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Kinetics and interrelation of [beta]-carotene and canthaxanthin transport in human plasma lipoproteins

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Kinetics and interrelation of β-carotene and canthaxanthin transport

in human plasma lipoproteins

by

Inke Paetau

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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has met the dissertation requirements of Iowa State University

Major Professor

For the Majór Department

For the Graduate College

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1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is divided into four sections. The first section is a general

introduction providing background information on the occurrence and functions of

carotenoids in both the plant and animal kingdoms. The introduction addresses aspects of

carotenoid research and emphasizes intestinal absorption of carotenoids and lipid

metabolism in greater detail. The second section of the dissertation is a manuscript

prepared for submission to the *American Journal of Clinical Nutrition*. The paper

describes the distinct kinetics of appearance of β-carotene (β,β-carotene) and

canthaxanthin (β,β-carotene-4,4'-dione), a model oxycarotenoid, in human plasma and

plasma lipoprotein fractions after ingestion of an oral dose. The methods used to isolate

lipoprotein fractions and to verify their purity by sodium dodecyl sulphate polyacrylamide

gradient gel electrophoresis (SDS-PAGE) are described. Relations of concentrations of

cholesterol and concentrations of predominant carotenoids, retinol, and α-tocopherol in

total plasma as well as in plasma lipoprotein fractions are discussed. Section three is a

manuscript prepared for submission to the *American Journal of Clinical Nutrition*. The

paper describes the interactive effects of concurrent ingestion of two carotenoids, β-

carotene and canthaxanthin, on the kinetics of these carotenoids in human plasma and

plasma lipoproteins. The fourth section is a general summary of the findings of both

papers and conclusions that can be derived. Cited references are included at the end of

each section. An appendix follows as section five.

Natural Occurrence and Biological Activity of Carotenoids

Carotenoids are a class of lipophilic natural pigments widely distributed throughout

the plant and animal kingdoms. In plants, they play essential light-harvesting roles during

photosynthetic events and protect membranes against photooxidative damage by bright

light [1, 2]. Carotenoids are responsible for the striking colors of many yellow, orange,

and red fruits and vegetables. A well-established biological function of carotenoids with at

least one unsubstituted β-ionone ring in animals is to serve as vitamin A precursors [3].

Carotenoids can be divided into hydrocarbon carotenoids, carotenes, and oxygenated

derivatives, oxycarotenoids or xanthophylls. The best known carotene is β-carotene (Fig.

1-1) which is the orange pigment with provitamin A activity in carrot roots. Canthaxanthin

(Fig. 1-2), the 4,4'-diketo analog of β-carotene without provitamin A activity, occurs

naturally in the edible chanterelle mushroom *Cantharellus cibarius*, in crustacea, salmon,

and the plumage of birds, including the pink flamingo [1]. The location within tissues and

the functional properties of carotenoids are determined by the chemical and physical

properties of the molecules. Carotenoids are located in lipophilic regions in the cell, such

as lipid globules, crystalline structures, and inner membranes [1]. In general, carotenoids

are polyisoprenoid compounds with a long chain of conjugated double bonds in the central

portion and modifications such as a six-membered ring at both ends of the molecule, e.g.

β-carotene. Lycopene, the open-chain structural isomer of β-carotene, is an exception.

The polyene chain gives these compounds their distinctive molecular shape, chemical

reactivity, and light absorbing properties [4].
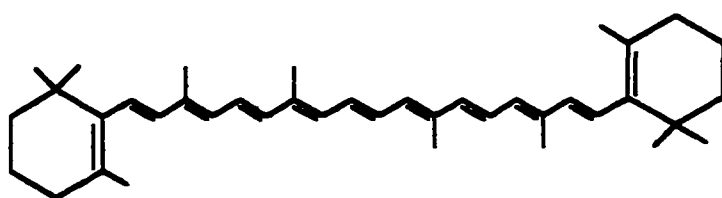
Figure 1-1. Structure of all-*trans* β-carotene.
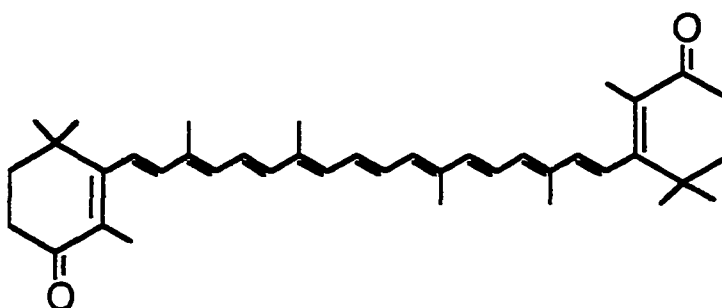
Figure 1-2. Structure of all-*trans* canthaxanthin (β,β-carotene-4,4'-dione)

The protective role of carotenoids in tissues has been attributed to their antioxidant

activity. In the etiology of many chronic diseases, including cancer, free radical-induced

damage is implicated. Beta-carotene is an effective antioxidant at low O_2 tensions (< 20

torr), such as found in mammalian tissues, but at higher (supraphysiological) O_2 tensions it

can act as a pro-oxidant [5, 6]. Singlet molecular oxygen ($^1O_2$) is an electronically excited molecule generated by photochemical reactions, or enzymatically, or by lipid peroxidation of biomembranes, which can be quenched (inactivated) by β-carotene [7, 8] and other carotenoids [5]. Carotenoids may also play a role in free radical reactions. Lim et al. [9] demonstrated the ability of xanthophylls (canthaxanthin, zeaxanthin, and astaxanthin) to act as chain-breaking antioxidants in the peroxidation of membranous phospholipids. Canthaxanthin inhibits peroxidation in liposomes, possibly by quenching singlet oxygen and other free radical species [10, 11].

Carotenoids in Human Health

Generally considered to be nontoxic, β-carotene has been applied as a colorant in foods, cosmetics and drugs. Its safety has been reviewed by Bendich [12]. Innocuous hypercarotenemia may occur in individuals taking more than 30 mg β-carotene per day, e.g. in patients treated for erythropoietic protoporphyria and other photosensitivity skin disorders [13, 14]. Canthaxanthin has been used as food colorant, oral tanning agent and has also found some medical application in the treatment of certain photosensitivity skin disorders [15]. In contrast to β-carotene, ingestion of large doses of canthaxanthin over prolonged periods can result in crystalline retinopathy [16] which reverses on discontinuation of canthaxanthin ingestion [17].

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Carotenoid research in relation to human health gained immense interest when epidemiological studies indicated that increased intake of vegetables and fruits, which contain high concentrations of carotenoids, and elevated blood concentrations of β-carotene were associated with reduced risk of some types of cancer, especially lung cancer [18-23]. It is not clear if β-carotene or other prominent carotenoids are responsible for the protective effects observed in epidemiological studies or if the carotenoids are simply indicators for recent vegetable and fruit intake [24], and thus for other potential protective phytochemicals or for low-fat diets or other healthy lifestyles. It is speculated that carotenoids may act as antioxidants preventing free radical and reactive-oxygen induced tissue damage implicated in carcinogenesis.

Carotenoids other than β-carotene may be responsible for observed protective effects. Despite high cigarette smoking rates, lung cancer incidence is lower on the Fiji islands compared to other countries of the South Pacific with comparable smoking rates [25]. In this study, intake of the oxycarotenoid, lutein (β,ε-carotene-3,3'-diol), was found to explain 14% of the variability in incidence. Increased consumption of dark-green, leafy vegetables was inversely related to lung cancer incidence [25, 26]. These commodities are good sources of lutein as well as of β-carotene. A study conducted in Italy showed a protective effect of raw tomato intake against cancers of the digestive tract [27]. The prominent carotenoid in tomatoes is lycopene which has no provitamin A activity but has been shown to have strong antioxidant activity [5]. In a recent study by Giovannucci et al. [28] it was found that lycopene was the only carotenoid intake that had a significant inverse trend with risk of prostate cancer. Overall intake of fruits and vegetables was not

related to risk of prostate cancer, and tomato-based food products were the only high-carotenoid foods related to risk with tomato sauce having the strongest inverse association.

There is also evidence that the risk of other age-related degenerative diseases, such as cardiovascular disease, immune-system decline, and cataracts is increased in populations with poor antioxidant status and low intake of fruit and vegetables [29-34]. The Eye Disease Case-Control Study [34] demonstrated a significant 43 % reduction in risk for age-related macular degeneration (AMD) among people in the highest quintile of carotenoid intake compared with those in the lowest quintile. Among specific carotenoids, the strongest association with a reduced risk for AMD was found for lutein and zeaxanthin which are primarily obtained from green, leafy vegetables. In terms of food items, a high intake of spinach or collard greens was associated with a lower risk for AMD. These findings are particularly interesting considering that lutein and zeaxanthin are selectively accumulated in the retina from the plasma. Lutein and zeaxanthin as the dominant pigments in the macula may serve as antioxidants to protect the retina from photo-oxidative damage.

Observational studies cannot establish a cause-and-effect relation, primarily because of the difficulty of confounding factors. Clinical intervention trials are necessary to establish "true" relations between purified food constituents and chronic diseases. During the past few years, several micronutrient and carotene intervention trials have been completed. The findings of these intervention trials are, in general, not consistent with a protective effect of β-carotene supplementation. A notable exception is a large study

conducted in China in a remote, nutritionally deprived area with one of the world's highest rates of esophageal and stomach cancer [35]. In this intervention trial, which ran for 5¼ years, it was found that subjects taking a combination of β-carotene (15 mg/d), α-tocopherol (30 mg/d), and selenium (50 μg/d) had a significant reduction in mortality from stomach cancer, total cancer and all causes combined. A subpopulation of older subjects from the same treatment group had a significant 44% reduction in nuclear cataract. Extrapolation of these findings to a well-nourished Western society with different ethnic background may not be valid.

Surprisingly, β-carotene failed to show protective effects against lung cancer in the Alpha Tocopherol Beta-Carotene Prevention Study (ATBC Study) conducted in Finland [36]. The male subjects were at high risk for lung cancer because they were cigarette smokers who had smoked an average of 20.4 cigarettes/day for an average of 35.9 years. Subjects taking 20 mg β-carotene/day for 6 years had a statistically significant 18% increase in lung cancer incidence. Mortality due to ischemic heart disease was also increased in the β-carotene group.

When these results were published, two large trials were still ongoing in the United States, the Physicians' Health Study (PHS) and the β-Carotene and Retinol Efficacy Trial (CARET). The PHS, which extended over 12 years and terminated in December 1995, randomly assigned participants to either take 50 mg β-carotene on alternate days or placebo. The study population, 22,071 male physicians aged 40-84 y, consisted primarily of "healthy" non-smokers, and was thus a low-risk population. The results indicate neither

beneficial nor harmful effects of β-carotene supplementation on cancer rates or cardiovascular disease [37]. Of the study population, 11 % were current smokers and 39 % were former smokers at time of enrollment in the study. Among current and former smokers, there were no significant differences between the β-carotene and the placebo group in any of the end points studied, such as lung cancer incidence, numbers of deaths from cancer, deaths from any cause, deaths from cardiovascular disease, incidence of myocardial infarction, and stroke. Among the participants who were smokers at the time of enrollment in the study, the relative risk of lung cancer was 0.90 for the β-carotene group as compared with the placebo group.

The subjects in the CARET study were at high-risk of lung cancer, either because they were current smokers or former smokers or because they had occupational exposure to asbestos. The trial was prematurely terminated in January 1996 after 4 years when preliminary results showed possible adverse effects comparable to those observed in the Finland study. The treatment of 30 mg β-carotene and 25,000 IU retinol per day in the form of retinyl palmitate resulted in a statistically significant 28% increase of lung cancer and 17% increase of total mortality [38].

Focusing on the remission or suppression of premalignant lesions is a good approach to study the cancer preventive activity of micronutrients or other compounds. A clinical trial conducted to test the efficacy of β-carotene (25 mg/d for 4 years) in preventing colorectal adenoma, a precursor of invasive cancer, did not find any effect in preventing new colorectal adenomas in patients with a history of past adenomas [39].

Supplementation with 50 mg β-carotene/day for five years had no effect on the occurrence

of new basal or squamous cell carcinoma in well-nourished patients with previous skin

cancer [40]. However, reversal of oral leukoplakia, a premalignant lesion of the oral

cavity, upon supplementation with β-carotene was observed in several studies [41-43].

Is the final conclusion that β-carotene supplementation is harmful and dietary β-

carotene not responsible for the protective effect observed with high fruit and vegetable

intakes? The current recommendation for cigarette smokers is to avoid β-carotene

supplements [CARIG Annual Meeting, 4/14/96, Washington, DC], but intake of β-

carotene in form of fruits and vegetables does not impose harm to smokers. The findings

of the above mentioned large intervention trials definitely alarmed lay people as well as the

scientific community. But a closer look at the results of the Finland study reveals that the

increase of lung cancer in the β-carotene group was primarily evident among men with

higher alcohol consumption suggesting an interaction of alcohol and β-carotene[44],

which was reported in earlier studies [45, 46]. In addition, a lower risk of lung cancer was

observed in men with higher levels of both serum and dietary β-carotene at baseline,

suggesting that these subjects had higher intakes of fruits and vegetables, and thus of

carotenoids, for a prolonged period exceeding the period of β-carotene supplementation.

Epidemiological studies indicate that lifetime intake of diets rich in phytochemicals,

including carotenoids, prevents rather than cures cancer. Cancer development is a

multiple-step process occurring over a (life-)long time period, so that late intervention

with preventive compounds is ineffective in stopping the process. Studies are necessary to

determine if supplementation with β-carotene and other carotenoids is beneficial during the early stages of cancer development; theoretically younger subjects should be recruited and followed over several decades.

The epidemiological evidence for a protective effect of dietary intakes of carotenoids, and thus of vegetables and fruits, against lung and other cancers is consistently strong. A recent review by Ziegler et al. [47] discusses in detail the evidence for the relation of nutrition and lung cancer. Despite the negative outcome of the ATBC Study and the other large intervention studies which do not indicate beneficial effects of β-carotene supplementation, carotenoid research needs to continue. It may be that dietary levels are protective but that pharmacological doses might be harmful. There is still much to learn about the effects and metabolism of carotenoids and interrelations with other nutrients and/or diet constituents.

Most of the earlier epidemiological studies relating vegetable, fruit, and carotenoid intakes with risk of certain diseases focused on β-carotene or total carotenoids provided with the diet. Often plasma concentration were measured by spectrophotometry which provides concentration values for total carotenoids but not for specific ones. With the recent reevaluation of fruits and vegetables for contents of individual carotenoids and the generation of a carotenoid food composition database [48] it is now possible to look at correlation between dietary intake of specific carotenoids and disease indices. For certain diseases, the association of reduced risk with increased intake of specific carotenoids was stronger for lutein or lycopene than for β-carotene. Lutein was associated with a decreased risk of lung cancer [25, 26], and it was the only carotenoid that had a strong

inverse relation with reduced risk of AMD [34]. Increased intake of lycopene was associated with reduced risk of prostate cancer [28] and cancers of the digestive tract [27]. These findings emphasize the importance of learning about the metabolism and mode of action of carotenoids other than β-carotene.

The association of vegetable and fruit consumption with reduced risk of degenerative diseases is strong and may involve other protective phytochemicals besides carotenoids, such as flavenoids, phenols, isothiocyanates and others. The importance of these dietary phytochemicals cannot be easily assessed in epidemiological studies because of a paucity of data regarding contents of these compounds in specific foods. Plasma carotenoids reflect recent intake of fruits and vegetables and may therefore serve as biomarkers for intake of other protective compounds present in the diet in order to identify low-risk individuals and to validate self-reported fruit and vegetable intakes.

The evaluation of specific carotenoids as protective agents requires knowledge of their distinct metabolism. The present dissertation will contribute to the understanding of intestinal absorption and metabolic interrelation of carotenoids in humans.

Absorption and Transport of Carotenoids in Humans

There are significant species differences in lipoprotein and carotenoid metabolism; therefore no good animal model exists to replace a human study. Most species convert β-

carotene extensively to retinoid metabolites in the small intestine and virtually none is

absorbed intact [49]. For example, chicks and rats are efficient converters of β-carotene to

retinoids, and thus do not accumulate intact β-carotene in their tissues. Chicks selectively

absorb oxycarotenoids, cats are unable to convert β-carotene to vitamin A, and adult

cattle accumulate mostly intact β-carotene and seem to absorb it specifically. Humans and

non-human primates indiscriminately absorb intact oxycarotenoids and hydrocarbon

carotenes and accumulate these carotenoids in tissues [50, 51]. The preruminant calf has

been postulated to be an appropriate animal model for the study of carotenoid uptake and

tissue distribution because preruminant calves absorb both oxycarotenoids and

hydrocarbon carotenes [52]. The calf model has a limitation in that the plasma lipoprotein

profiles are different for humans and calves. Whereas humans have an average low-density

lipoprotein:high-density lipoprotein (LDL:HDL) ratio of 1.1:1, calves have a ratio of 1:8.

Preruminant calves carry the majority of plasma carotenoids in the HDL fraction, with a

small amount associated with LDL [52], whereas, in humans, the majority of hydrocarbon

carotenes is associated with the LDL fraction [53-55]. The ferret is another animal model

that has been applied for the study of uptake and tissue distribution of carotenoids [56-

58]. Ferrets may be discriminate accumulators because low serum canthaxanthin

concentrations were reported in ferrets after daily ingestion of 50 mg/kg body weight [59]

A study by White et al. [60] revealed that serum accumulation of β-carotene exceeded that

of canthaxanthin in ferrets and a concurrent dose of canthaxanthin antagonized the serum

appearance and tissue distribution of an equal dose of β-carotene. In humans, a concurrent

dose of canthaxanthin did not affect the serum appearance of β-carotene, but β-carotene

inhibited the appearance of canthaxanthin in serum [61]. These apparent species

differences emphasize the importance of studying metabolism of diverse carotenoids in the

organism of interest, in our case, the human.

Information on the intestinal absorption of carotenoids in humans is mostly limited

to plasma appearance curves following a test dose. The movement of carotenoids and

metabolites from the luminal content in the small intestine into the lymphatic or portal

systems can be defined as absorption. Factors influencing the efficiency of absorption are

[66]:

* idiosyncrasy
* food matrix
* fat content of the meal
* formation of mixed micelles, i.e. presence of bile salts
* uptake of carotenoids by enterocytes
* packaging of carotenoids into lipoproteins and transport to the lymphatic system.

Several studies have suggested that individuals on identical diets have different

serum increments in response to equimolar doses of β-carotene, suggesting the existence

of *responders* and *non-responders* [62-64]. Nierenberg et al. [62] reported that the best

predictor for response was the initial plasma β-carotene concentration. In a study by

Wahlqvist et al. [65], the existence of gender differences was observed in regard to the

relation between baseline serum β-carotene concentrations and change in serum

concentrations after ingestion of a β-carotene dose. In men, the baseline serum β-carotene

concentrations were correlated with changes in serum concentrations, whereas, in women,

the baseline serum β-carotene concentrations were not related to changes in serum

concentrations. Kostic et al. [138] found that the β-carotene response to an oral β-carotene dose was not predictive of the serum response to other carotenoids, in this case lutein. In their study, subjects who would have been classified as *non-responders* relative to their intestinal β-carotene absorption showed good responses to a dose of lutein.

Erdman et al. [66] reviewed dietary and non-dietary factors affecting carotenoid absorption in humans. In general, purified β-carotene and β-carotene from processed foods have a higher bioavailability than similar amounts present in raw, uncooked vegetables resulting in varying absorption rates in the range of 1-50%. Since carotenoids are lipophilic compounds, concurrent ingestion of fat facilitates intestinal carotenoid absorption. Most investigators state that fat is essential for the absorption of β-carotene, whereas others observed limited absorption when the β-carotene dose was ingested with a no-fat or low-fat diet [64, 67-69]. The latter findings suggest that the chylomicron-lymphatic transport of β-carotene may not be the only pathway for utilization and that endogenously secreted fat may play a role in absorption of β-carotene with a fat-free diet.

The formation of bile micelles occurs when a critical micellar concentration (CMC) of bile acids and phospholipids is achieved. The aggregation of bile salts into micelles, and the formation of mixed micelles with the products of lipid digestion and other lipid-soluble food constituents are essential in facilitating the absorption of lipophilic compounds from the intestine. Micelles are sufficiently soluble to allow the transport of fat-soluble compounds through the aqueous environment and unstirred water layer in the small intestine. At the brushborder, micelles interact with the enterocytes and the lipophilic

contents of the micelles diffuse out of the micelles and across the cell membrane.

Hollander and Ruble [70] demonstrated that bile salts are necessary for micellar

solubilization of β-carotene, but once the CMC is reached, further increase in bile salt

concentration does not increase the β-carotene absorption rate in rats. It is believed that

the uptake of carotenoids by the enterocyte occurs passively and is not carrier-mediated.

The β-carotene absorption rate remained linear as concentrations of the β-carotene dose

were varied from 0.5-11μM [70]. Newly absorbed fatty acids with more than 14 carbon

atoms are re-esterified into triacylglycerols (TG) in the enterocyte. These resynthesized

TGs, together with fat-soluble vitamins and probably carotenoids, are collected in the

cell's endoplasmic reticulum where they acquire apolipoproteins and are then finally

assembled into chylomicrons in the Golgi apparatus. Chylomicrons then migrate to the

basal-lateral cell membrane where they are exocytosed into the intracellular space for

passage to the lymphatic system. Since β-carotene appears simultaneously in lymph with

newly absorbed dietary fat, it is assumed that β-carotene follows the same route during

absorption in the enterocyte.

Lipoproteins secreted by the enterocyte during the fasting state carry endogenous

lipids [71]. These lipoproteins, which are called intestinal very low density lipoprotein

(VLDL), may be responsible for β-carotene absorption after ingestion of a β-carotene

dose with a no-fat meal or no meal at all. Experiments by Ockner and Jones [72]

supported the theory that lymph VLDL contain endogenous lipid which is reabsorbed

from the intestinal lumen. They demonstrated that cholestyramine administration (a bile

sequestrant) or bile diversion resulted in decreased lymph lipid output with a marked

reduction in VLDL. The intestinal VLDL resembled plasma VLDL in composition,

Svedberg flotation rate (S_f), and electrophoretic mobility.

Tso and Fujimoto [73] propose two pathways for the formation of chylomicrons

and intestinal VLDL. By administering Pluronic-L81 (a hydrophobic surfactant) to rats

they were able to inhibit formation of intestinal chylomicrons, but not of intestinal VLDL

particles. Intraduodenal infusion of phosphatidyl choline resulted in the lymphatic

transport of VLDL and was not affected by Pluronic-L81. They hypothesized that

absorbed fatty acids and fatty acids derived from the hydrolysis of absorbed

lysophosphatidyl choline are used to form triglyceride via the α-glycerophosphate pathway

which is then packaged mostly into VLDL particles. During lipid absorption in the fed

state, the monoacylglycerol pathway is the predominant pathway for triglyceride formation

from absorbed 2-monoacylglycerol (2-MG) and fatty acids. If little or no 2-MG is present

in the enterocyte, the α-glycerophosphate pathway becomes the more important pathway

for triglyceride formation.

The type of dietary fat has an effect on lymph lipoprotein particle size and lipid

content. Feldman et al [74] demonstrated in a rat model that lymph chylomicron particle

size and lipid content were greater with an unsaturated-fat diet. Feeding specific fatty

acids revealed that saturated fatty acids appeared primarily in VLDL and unsaturated fatty

acids were recovered in chylomicrons. If we assume the existence of two pathways,

perhaps locally separated, for the formation of chylomicron and VLDL particles, it might

be that carotenoids that differ in hydrophobicity, e.g., β-carotene and canthaxanthin, are preferentially packaged in one of the two lipoprotein particles.

One of the integral apolipoproteins present in chylomicron and VLDL particles is apolipoprotein B (apo-B) which is essential for the assembly of these lipoproteins. Apo-B does not exchange between lipoprotein particles, it remains associated with the same lipoprotein particle in the circulation [75, 76]. In humans, apo-B48 is exclusively associated with chylomicrons, and thus is of intestinal origin, whereas apo-B100 is primarily synthesized in the liver and thus associated with VLDL and LDL [77, 78]. Apo-B100 is the ligand that mediates the clearance of LDL from the circulation by interaction with LDL receptors. Apo-B48 is essential for chylomicron formation.

