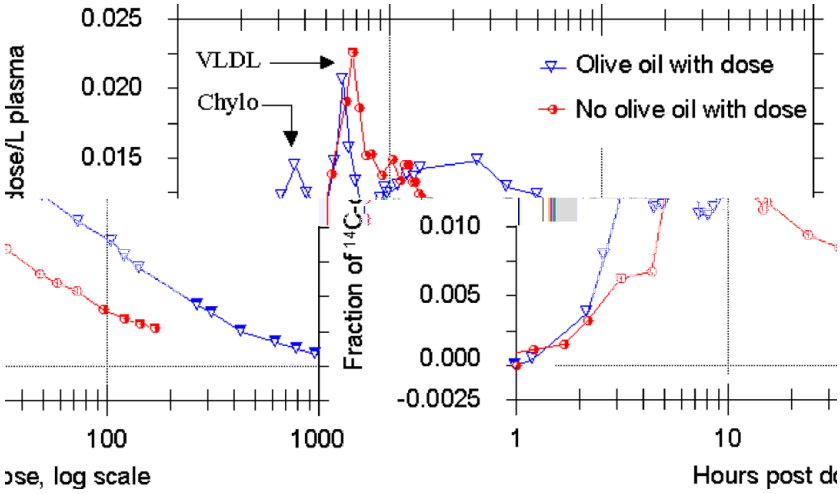

Fig. 4. Patterns of ^{14}C in plasma following a small (1 nmol) oral dose of [^{14}C]β-carotene in an oil-based banana cocktail and 20 weeks later a repeat dose in an oil-free banana cocktail to the same adult woman (*subject 06*). In the absence of oil the chylomicron retinyl ester peak at 2–3 h is small, the VLDL retinyl ester peak at 6–7 h is unaffected, and the β-carotene peak is smaller also.

that adding fat to the bolus dose may change the absorption route of [^{14}C]β-carotene, so that absorption is faster and more efficient as suggested also by Borel [102].

β-Carotene is thought to diffuse from mixed micelles in the small intestine through the unstirred water layer into the enterocyte [103]. Upon crossing the gut wall, β-carotene is either cleaved to vitamin A and related metabolites, returned to the intestinal lumen via bile, or absorbed intact into circulation with lymph chylomicrons. Most scientists believe that β-carotene uptake is by passive diffusion. This belief is based on radioisotope work in rats [43–45,104]. This important research was conducted at a time when isotope studies in normal humans were not feasible and much less basic information about carotenoids was known. Rats are 'white' fat animals; while humans have 'yellow' fat because they store carotenoids in fat. Therefore, we know that studies on carotenoid absorption and metabolism in rats may not mimic that process in humans.

Carotenoid absorption mechanisms

The mechanism of carotenoid absorption may need to be re-examined for several reasons. First, some carotenoids are accumulated and stored selectively in tissues, lutein in macula [9], lycopene in prostate [105], and β-carotene in bovid corpus luteum [106]. The mechanisms for the selectivity might involve selective absorption and transport processes. Second, human cell lines, such as $CaCo_2$ cells, appear to absorb β-carotene by active mechanisms [14]. Third, after a single dose of [^{14}C]β-carotene, the isotope appears in the blood in a

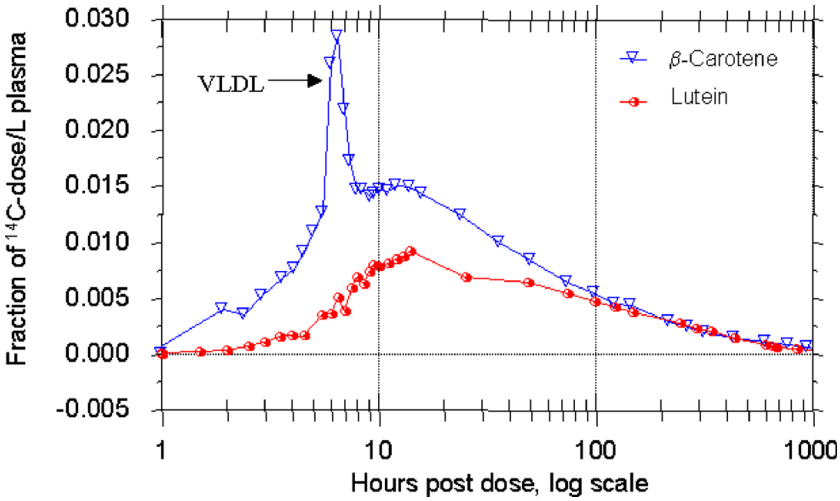

Fig. 5. Patterns of ^{14}C in plasma from oral [^{14}C]β-carotene (1 nmol) and, 20 weeks later, [^{14}C]lutein (125 nmol) each in an oil-based banana cocktail to the same adult woman (*subject 02*). The ^{14}C from lutein does not show peaks in the region of plasma chylomicrons at 2–3 h or VLDL 6–7 h.