Both apo-B proteins are products of a single gene, the aminoterminal half of apo-B100 shows colinearity with apo-B48 [79]. The intestinal apo-B mRNA undergoes posttranslational cytosine deamination resulting in the production of an in-frame stop codon and translation to apo-B48 [79-82]. Higuchi et al. [80] were able to detect two distinct apo-B mRNAs in both human intestine and liver. One apo-B mRNA coded for apo-B100 and the second mRNA contained an in-frame stop codon that provides the mechanism for the biosynthesis of apo-B48. But human liver appears to secrete almost exclusively apo-B100 suggesting that the predominant apo-B mRNA in hepatic tissue contains the whole coding sequence resulting in translation to apo-B100. It has been shown that human intestine secretes both apo-B48 and apo-B100 [81]. Intestinal biopsies from two normal subjects indicated that 84 ± 3 % of intestinal apoB mRNA encoded apo-B48, while 16 ± 3 % coded for apo-B100. But the majority of newly synthesized intestinal

apo-B protein was apo-B48, only 3-5 % being apo-B100 under the study conditions, indicating increased degradation of apo-B100 mRNA. The secretion of apo-B100 with intestinal VLDL by human intestine may lead to the formation of intestinal apo-B100 remnants which resemble LDL particles.

We need to be aware of the fact that it is not possible to distinguish between intestinal VLDL and hepatic VLDL, because they carry the same apo-B protein, B100, and they resemble each other in composition and S_f rates. The increase in triglyceride-rich lipoprotein (TRL) apo-B concentrations during postprandial triglyceridemia has been shown to be due to an increase in both TRL apo-B48 and apo-B100 concentrations [83-85]. Since the contribution of intestinal VLDL is considered to be insignificant [81], the postprandial increase in VLDL apo-B100 after a fat load is thought to be due to VLDL of hepatic origin. In one study the increase in the number of TRL was to 80 % explained by an increase in the number of VLDL particles estimated by an increase in apo-B100 concentration [84]. But considering the massive triglyceride load of each chylomicron particle, most of the postprandial lipemia was accounted for by apo-B48 containing particles. The increase in VLDL may be explained by the preferential metabolism of incoming chylomicron particles by lipoprotein lipase because it is believed that exogenous triglyceride, associated with chylomicron particles, compete with endogenous triglyceride, associated with VLDL, for a common, saturable, plasma triglyceride removal system related to lipoprotein lipase [86].

Not all TRL are metabolized in the same manner; TRL are a heterogeneous class of lipoprotein particles with distinct sites of synthesis, structure and metabolism. The

VLDL metabolism involves hydrolysis of core triglycerides by lipoprotein lipase, resulting in particles of decreasing size, enriched in cholesterol and protein which could end up in LDL, so that VLDL serves as a precursor for LDL. Even within the VLDL fraction there exists substantial heterogeneity. In terms of Svedberg flotation rates (S_f), VLDL particles are classified in a range of S_f 20-400. It has been reported that the postprandial increase in VLDL particles was primarily due to an increase in large VLDL (S_f 60-400 apo-B100), whereas the plasma concentration of small VLDL (S_f 20-60 apo-B100) was not affected by an oral fat load [84]. In the study it was proposed that large VLDL compete with chylomicrons for a common lipolytic pathway, and that chylomicrons are rapidly cleared from the plasma, indicating that smaller intestinal lipoproteins do not primarily originate from larger S_f > 400 chylomicrons, but are instead secreted directly into the S_f 20-400 fraction and subsequently converted to smaller remnants.

By subfractionation of the VLDL fraction (S_f 20-400) it may be possible to elucidate the fate of the different-sized VLDL particles. In a study by Packard et al. [87] using radioactively-labeled apo-B100 they demonstrated that particles from the S_f 100-400 flotation range were monoexponentially converted into S_f 12-100 particles, which are considered intermediate density lipoproteins (IDL). Less than 10 % of the S_f 12-100 fraction ended up in LDL, the remaining 90 % were slowly cleared by the liver. Further subfractionation of the S_f 12-100 revealed that the S_f 40-60 flotation interval contained particles that decayed slowly and were not converted to LDL. The S_f 20-40 lipoproteins were more rapidly and completely converted into LDL than the S_f 40-60 particles. Their

conclusion was that apo-B in LDL is mostly derived from small VLDL which are excluded

from the large-VLDL particle fraction (S_f 100-400).

Retinyl esters have been used as markers for intestinal lipoproteins because it is

believed that they remain associated with chylomicrons during triglyceride hydrolysis and

are taken up by the liver within the chylomicron remnants [88, 89]. Dietary retinol is

absorbed by the enterocyte and esterified by transacylation from, predominantly the α-

position of phosphatidyl choline (lecithin), by the action of lecithin:retinol acyltransferase

(LRAT) [90]. The resultant retinyl esters are then incorporated into chylomicrons and

released into the lymph. Chylomicron remnants containing the esters are taken up by the

liver and retinyl esters are either stored in the hepatic tissue or resecreted in form of retinol

bound to retinol binding protein. The normal liver does not resecrete esterified retinol.

Berr and Kern [88] estimated the exchange of retinyl esters with other lipoproteins to be

insignificant. They demonstrated that 4.3 % was transferred from chylomicrons to other

lipoprotein classes during in vitro incubation for 5 h. During 12 h post-dosing in human

subjects, only 6.4 ± 1.5 % of the retinyl palmitate absorbed was found in the LDL fraction

and 3.1 ± 3.8 % in the higher density lipoproteins.

Recently, the usefulness of retinyl esters as a marker for intestinally derived

lipoproteins has been questioned because retinyl esters have been detected in LDL and

HDL particles [91]. In this study, the investigators detected 34 % of plasma retinyl esters

in the LDL fraction at baseline. In the first 6 h postprandially, retinyl esters were mostly

associated with the TRL fraction (75 %) and less than 10 % were found in LDL. After 9

and more hours, retinyl esters increased gradually in LDL and 5 % were detected in HDL,

which could be of intestinal origin. The source of the retinyl esters in the LDL fraction is not known. Since LDL is believed to originate from the catabolism of small VLDL particles the authors hypothesized that retinyl ester-containing LDL derived from apo-B100-containing lipoproteins of intestinal origin, or the liver secretes retinyl ester within apo-B100-containing VLDL, or the esters are transferred to LDL from other lipoproteins. The authors concluded with the recommendation that retinyl esters should not be used as markers for intestinal TRL and that apo-B48 is better suited to identify intestinal lipoproteins.

Another interesting observation in the study by Krasinski et al. [91] was that the mean plasma triglyceride and apo-B48 concentrations peaked at 3 h after the meal, whereas the mean plasma retinyl ester concentration peaked at 6 h in TRL. This delay in plasma retinyl ester concentration peak has been reported by others [89]. Apo-B48 peaked at 3 h postprandially, i.e. the highest number of new chylomicron particles occurs in the plasma at this point, indicating that the incorporation of retinyl esters into chylomicrons in the enterocyte must be delayed.

Nonprovitamin A carotenoids are not cleaved to retinoids within the enterocyte and are thus absorbed intact in humans. The present study will follow the kinetics of two carotenoids, one provitamin A and one nonprovitamin A carotenoid, in the major lipoprotein classes and VLDL subfractions and will thus add to the body of knowledge about transport and time course of carotenoids in lipoproteins. Alternatively to intact carotenoid intestinal absorption, provitamin A carotenoids can be converted to retinoid metabolites in the enterocyte. A tracer study has shown that 64% of absorbed ^{13}C-β-

carotene entered the plasma as retinyl esters, 21% as retinol, and 14% as intact β-carotene

[92]. Similar results were found in earlier studies using radioactive β-carotene where 8-

17% of administered radioactivity was recoverable in lymph, of which 2-28% accounted

for β-carotene, and 61-90% of absorbed radioactivity was detected in form of retinyl

esters [93, 94].

It is not known how carotenoids are transported from the brush border membrane

to the cell organelles. The existence of specific intracellular transport mechanisms can not

be ruled out. Hollander and Ruble [70] examined the effect of different fatty acids on the

absorption rate of β-carotene and they showed that the rate was higher when the meal

contained short- and medium-chain fatty acids compared to long-chain polyunsaturated

fatty acids (LCPUFAs). The decreased β-carotene absorption in presence of LCPUFAs,

which have high binding affinities for fatty acid binding proteins (FABP), may be due to

competition between the carotenoid and LCPUFAs for binding to FABPs. FABPs may

serve as a transport vehicle for β-carotene through the aqueous cytosol. In a recent study,

Gugger and Erdman used bovine liver and intestine to study the possible cytosolic protein-

mediated carotenoid transfer between liposomes and mitochondria in vitro [95]. They

were not able to demonstrate transfer of β-carotene under the experimental conditions and

concluded that cytosolic intracellular transport of β-carotene is not mediated by proteins.

They hypothesized that intracellular movement of carotenoids may occur by vesicular

transport or by membrane-bound proteins.

Chylomicrons and intestinal VLDL are transported through the lymphatic system to the thoracic duct which empties into the subclavian vein and enters the main circulation. As the blood circulates, lipoprotein lipase, located at the luminal surface of the endothelium and fat cells, acts repeatedly on the chylomicrons removing most of the triacylglycerols and leaving chylomicron remnants which are quickly taken up by the liver. Carotenoids transported in chylomicrons can find their way into hepatic and extrahepatic tissues by virtue of chylomicron catabolism. In hepatic tissue, β-carotene can either be stored, converted to retinol, or repackaged into VLDL and released into the circulation.

Under fasting conditions all hydrocarbon carotenes are carried predominantly by LDL, whereas, among the xanthophylls, cryptoxanthin is equally distributed between LDL and HDL, and lutein plus zeaxanthin are primarily associated with HDL [53-55]. A recent study explained these distribution patterns with the solubility characteristics of polar and apolar carotenoids in biological emulsions [96]. The oxycarotenoids were preferentially solubilized in phospholipids, whereas the hydrocarbon carotenes preferentially solubilized in triglycerides. So that oxycarotenoids can be expected to be associated with lipoproteins with a high phospholipid/apolar lipid mass ratio such as found in HDL.

The appearance of β-carotene in serum after ingestion of a single dose is biphasic with an initial peak at 4-7 h post-dosing and a second, larger peak at 24-48 h post-dosing [61, 69]. The early postprandial rise in serum β-carotene had been attributed to chylomicron influx and the removal of chylomicron remnants by the liver results in disappearance of β-carotene [69, 97]. The β-carotene peak increment in the VLDL fraction occurring at 6 h exceeded the coinciding peak in the chylomicron fraction [69]

which could be due to contamination of the VLDL fraction with chylomicron remnants or intestinal input of small chylomicrons. The latter is the more likely explanation because chylomicron remnants are rapidly cleared from the plasma by the liver having a half-life of several minutes [88]. The second peak, occurring at 24 to 48 h post-dosing, was associated with the LDL fraction probably due to hepatic secretion of VLDL particles which are converted to LDL particles by action of lipoprotein lipase.

A study by Kübler [98] reported on the pharmacokinetics of a single canthaxanthin dose in serum. Two or more canthaxanthin peaks were observed in the combined chylomicron plus VLDL fraction within 8 h post-dosing. Within 5 h post-dosing, the canthaxanthin concentration in LDL exceeded that in chylomicron plus VLDL, and peaked approximately 8 h post-dosing. The observed rapid increase of canthaxanthin content in the LDL fraction may result from either transfer from chylomicrons or incorporation into intestinal VLDL particles which are subsequently metabolized to LDL [61]. White et al [61] attributed the monophasic appearance of oxycarotenoids in serum to coincident peaks of the carotenoids in TRL and LDL.

It is not known if carotenoids exchange between lipoproteins. Certain apolipoproteins, cholesterol, and phospholipids are able to transfer spontaneously among different lipoproteins, whereas more hydrophobic lipoprotein components, such as cholesterol esters and triacylglycerols require specific transfer proteins. The interchange of lipids between circulating lipoprotein particles is facilitated by the plasma enzymes lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) [75]. Borel et al. [96] studied the behavior of polar and apolar carotenoids in a model of

phospholipid-stabilized triglyceride emulsions. They demonstrated that zeaxanthin, a carotenoid with two hydroxyl groups, was preferentially solubilized in the surface phospholipids while β-carotene was preferentially solubilized in the core triglyceride. They also showed that the more polar carotenoid was transferred between a model emulsion and mixed micelles without triglyceride lipolysis, while the apolar carotenoid absolutely required triglyceride lipolysis to be transferred. They therefore suggested that polar carotenoids located at the lipid droplet's surface can spontaneously transfer.

These findings suggest that transfer of polar carotenoids between TRL and other lipoproteins may occur, which could explain the early rise of polar carotenoids in VLDL and LDL. Tocopherols are readily transferred from HDL to LDL and VLDL without the involvement of lipid transfer proteins [99]. In contrast, Romanchik et al. were not able to show transfer of α-tocopherol and carotenoids among lipoproteins when plasma was incubated in vitro [100]. If we assume that canthaxanthin is not exchanged between lipoproteins, the rapid increase in LDL-canthaxanthin may result from incorporation into intestinal VLDL which undergo subsequent catabolism to LDL [61]. The analysis of the VLDL fraction for apolipoproteins B48 and B100 would provide information on the source of carotenoids in the early carotenoid rise. It is very possible that intestinal VLDL entering the circulation are catabolized to LDL without being first internalized by the liver which could explain the rapid rise of canthaxanthin in the LDL fraction if canthaxanthin is incorporated into intestinal VLDL.

Another interesting point regarding intestinal carotenoid absorption is the apparent discrimination between geometrical isomers of carotenoids, i.e. trans and cis isomers, as

they occur in the diet and human tissues. The geometrical isomers can be interconverted by light, thermal energy or chemical reactions [101]. Despite the vast number of possible configurations, only a limited number are preferentially formed. For example, β-carotene primarily exists in the all-trans, 9-cis, 13-cis and 15-cis forms [4]. It is not known if geometrical isomers posses isomer-specific functions. In human plasma, the prominent isomer is all-trans β-carotene and less than 5 % of total β-carotene occurs as cis isomers, especially 13- and 15-cis β-carotenes with 9-cis β-carotene being absent from plasma. In tissue, greater than 60 % of total β-carotene is found as all-trans, 10-20 % as 13- and 15-cis, and 10-20 % as 9-cis β-carotene [102].

Several investigators studied the serum response to the ingestion of an isomer mixture of all-trans and 9-cis β-carotene from a natural source [103-105]. The ratio at which the two isomers appear in plasma did not reflect the composition of geometrical isomers in the dose. The 9-cis isomer was either not detected in serum or showed a slight, statistically not significant increase which did not reflect the ingested isomer ratio. The carotenoid pattern in chylomicrons revealed preferential accumulation of all-trans β-carotene over its 9-cis isomer [104]. These data suggest that the isomer discrimination occurs in the enterocyte, either specific uptake, or incorporation into lipoproteins. Cis-trans bioisomerization in lumen or enterocyte has also been indicated to occur in humans [106]. An animal study, done in ferrets, demonstrated that both all-trans and 9-cis were absorbed equally well through the lymphatic system after intestinal perfusion [107]. The investigators did not observe preferential transport through lymph regardless of the isomer

perfused, but no 9-cis β-carotene was detectable in ferret serum. They observed high intestinal affinity for 9-cis β-carotene and suggested that, after its absorption, 9-cis β-carotene undergoes more rapid peripheral tissue uptake than the all-trans isomer. After perfusion with 9-cis β-carotene a rise in 9-cis retinoic acid was observed. The conversion of 9-cis β-carotene to 9-cis retinoic acid had been shown before [108, 109]. The high concentration of 9-cis β-carotene in hepatic tissue may also be due to isomerization of all-trans β-carotene. The mechanism responsible for the high cis-isomer content in tissues needs to be elucidated.

In contrast, discrimination of vitamers and stereoisomers of tocopherol occurs postabsorptively at the level of hepatic VLDL secretion [110, 111]. The vitamers, γ- and α-tocopherol, and the stereoisomers, RRR- and SRR-α-tocopherols, are equally well-absorbed from the intestinal lumen and secreted within chylomicrons into lymph, but subsequently RRR-α-tocopherol is the prominent stereoisomer in plasma. After the internalization of chylomicron remnants, containing both isomers by the liver, α-tocopherol becomes preferentially incorporated into VLDL for secretion into the plasma, whereas γ-tocopherol is excreted by the liver via bile. Tocopherol-binding protein (TBP) has been isolated from rat and human hepatocytes [112-114]. It has been suggested that the TBP, which binds specifically RRR-α-tocopherol, is responsible for hepatic discrimination of tocopherols by preferential incorporation of RRR-α-tocopherol into nascent VLDL [115, 116].

Dietary and Non-Dietary Determinants of Serum Carotenoid Concentrations

Serum carotenoid concentrations are not homeostatically controlled by the human body, they show inter-individual variation over a wide concentration range. Table 1-1 shows reported ranges of the predominant carotenoids, retinol, and α-tocopherol in human serum [117-119]. Dietary intake is a major determinant of serum carotenoid concentrations with seasonal variations reflecting the diet [24, 120-122]. The highest serum β-carotene concentrations have been observed in fall, whereas the highest dietary β-carotene concentrations occurred in summer [122]. This contradiction may be related to the increased amount of light exposure during the summer months, because photodegradation of carotenoids has been shown to occur in humans [123]. No seasonal variation of plasma α-tocopherol was observed [122].

Table 1-1. Reference ranges of serum carotenoids, retinol, and α-tocopherol in humans.

	μmol/L		
	NHANES III [117]	Kaplan et al. [118]	Stacewicz-Sapuntzakis et al.[119]
Lutein/Zeaxanthin	0.16-0.72	----	0.08-0.79
β-Cryptoxanthin	0.05-0.38	----	0.04-0.49
Lycopene	0.13-0.82	0.23-1.21	0.03-1.32
α-Carotene	0.02-0.22	0.01-0.40	0.00-0.21
β-Carotene	0.09-0.91	0.07-1.39	0.05-1.29
Retinol	----	1.12-3.25	1.31-4.45
α-Tocopherol	----	11.61-40.17	----

In general, women have higher plasma concentrations of α- and β-carotene, β-cryptoxanthin, and lutein plus zeaxanthin, whereas men have higher concentrations of retinol [118]. There were no significant differences in the concentrations of lycopene and α-tocopherol in men as compared to women. Lower serum carotenoid concentrations have been reported to be related to alcohol consumption, smoking, high body mass index (BMI), and low serum cholesterol concentrations [124-127]. A recent study showed that the differences in serum carotenoids between women and men, smokers and non-smokers, and with alcohol consumption were of similar extent as the differences in carotenoid intake in these groups, suggesting that observed differences are due to diet and not to an effect of these factors on carotenoid absorption and metabolism [127]. In contrast, serum carotenoids but not dietary carotenoids were related to BMI, HDL and non-HDL cholesterol. Significant positive correlations between total plasma carotenoids and plasma total- and LDL-cholesterol have been reported in earlier studies [118, 122, 128, 129], and, addressing individual carotenoids, lycopene [129, 130] and lutein/zeaxanthin [131] appear to be more highly correlated with total- and low-density lipoprotein-cholesterol than β-carotene. In contrast to plasma α-tocopherol and retinol, β-carotene and lycopene were not correlated with total plasma lipids [54, 131].

Since carotenoids are excellent singlet oxygen quenchers and additionally have the ability to react with peroxyl radicals involved in lipid peroxidation [5, 7-9], supplementation with β-carotene may be beneficial in reducing oxidative stress in organisms. Of concern is the possible effect that supplementation with one specific nutrient might have on the serum level of other micronutrients. Results from two large

trials show that long-term oral supplementation with β-carotene does not have a detrimental effect on serum α-tocopherol concentrations [132, 133]; in fact, significantly increased α-tocopherol concentrations were observed at the end of the intervention period.

Results from studies that examined the effect of β-carotene supplementation on other serum carotenoids are not consistent. A study examining the accumulation of β-carotene in serum and skin observed that with the highest β-carotene dose administered (100 mg / 3 times per day) the serum concentrations of other carotenoids would decrease [67]. In a random sample of subjects from the participants in the Physicians Health Study it was found that 50 mg β-carotene on alternate days did not affect the plasma concentrations of other carotenoids or tocopherols [134]. But differences in cellular concentrations of carotenoids in peripheral blood mononuclear cells (PBMNC) and red blood cells (RBC) after supplementation were observed. In the placebo group, lutein was the most abundant carotenoid in PBMNCs and RBCs, whereas in the supplemented group lutein was second to β-carotene in PBMNCs and a statistically non-significant decrease in lutein concentrations was observed in both PBMNCs and RBCs. Wahlqvist et al. [65] reported statistically significant increases in serum concentrations of lycopene and α-carotene, besides the expected increases in β-carotene concentrations, after supplementation with 20 mg of β-carotene for 24 months. They hypothesized a common pathway for absorption of lycopene, α-carotene, and β-carotene, all of which are hydrocarbon carotenes, that may be different from that of oxycarotenoids.

Supplementation with β-carotene may boost the absorption pathway so that the

absorption of other carotenes may be elevated. Another possible explanation is a sparing

effect. It is plausible that β-carotene and other hydrocarbon carotenes may be able to

substitute for each other in biological functions, so that supplementation with β-carotene

may suppress the utilization or catabolism of other carotenoids with preferential use of β-

carotene. An increase in serum α-carotene concentrations upon supplementation with

purified β-carotene was observed in previous studies [135, 136].

Reduced plasma lutein concentrations were observed upon supplementation with

purified β-carotene, suggesting a competition of the two carotenoids during intestinal

absorption [135, 136]. The effects might be different when the carotenoids are ingested in

the form of food and not as purified compounds. In a study reporting on the plasma

carotenoid response to daily intake of β-carotene in the form of a single supplement of

selected foods or purified β-carotene in the context of a controlled low-carotenoid diet it

was observed that ingestion of β-carotene supplement in the form of a selected food did

not interfere with the appearance of dietary lutein in plasma, whereas a reduced

concentration of lutein in plasma was observed for the group that ingested an equivalent

amount of β-carotene as a purified β-carotene supplement relative to the placebo group

[136].

The pilot study for the current study was the first human metabolic study that

addressed specifically the interrelation of a hydrocarbon carotene and an oxycarotenoid

after concurrent ingestion of an equimolar dose of β-carotene and canthaxanthin [61]. In

this study, the peak serum concentration of canthaxanthin was reduced by concurrent ingestion of an equimolar β-carotene dose to 61% of the value for canthaxanthin when administered in absence of β-carotene. The appearance of β-carotene in plasma after an oral dose was not antagonized by concurrent ingestion of an equimolar canthaxanthin dose. In contrast, in a ferret model, it was demonstrated that concurrent dosing of β-carotene and canthaxanthin antagonized the serum appearance of β-carotene [60]. Tissue β-carotene concentrations were also lower in the ferrets that received the combined dose compared with the group receiving a single dose of β-carotene. These apparent species differences stress the importance of human metabolic studies when studying human metabolism.

A recent study, also using a ferret model, found that canthaxanthin supplementation resulted in significantly higher α- plus β-carotene concentration in hepatic tissue compared with the placebo group [137]. Concentrations of α-tocopherol in liver and lungs and of lutein/zeaxanthin in adipose tissue were significantly lower in ferrets fed canthaxanthin compared with the placebo group. Depending on the specific tissue assayed, the effects of canthaxanthin supplementation on other carotenoids and micronutrients demonstrated either synergistic or antagonistic relations. The ferret as a model to examine intestinal absorption and tissue distribution of carotenoids in humans might not be ideal because it appears the ferret is not an indiscriminate absorber of carotenoids. In ferrets, serum accumulations of β-carotene exceeded those of

canthaxanthin after an equimolar dose, whereas in humans the opposite has been observed [61].

The postulated theory of interaction between β-carotene and lutein during intestinal absorption was supported by the finding that concurrent administration of an equimolar dose of β-carotene and lutein inhibits the appearance of lutein in serum, which was shown by a reduction of the mean area under the curve (AUC) for lutein to 54-61% of its value when given alone [138]. The effect of lutein on β-carotene response showed broad interindividual variation with individual AUC values for β-carotene showing fivefold enhancement to 69 % reduction compared with the AUC values after a single dose of β-carotene without lutein, so that the mean AUC values for β-carotene were unaffected by the presence of lutein. Subjects with low β-carotene AUC values after a single dose of β-carotene, showed increased AUC values for β-carotene after a combined dose of β-carotene and lutein. One possible explanation is that subjects with low β-carotene AUC values after a single dose are efficient converters of β-carotene to vitamin A in the intestinal mucosa, i.e. little β-carotene is absorbed intact and appears in serum, and that lutein might inhibit the conversion so that more intact β-carotene is absorbed. An enzyme responsible for conversion of provitamin A carotenoids in the intestinal mucosa is β-carotene-15,15'-dioxygenase. Ershov et al [139] demonstrated that lutein, lycopene, and astaxanthin, all of which are nonprovitamin A carotenoids, can form enzyme-pseudosubstrate complexes and inhibit the enzyme competitively.

The observed interrelations between carotenoids in humans may be confined to occurrence between hydrocarbon carotenoids and oxycarotenoids. The findings from the above mentioned studies examining effects of combined doses of β-carotene plus canthaxanthin and β-carotene plus lutein [61, 138] were consistent, an oral dose of β-carotene reduced the appearance of the dose of the oxycarotenoids in serum. A recent study was not able to demonstrate an effect of a combined dose of β-carotene and lycopene, two hydrocarbon carotenoids, on the serum response of either carotenoid [140].

The response to combined ingestion of carotenoids may be markedly different when mixtures of carotenoids are ingested in food. A recent study by DePee et al. [141] compared serum β-carotene and retinol responses of lactating women with marginal vitamin A status to a dark-green leafy vegetable serving and an enriched wafer, both of which provided the same amount of β-carotene. The serum β-carotene concentration increased by 17 % in the vegetable group, whereas in the wafer group, the increase was 390 %. The retinol status of the women in the wafer group improved and no significant change was observed for the vegetable group. The authors give several potential explanations for these observations, one of which is that competition of β-carotene with other carotenoids present in the vegetable supplement may have inhibited β-carotene absorption and conversion to retinol. Of course, the food matrix is a very complex entity and it might be that specific carotenoids are located in different compartments within the cell making them more or less available. Dark-green leafy vegetables usually contain high concentrations of lutein, which is more hydrophilic than β-carotene, and thus may be

better bioavailable and already be present in the enterocyte once β-carotene has been freed

from the food matrix and absorbed by the enterocyte. Possible interaction of lutein and β-

carotene in the enterocyte may reduce the bioavailability of the carotenoids and/or the

conversion of β-carotene into retinoids.

If we think back to the discussion on carotenoids in human health, especially the

epidemiological studies reporting a reduced risk of lung cancer with high intakes of

carotenoids, it was found that especially lutein showed strong association with the risk of

lung cancer [25, 26]. In light of the recent findings regarding interaction of purified β-

carotene and lutein, i.e. the diminished appearance of lutein in serum after concurrent

ingestion of the two carotenoids, it could be speculated that the results of the Finland

study, i.e. increase in lung cancer incidence with β-carotene supplementation (20 mg/day)

in male smokers, are due to reduced utilization of lutein in the presence of purified β-

carotene. For more discussion on possible explanations of the negative outcome of the

latest intervention trials the reader is referred to the recent review by Ziegler et al. [47].

Because other carotenoids are potentially preventive agents it is implicit that their

metabolism is understood and possible interaction with other micronutrients are explored.

Since it is believed that uptake of carotenoids by the enterocyte is via passive diffusion

[70], the apparent interactions of specific carotenoids are likely to occur within the

enterocyte. Possible sites of interaction or competition could be at the level of β-carotene-

15,15'-dioxygenase, as discussed above, or at level of incorporation of carotenoids into

intestinal lipoproteins. The present dissertation will contribute to the knowledge of

interaction between hydrocarbon carotenes and oxycarotenoids during intestinal

absorption by investigating the kinetics of the appearance and disappearance of single and

combined oral doses of β-carotene and canthaxanthin in plasma and plasma triglyceride-

rich lipoprotein subfractions.

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2. DISTINCT KINETICS OF β-CAROTENE AND CANTHAXANTHIN APPEARANCE IN HUMAN PLASMA LIPOPROTEINS

Inke Paetau, Huiping Chen, Natalie M.-Y. Goh, and Wendy S. White

Abstract

The kinetics of single equimolar doses of β-carotene and canthaxanthin (47 μmol and 44 μmol, respectively) in plasma and plasma lipoproteins were investigated and compared in healthy premenopausal women. The lipoproteins were separated by cumulative rate ultracentrifugation which isolated four subfractions of triglyceride-rich lipoproteins (TRL) with progressively decreasing diameter and increasing density, i.e. chylomicron and three very-low density lipoprotein (VLDLA, B, and C) fractions, and also intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). In plasma, β-carotene kinetics were biphasic with a minor peak increment at 5 h post-dosing $(0.36 \pm 0.06$ μmol/L), after which concentrations fell and then increased again to a larger peak at 48 h post-dosing $(0.82 \pm 0.21$ μmol/L). The minor plasma peak coincided with peak β-carotene increments in TRL. The major peak was associated with appearance of β-carotene in LDL. The peak increment at 6 h in VLDLA $(0.12 \pm 0.05$ μmol/L) exceeded that in chylomicrons $(0.07 \pm 0.03$ μmol/L) although the apolipoprotein profile by sodium

dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was not consistent with

significant contamination of VLDLA by chylomicron remnants. Plasma kinetics of the

oxycarotenoid, canthaxanthin, were monophasic with a peak increment at 12 h post-

dosing (1.41 ± 0.11 μmol/L) followed by a steady decline. Canthaxanthin increments

peaked at 6 h post-dosing in chylomicrons and VLDLA (0.17 ± 0.03 μmol/L and 0.21 ±

0.04 μmol/L, respectively), whereas the peak increment was delayed in VLDLB and

VLDLC to 8 h post-dosing. There was a rapid canthaxanthin accumulation in LDL which

began 2 h post-dosing. It is concluded that concurrent increases of canthaxanthin in TRL

and LDL are responsible for the observed monophasic plasma canthaxanthin response and

may thus account for the monophasic response of other oxycarotenoids. Rapid

postprandial accumulation of oxycarotenoids in LDL, in contrast to delayed appearance of

β-carotene, may have profound health implication if we consider that carotenoids other

than β-carotene may potentially exert beneficial effects as antioxidants.

Introduction

Carotenoid research in relation to human health gained interest when

epidemiological studies indicated that intake of carotenoid-rich fruits and vegetables and

elevated blood concentration of β-carotene were inversely associated with risk of some

types of cancer, particularly lung cancer [1, 2]. The carotenoid investigated and associated

with beneficial effects was β-carotene, although fruits and vegetables are also sources of other carotenoids.

Recent findings from large intervention trials in Western populations are not consistent with a protective effect of β-carotene supplementation [3-7]. Carotenoid intake in the form of fruits and vegetables continues to be recommended as dietary levels of β-carotene, other carotenoids, other phytochemicals, or associated food patterns may account for the consistent protective associations in observational studies [2]. Oxycarotenoids such as lutein, zeaxanthin, and cryptoxanthin are present in the diet, and, as a result, in serum and tissues. A stronger relation of lutein intake than of β-carotene intake with reduced risk of lung cancer was recently reported [8,9]. Lutein-rich foods are also associated with a lower risk of age-related macular degeneration [10]. Lutein and zeaxanthin are selectively accumulated from plasma in the retina where they may protect from photooxidative damage in their action as antioxidants. Thus distinct metabolism of individual carotenoids may have important health implications.

This study investigated the kinetics of a model oxycarotenoid, canthaxanthin, and a hydrocarbon carotene, β-carotene, in intestinal- and hepatic-derived lipoproteins after ingestion of single equimolar oral doses of each carotenoid. Individual carotenoids show metabolic heterogeneity that is reflected in their lipoprotein distribution. Plasma carotenoids are transported in lipoproteins with the majority of hydrocarbon carotenes associated with low density lipoproteins (LDL) and the oxycarotenoids approximately equally distributed among LDL and high density lipoproteins (HDL) [11-14]. Triglyceride-rich lipoproteins (TRL) are a heterogeneous class of lipoprotein particles with distinct

sites of synthesis and metabolism [15-18]. The very-low density lipoprotein (VLDL) fraction is defined as having Svedberg flotation rates (S_f) of 20 - 400 [15]. It was shown that smaller VLDL particles (S_f 20-40) are almost completely converted to LDL in the circulation by action of hepatic triglyceride lipase, whereas larger VLDL particles are hydrolyzed by lipoprotein lipase and cleared by the liver [16]. Chylomicrons secreted by the enterocyte have flotation rates of $S_f > 400$. The possibility that the intestine is also secreting smaller sized particles ($S_f < 400$) has been proposed [17]. In light of the heterogeneity of VLDL particles, we separated the TRL into four subfractions to be able to reveal distinct kinetics of β-carotene and the oxycarotenoid, canthaxanthin, in different lipoprotein particles after ingestion of a single oral dose.

Materials and Methods

Subjects. Ten healthy, non-smoking, premenopausal women aged 20-36 y participated in the study. Subjects underwent a screening procedure that included a health and lifestyle questionnaire, physical examination, and complete blood count and blood chemistry profile. Criteria for exclusion were: history of chronic disease, lipid malabsorption or intestinal disorders, use of medications that may affect lipid absorption or transport (including antibiotics), hyperlipidemia indicated by plasma lipid and lipoprotein profile, lactose intolerance, history of anemia or excessive bleeding, history of photosensitivity disorders, history of eating disorders, hyper- or hypothyroidism indicated by serum thyroxine (T_4) and thyroid stimulating hormone (TSH), menstrual cycle

irregularities or abnormalities, current or planned pregnancy, use of oral contraceptive

agents, current use of vitamin or mineral supplements, vegetarianism, current or recent

cigarette smoking, and frequent consumption of alcoholic beverages (> 1 drink/day).

Before enrollment in the study, the subjects were instructed to complete a three-day

written food record to screen for unusual dietary behaviors, such as restrained eating and

vegetarian diet. Percent body fat at the commencement and the termination of the study

was determined by total-body electrical conductivity (TOBEC, EM-SCAN Inc.,

Springfield, IL). Informed consent was obtained from all subjects, and the study

procedures were approved by the Human Subjects Research Review Committee of Iowa

State University.

A total of twelve subjects participated in the study. Subject ten met the diagnostic

criteria for hypolipidemia [19] and her data were excluded from the statistical analyses and

calculations of the mean area under the plasma concentration-time curves. Subjects eleven

and twelve were excluded because they did not complete the third period of the study.

Diet. Subjects were provided a list of foods to exclude and instructed to avoid

consumption of carotenoid-rich fruits and vegetables for 5 days before each study period.

During the dosing periods, the subjects consumed a controlled low-carotenoid diet for 1

day before and 4 days after dosing. A single daily menu of weighed food portions was

provided. The meals were prepared and consumed in the Human Nutrition Metabolic Unit

of the Center for Designing Foods to Improve Nutrition at Iowa State University except

for the carry-out lunches and evening snacks on weekdays. Duplicate aliquots of a 24-

hour diet composite were analyzed by high performance liquid chromatography (HPLC)

for carotenoid, retinol, and α-tocopherol contents during each study period. The

extraction of the 24-hour food composite followed the protocol described previously [20].

On average, across three study periods, the diet provided 504.8 ± 52.8 µg/d lutein, $18.9 \pm$

0.4 µg/d cryptoxanthin, 145.3 ± 8.6 µg/d β-carotene, no detectable α-carotene and

lycopene, 591.7 ± 64.6 µg/d retinol, and 5.5 ± 0.2 mg/d α-tocopherol. The macronutrient

composition of the diet was estimated using Nutritionist IV software (N-Squared

Computing Inc., Salem, OR). The daily diet of 8.8 MJ was distributed as 14% of total

energy from protein, 63% of total energy from carbohydrates, and 23% of total energy

from fat.

Carotenoid dose. Water-dispersible 10% (wt:wt) β-carotene, 10% canthaxanthin,

and placebo beadlets were provided by Hoffmann-La Roche (Nutley, NJ). Canthaxanthin

is the naturally occurring color pigment in the chanterelle mushroom, in crustacea, and in

wild salmon; synthetic canthaxanthin is approved as a food colorant in the United States

[21]. Canthaxanthin is one of three carotenoids approved for human ingestion and is

commercially available as a water-miscible palatable formulation. Limited data suggest

similar kinetics with other oxycarotenoids [22]. For the preparation of the dose providing

25 mg (47 µmol) β-carotene or 25 mg (44 µmol) canthaxanthin, 250 mg of the respective

beadlets were dissolved in 100 ml of warm whole milk (40°C) to which 296 ml cold whole

milk were added. Results presented here are part of an interaction study investigating

effects of concurrent ingestion of equimolar amounts of β-carotene and canthaxanthin on

their individual plasma kinetics. To compensate for the vehicle effect, when administering

the single dose, an equal amount (250 mg) of placebo beadlets was added. The carotenoid

dose in milk (396 ml) containing an estimated 13 g fat was administered with the breakfast

containing an additional 20 g fat to facilitate intestinal absorption.

Study protocol. During each of three five-day study periods, subjects ingested

either a β-carotene or canthaxanthin dose, separated by a washout period of ten or more

weeks to minimize residual effects of the previous carotenoid dose. On the day of dosing,

the second day on the low carotenoid diet, subjects arrived at the metabolic unit after an

overnight (12-hour) fast, and a baseline blood sample (7 ml) was drawn via a catheter

placed in a forearm vein by a registered nurse. After administration of the carotenoid dose,

followed by the breakfast, blood samples were drawn at hourly intervals for 12 h post-

dosing via the intravenous catheter into a syringe. The patency of the catheter was

maintained by flushing with sterile physiological saline; 3 ml of sterile saline was injected

after each blood draw and withdrawn immediately before the next blood collection.

Additional blood samples were drawn from the antecubital vein via venipuncture after an

overnight fast at 24, 48, 72, 96, 192, 360, and 528 h post-dosing. The blood samples were

immediately placed on ice, protected from light, and then centrifuged (1380 x g, 4° C, 20

min) to separate the plasma. Aliquots of the plasma were stored at -80°C until analyzed,

except for the samples used for lipoprotein fractionation.

Lipoprotein fractionation. Blood draws obtained at 0, 2, 4, 6, 8, and 10 h post-

dosing from subjects 1 to 5 were used immediately for lipoprotein fractionation.

Lipoprotein fractions were separated by cumulative rate ultracentrifugation to obtain

chylomicrons, three VLDL subfractions, and LDL [23]. Plasma density was adjusted to

1.10 g/ml by addition of solid potassium bromide (KBr) (0.14 g/ml). Four ml of plasma

were overlayered with salt solutions of decreasing density, 3 ml each of 1.065 g/ml and

1.020 g/ml, and 3.4 ml of 1.006 g/ml, in Beckman Ultra-Clear™ 14 x 95 mm centrifuge

tubes (Beckman Instruments, Palo Alto, CA). Preparation of the density solutions was

according to Pitas et al. [24]. The SW40i swinging bucket rotor of the Beckman L8-M

ultracentrifuge was used for the cumulative rate centrifugation at 20°C. Centrifugation

was for 43 min at 28,300 rpm (4.5 x 10^6 g-min, chylomicron fraction, $S_f > 400$), then for

67 min at 40,000 rpm (17.5 x 10^6 g-min, VLDLA fraction, S_f 175-400), then for 71 min at

40,000 rpm (31.2 x 10^6, VLDLB fraction, S_f 100-175), and finally for 18 h at 37,000 rpm

(152 x 10^6, VLDLC, S_f 20-100, and LDL, S_f 0-12, fractions). After the first three

sequential centrifugations, each fraction was carefully aspirated from the top of the tube

and the tube was refilled with density 1.006 g/ml salt solution. After the 18 h

centrifugation, the gradient was fractionated from top into 2.0 ml of VLDLC fraction, 3.0

ml of IDL fraction, 2.5 ml of visible LDL fraction, and plasma infranatant. Procedures

were performed in yellow light. Aliquots of the lipoprotein fractions for carotenoid

analysis were stored at -80°C until analyzed.

Electron microscopy of lipoprotein fractions. Negative stain technique was

applied to determine the particle size distribution of the lipoproteins by electron

microscopy [25]. Isolated lipoprotein fractions were dialyzed overnight at 4°C in

Spectra/Por molecular porous dialysis membrane tubing with a molecular weight cut-off of

12 - 14 K (Baxter Diagnostics Inc., McGaw Park, IL). The dialysis buffer (pH 7.4)

contained 5 mM ammonium bicarbonate, 0.02% (wt:vol) ethylenediamine-tetraacetic acid

(EDTA), and 0.02% (wt:vol) sodium azide (N_3Na). Dialyzed fractions were stained with phosphotungstic acid (pH 7.3), a small droplet was placed on the Formvar-carbon-coated grid, and excess fluid was removed by blotting with a paper tissue. The LDL fraction was diluted with deionized water (1:4) before staining. The electron microscope (1200 EX STEM, JOEL, Japan) was set at 80 KV. Particle diameters were determined by image analysis.

Apolipoprotein composition of lipoprotein fractions. Apolipoproteins of the individual lipoprotein fractions were separated by sodium dodecyl sulphate polyacrylamide gradient gel electrophoresis (SDS-PAGE) [24]. To prepare the samples for electrophoresis, lipoprotein fractions were concentrated in Centricon-100 concentrators (MW cut-off 100,000, Amicon Corp., Beverly, MA). Sample (2 mL) was placed in the upper chamber of the concentrator, which was then centrifuged in a fixed-angle rotor (714 x g, 20°C, 30 min) until the desired volume was reached. The final volumes for each fraction were 1.0 ml of chylomicron, 2.0 ml of VLDLA, 1.2 ml of VLDLB, 1.3 ml of VLDLC, and 1.6 ml of LDL. Before running the gel, 0.25 ml 1% SDS and 0.25 ml tracking dye (3.03% Tris, 14.4% glycine, 2% sucrose, 0.05% bromophenol blue) were added to the concentrated fractions. The volumes of the fractions loaded onto the gel were ~60 µl chylomicron, ~60 µl VLDLA, ~40 µl VLDLB, ~40 µl VLDLC, and ~20 µl LDL with a protein content of 35 µg, 35 µg, 25 µg, 20 µg, and 20 µg, respectively, as determined using a commercial assay for quantitative microdetermination of total protein (Sigma Diagnostics, St. Louis, MO). A 1-10 % linear gradient gel was prepared, the samples loaded into the wells, carefully overlayed with running buffer (3.03% Tris, 14.4%

glycine, 0.1% SDS) and run overnight at 45 V. A high molecular weight standard (SDS-6H, Sigma, St. Louis, MO) was run for comparison.

Carotenoid and lipid content of plasma and plasma lipoproteins. Procedures were performed under yellow light. Following the method by Stacewicz-Sapuntzakis [26], duplicate 200-μl or 500-μl aliquots of plasma or plasma lipoproteins, respectively, were denatured by addition of an equal volume of absolute ethanol containing 0.01% BHT and retinyl acetate as the internal standard. The samples were then extracted twice with hexane containing 0.01% BHT and the combined hexane layers were evaporated to dryness under vacuum. The residues were reconstituted with ethyl ether and mobile phase (1:3; vol:vol), and 20-μl aliquots were injected into the HPLC system.

The components of the HPLC system were Waters instruments (Waters Chromatography, Milford, MA), and consisted of the 717Plus Autosampler with temperature control set at 5°C, the 510 Solvent Delivery System, and the 996 Photodiode Array Detector. The system operated with the Millennium 2010 Chromatography Manager software. Data were collected at 290, 325, and 453 nm. Separation of analytes was performed on a 5 μm Nova-Pak C_{18} (Waters) 3.9 x 150 mm analytical column protected by a guard column and eluted with methanol-acetonitrile-tetrahydrofuran (50:45:5) containing 0.1% ammonium acetate. Solvents were HPLC grade; methanol, acetonitrile, and ammonium acetate were purchased from Fisher Scientific (Chicago, IL), tetrahydrofuran (OmniSolv®) was purchased from Baxter (McGaw Park, IL). The mobile phase was filtered (Nylon-66 filter, 0.2 μm, Rainin Instruments Co., Woburn, MA) and

degassed before use. The flow rate was 1.0 ml/min and carotenoids were eluted within 20 min.

Retinyl acetate, retinol, α-tocopherol, α-carotene, β-carotene, and canthaxanthin standards were purchased from Fluka Chemical (Ronkonkoma, NY) and lycopene standard from Sigma Chemical. Lutein was donated by Kemin Industries (Des Moines, IA) and β-cryptoxanthin by Hoffmann-La Roche (Nutley, NJ). Calibration curves were generated from the ratios of the peak height of the carotenoid standards to the peak height of the internal standard plotted against the carotenoid concentration. The presented concentrations (μmol/L) in lipoprotein fractions are based on the original volume of plasma used for the separation of the lipoproteins. Accuracy and precision of the analyses were verified using a standard reference material (SRM 968a, Fat-Soluble Vitamins in Human Serum) from the National Institute of Standards and Technology. Quality control included routine analysis of a plasma pool. Inter-assay coefficients of variation were below 5% for all carotenoids, retinol, and α-tocopherol.

Total plasma cholesterol and LDL-cholesterol were measured enzymatically using a commercial assay from Diagnostic Chemicals Ltd. (Oxford, CT). Accuracy and precision were confirmed using fresh frozen human serum pools (Pacific Biometrics Research Foundation, Seattle, WA). Total plasma triglycerides and chylomicron-triglycerides were determined enzymatically using a commercial assay (Triglycerides/GPO, Boehringer Mannheim Corporation, Indianapolis, IN).

Pearson correlation coefficients were calculated to determine the relation of LDL-cholesterol and LDL-carotenoid concentrations and of plasma total cholesterol and plasma

carotenoid concentrations at baseline. The post-dosing areas under the carotenoid

concentration versus time curves (AUC) were calculated by trapezoidal approximation

after adjustment for the baseline carotenoid concentrations. The statistical significance of

differences between mean values were analyzed by paired t-test.

Results

Subject Characteristics. Table 2-1 summarizes characteristics of the individual

subjects, such as age, Body Mass Index (BMI), and percent body fat determined by

TOBEC. The mean (± SEM) baseline plasma concentrations of prominent carotenoids,

retinol, and α-tocopherol for nine subjects are given in Table 2-2.

Table 2-1. Subject characteristics.

| Subject | Characteristic | | | |
	Age (y)	BMI (kg/m^2)	% Body Fat at Start	% Body Fat at End
1	21	19.3	24.6	24.6
2	36	22.5	35.4	33.6
3	32	24.2	30.5	27.4
4	20	21.6	26.7	27.6
5	23	25.5	36.1	35.8
6	20	21.0	23.6	27.3
7	21	18.7	22.1	24.3
8	35	21.0	28.6	29.6
9	21	28.3	34.1	35.8
10	25	24.0	32.0	31.4
Mean ± SEM	25.4 ± 6.4	22.6 ± 2.9	29.4 ± 5.0	29.3 ± 4.3

The plasma carotenoid concentrations were in the normal range based on the NHANES

III data [27], but at the lower end, which suggests good compliance with instructions to

avoid carotenoid-rich food for 5 days prior to the study period.

As expected, there was a significant positive correlation of BMI and percent body

fat (r = 0.81, p < 0.01). The post-dosing areas under the concentration-time curves

(AUCs) for β-carotene and canthaxanthin (data not shown) were not correlated with BMI

or percent body fat. Baseline plasma β-carotene concentrations were not correlated with

the β-carotene AUCs.

Table 2-2. Baseline plasma concentrations
of carotenoids, α-tocopherol, and retinol.
Values are means for nine subjects ± SEM.

	Concentration (μmol/L)
α-Carotene	0.15 ± 0.04
β-Carotene	0.41 ± 0.02
β-Cryptoxanthin	0.08 ± 0.02
Lutein	0.17 ± 0.02
Lycopene	0.42 ± 0.04
Retinol	1.45 ± 0.10
α-Tocopherol	15.33 ± 0.51

Particle size distribution and purity of the lipoprotein fractions. Cumulative rate

ultracentrifugation fractionated plasma triglyceride-rich lipoproteins into four subfractions

of progressively decreasing diameter and increasing density, i.e. chylomicrons, VLDLA,

VLDLB, and VLDLC (Figure 2-1). The size distribution of lipoprotein particles from two

Figure 2-1. Representative electron microscope photograph of chylomicrons, very-low density lipoprotein (VLDL) subfractions, and low density lipoproteins (LDL) isolated from the plasma of a normotriglyceridemic subject 6 hours after a fat-rich meal.
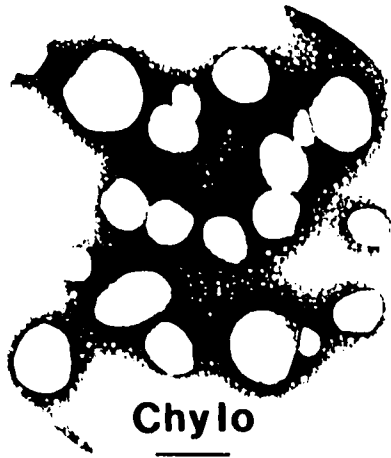
Chylo
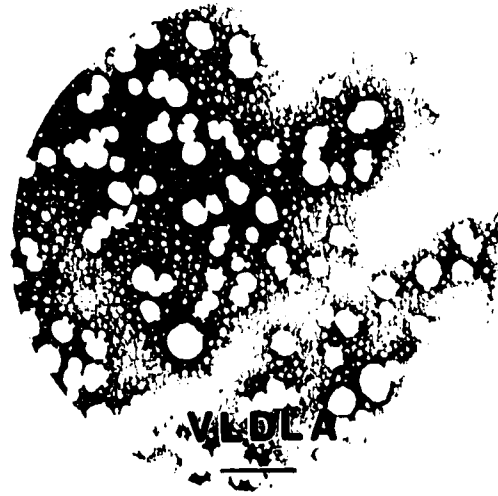
VLDL A
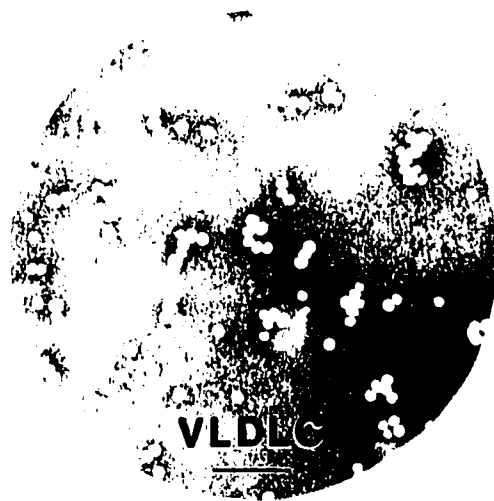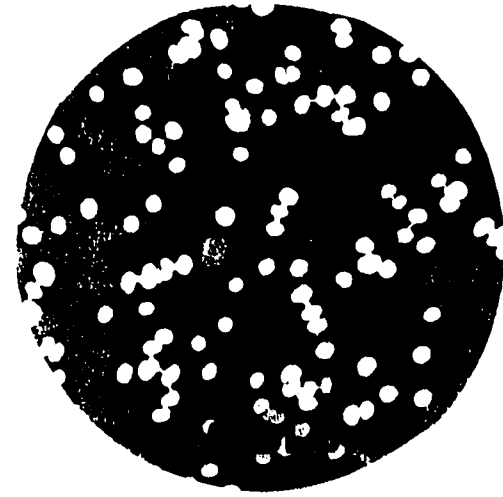
VLDL c

LDL

subjects (6-h postprandial samples) was determined by electron microscopy and image

analysis. The mean diameter (± SEM) was 100.0 ± 0.0 nm, chylomicrons; 46.0 ± 2.4 nm,

VLDLA; 37.8 ± 2.6 nm, VLDLB; 24.8 ± 3.3 nm, VLDLC; and 14.0 ± 0.1 nm, LDL. The

purity of the fractions was evaluated by visualization of the apolipoproteins by SDS-

PAGE (Figure 2-2). Apolipoprotein B48 (apo-B48) was the major apo-B detected in the

chylomicron fraction with a faint band of apo-B100 present as noted by other investigators

[28]. Apo-B48 was not detected in the other TRL fractions or in LDL.

Plasma carotenoid concentration-time curves. The plasma increments of β-

carotene and canthaxanthin measured for 528 h after ingestion of a 25-mg single oral dose

of either carotenoid are shown in Figure 2-3. The presented concentrations are the means

(± SEM) of 9 subjects adjusted by subtraction of the baseline carotenoid concentrations.

The plasma canthaxanthin increment was monophasic with a rapid increase in

concentration to a single peak at 12 h post-dosing (1.41 ± 0.11 μmol/L) followed by a

steady decline to concentrations near baseline at 360 h. The plasma β-carotene increment

was biphasic with a minor peak at 5 h (0.36 ± 0.06 μmol/L) after which the concentrations

declined and then increased with a major peak at 48 h (0.82 ± 0.21 μmol/L) post-dosing.

The β-carotene concentrations then decreased slowly to values close to baseline

concentration at 528 h. These findings confirm the kinetics observed in an earlier pilot

study (20).

β-Carotene and canthaxanthin kinetics in plasma lipoprotein fractions.

Lipoproteins were isolated from the plasma of five subjects. The mean concentration-time

Figure 2-2. Separation of apolipoproteins B48 and B100 by sodium dodecyl sulphate polyacrylamide gradient gel electrophoresis (1-10% gradient). The plasma lipoproteins were fractionated by cumulative rate ultracentrifugation. Abbreviations: MW, standard of molecular weight markers; Chy, chylomicron; V_A, very-low density lipoprotein (VLDL) A; V_B, VLDLB; V_C, VLDLC; LDL, low density lipoprotein.

MW   Chy V_A V_B V_C LDL

KD

205

116
97

66

45

29
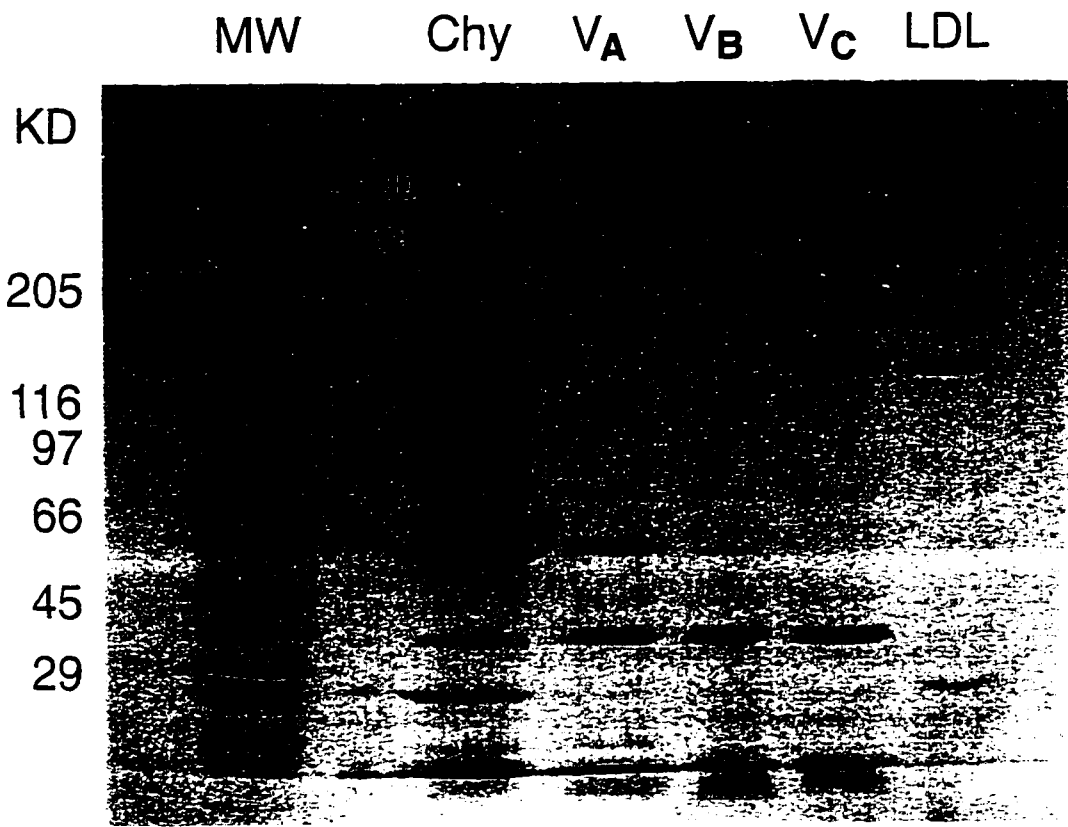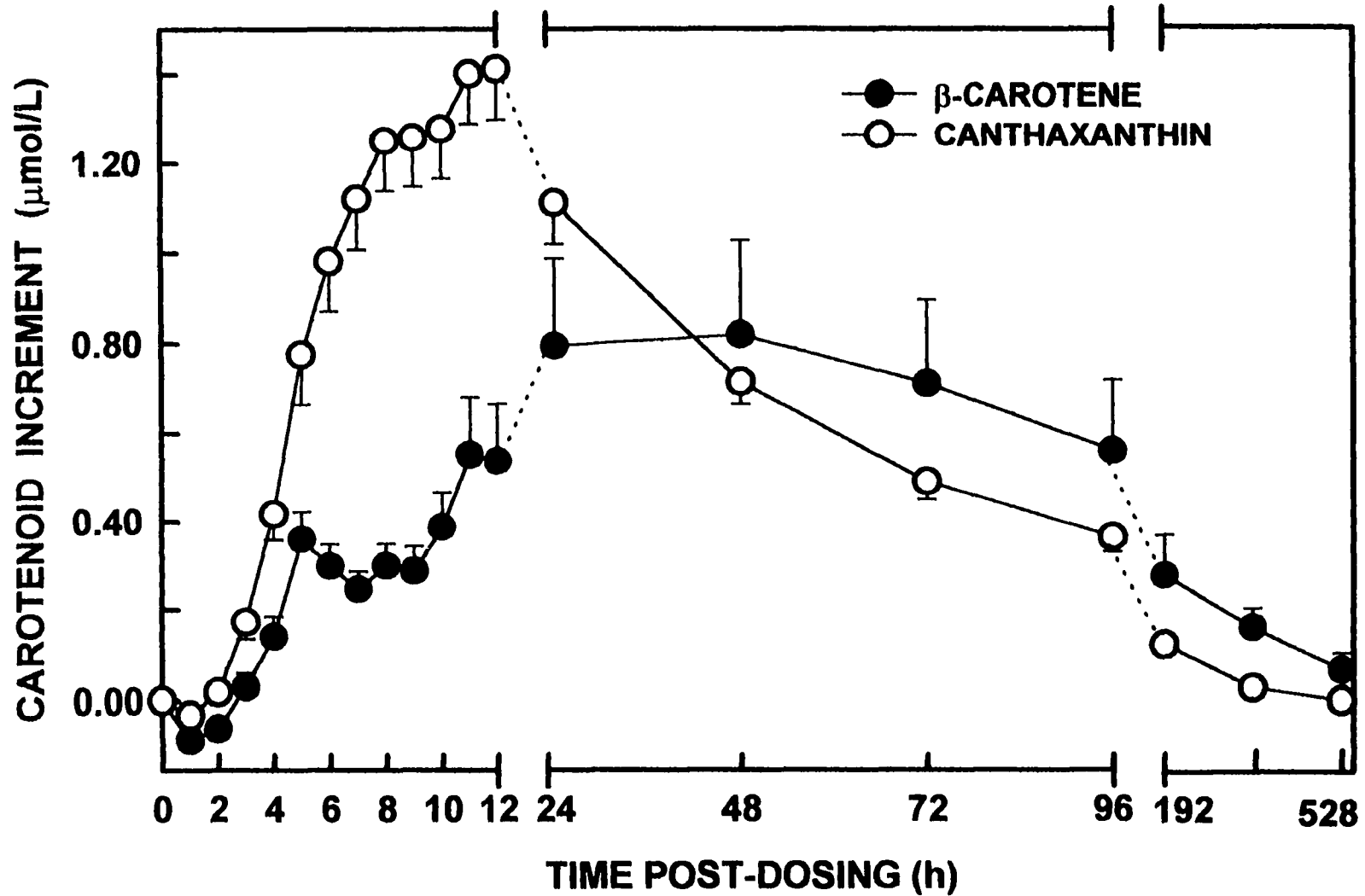
Figure 2-3. Plasma increments after ingestion of a single oral dose of 25 mg of β-carotene or canthaxanthin. Concentrations shown are mean values for nine subjects ± SEM adjusted by subtraction of the respective baseline carotenoid concentrations.

curves of β-carotene appearance in the plasma lipoprotein fractions for 10 h after

ingestion of 25 mg β-carotene are shown in Figure 2-4. There were coincident peak β-

carotene increments at 6 h post-dosing in chylomicrons and each VLDL subfraction. The

mean peak lipoprotein β-carotene concentration increment in the VLDLA subfraction at 6

h exceeded that in chylomicrons by a factor of 1.7 (0.12 ± 0.05 μmol/L and 0.07 ± 0.03

μmol/L, respectively). An early decrease in β-carotene concentration was observed in the

LDL fraction which was followed by a gradual increment starting 4 to 6 h post-dosing

(Figure 2-5). The decrease in LDL-β-carotene concentrations coincides with an observed

decrease in total plasma β-carotene concentrations. The timing of the β-carotene

increment was similar in IDL and LDL; β-carotene concentrations started to rise 4 to 6 h

post-dosing and were still increasing at 10 h.

The mean canthaxanthin concentration-time curves in the individual lipoprotein

fractions for 10 h after ingestion of 25 mg canthaxanthin are shown in Figure 2-4. The

mean canthaxanthin concentration (± SEM) peaked 6 h post-dosing in the chylomicron

and VLDLA fractions (0.17 ± 0.03 μmol/L and 0.21 ± 0.04 μmol/L, respectively). The

canthaxanthin mean peak increment in VLDLB and VLDLC fractions occurred at 8 h

post-dosing (0.09 ± 0.02 μmol/L and 0.10 ± 0.03 μmol/L, respectively). Canthaxanthin

concentrations in LDL started to rise 2 h post-dosing, and the increment in LDL at 6 h

post-dosing (0.25 ± 0.05 μmol/L) exceeded that in chylomicrons and in VLDLA. In the

IDL fraction a gradual increase in canthaxanthin concentration was observed starting at 2

h and continuing at 10 h post-dosing.
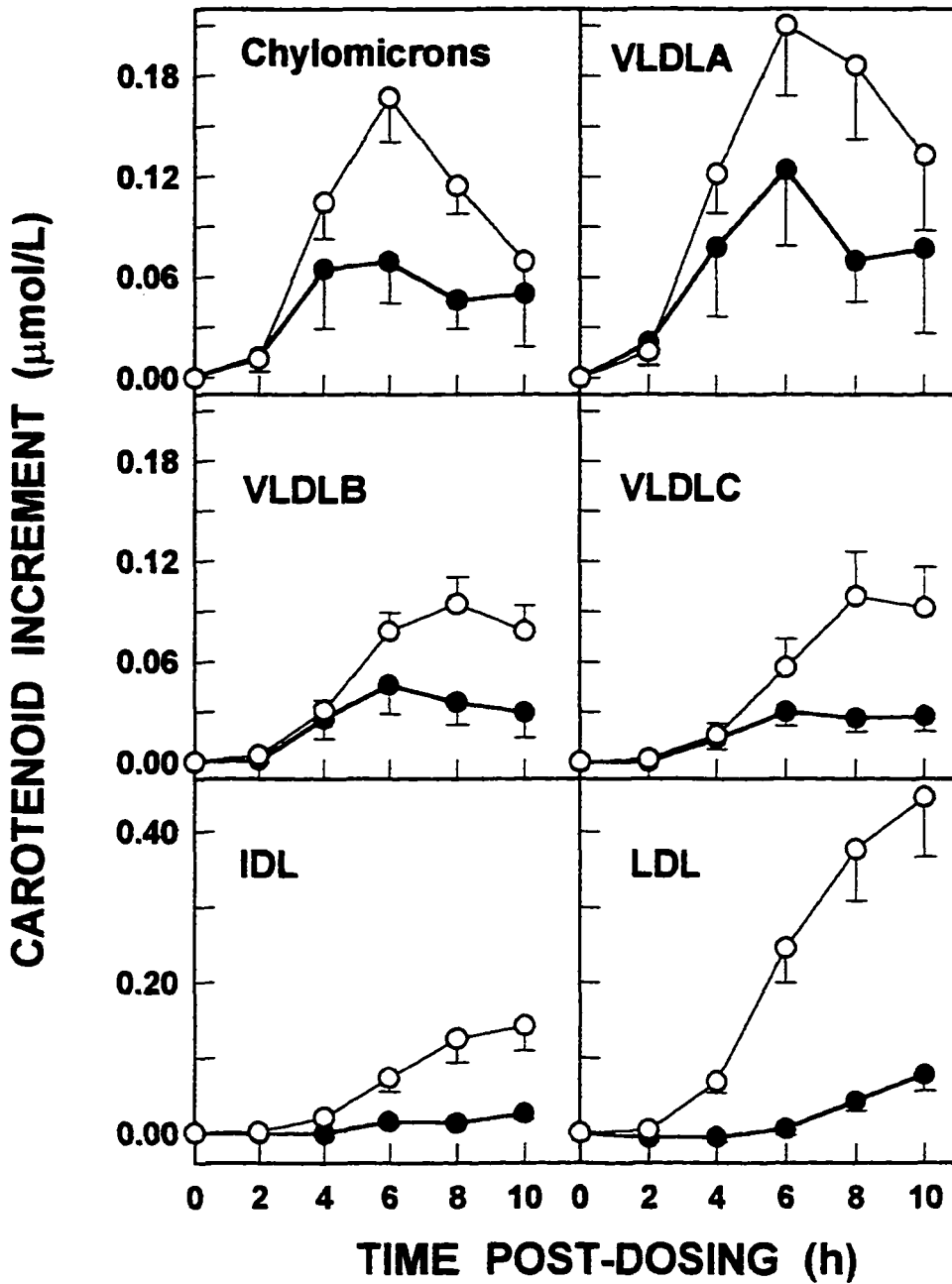
Figure 2-4. Mean lipoprotein carotenoid increments (μmol/L) in the chylomicron, very-low density A (VLDLA), VLDLB, VLDLC, intermediate density (IDL), and low density lipoprotein (LDL) fractions after ingestion of a single oral dose of 25 mg canthaxanthin (O) or β-carotene (●). Data points presented are mean values for five subjects ± SEM.
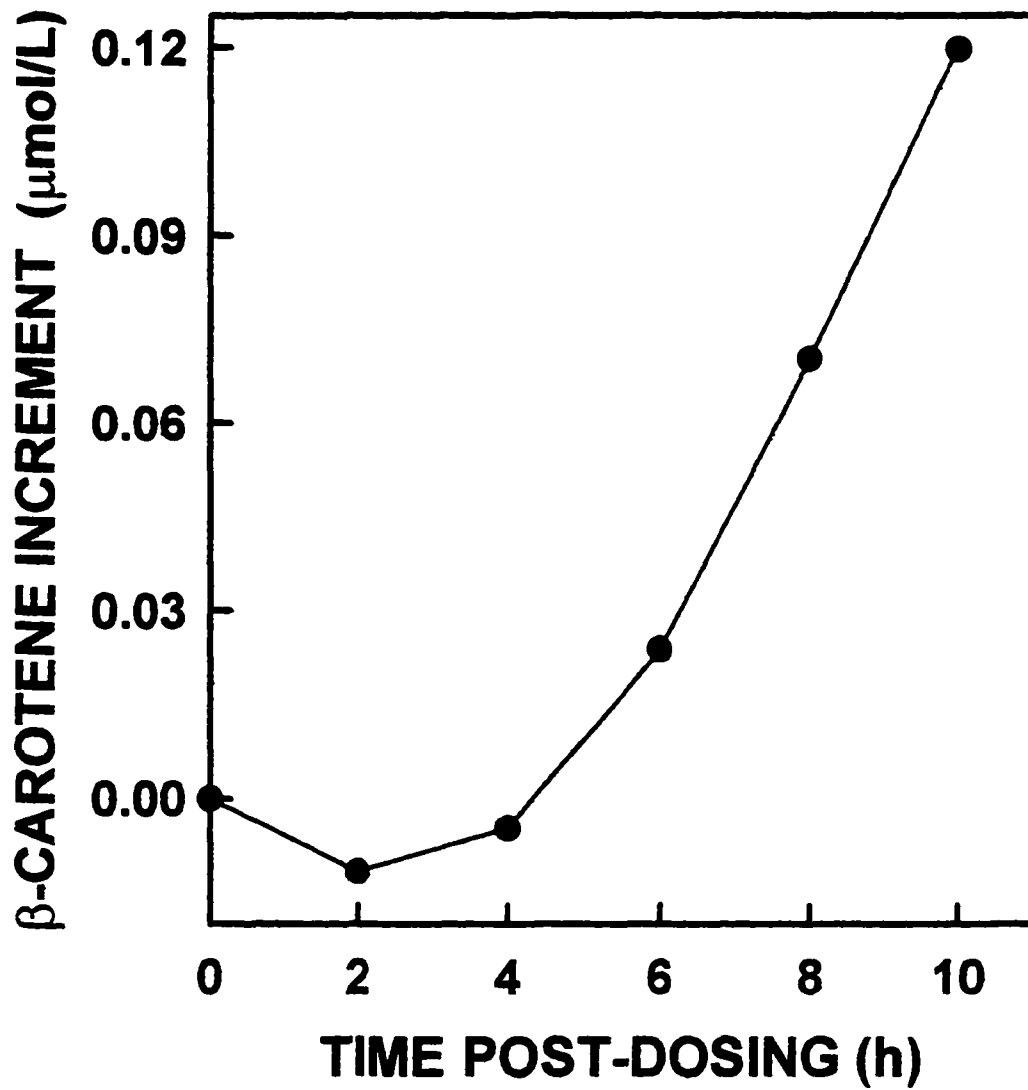
Figure 2-5. LDL-β-carotene increment (μmol/L) of a representative subject after ingestion of a single β-carotene dose. Note the initial decrease below baseline LDL concentration.

Figure 2-6 presents the distribution of β-carotene and canthaxanthin increments in

lipoproteins at three time points during 10 h post-dosing. At 4 h post-dosing, most of the

newly absorbed carotenoids were associated with chylomicrons and VLDLA (β-carotene,

$29.8 \pm 4.7\%$ and $37.8 \pm 4.2\%$, respectively; canthaxanthin, $26.5 \pm 5.3\%$ and $29.1 \pm 5.7\%$,

respectively). There was no detectable β-carotene in LDL at 4 h post-dosing; interestingly,

$11.0 \pm 4.6\%$ was associated with the infranatant, which is basically HDL and plasma

proteins. In contrast, $15.8 \pm 2.3\%$ of newly absorbed canthaxanthin appeared in LDL and

$16.5 \pm 3.4\%$ in the infranatant at 4 h post-dosing. At 6 h post-dosing, β-carotene

increments in the infranatant were significantly greater than the increments in LDL ($p <$

0.05), whereas, at 10 h post-dosing, the difference did not reach statistical significance (p

$= 0.094$). Canthaxanthin increments in LDL and infranatant were approximately equal at

each time point. At 10 h post-dosing, $32.4 \pm 2.9\%$ of newly absorbed β-carotene was

associated with TRL (sum of individual fractions), $8.3 \pm 2.9\%$ with IDL, $19.8 \pm 3.3\%$ with

LDL, and $39.3 \pm 8.0\%$ with infranatant. In contrast, a greater percentage of canthaxanthin

accumulated in LDL ($32.4 \pm 3.6\%$, $p < 0.05$) and a lesser percentage, although the

difference was not statistically significant, in the infranatant ($30.1 \pm 2.1\%$, $p = 0.31$) at 10

h post-dosing. Lipoprotein distribution of β-carotene and canthaxanthin during the 10-h

postprandial phase demonstrates the rapid accumulation of canthaxanthin and the delayed

appearance of β-carotene in LDL.

Comparing the distribution of β-carotene and canthaxanthin at 10 h post-dosing in

lipoproteins of density ≥ 1.06 g/mL as a group (LDL plus HDL) with triglyceride-rich
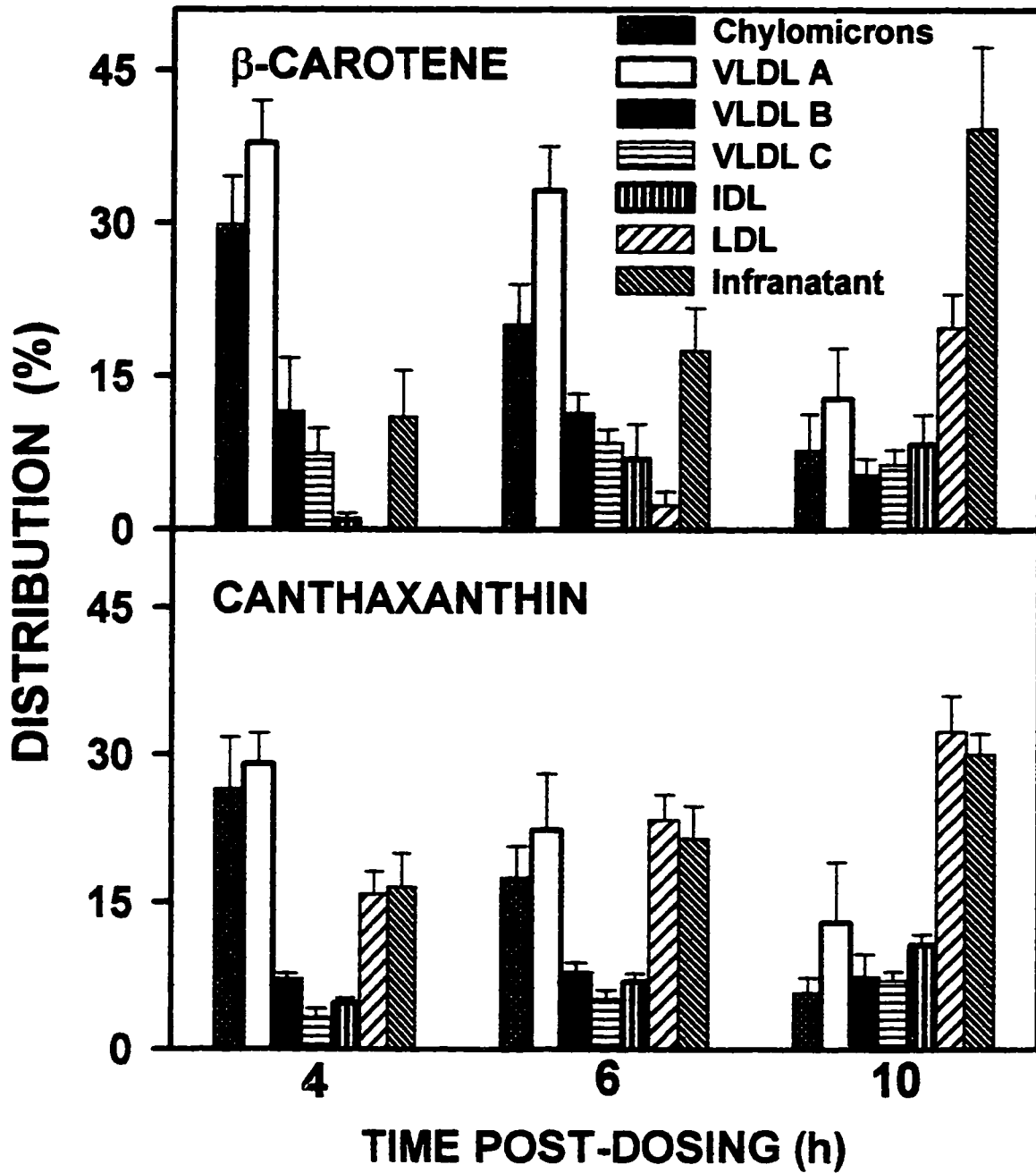
Figure 2-6. Distribution of β-carotene and canthaxanthin in lipoproteins at 4, 6, and 10 hours after ingestion of single equimolar doses. Data represent mean values for 5 subjects ± SEM.

lipoproteins as a group (chylomicrons plus VLDL subfractions) we found similar

distribution for both carotenoids. At 10 h post-dosing, $32.4 \pm 2.9\%$ of β-carotene and

$33.3 \pm 2.8\%$ of canthaxanthin were associated with TRL and $59.0 \pm 5.7\%$ of β-carotene

and $62.4 \pm 2.9\%$ of canthaxanthin with higher density lipoproteins.

Distribution of carotenoids, α-tocopherol, and retinol in the lipoprotein

fractions at baseline. The distribution of carotenoids, α-tocopherol, and retinol at

baseline in the lipoprotein fractions is presented in Table 2-3. The hydrocarbon carotenes,

lycopene, α-carotene, and β-carotene, were primarily (> 50%) associated with LDL,

whereas the majority (> 50%) of the xanthophylls, lutein/zeaxanthin and cryptoxanthin,

was found in the infranatant fraction which includes HDL. Retinol was 99% associated

with the infranatant fraction, 1% was in LDL. The majority of α-tocopherol (38%) was

recovered in the infranatant fraction, which includes HDL, and approximately equal

amounts were distributed between VLDL and LDL, 25 and 28%, respectively.

Table 2-3. Relative lipoprotein distribution of carotenoids, α-tocopherol, and retinol at baseline. Values are means for five subjects $\pm$ SEM.

	Distribution (%, Mean ± SEM)					
	VLDLA	VLDLB	VLDLC	IDL	LDL	Infranatant
Lutein	4.9 ± 2.3	5.6 ± 1.1	4.0 ± 1.2	5.1 ± 0.4	17.0 ± 2.5	62.1 ± 2.6
β-Cryptoxanthin	2.9 ± 1.9	2.7 ± 1.7	4.1 ± 2.0	5.8 ± 2.6	31.4 ± 5.4	53.1 ± 1.5
Lycopene	1.5 ± 1.0	4.3 ± 2.2	4.2 ± 1.8	12.0 ± 1.4	60.1 ± 3.8	18.0 ± 2.8
α-Carotene	0.6 ± 0.6	3.8 ± 3.8	0.4 ± 0.4	5.1 ± 0.7	61.8 ± 5.5	28.3 ± 2.6
β-Carotene	1.6 ± 1.0	1.9 ± 0.8	1.9 ± 1.3	13.5 ± 2.2	54.0 ± 3.5	27.2 ± 2.4
α-Tocopherol	8.6 ± 3.5	7.3 ± 3.0	8.8 ± 1.5	9.3 ± 1.7	28.3 ± 2.0	37.6 ± 7.9
Retinol	0	0	0	0	0.8 ± 0.3	99.2 ± 0.3

Baseline correlations of carotenoid, α-tocopherol, and retinol concentrations

with plasma and LDL-cholesterol. No correlation was found between the fasting baseline

LDL concentrations of cholesterol and the concentrations of β-carotene, lutein/zeaxanthin,

or cryptoxanthin in LDL. In our five subjects, the LDL concentrations of cholesterol

tended to be correlated with LDL concentrations of lycopene ($r = 0.60$, $p = 0.07$) and α-

carotene ($r = 0.59$, $p = 0.08$). There was a highly significant positive correlation of

baseline concentrations of α-tocopherol and cholesterol in LDL ($r = 0.78$, $p = 0.008$). If

we examine the relation of concentrations of total cholesterol and of individual

carotenoids in fasting plasma at baseline, only the correlation of plasma cholesterol with

plasma lycopene was statistically significant ($r = 0.65$, $p = 0.022$). Plasma cholesterol and

plasma α-tocopherol concentrations were also positively correlated ($r = 0.64$, $p = 0.025$).

Table 2-4 presents significant correlations of fasting carotenoid concentrations in

LDL. Concentrations of the oxycarotenoid, lutein, were correlated with those of lycopene,

α-carotene, and β-carotene in LDL. The concentrations of the hydrocarbon carotenes in

LDL, α-carotene, β-carotene, and lycopene were significantly correlated. Baseline total

plasma concentrations of lutein were significantly correlated with those of each of the

other carotenoids.

Triacylglycerol kinetics in plasma and chylomicrons. Figure 2-7 shows the

kinetics of triacylglycerol, β-carotene, and canthaxanthin in plasma and chylomicrons

during the postprandial phase. The baseline-corrected mean ($\pm$ SEM) plasma triglyceride

curve ($n = 9$) has a peak increment 4 to 7 hours post-dosing (0.50 ± 0.09 mmol/L)
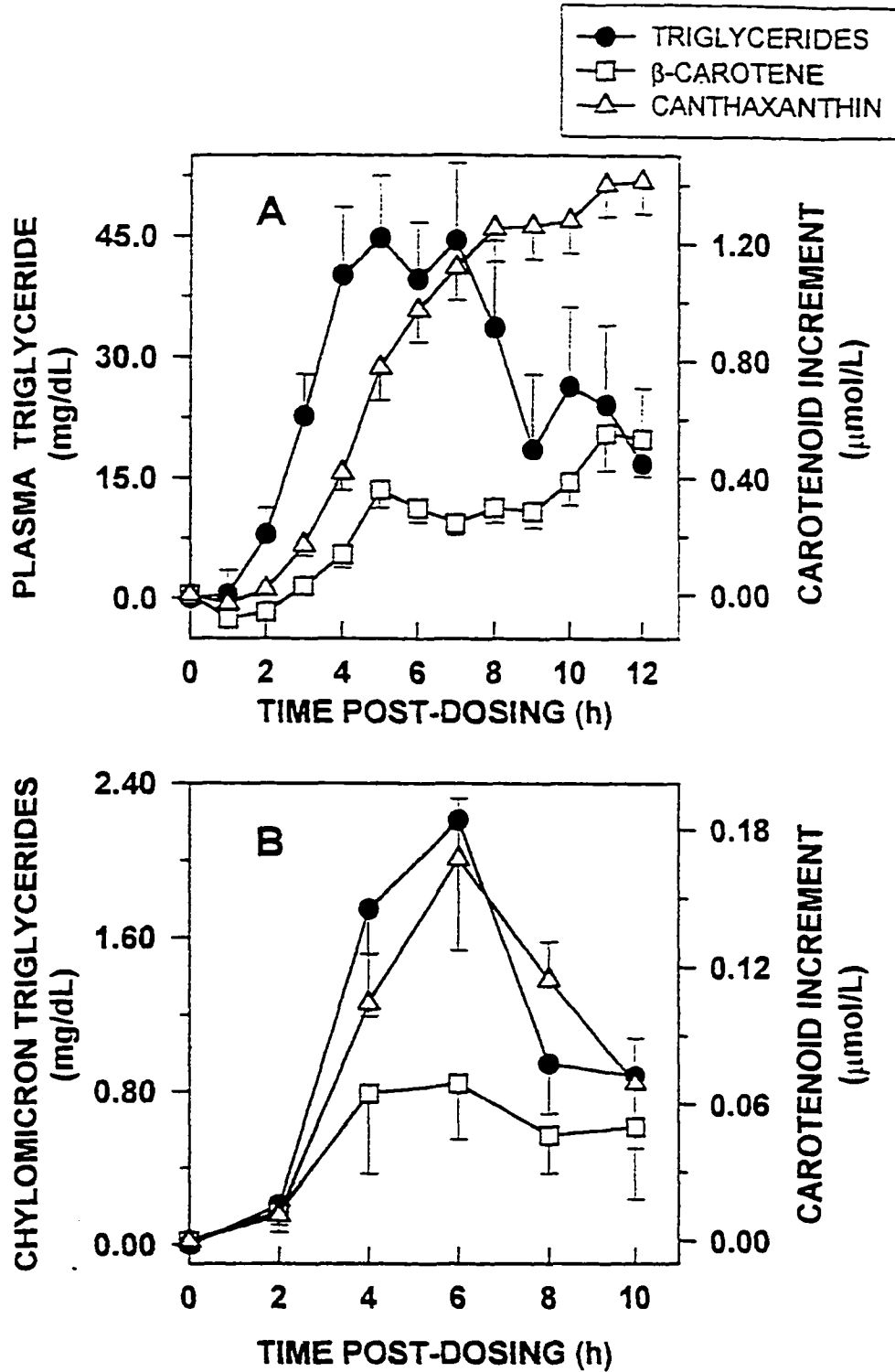
Figure 2-7. Triglyceride, β-carotene, and canthaxanthin increments in plasma (A) and chylomicrons (B) after ingestion of a β-carotene or canthaxanthin dose with a fat-rich meal. Data represent mean (± SEM) values of nine (A) and five subjects (B).

coinciding with the initial minor peak increment of β-carotene. The kinetics of

triglycerides and canthaxanthin in plasma are not similar which is explained by the

coincident increments of canthaxanthin in TRL and LDL. In chylomicrons, the mean

triglyceride curve (n = 5) peaks 6 h post-dosing (0.02 ± 0.01 mmol/L) coincident with β-

carotene and canthaxanthin increments.

Table 2-4. Correlations of fasting plasma carotenoid concentrations in LDL.

	Correlation Coefficient (r)	p Value
LDL-Lutein and LDL Lycopene	0.66	0.05
LDL α-Carotene	0.71	0.05
LDL β-Carotene	0.63	0.05
LDL Lycopene and LDL α-Carotene	0.62	0.05
LDL β-Carotene	0.72	0.05
LDL α-Carotene and LDL β-Carotene	0.75	0.01

Discussion

The kinetics of the oxycarotenoid, canthaxanthin, and the hydrocarbon carotene,

β-carotene, in plasma are distinct (Figure 2-3). We observed a monophasic time course for

canthaxanthin with rapid accumulation in plasma and a peak concentration at 12 h post-

dosing, which is similar to preliminary reports of the pharmacokinetics of canthaxanthin

[20, 29] and other oxycarotenoids [22, 30]. Thus canthaxanthin is a suitable model for study of the metabolism of oxycarotenoids in humans.

Investigation of the kinetics of canthaxanthin in lipoprotein fractions is informative regarding the mechanism underlying the monophasic increment of canthaxanthin in plasma. There was a rapid accumulation of canthaxanthin in chylomicrons and VLDLA with a peak increment in the VLDLA fraction at 6 h post-dosing that exceeded that in chylomicrons (Figure 2-4). The inability to detect apo-B48 in the VLDLA fraction by SDS-PAGE (Figure 2-2) suggests that significant contamination by chylomicron remnants is an unlikely explanation for the rapid canthaxanthin increment in VLDLA. Canthaxanthin concentrations increased rapidly in the LDL fraction; the 6-h post-dosing increment in LDL exceeded the 6-h canthaxanthin increment in chylomicrons and in VLDLA. These findings, the rapid increase of canthaxanthin concentrations in both TRL and LDL, confirm the hypothesis that the monophasic plasma increment is due to coincident peaks in TRL and LDL [20].

The early increase of canthaxanthin content in the LDL fraction may result from canthaxanthin transfer from TRL, incorporation into intestinal VLDL particles which are subsequently metabolized to LDL, or rapid incorporation into hepatic VLDL. Although our results are highly suggestive of canthaxanthin transfer from TRL to LDL, an in vitro study could not demonstrate transfer of lutein/zeaxanthin, oxycarotenoids which have similar polarity to canthaxanthin, among lipoprotein fractions in chylomicron-free plasma [31]. Canthaxanthin, an oxycarotenoid, is more polar than β-carotene and is likely to be solubilized preferentially in the surface phospholipids of lipoproteins [32]. Localization at

the surface may make oxycarotenoids prone to transfer to lipoproteins with higher phospholipid/apolar lipid ratios, such as LDL or HDL. Intestinal VLDL cannot be separated from hepatic VLDL because they have the same flotation characteristics and both carry apo-B100 as their integral apolipoprotein. It is believed that the contribution of intestinal VLDL during postprandial lipemia is insignificant [33], and thus intestinal VLDL would not be expected to be a major route for intestinal carotenoid absorption. At 4 h post-dosing, canthaxanthin concentrations began to rise slowly in VLDLC, the fraction thought to contain the majority of particles which are converted to LDL [34]. The canthaxanthin content in LDL exceeded that in VLDLC at 4 h post-dosing, so that conversion of VLDLC particles into LDL does not appear to be responsible for the early appearance of canthaxanthin in LDL.

The biphasic kinetics of β-carotene appearance in plasma after a single oral dose were demonstrated previously [20]. The early peak occurring 4-7 h post-dosing has been attributed to chylomicron influx and to occurrence of β-carotene in VLDL [20]. Cornwell et al. [12] showed that the β-carotene peak increment in the VLDL fraction at 6 h post-dosing exceeds that in the chylomicron fraction. The investigators attributed the large VLDL increment to contamination of the VLDL fraction with chylomicron remnants. The second larger peak at 24 to 48 h post-dosing was associated with the LDL fraction and was attributed to hepatic secretion of VLDL particles which are converted to LDL particles by action of lipoprotein lipase.

In our study we observed that the rapid postprandial plasma increment of β-carotene, as well as of canthaxanthin, was most marked in the large VLDL subfraction (S_f

175-400) (Figure 2-4). The large β-carotene increment in VLDLA at 6 h post-dosing exceeded the β-carotene increment in chylomicrons; this is consistent with the earlier findings of Cornwell et al. [12] for total VLDL. Apo-B48, the apolipoprotein associated with chylomicrons, could not be detected in the VLDL subfractions by SDS-PAGE (Figure 2-2); Apo-B100 was the only apolipoprotein B detected in these fractions. An increase in the number of VLDL particles during postprandial lipemia has been observed recently by other investigators [17, 18] and was attributed to VLDL particles of hepatic origin. In a recent study [35], of newly absorbed β-carotene and retinyl esters derived from cleavage of β-carotene in TRL, it was concluded, using β-carotene and retinyl esters as indicators for intestinal lipoproteins, that the increase in TRL was primarily due to lipoproteins of intestinal origin.

If the VLDL particles in fraction A are of hepatic origin, how did the carotenoids accumulate so rapidly in this fraction? One could speculate that there was transfer of carotenoids from chylomicrons to hepatic VLDL. This is unlikely to be the case for β-carotene which is very hydrophobic and solubilized in the core triacylglycerol of lipoproteins [32]. An in vitro study was unable to demonstrate transfer of β-carotene or other carotenoids among lipoprotein fractions during incubation of chylomicron-free plasma [31]. The delayed appearance of β-carotene in the LDL fraction also indicates that transfer is unlikely [14].

Since we did not detect apo-B48 in the VLDLA subfraction (Figure 2-2), we cannot conclude that the VLDLA fraction contains remnants from catabolism of

chylomicrons, i.e. removal of triglyceride by action of lipoprotein lipase. Chylomicron remnants with the incorporated carotenoids are quickly taken up by the liver. Berr et al. [34] reported mean half-lives for chylomicrons in plasma of 29 ± 16 min. In hepatic tissue, β-carotene can either be stored, converted to retinol, or repackaged into VLDL and released into the circulation. If the early rise of β-carotene in the VLDLA fraction is due to hepatic secretion of nascent VLDL particles it would imply a rapid turnover of β-carotene in hepatic tissue.

As discussed above, small VLDL particles (S_f 20-40), which would be found in VLDLC using our fractionation method, are preferentially converted to LDL. We observed an increase of β-carotene concentration in the VLDLC fraction that reached a plateau between 6 and 8 h post-dosing. The concentration of β-carotene in LDL started to rise at 6 h post-dosing coinciding with the plateau in VLDLC, indicative of steady influx of particles into the S_f 20-100 range fraction and subsequent conversion to LDL particles. It is likely that the majority of VLDLC particles are of hepatic origin and that these small VLDL particles are subsequently converted to LDL.

We observed an early decrease in plasma β-carotene concentrations below baseline values before concentrations began to rise at 4 to 6 h post-dosing (Figure 2-5). Addressing β-carotene kinetics in individual lipoprotein fractions revealed that the early decline in β-carotene concentrations was associated with the LDL fraction. It is plausible that incoming intestinal lipoproteins compete with hepatic lipoproteins for catabolism by lipoprotein lipase so that less hepatic VLDL particles are converted to LDL particles.

It is interesting to note that the triglyceride increments peaked 6 h post-dosing in TRL coinciding with β-carotene and canthaxanthin (Figure 2-7). A delay of β-carotene peak increments was observed by VanVliet et al. [35]. They observed early triglyceride peak increments (2 h post-dosing) and β-carotene peak increments 5 h post-dosing. These discrepancies may stem from the type of fat in the experimental meal. VanVliet used a highly unsaturated fat, whereas our subjects derived the dietary fat from dairy products, with a corresponding higher intake of saturated fatty acids.

Under fasting conditions, hydrocarbon carotenes are carried predominantly by LDL, whereas, of the xanthophylls, β-cryptoxanthin is equally distributed between LDL and HDL, and lutein plus zeaxanthin are primarily associated with HDL [11, 13,14, 31, 36, 37]. Borel et al. [32] demonstrated in experiments on the solubility of polar and apolar carotenoids that xanthophylls are preferentially solubilized in phospholipid, so that these carotenoids are expected to be associated with lipoproteins of high phospholipid:apolar-lipid ratio such as HDL. Plasma β-carotene has been reported to be associated 5 - 12% with VLDL, 63 - 71% with LDL, and 15 to 28% with HDL [13, 14, 37]. In studies investigating the relative distribution of several carotenoids, some found that the relative amount of plasma lycopene associated with LDL was higher than the relative amount of plasma β-carotene carried in LDL [14, 37]. Lycopene was found to be associated 10% with VLDL, 73% with LDL, and 17% with HDL [14]. The distribution of α-tocopherol is more equal among the lipoprotein fractions. Bjornson et al [13] found 18 - 19% of plasma α-tocopherol associated with VLDL, 39 - 58% with LDL, and 33 - 43% with HDL.

Our data on the relative distribution of carotenoids is somewhat different to previous studies in that we found smaller percentages of plasma lycopene and β-carotene associated with LDL; 60 and 54 %, respectively (Table 2-3). Discrepancies with previous reports may stem from different lipoprotein fractionation methods used in the studies. We determined the amount of the carotenoids in IDL, whereas previous reports were limited to VLDL, LDL, and HDL. Assuming that the IDL fraction separated in our study contributed to the LDL fraction in other studies, we find that our data agree well. Alternatively, the avoidance of carotenoid-rich foods might have had an impact on the relative distribution of carotenoids in lipoproteins. To our knowledge, this has not been studied before. Our subjects were instructed to avoid ingestion of carotenoid-rich foods during the week before collection of the baseline blood sample. In contrast, it has been shown that supplementation with β-carotene does not change its relative distribution in lipoproteins [13, 37].

It is interesting to note that plasma lycopene but not β-carotene was significantly correlated with plasma cholesterol in the present study. There was a tendency of lycopene and α-carotene in LDL to be correlated with LDL-cholesterol ($p < 0.10$). The correlations of plasma cholesterol with plasma β-carotene ($p = 0.97$) and of LDL-cholesterol with LDL-β-carotene ($p = 0.28$) were not significant. One possible mechanism by which carotenoids may exert beneficial effects on human health is via their antioxidant properties. Lycopene has been shown to be a more efficient singlet oxygen quencher than β-carotene [38] and since lycopene is transported primarily in LDL it could be speculated that it is the

major singlet oxygen quencher in LDL-cholesterol. Recently, a metabolite resulting from oxidation of lycopene has been identified in human plasma [39]. There are inconsistent findings regarding correlations of LDL-cholesterol with plasma carotenoids. Some studies demonstrate significant positive correlation of β-carotene with total plasma cholesterol [37, 40], others fail to find significant correlations [37]. Addressing individual carotenoids, lycopene appears to be more highly correlated with plasma and low-density lipoprotein-cholesterol than β-carotene [41, 42].

The results of this study show distinct kinetics of β-carotene and canthaxanthin in human plasma lipoproteins. Early increments of β-carotene and canthaxanthin in plasma reflect rapid incorporation into TRL; we observed early increments of β-carotene and canthaxanthin in both chylomicrons and VLDL. The peak β-carotene increment in LDL was delayed, suggesting that β-carotene is not readily transferred from TRL to LDL. In contrast, canthaxanthin peak increments in TRL coincide with rising concentrations in LDL. The rapid rise of canthaxanthin concentration in LDL may be the result of influx from intestinal VLDL particles which are subsequently converted to LDL, rapid secretion in hepatic VLDL followed by catabolism to LDL, or transfer of canthaxanthin from TRL to LDL. We demonstrated the underlying mechanism for the monophasic plasma kinetics of oxycarotenoids, that is, coincident peak increments in TRL and LDL. Rapid accumulation of oxycarotenoids in LDL particles may have profound health implication if we consider their potential action as antioxidants.

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3. INTERACTIONS OF THE POSTPRANDIAL APPEARANCE OF β-CAROTENE AND CANTHAXANTHIN IN HUMAN PLASMA TRIGLYCERIDE-RICH LIPOPROTEINS

Inke Paetau and Wendy S. White

Abstract

The effects of ingestion of a combined equimolar dose of β-carotene (47 μmol)

and canthaxanthin (44 μmol) with a fat-rich meal on their individual appearance in plasma

and postprandial plasma lipoproteins were investigated in healthy premenopausal women.

During three study periods the subjects (n = 9) ingested either an individual β-carotene or

canthaxanthin dose or a combined β-carotene plus canthaxanthin dose. Blood samples

were taken at 0 h and then hourly for 12 h post-dosing; additional blood samples were

drawn after an overnight fast at 24, 48, 72, 96, 192, 360, and 528 h post-dosing. At six

time points, i.e. 0, 2, 4, 6, 8, and 10 h post-dosing, for a subset of subjects (n = 5) plasma

lipoproteins were separated by cumulative rate ultracentrifugation into chylomicrons, three

VLDL subfractions of decreasing size and increasing density (VLDL A, B and C,

respectively), IDL, and LDL. The mean plasma β-carotene concentration peaked at 5 h

and 48 h, whereas the mean plasma canthaxanthin concentration peaked once at 12 h post-

dosing. The ingestion of a combined dose of β-carotene and canthaxanthin inhibited the

appearance of the canthaxanthin dose ($p < 0.01$), but did not significantly affect the appearance of the β-carotene dose in plasma. Ingestion of the combined dose significantly inhibited the appearance of canthaxanthin in triglyceride-rich lipoproteins (TRL), but did not affect the rapid accumulation of canthaxanthin in LDL at 10-h post-dosing. The mean ($\pm$ SEM) areas under the plasma lipoprotein canthaxanthin concentration-time curves (AUC) were significantly reduced in chylomicrons by $37.8 \pm 7.1\%$ ($p < 0.01$), in VLDLA by $43.0 \pm 8.1\%$ ($p < 0.05$), in VLDLB by $32.7 \pm 8.1\%$ ($p < 0.05$), and in VLDLC by $30.3 \pm 7.6\%$ ($p < 0.001$). In contrast, concurrent ingestion of the canthaxanthin dose did not significantly affect the appearance of the β-carotene dose in TRL or LDL. These results confirm and extend earlier reports of specific interactions of β-carotene and the oxycarotenoids canthaxanthin and lutein during intestinal absorption.

Introduction

There is consistent epidemiological evidence for an association of high intake of carotenoid-rich fruits and vegetables and high serum β-carotene concentrations with reduced risk of lung cancer [1-4]. It is not clear if β-carotene is the beneficial agent or if β-carotene is simply a marker for other protective dietary components, including other carotenoids. Recently, an oxycarotenoid, lutein, was found to have stronger associations with reduced risk of certain degenerative diseases than β-carotene [5-7]. High consumption of dark-green leafy vegetables and corresponding high lutein intake had

stronger association with reduced risk of lung cancer than dietary β-carotene [2]. In the

Eye Disease Case-Control Study, men with the highest lutein intakes had significantly

reduced risk of age-related macular degeneration [7].

Observational studies cannot establish a cause-and-effect relation, therefore clinical

trials are necessary to establish a direct relation between purified food constituents and

chronic disease. Several β-carotene intervention trials have recently concluded and the

findings of these studies are, in general, not consistent with a protective effect of β-

carotene supplementation. In these trials β-carotene supplementation had detrimental

effects [8, 9], no effects [10-12], or beneficial effects [13, 14] on selected degenerative

diseases. Supplementation of heavy smokers with β-carotene resulted in significant

increases in the incidence of lung cancer [8, 9]. An interaction of β-carotene with ethanol

may be one explanation for the negative outcome in this study because the increased

incidence of lung cancer was primarily evident among men with the highest alcohol

consumption [15]. The serum β-carotene concentrations achieved in these studies were

extremely high, which may have resulted in a prooxidative activity of β-carotene in the

lung [16]. Possible negative effects of supplemental β-carotene on other micronutrients

are another potential mechanism underlying the observed increase in risk [4].

Limited data suggest that hydrocarbon carotenes and oxycarotenoids are distinct in

their intestinal absorption and metabolism. In humans, carotenes and oxycarotenoids show

distinct kinetics [17, 18]. The oxycarotenoids, lutein and canthaxanthin, rapidly appear in

serum and have a single peak increment 12 -16 h post-dosing, whereas β-carotene has

biphasic kinetics with a minor peak at 5-7 h post-dosing and a larger, sustained peak at 24

to 48 h post-dosing. Canthaxanthin is a good tracer oxycarotenoid because it is not

prominent in the human diet and not present in significant concentrations in human plasma.

The kinetics of appearance and disappearance in plasma after a single canthaxanthin dose

are comparable with the kinetics of the oxycarotenoid lutein [18] and other oxycarotenoid

analogs [19].

The basis of the current investigation is a pilot study by White et al. [17]

addressing the interrelation of a hydrocarbon carotene, β-carotene, and the oxycarotenoid,

canthaxanthin, after concurrent ingestion and during the appearance of each in the serum

of human subjects. Comparison of the kinetics of a single β-carotene or canthaxanthin

dose with that of a combined β-carotene plus canthaxanthin dose within each of two

subjects indicated that the 72-h area under the serum concentration-time curve for

canthaxanthin was reduced 34% by concurrent ingestion of an equimolar β-carotene dose.

A recent study by Kostic et al. [18] reported inhibition of the serum appearance of lutein

by β-carotene when the two carotenoids were ingested concurrently by human subjects.

Concurrent ingestion of lutein and β-carotene reduced the mean area under the curve for

lutein 39% compared to that for lutein when administered alone. Johnson et al. [20] did

not find an inhibition of β-carotene serum appearance after concurrent ingestion of β-

carotene and lycopene, two hydrocarbon carotenes, which supports the hypothesis that

observed interactions among carotenoids are confined to occurrence between hydrocarbon

carotenes and oxycarotenoids [17].

The mechanism and site of the inhibitory effect of β-carotene is not known. Early

inhibition observed in previous studies suggests an effect during intestinal absorption. It

may occur in the enterocyte during incorporation of carotenoids into intestinal lipoproteins

for transport via the lymphatic system to the circulation. The current study extends our

knowledge of interactions of β-carotene and oxycarotenoids by investigating the kinetics

of the appearance and disappearance of single and combined oral doses of β-carotene and

canthaxanthin, a model oxycarotenoid, in human postprandial plasma TRL.

Material and Methods

Subjects. Ten healthy, non-smoking premenopausal women aged 20-36 y

participated in the study. Subjects underwent a screening procedure that included a health

and lifestyle questionnaire, physical examination, complete blood count, and blood

chemistry profile. Criteria for exclusion were: history of chronic disease, lipid

malabsorption or intestinal disorders, use of medications that affect lipid absorption or

transport (including antibiotics), hyperlipidemia indicated by plasma lipid and lipoprotein

profile, lactose intolerance, history of anemia or excessive bleeding, history of

photosensitivity disorders, history of eating disorders, hyper- or hypothyroidism indicated

by measured serum thyroxine (T_4) and thyroid stimulating hormone (TSH), menstrual

cycle irregularities or abnormalities, current or planned pregnancy, use of oral

contraceptive agents, current use of vitamin or mineral supplements, vegetarianism,

current or recent cigarette smoking, and frequent consumption of alcoholic beverages (> 1

drink/day). Before enrollment in the study, the subjects were instructed to complete a

three-day written food record to screen for unusual dietary behaviors such as restrained

eating. Informed consent was obtained from all subjects and study procedures were

approved by the Human Subjects Research Review Committee of Iowa State University.

A total of twelve subjects participated in the study. Subject 10 met the diagnostic

criteria for hypolipidemia [21] and her data were excluded from the statistical analyses and

calculations of the mean area under the plasma concentration-time curves. Subjects 11 and

12 were excluded because they did not complete the third period of the study.

Diet. The subjects were instructed to avoid consumption of fruits and vegetables

high in carotenoids and were provided a list of foods to exclude from the diet for 5 days

before each study period. During the dosing periods, the subjects consumed a controlled

low-carotenoid diet for 1 day before and 4 days after dosing. A single daily menu of

weighed food portions was provided. The meals were prepared and consumed in the

Human Nutrition Metabolic Unit of the Center for Designing Foods to Improve Nutrition

at Iowa State University except for the carry-out lunches and evening snacks. A 24-hour

diet composite was analyzed by high performance liquid chromatography (HPLC) for

carotenoid, retinol, and α-tocopherol contents [17]. On average, the diet provided 504.8 ±

52.8 µg/d lutein, 18.9 ± 0.4 µd/d cryptoxanthin, 145.3 ± 8.6 µg/d β-carotene, no

detectable α-carotene and lycopene, 591.7 ± 64.6 µg/d retinol, and 5.5 ± 0.2 mg/d α-

tocopherol. The macronutrient composition of the diet was estimated using Nutritionist IV

software (N-Squared Computing Inc., Salem, OR). The estimated macronutrient

distribution of the 8.8 MJ daily diet was 14% of total energy from protein, 63% of total energy from carbohydrate, and 23% of total energy from fat.

Carotenoid dose. Water-dispersible 10% (wt:wt) β-carotene, 10% canthaxanthin, and placebo beadlets were provided by Hoffmann-La Roche (Nutley, NJ). Beadlets were used because of the commercial availability of these formulations approved for human ingestion [22] and because of the high bioavailability of the carotenoids [23] which would be expected to provide a more consistent plasma response. For the preparation of the dose providing 25 mg (47 μmol) β-carotene, 25 mg (44 μmol) canthaxanthin, or a combined dose of 25 mg of each, 250 mg of the respective beadlets were dissolved in 100 ml of warm whole milk (40°C) and 296 ml of cold milk were added. For the individual dose, β-carotene or canthaxanthin was administered with an equal weight of placebo beadlets. The carotenoid dose in 396 ml whole milk (13 g fat) was administered with the standard breakfast containing an additional 20 g fat to facilitate intestinal absorption.

Study protocol. During each of three five-day study periods, separated by 10-week washout periods to minimize residual effects of the previous carotenoid dose, subjects ingested either a β-carotene plus placebo dose, a canthaxanthin plus placebo dose, or a β-carotene plus canthaxanthin dose. On the day of dosing, the second day of the low-carotenoid diet, subjects arrived at the metabolic unit after an overnight fast and a baseline blood sample (7 ml) was drawn via a catheter placed in a forearm vein by a registered nurse. After administration of the carotenoid dose followed by the standard breakfast, blood samples were drawn at hourly intervals for 12 h post-dosing via the intravenous catheter into a syringe. The patency of the catheter was maintained by flushing with sterile

physiological saline; 3 ml of sterile saline was injected after each blood draw and withdrawn immediately before the next blood collection. Additional blood samples were drawn from the antecubital vein via venipuncture after an overnight fast at 24, 48, 72, 96, 192, 360, and 528 h post-dosing. The blood samples were immediately placed on ice, protected from light, and then centrifuged (1380 x g, 4° C, 20 min) to separate plasma. Aliquots of plasma were stored at -80° C until analyzed.

Blood draws obtained at 0, 2, 4, 6, 8, and 10 h post-dosing from subjects 1 to 5 were used immediately for lipoprotein fractionation. Lipoprotein fractions were separated by cumulative rate ultracentrifugation to obtain chylomicrons, three VLDL subfractions, and LDL according to the method of Redgrave et al. [24] as modified by Berr [25]. Procedures were performed in yellow light. Aliquots of the isolated fractions for carotenoid analysis were stored at -80°C until analyzed.

Extraction and HPLC analysis of plasma and plasma lipoproteins. Procedures were performed in yellow light. Duplicate 200-µl or 500-µl aliquots of plasma or suspended plasma lipoproteins, respectively, were denatured by addition of an equal volume of absolute ethanol containing 0.01 % BHT and retinyl acetate as an internal standard according to the method of Stacewicz-Sapuntzakis et al. [26]. The samples were then extracted twice with hexane containing 0.01 % BHT and the combined hexane layers were evaporated to dryness under vacuum (AS160 SpeedVac, Savant Instruments, Farmingdale, NY). The residues were reconstituted with ethyl ether and mobile phase (1:3; vol:vol), and 20-µl aliquots were injected into the HPLC system.

Retinyl acetate, retinol, α-tocopherol, α-carotene, β-carotene, and canthaxanthin

standards were purchased from Fluka Chemical (Ronkonkoma, NY) and lycopene

standard from Sigma Chemical (St. Louis, MO). Cryptoxanthin was donated by

Hoffmann-La Roche (Nutley, NJ) and lutein by Kemin Industries (Des Moines, IA).

Calibration curves were generated from the ratios of the peak height of the carotenoid

standards to the peak height of the internal standard plotted against the carotenoid

concentration. Accuracy and reproducibility of the analyses were verified using a standard

reference material (SRM 968a, Fat-Soluble Vitamins in Human Serum) from the National

Institute of Standards and Technology (Gaithersburg, MD). Quality control included

routine analysis of a plasma pool. Inter-assay coefficients of variation were below 5% for

all carotenoids, retinol, and α-tocopherol.

Statistical analysis. The data were analyzed as a randomized block experiment

with subjects considered as blocks. Plasma carotenoid concentrations at individual time

points were regarded as repeated measures. Significant differences between treatments

were analyzed by the general linear models procedure of SAS [27]. The area under the

plasma concentration versus time curve (AUC) was calculated by trapezoidal

approximation after adjustment for the baseline plasma concentrations of the carotenoids.

Significant differences of the AUC values for single versus combined carotenoid dose

were analyzed by paired t-test.

Results

Effects of a concurrent dose of β-carotene and canthaxanthin on the kinetics of the individual carotenoids in plasma lipoprotein fractions. The mean canthaxanthin concentration-time curves in the individual lipoprotein fractions during 10 h after ingestion of either an individual 25 mg canthaxanthin dose plus placebo or a combined 25 mg canthaxanthin plus 25 mg β-carotene dose are presented in Figure 3-1. Repeated measures ANOVA indicated that concurrent ingestion of a β-carotene dose inhibited the appearance of canthaxanthin in chylomicrons ($p < 0.01$), VLDLA ($p < 0.05$), VLDLB ($p < 0.05$), and VLDLC ($p < 0.01$). The rapid accumulation of canthaxanthin in LDL was not affected by concurrent ingestion of β-carotene ($p = 0.25$) during the first postprandial 10 hours. Table 3-1 gives the mean areas under the lipoprotein concentration-time curves (AUC) for 0 to 10 h after an individual dose of β-carotene or canthaxanthin or a combined dose of β-carotene and canthaxanthin. The AUC for canthaxanthin after concurrent ingestion of β-carotene was reduced in chylomicrons $37.8 \pm 7.1\%$ ($p < 0.01$), in VLDLA $43.0 \pm 8.1\%$ ($p < 0.05$), in VLDLB $32.7 \pm 8.1\%$ ($p < 0.05$), and in VLDLC $30.3 \pm 7.6\%$ ($p < 0.001$).

The mean β-carotene plasma lipoprotein fraction response curves after ingestion of either an individual 25 mg β-carotene dose plus placebo or a combined 25 mg β-carotene plus 25 mg canthaxanthin dose are shown in Figure 3-2. The timing of the peak lipoprotein β-carotene increments was not affected by concurrent ingestion of canthaxanthin, and the effect of concurrent canthaxanthin ingestion on β-carotene
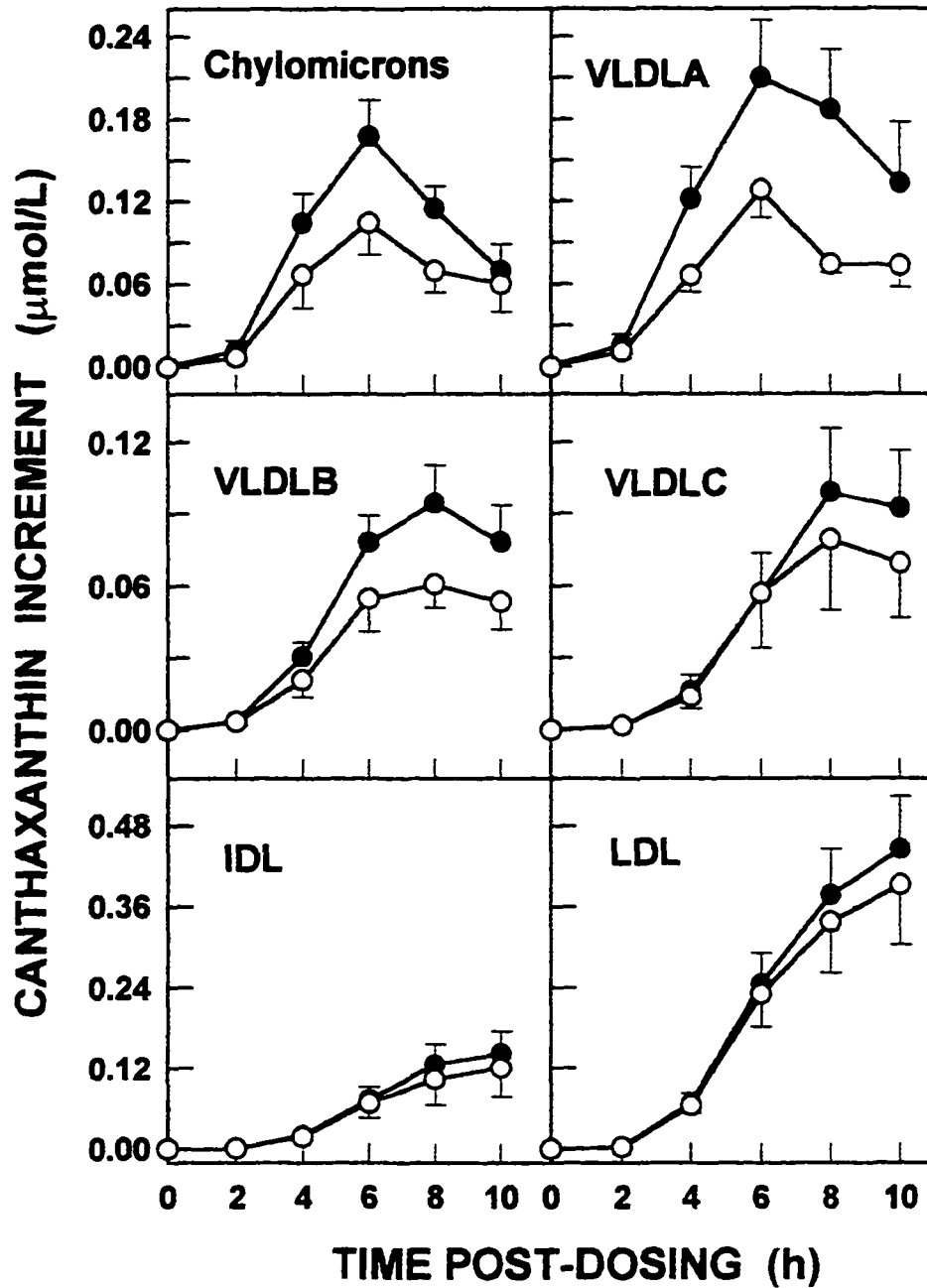
Figure 3-1. Mean (± SEM, n = 5) plasma lipoprotein canthaxanthin increments after ingestion of either an individual 25 mg canthaxanthin dose plus placebo (●) or a combined 25 mg canthaxanthin plus 25 mg β-carotene dose (O). Lipoprotein canthaxanthin concentrations were adjusted by subtraction of baseline concentrations.

Table 3-1. Mean (± SEM) areas under the plasma lipoprotein concentration-time curves (AUC) for 0 to 10 h after an individual dose of β-carotene or canthaxanthin or a combined dose of β-carotene and canthaxanthin. Between and within subject comparisons for each of five subjects.

Fraction	AUC for β-Carotene (μmol ·h/L)			AUC for Canthaxanthin (μmol ·h/L)		
	β-Carotene plus Placebo	β-Carotene plus Canthaxanthin	Within subject difference	Canthaxanthin plus Placebo	Canthaxanthin plus β-Carotene	Within subject difference
Chylomicrons	0.43 ± 0.20	0.32 ± 0.11	-0.12 ± 0.09	0.86 ± 0.15	0.55 ± 0.14	-0.31 ± 0.05 [b]
VLDLA	0.66 ± 0.29	0.49 ± 0.12	-0.17 ± 0.17	1.20 ± 0.23	0.63 ± 0.08	-0.57 ± 0.19 [c]
VLDLB	0.25 ± 0.10	0.22 ± 0.07	-0.03 ± 0.03	0.49 ± 0.07	0.33 ± 0.06	-0.16 ± 0.04 [c]
VLDLC	0.13 ± 0.05	0.15 ± 0.05	+0.02 ± 0.01	0.49 ± 0.15	0.37 ± 0.14	-0.12 ± 0.01 [a]
LDL	0.11 ± 0.08	0.08 ± 0.04	-0.04 ± 0.09	1.83 ± 0.33	1.66 ± 0.36	-0.17 ± 0.11

[a] $p < 0.001$

[b] $p < 0.01$

[c] $p < 0.05$

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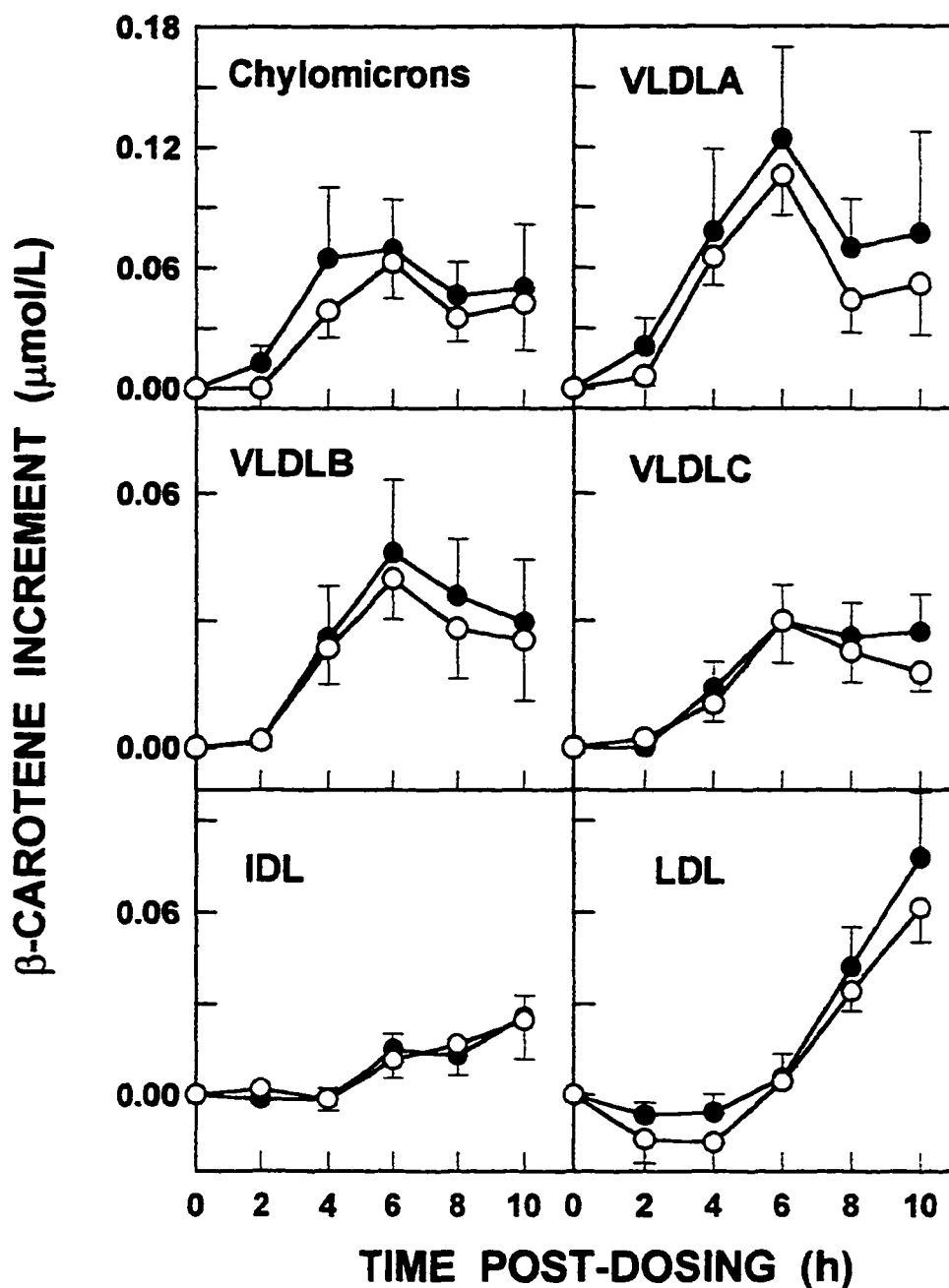
Figure 3-2. Mean (± SEM, n = 5) plasma lipoprotein β-carotene increments after ingestion of either an individual 25 mg β-carotene dose plus placebo (●) or a combined 25 mg β-carotene plus 25 mg canthaxanthin dose (○). Lipoprotein β-carotene concentrations were adjusted by subtraction of baseline concentrations.

concentrations was not statistically significant when compared within subjects by repeated measures ANOVA.

Effects of a concurrent dose of β-carotene and canthaxanthin on the kinetics of the individual carotenoids in plasma. Figure 3-3 presents the postprandial plasma appearance of β-carotene and canthaxanthin during the first 10 h after ingestion of individual doses or a combined dose. The appearance of canthaxanthin was significantly inhibited by concurrent ingestion of β-carotene, whereas the effect of concurrent ingestion on the appearance of β-carotene was not significant. Shown in Figure 3-4 are the mean plasma canthaxanthin concentration-time curves for 528 hours after ingestion of either a single oral 25 mg canthaxanthin dose or a combined 25 mg canthaxanthin plus 25 mg β-carotene dose. The data are mean values for nine subjects adjusted by subtraction of baseline canthaxanthin concentrations. The plasma canthaxanthin response curve is monophasic; canthaxanthin concentrations increased rapidly and peaked 8-12 hours post-dosing after which plasma concentrations declined steadily to reach values near baseline concentrations at 360 h.

Repeated measure ANOVA across the individual time points indicated that ingestion of a concurrent β-carotene dose inhibited the appearance of canthaxanthin significantly ($p < 0.01$).

The AUCs for individual subjects for a single dose of β-carotene or canthaxanthin and for a combined dose of β-carotene plus canthaxanthin for 0 to 96 h post-dosing are shown in Table 3-2. We present the AUCs for 0 to 96 h and ignore the AUCs for 192 to
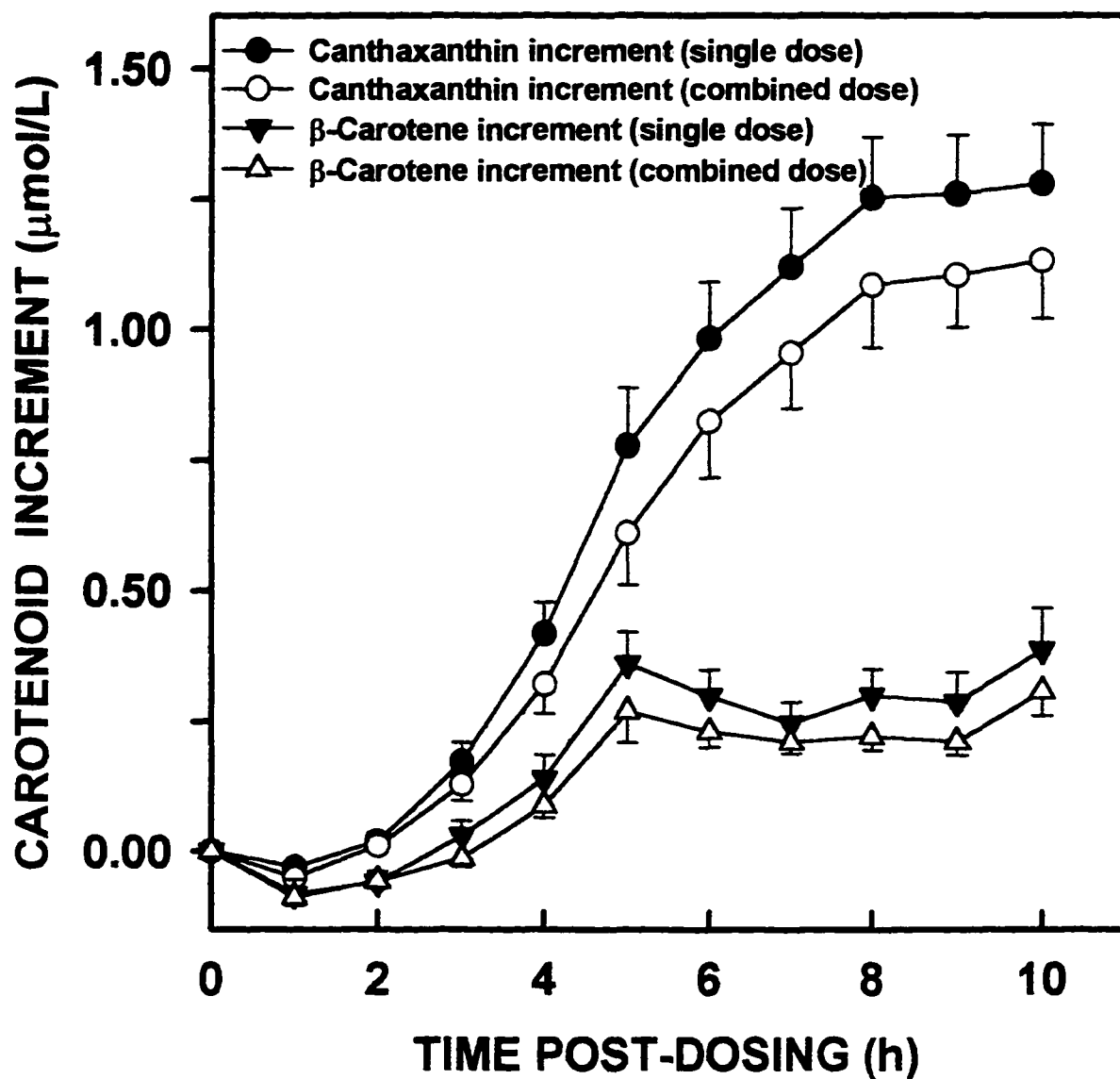
Figure 3-3. Mean (± SEM, n = 9) plasma carotenoid concentration-time curves for 10 h after ingestion of either a single oral dose of 25 mg of canthaxanthin or β-carotene or a combined dose of 25 mg each of canthaxanthin and β-carotene.
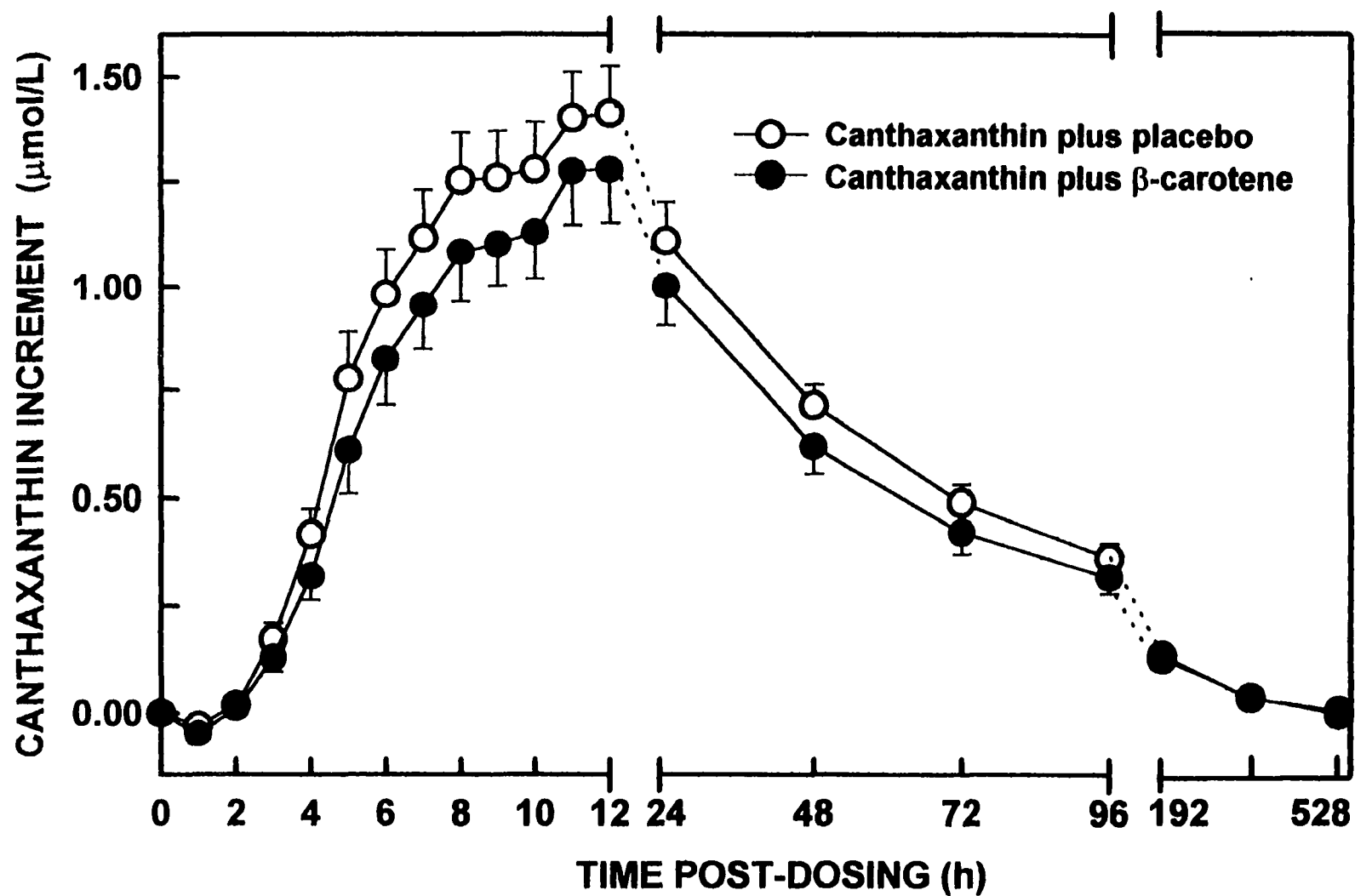
Figure 3-4. Mean (± SEM, n = 9) plasma canthaxanthin concentration-time curves for 528 h after ingestion of either 25 mg of canthaxanthin plus placebo or 25 mg each of β-carotene and canthaxanthin. Note the changes in the time scale between 12 and 24 h and between 96 and 192 h which are indicated by a dashed line.

Table 3-2. Within-subject comparison of the areas under the plasma concentration-time curves (AUC) for 0 to 96 h after an individual dose of β-carotene or canthaxanthin or a combined dose of β-carotene and canthaxanthin.

| Subject | Area Under Curve (μmol • h/L) | | | | | |
	β-Carotene alone	β-Carotene with canthaxanthin	Within subject difference	Canthaxanthin alone	Canthaxanthin with β-carotene	Within subject difference
1	36.2	40.4	+4.2	98.1	87.1	-11.0
2	113.0	90.0	-23.0	79.9	76.9	-3.1
3	149.0	96.1	-52.6	79.4	65.8	-13.6
4	2.6	26.7	+24.1	46.4	32.2	-14.2
5	23.1	34.3	+11.1	51.1	38.5	-12.6
6	47.9	12.4	-35.5	62.4	53.2	-9.2
7	34.6	57.7	+23.1	78.2	77.0	-1.2
8	102.0	53.0	-49.3	71.4	63.1	-8.3
9	65.0	46.3	-18.7	73.0	69.6	-3.4
Mean ± SEM	63.7 ± 16.0	50.8 ± 9.2	-13.0 ± 9.9	71.1 ± 5.3	62.6 ± 6.1	-8.5 ± 1.6

528 h because the subjects were on the controlled diet until 96 h post-dosing. Comparison

within subjects by repeated measures ANOVA indicated that the effect of canthaxanthin

on the β-carotene AUC was not statistically significant, whereas the reduction in mean

canthaxanthin AUC was significant ($p < 0.01$).

Shown in Figure 3-5 are the mean plasma β-carotene concentration-time curves

for 528 hours after ingestion of either an individual 25 mg β-carotene dose plus placebo or

a combined dose of 25 mg β-carotene plus 25 mg canthaxanthin. The presented

concentrations are mean values for nine subjects adjusted by subtraction of baseline β-

carotene concentrations. The curve has biphasic kinetics with a minor peak at 5 h post-

dosing after which the plasma concentrations fell and then increased with a major peak at

48 h post-dosing. The plasma β-carotene concentrations then declined slowly to values

near baseline concentrations at 528 h.

The kinetics of β-carotene plasma appearance and disappearance after ingestion of

an oral β-carotene dose were not consistently affected by concurrent ingestion of

canthaxanthin. Although the magnitude of the mean plasma response to a combined β-

carotene and canthaxanthin dose is reduced in the composite response curve (Figure 3-5)

relative to the response to an individual β-carotene dose, the reduction in plasma β-

carotene concentrations across all time points after ingestion of an equimolar dose of β-

carotene and canthaxanthin was not statistically significant when compared within

subjects. The composite curve is the mean response curve of all subjects, the statistical

comparison of the treatments was within subjects.
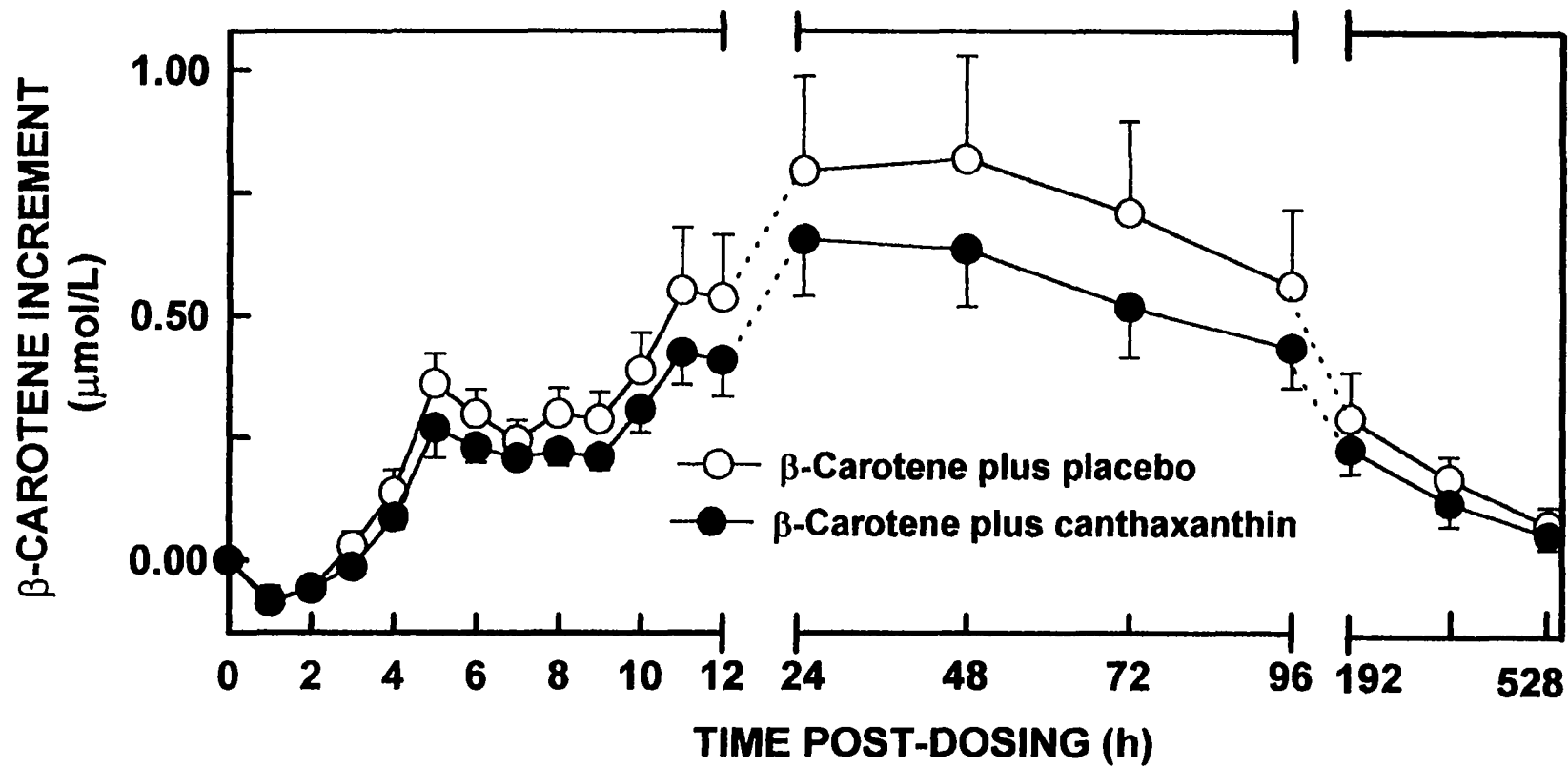
Figure 3-5. Mean (± SEM, n = 9) plasma β-carotene concentration-time curves for 528 h after ingestion of either 25 mg of β-carotene plus placebo or 25 mg each of β-carotene and canthaxanthin. Plasma β-carotene concentrations were adjusted by subtraction of baseline plasma concentrations. Note the changes in the time scale between 12 and 24 h and between 96 and 192 h which are indicated by a dashed line.

The effect of combined dosing on the plasma appearance of β-carotene is more accurately presented by the within subject comparison in Table 3-2. The subjects showed extensive variation in response to the coinciding dose; five of nine subjects had reduced plasma β-carotene increments after ingestion of the concurrent canthaxanthin dose whereas the remaining four subjects had increased plasma β-carotene increments compared to the response to the single oral β-carotene dose. The individual peak plasma β-carotene increments after the combined dose varied from a 70% reduction to a 400% increase (data not shown). When calculating the within subject ratios of the peak plasma β-carotene increments after ingestion of an individual or combined dose (β-carotene:β-carotene plus canthaxanthin), the mean effect of the combined β-carotene and canthaxanthin dose was a 27% increase in peak increment compared with the individual β-carotene dose.

The kinetics of single oral doses of β-carotene and canthaxanthin in plasma are distinct. Canthaxanthin has a monophasic response with a single peak concentration occurring 8 to 12 h post-dosing, whereas β-carotene has a biphasic response with a minor peak concentration at 5 h post-dosing and a larger sustained peak at 48 h post-dosing. We discussed the kinetics of β-carotene and canthaxanthin in plasma and plasma lipoprotein fractions in an earlier paper. Our findings are in agreement with previous reports on the kinetics of β-carotene [17, 28] and canthaxanthin [17, 29].

Discussion

The mechanism and site of the apparent interactions of β-carotene and

oxycarotenoids are not known. In the present study we investigated the effect of

coinciding ingestion of β-carotene and canthaxanthin on their incorporation into transport

lipoproteins. We demonstrated that coincident ingestion of the β-carotene dose

significantly inhibited the appearance of the canthaxanthin dose in chylomicrons and in

VLDL subfractions, but not the rapid accumulation of canthaxanthin in LDL. In contrast,

the kinetics and appearance of β-carotene in the lipoprotein fractions were not consistently

affected by ingestion of canthaxanthin.

The pilot study for the current study [17] indicated that coincident ingestion of an

equimolar dose of β-carotene and canthaxanthin reduced the peak serum concentration of

canthaxanthin by 39% compared to the value for canthaxanthin when administered in

absence of β-carotene. The appearance of β-carotene in plasma after an oral dose was not

antagonized by concurrent ingestion of an equimolar canthaxanthin dose. The inhibition

occurred early during the postprandial phase suggesting an interaction of the carotenoids

during intestinal absorption.

Our findings support the hypothesis that one site of interaction of β-carotene and

canthaxanthin is within the enterocyte or during micellar solubilization in the lumen. It

appears that the incorporation of canthaxanthin into TRL is reduced in presence of β-

carotene. The rapid accumulation of the canthaxanthin dose in LDL is not affected by the

β-carotene dose. There are two possible explanations for this observation. One, a

hypothetical transfer of canthaxanthin from TRL to LDL which occurs at the same rate as when administered in a single oral dose. Two, incorporation of canthaxanthin into intestinal or hepatic VLDL particles which are subsequently converted to LDL particles, and that this incorporation is not affected by the presence of β-carotene in the enterocyte. The first possibility is supported by Borel et al.[30] who showed that oxycarotenoids are preferentially solubilized in phospholipids, i.e. at the surface of lipoproteins, rendering them prone to transfer among lipoproteins and to accumulate in lipoproteins with a high phospholipid:apolar lipid ratio, such as LDL or HDL.

The second hypothesis is supported by the finding that the small intestine is capable of secreting VLDL particles; however, their contribution to postprandial influx of intestinal lipoproteins is considered insignificant [31]. It is unlikely that intestinal VLDL is responsible for the rapid accumulation of canthaxanthin in LDL because we observed that coincident ingestion of β-carotene significantly inhibited the appearance of the canthaxanthin dose in each VLDL subfraction, i.e. the fractions in which one would expect canthaxanthin to appear first before the particles were catabolized to LDL particles.

Our data confirm the findings from the pilot study [17] that coincident ingestion of an oral equimolar dose of β-carotene and canthaxanthin significantly inhibits the plasma appearance of canthaxanthin, but not that of β-carotene. Individual peak plasma canthaxanthin increments after the combined dose varied from 36% reduction to essentially no change relative to an individual canthaxanthin dose. Eight of nine subjects showed reduced peak plasma canthaxanthin concentration, only one subject showed essentially unchanged plasma appearance of canthaxanthin after ingestion of the combined

dose compared with ingestion of the single dose. The effect of combined dosing on peak plasma β-carotene concentration showed extensive variation across subjects ranging from a 70% reduction to a fourfold increase relative to ingestion of the single dose. For five of nine subjects the peak plasma concentrations were reduced, whereas, for the remaining four subjects, the peak plasma β-carotene increments were enhanced after ingestion of the combined dose.

We observed enhanced appearance of intact β-carotene after ingestion of a combined dose of β-carotene and canthaxanthin in subjects who had low AUC values for a single oral β-carotene dose. Subjects with low absorption of β-carotene may be efficient converters of β-carotene to retinoids [32]. The enhanced absorption of the β-carotene dose when ingested coincident with an equimolar canthaxanthin dose may be due to interaction of canthaxanthin with β-carotene-15,15'-dioxygenase, the enzyme responsible for the conversion of β-carotene into retinoids in the enterocyte. Ershov et al [33] demonstrated that the nonprovitamin A carotenoids, lutein and lycopene, can form enzyme-pseudosubstrate complexes and inhibit the enzyme so that less β-carotene is converted to retinoids and more is absorbed intact.

Our findings are similar to those of Kostic et al. [18]. It was demonstrated that concurrent administration of an equimolar dose of β-carotene and lutein inhibits the appearance of lutein in serum as shown by a reduction of the mean area under the curve (AUC) for lutein by 39% compared to its value when given alone. In their study, seven of eight subjects showed this effect, one subject showed a 15% increase in lutein absorption

in presence of β-carotene. In contrast, the effect of lutein on the β-carotene response showed broad interindividual variation; for five of eight subjects, the absorption of β-carotene was reduced after ingestion of the combined dose compared with a single oral β-carotene dose, three subjects showed enhanced β-carotene absorption in presence of lutein. Subjects with low β-carotene absorption after a single oral dose of β-carotene showed enhanced absorption of β-carotene with concurrent ingestion of lutein.

The observed interrelations of carotenoids in humans may be confined to occurrence between hydrocarbon carotenes and oxycarotenoids [17]. Johnson et al. [20] were not able to demonstrate an effect of a combined oral dose of β-carotene and lycopene, two hydrocarbon carotenes, on the serum response of the two. Our data confirm and extend earlier reports on the interference of β-carotene with intestinal absorption of oxycarotenoids during coincident ingestion. Here we demonstrate antagonistic effects of a coincident β-carotene dose on the appearance of an equimolar canthaxanthin dose in TRL, but not in LDL. Canthaxanthin did not have a consistent effect on the intestinal absorption of β-carotene. If canthaxanthin and β-carotene share a common transport mechanism through the cytosol to the endoplasmic reticulum of the enterocyte for incorporation into intestinal lipoproteins, it is possible that this transport mechanism has a higher affinity for β-carotene. The nature of the interactions among carotenoids deserves further research, especially in the light of health protective effects of carotenoids other than β-carotene. In recent epidemiological studies [2, 5, 6], lutein intakes were more strongly associated with risk of lung cancer than β-carotene intakes. If the intestinal absorption of other protective

agents is inhibited by supplemental β-carotene the long-term effect may be detrimental

rather than beneficial.

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4. GENERAL CONCLUSIONS

As provitamin A, β-carotene is present in a variety of vitamin supplements available over the counter in the United States, and, as a colorant, is found in various foods. In recent years, β-carotene has made the news as a potential health protective agent. Findings from recent large intervention trials are not consistent with a protective effect of supplemental β-carotene. It is crucial to understand the metabolism of a purified compound and its interaction with other nutrients if the compound is ingested as a supplement. Foods contain a variety of carotenoids other than β-carotene which may exert potential protective effects to human health. If β-carotene interferes with the utilization of other carotenoids, especially oxycarotenoids, supplementation with β-carotene may have detrimental effects on human health.

We investigated the intestinal absorption and the lipoprotein transport of single equimolar oral β-carotene and canthaxanthin doses, as well as those of a combined β-carotene and canthaxanthin dose in healthy premenopausal women to confirm and extend the findings of a pilot study. We compared treatment effects within subjects, i.e. each subject served as her own control. The kinetics of β-carotene, a hydrocarbon carotene, and canthaxanthin, a model oxycarotenoid, in plasma were determined to be distinct. Canthaxanthin concentrations in plasma increased rapidly after ingestion of an oral dose and peaked 12 hours post-dosing, whereas the appearance of β-carotene in plasma was biphasic with a lesser peak 5 hours post-dosing and a larger sustained peak 48 hours post-

dosing. Canthaxanthin appears to be a good model for other oxycarotenoids, lutein and cryptoxanthin, which also show rapid appearance and monophasic kinetics in human serum after ingestion.

Underlying these distinct kinetics is the transport of the investigated carotenoids in plasma lipoproteins. The early rise in β-carotene concentrations in plasma was determined to be due to influx of β-carotene transported in chylomicrons and VLDL; the latter was thought by other investigators to represent VLDL contamination by chylomicron remnants, however, we did not detect apo-B48, the integral apolipoprotein of chylomicrons and chylomicron remnants. Chylomicrons and VLDL particles are catabolized by lipoprotein lipase in the circulation and the remnant particles are cleared by the liver, thus upon their removal, plasma β-carotene concentrations decreased. The second β-carotene peak was due to delayed appearance in LDL particles. It is likely that hepatocytes secreted nascent VLDL particles containing β-carotene which were subsequently catabolized to LDL. We observed that the concentration of ingested β-carotene in LDL began to rise 6 hours post-dosing after concentrations in TRL had peaked. In contrast, canthaxanthin concentrations rose concurrently in all lipoprotein fractions after ingestion of a single oral dose. Interesting was the rapid accumulation of the oxycarotenoid, canthaxanthin, in LDL. For the first time, we have demonstrated that the coincident appearance of canthaxanthin in TRL and LDL explains the monophasic canthaxanthin response in plasma after an oral dose of canthaxanthin and likely that of other oxycarotenoids prominent in the human diet.

Our findings indicate that β-carotene and canthaxanthin interact when ingested together in one oral dose. The plasma appearance of canthaxanthin was significantly inhibited by coincident ingestion of β-carotene. We observed a mean within-subject reduction in the area under the plasma canthaxanthin concentration-time curve (AUC) of 8.5% which was statistically significant. The antagonistic effect of the β-carotene dose on the absorption of the canthaxanthin dose was more marked in individual lipoprotein fractions during the immediate postprandial period. The reduction in the lipoprotein canthaxanthin AUC for 0 to 10 h after concurrent ingestion of β-carotene and canthaxanthin was significant in TRL but not in LDL. The reduction in AUC values for canthaxanthin in TRL subfractions ranged from 30.3 ± 7.6% to 43.0 ± 8.1% compared with the AUC after an individual canthaxanthin dose. The rapid accumulation of canthaxanthin in LDL was not affected by coincident ingestion of the β-carotene dose. One possible explanation for this observation is transfer of the oxycarotenoid from TRL to LDL and that the transfer rate is not affected by coincident ingestion of β-carotene.

We did not observe a statistically significant effect of concurrent ingestion of the canthaxanthin dose on the appearance of the β-carotene dose in either plasma or plasma lipoproteins. It is important to note that, in contrast to the canthaxanthin response, the subjects varied in their plasma β-carotene response after ingestion of the combined β-carotene plus canthaxanthin dose. Of nine subjects, five showed a reduced plasma appearance of the β-carotene dose when ingested concurrently with canthaxanthin, and the remaining four showed an enhanced plasma β-carotene increment. Subjects with AUC

values for β-carotene after a single oral dose below 86 μmol/L·h showed enhanced

absorption of β-carotene in presence of canthaxanthin, whereas subjects with AUC values

above 86 μmol/L·h showed reduced absorption of β-carotene when ingested

coincidentally with canthaxanthin. These findings are consistent with a study by Kostic et

al. investigating effects of a combined equimolar dose of β-carotene and lutein. The

interaction may be limited to inhibition of intestinal cleavage of β-carotene.

This study confirms and extends earlier reports of specific interactions of β-

carotene and the oxycarotenoids, canthaxanthin and lutein, during intestinal absorption.

These findings have potential health implication if β-carotene supplementation interferes

with the utilization of other potentially beneficial dietary carotenoids.

APPENDIX

Table I. Recipe for 1-10 % linear gradient gel.

	Acrylamide (30%)	1.5 M Tris-HCL	SDS (10%)	Glycerol	Deionized Water	TEMED	Ammonium Persulfate (10%)
1 %	0.54 ml	4.0 ml	0.16 ml	—	11.22 ml	6.0 µl	80 µl
10 %	5.33 ml	4.0 ml	0.16 ml	6.46 ml	—	6.0 µl	48 µl

For the coomassie blue staining, the gel was immersed in stain overnight in a closed container to prevent evaporation. The staining solution contained 0.1% coomassie blue R-250, 47.5% methanol, 5.0% acetic acid, and 47.5% deionized water. The solution was stirred to dissolve all components and then filtered (Whatman #1 paper). The destaining solution contained 7.5% acetic acid, 5.0% methanol, and 87.5% deionized water. The gel was immersed in the destaining solution until the background on the gel was clear.

Table II. Within-subject comparison of the areas under the chylomicron carotenoid concentration-time curves (AUC) after an individual dose of either β-carotene or canthaxanthin or a combined dose of β-carotene and canthaxanthin.

Subject	Mean AUC ± SEM for β-Carotene in Chylomicron (μmol/L·h)		Difference (%)	Mean AUC ± SEM for Canthaxanthin in Chylomicron (μmol/L·h)		Difference (%)
	β-Carotene plus Placebo	β-Carotene plus Canthaxanthin		Canthaxanthin plus Placebo	Canthaxanthin plus β-Carotene	
1	1.16	0.70	-39.9	1.09	0.67	-38.3
2	0.25	0.14	-45.0	0.65	0.28	-56.1
3	0.06	0.17	+192.0	0.74	0.37	-50.6
4	0.50	0.40	-19.2	· 1.33	1.05	-20.7
5	0.21	0.17	-17.5	0.51	0.39	-23.1

Table III. Within-subject comparison of the areas under the VLDLA carotenoid concentration-time curves (AUC) after an individual dose of either β-carotene or canthaxanthin or a combined dose of β-carotene and canthaxanthin.

Subject	Mean AUC ± SEM for β-Carotene in VLDLA (μmol/L·h)		Difference (%)	Mean AUC ± SEM for Canthaxanthin in VLDLA (μmol/L·h)		Difference (%)
	β-Carotene plus Placebo	β-Carotene plus Canthaxanthin		Canthaxanthin plus Placebo	Canthaxanthin plus β-Carotene	
1	1.76	0.94	-46.5	1.95	0.72	-63.1
2	0.32	0.26	-18.8	0.77	0.43	-44.4
3	0.10	0.35	+247.0	1.08	0.50	-53.4
4	0.69	0.48	-30.3	1.47	0.89	-39.6
5	0.43	0.43	-0.4	0.73	0.62	-14.8

Table IV. Within-subject comparison of the areas under the VLDLB carotenoid concentration-time curves (AUC) after an individual dose of either β-carotene or canthaxanthin or a combined dose of β-carotene and canthaxanthin.

Subject	Mean AUC ± SEM for β-Carotene in VLDLB (μmol/L·h)		Difference (%)	Mean AUC ± SEM for Canthaxanthin in VLDLB (μmol/L·h)		Difference (%)
	β-Carotene plus Placebo	β-Carotene plus Canthaxanthin		Canthaxanthin plus Placebo	Canthaxanthin plus β-Carotene	
1	0.57	0.45	-20.4	0.56	0.33	-41.4
2	0.07	0.04	-37.9	0.28	0.16	-41.1
3	0.02	0.11	+386.0	0.53	0.25	-53.0
4	0.26	0.21	-15.6	0.66	0.53	-19.4
5	0.31	0.23	-27.6	0.43	0.39	-8.7

Table V. Within-subject comparison of the areas under the VLDLC carotenoid concentration-time curves (AUC) after an individual dose of either β-carotene or canthaxanthin or a combined dose of β-carotene and canthaxanthin.

Subject	Mean AUC ± SEM for β-Carotene in VLDLC (μmol/L·h)		Difference (%)	Mean AUC ± SEM for Canthaxanthin in VLDLC (μmol/L·h)		Difference (%)
	β-Carotene plus Placebo	β-Carotene plus Canthaxanthin		Canthaxanthin plus Placebo	Canthaxanthin plus β-Carotene	
1	0.30	[1]NA	NA	0.22	NA	NA
2	0.06	0.04	-31.2	0.22	0.12	-45.5
3	0.03	0.08	+142.0	0.31	0.18	-41.2
4	0.21	0.22	+2.0	0.87	0.72	-16.5
5	0.24	0.26	+8.4	0.57	0.47	-18.1

[1] not available

Table VI. Within-subject comparison of the areas under the LDL carotenoid concentration-time curves (AUC) after an individual dose of either β-carotene or canthaxanthin or a combined dose of β-carotene and canthaxanthin.

Subject	Mean AUC ± SEM for β-Carotene in LDL (μmol/L·h)		Difference (%)	Mean AUC ± SEM for Canthaxanthin in LDL (μmol/L·h)		Difference (%)
	β-Carotene plus Placebo	β-Carotene plus Canthaxanthin		Canthaxanthin plus Placebo	Canthaxanthin plus β-Carotene	
1	0.29	[1]NA	NA	NA	NA	NA
2	0.11	0.09	-21.6	1.74	1.24	-28.6
3	-0.09	0.06	+168.0	1.00	0.97	-2.4
4	0.16	0.17	+10.5	2.01	1.88	-6.7
5	0.28	-0.01	-104.0	2.58	2.57	-0.7

[1] not available

Table VII. Plasma β-carotene increments (μmol/L) after ingestion of a β-carotene plus placebo dose (trt A) and after a β-carotene plus canthaxanthin dose (trt C).

HOUR	1 β-C (trt A)	1 β-C (trt C)	2 β-C (trt A)	2 β-C (trt C)	3 β-C (trt A)	3 β-C (trt C)	4 β-C (trt A)	4 β-C (trt C)	5 β-C (trt A)	5 β-C (trt C)
0	0	0	0	0	0	0	0	0	0	0
1	-0.0147	-0.0598	-0.135	-0.1009	-0.1068	-0.0973	-0.1542	-0.0911	-0.0971	-0.0859
2	-0.0149	-0.0073	-0.1284	-0.0711	-0.0427	-0.1161	-0.1426	-0.0681	-0.0759	-0.0679
3	0.0416	0.0920	0.0153	-0.0080	0.2067	-0.0381	-0.1186	-0.0281	0.0167	0.0144
4	0.1356	0.2288	0.1323	0.0987	0.3957	0.0725	-0.097	0.0312	0.0212	0.0068
5	0.4946	0.4915	0.2701	0.2675	0.5619	0.2043	0.0200	0.1997	0.1352	0.0968
6	0.4076	0.3923	0.2412	0.3212	0.4847	0.2452	-0.0139	0.1527	0.1625	0.1274
7	0.2884	0.2446	0.2479	0.2487	0.2955	0.2372	-0.0134	0.1326	0.1277	0.1633
8	0.2759	0.2690	0.3529	0.3195	0.3786	0.2324	-0.0252	0.1287	0.1430	0.1286
9	0.2016	0.1972	0.3231	0.3048	0.5142	0.3356	-0.0315	0.1302	0.1211	0.1193
10	0.2632	0.3144	0.4731	0.4364	0.7838	0.5840	-0.0055	0.1644	0.1147	0.1392
11	0.3529	0.4601	1.0093	0.5544	1.2410	0.7978	0.0266	0.2187	0.1761	0.2601
12	0.3831	0.4082	0.8715	0.6756	1.3010	0.8318	0.0065	0.2215	0.1681	0.2127
24	0.4357	0.5340	1.5119	1.1765	1.7779	1.2334	0.0897	0.3982	0.3238	0.4257
48	0.4468	0.4536	1.5176	1.1176	1.8856	1.2220	0.0508	0.3196	0.3135	0.4575
72	0.3854	0.4277	1.2191	--	1.7253	1.1121	0.0241	0.3027	0.2489	0.3915
96	0.2918	0.3628	0.9664	0.732	1.4123	0.8382	-0.0275	0.1823	0.1571	0.3272
192	0.1601	0.1824	0.3940	0.2315	0.7906	0.4691	-0.0794	0.0611	0.1241	--
360	0.1635	0.1826	0.2364	-0.0065	0.3743	0.3701	0.0184	-0.0165	0.1087	0.0436
528	--	0.0841	0.1161	--	0.2399	0.1495	-0.0750	-0.0979	0.1095	-0.0110

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HOUR	6		7		8		9		10	
	β-C (trt A)	β-C (trt C)	β-C (trt A)	β-C (trt C)	β-C (trt A)	β-C (trt C)	β-C (trt A)	β-C (trt C)	β-C (trt A)	β-C (trt C)
0	0	0	0	0	0	0	0	0	0	0
1	-0.1283	-0.1386	-0.1111	-0.1156	-0.0985	-0.1009	0.1168	0.0134	-0.1419	-0.1517
2	-0.0878	-0.1338	-0.0642	-0.0609	-0.0395	-0.0864	0.0605	-0.00627	-0.0835	-0.1124
3	0.0217	-0.1087	0.0005	-0.0313	0.0054	-0.0495	0.1003	0.00384	0.0015	-0.0820
4	0.1513	-0.0782	0.1767	0.0276	0.1021	0.1264	0.1589	0.0449	0.2304	0.1569
5	0.1834	0.1240	0.4175	0.0601	0.5366	0.5646	0.3712	0.1296	0.4185	0.4075
6	0.0763	0.0632	0.3960	0.1523	0.3086	0.2812	0.3806	0.1618	0.3103	0.2358
7	0.0225	0.0795	0.3927	0.2084	0.2568	0.3207	0.3443	0.1233	0.2707	0.2041
8	0.0726	0.1647	0.4193	0.1708	0.3058	0.3545	0.4755	0.1961	0.3606	0.1904
9	0.0657	0.1695	0.3122	0.1430	0.3066	0.2678	0.4809	0.2166	0.3508	0.1812
10	0.1173	0.2597	0.4336	0.2125	0.3647	0.3843	0.5967	0.2793	0.4529	0.2512
11	0.1607	0.4550	0.4906	0.1511	0.3794	0.4406	0.6138	0.4424	0.6806	0.4770
12	0.2980	0.4462	0.4667	0.1304	0.3692	0.3946	0.6045	0.3617	0.6355	0.4366
24	0.6666	1.0080	0.6037	0.1819	0.4680	0.7222	1.1927	0.6400	0.7570	0.6028
48	0.7346	0.9741	0.5573	0.1589	0.3966	0.7444	1.3519	0.6612	0.8557	0.6022
72	0.6035	0.8119	0.5325	0.1115	0.3673	0.6458	1.1893	0.6587	0.7065	0.4967
96	0.4940	0.6573 ·	0.4153	0.0703	0.2482	0.5191	1.0165	0.4881	0.5744	0.3738
192	0.2157	0.3029	0.1867	0.0667	0.1286	0.2137	0.6153	0.3272	0.1586	0.1693
360	0.0978	0.1252	0.1286	0.0559	0.0390	0.0555	0.3304	0.2005	0.0203	--
528	0.0167	-0.0045	0.0160	0.0135	-0.0222	0.0489	0.1386	0.1413	0.0024	0.0381

Table VIII. Plasma canthaxanthin increments (µmol/L) after ingestion of canthaxanthin plus placebo dose (trt B) and after a β-carotene plus canthaxanthin dose (trt C).

HOUR	1 CX (trt B)	1 CX (trt C)	2 CX (trt B)	2 CX (trt C)	3 CX (trt B)	3 CX (trt C)	4 CX (trt B)	4 CX (trt C)	5 CX (trt B)	5 CX (trt C)
0	0	0	0	0	0	0	0	0	0	0
1	-0.0264	-0.0254	-0.0316	-0.0421	-0.0394	-0.0273	-0.0321	-0.0326	-0.0314	-0.0313
2	0.0902	0.0572	-0.0110	-0.0222	-0.0240	-0.0230	-0.0013	0.0087	-0.0109	-0.0088
3	0.3700	0.3547	0.1117	0.1051	0.0629	0.0417	0.1469	0.093	0.1030	0.1064
4	0.6581	0.6748	0.5002	0.3098	0.2588	0.1480	0.3331	0.2051	0.2254	0.1921
5	1.3299	1.0825	0.8441	0.6412	0.4828	0.3351	0.6370	0.4066	0.4909	0.3438
6	1.3694	1.3991	1.2978	1.0777	0.6539	0.5183	0.7545	0.5289	0.7516	0.5219
7	1.5835	1.3947	1.5871	1.1367	0.8622	0.6594	0.9370	0.6348	0.9653	0.6082
8	1.7245	1.6102	1.6729	1.4143	0.8610	0.7783	1.0375	0.6401	1.1188	0.6638
9	1.7766	1.4464	1.6262	1.4493	0.8655	0.9020	0.9840	0.6834	1.1342	0.7039
10	1.8383	1.5456	1.6435	1.5060	0.9119	0.9672	0.9418	0.6363	1.0586	0.6441
11	2.0292	1.7872	1.7261	1.6821	1.0616	1.2230	1.0635	0.6481	1.0989	0.7875
12	2.0401	1.7262	1.7543	1.7873	1.2240	1.2982	0.9773	0.6754	1.0369	0.7775
24	1.5586	1.2741	1.3054	1.3579	1.3110	1.1064	0.6977	0.5092	0.7589	0.6189
48	0.8977	0.8530	0.7692	0.7243	0.8931	0.6910	0.4719	0.3096	0.5101	0.3731
72	0.6765	0.6274	0.4848	--	0.6351	0.4629	0.2943	0.2133	0.3536	0.2624
96	0.5243	0.5081	0.3315	0.2905	0.4378	0.3544	0.1952	0.1409	0.2575	0.2086
192	0.2104	0.1933	0.0542	0.0534	0.1798	0.1207	0.0630	0.0310	0.0757	--
360	0.0735	0.0723	0.0153	0.0193	0.0396	0.0669	-0.0010	-0.0143	--	-0.0009
528	--	0.0163	-0.0139	--	0.0617	-0.0056	0.0046	-0.0270	-0.0273	-0.0206

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HOUR	6		7		8		9		10	
	CX (trt B)	CX (trt C)	CX (trt B)	CX (trt C)	CX (trt B)	CX (trt C)	CX (trt B)	CX (trt C)	CX (trt B)	CX (trt C)
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	-0.0222	0.0000	-0.0263	-0.0375	-0.02611	-0.0200	-0.0295	-0.0329	-0.0330	-0.0308
2	-0.0222	0.0033	0.0483	0.0421	0.1029	0.0498	-0.0087	-0.0132	0.00723	0.0113
3	0.0312	0.0378	0.1815	0.1798	0.3563	0.1483	0.0787	0.0601	0.1339	0.0682
4	0.1150	0.0890	0.2934	0.3414	0.7182	0.3950	0.3133	0.1767	0.4497	0.4346
5	0.1939	0.2450	0.4828	0.4282	1.3128	0.9629	0.6300	0.3924	0.7724	0.8879
6	0.1992	0.3133	0.6096	0.7546	1.4950	1.1459	0.9394	0.5788	0.9626	0.8806
7	0.2210	0.3885	0.6464	1.0279	1.4464	1.3987	1.0417	0.7044	1.0000	1.0158
8	0.3152	0.4969	0.7652	1.0934	1.5429	1.5084	1.3180	0.9701	1.2343	1.0882
9	0.3742	0.5585	0.8090	1.1083	1.4449	1.4473	1.3980	1.0365	1.2955	1.1615
10	0.5406	0.6256	0.9124	1.2261	1.4321	1.3817	1.4096	1.0827	1.3646	1.1983
11	0.9350	1.0793	1.2735	1.1490	1.3433	1.4658	1.5284	1.2798	1.4891	1.4568
12	1.1559	0.9204	1.3390	1.1657	1.4710	1.4138	1.3104	1.2694	1.5633	1.3992
24	0.8457	0.9464	0.9448	0.8098	1.0981	1.1359	1.1330	1.0361	1.1941	1.1641
48	0.5603	0.5696	0.6828	0.5173	0.7771	0.7858	0.6849	0.6250	0.7425	0.6895
72	0.3810	0.3585	0.4228	0.3472	0.5668	0.5492	0.5258	0.4582	0.4580	0.4530
96	0.2915	0.2748	0.3421	0.2495	0.4570	0.4524	0.4017	0.3289	0.3249	0.3283
192	0.0801	0.0910	0.1406	0.1069	0.1372	0.1736	0.1619	0.1211	0.0572	0.0972
360	-0.0032	0.0225	0.0263	0.0216	0.0421	0.0535	0.0366	0.0112	0.0088	---
528	-0.0182	0.0000	-0.0133	-0.0170	0.0079	0.0055	0.00281	-0.0266	-0.0257	-0.0095

Table IX. Lipoprotein β-carotene increments (μmol/L) after ingestion of a β-carotene plus placebo dose (trtA) and after a β-carotene plus canthaxanthin dose (trt C).

		3		5		4		1		2	
		β-C (trt A)	β-C (trt C)	β-C (trt A)	β-C (trt C)	β-C (trt A)	β-C (trt C)	β-C (trt A)	β-C (trt C)	β-C (trt A)	β-C (trt C)
Chylo	0	0	0	0	0	0	0	0	0	0	0
	2	0.04388	0	0	0	0	0	0.01884	0	0	0
	4	0.19866	0.07651	0.02842	0.01974	0.00562	0.02028	0.07108	0.06493	0.01901	0.01134
	6	0.14894	0.12697	0.05588	0.02959	0.02194	0.03884	0.09983	0.07752	0.01904	0.04078
	8	0.10507	0.08012	0.0372	0.01438	0.00109	0.02138	0.04689	0.03998	0.04033	0.02004
	10	0.17158	0.13305	0.00265	0.00768	0	0.00651	0.02635	0.03903	0.04916	0.02551
VLDLA	0	0	0	0	0	0	0	0	0	0	0
	2	0.07141	0	0	0	0	0.00667	0.03326	0.02296	0	0
	4	0.23805	0.11403	0.03996	0.03391	0.00766	0.0399	0.06727	0.06644	0.03554	0.07215
	6	0.27796	0.17484	0.06809	0.06	0.02826	0.07909	0.17467	0.11804	0.07114	0.09679
	8	0.15633	0.10733	0.04502	0.02946	0.01206	0.03896	0.05576	0.01669	0.07885	0.02718
	10	0.2757	0.15134	0.01358	0.01274	0.00587	0.02384	0.03021	0.03427	0.05915	0.03618
VLDLB	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0.00604	0.00767	0	0
	4	0.06122	0.04846	0.00667	0.00219	0	0.0075	0.01275	0.02723	0.04839	0.0311
	6	0.10328	0.06356	0.01558	0.01138	0.00649	0.02482	0.06025	0.0505	0.04384	0.04843
	8	0.07906	0.0737	0.0111	0.00677	0.00394	0.01631	0.03994	0.01828	0.04424	0.02477
	10	0.08224	0.08168	0.00345	0.00294	0.00194	0.01356	0.02169	0.0119	0.03819	0.01646
VLDLC	0	0	lost	0	0	0	0	0	0	0	0
	2	0	lost	0	0	0	0.0021	0.00065	0.00452	0	0.00174
	4	0.03139	lost	0.00399	0.00223	0.00117	0.00406	0.00633	0.01127	0.02656	0.02351
	6	0.04869	lost	0.00999	0.00578	0.00839	0.01566	0.03892	0.04988	0.04301	0.04786
	8	0.04466	lost	0.00934	0.0069	0.00487	0.01133	0.04076	0.03063	0.03032	0.04194
	10	0.04792	lost	0.00878	0.00828	0.00331	0.01158	0.03658	0.02135	0.03939	0.02926
LDL	0	0	lost	0	0	0	0	0	0	0	0
	2	-0.00831	lost	0.00361	-0.0048	-0.01738	-0.00754	0.00109	-0.00536	-0.01159	-0.04022
	4	0.00019	lost	-0.02133	0.00005	-0.01595	-0.00374	0.01276	-0.00941	-0.00468	-0.04916
	6	0.02164	lost	0.00144	0.00353	-0.02065	0.0045	0.00193	0.00975	0.02384	-0.00015
	8	0.06819	lost	0.04556	0.02553	0.00234	0.01808	0.02481	0.04843	0.07025	0.04468
	10	0.12731	lost	0.05364	0.03936	0.0118	0.03986	0.07588	0.08682	0.11966	0.07956

Table X. Lipoprotein canthaxanthin increments (μmol/L) after ingestion of a canthaxanthin plus placebo dose (trt B) and after a canthaxanthin plus β-carotene dose (trt C).

		3		5		4		1		2	
		CX (trt B)	CX (trt C)	CX (trt B)	CX (trt C)	CX (trt B)	CX (trt C)	CX (trt B)	CX (trt C)	CX (trt B)	CX (trt C)
Chylo	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0.00429	0.00294	0.00751	0.01016	0.04211	0.01654	0.00229	0.0036
	4	0.14144	0.0643	0.06162	0.03965	0.09524	0.04405	0.16532	0.15807	0.0576	0.0261
	6	0.23588	0.13206	0.12523	0.05565	0.1294	0.06837	0.22717	0.18079	0.11808	0.08562
	8	0.1222	0.08063	0.11487	0.03459	0.11681	0.05039	0.16079	0.12266	0.05743	0.05809
	10	0.09378	0.12056	0.03414	0.01804	0.04432	0.0209	0.13526	0.09604	0.0389	0.04508
VLDLA	0	0	0	0	0	0	0	0	0	0	0
	2	0.00574	0	0.00755	0.00651	0.0125	0.01317	0.04737	0.02954	0.00486	0.00587
	4	0.12811	0.06634	0.05272	0.03972	0.10356	0.05111	0.19777	0.10957	0.12534	0.06322
	6	0.35963	0.13395	0.14161	0.0822	0.1625	0.09129	0.24363	0.19572	0.1426	0.13756
	8	0.33134	0.09829	0.15049	0.06627	0.19925	0.07244	0.19213	0.06352	0.05831	0.07029
	10	0.30518	0.12472	0.06175	0.03692	0.12298	0.04687	0.10673	0.09098	0.06637	0.06675
VLDLB	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0.00276	0.00271	0.00524	0.00391	0.00857	0.00854	0.00243	0.00274
	4	0.02461	0.00634	0.01304	0.00954	0.02745	0.01475	0.04653	0.04469	0.04112	0.02856
	6	0.08157	0.03844	0.03852	0.027	0.07637	0.03722	0.10875	0.10083	0.08507	0.07014
	8	0.11327	0.07189	0.06238	0.03025	0.10471	0.04723	0.13806	0.08527	0.05456	0.06909
	10	0.12346	0.09618	0.0453	0.02509	0.1059	0.04424	0.05708	0.05395	0.05979	0.04813
VLDLC	0	0	lost	0	0	0	0	0	0	0	0
	2	0	lost	0.00083	0.00047	0.00148	0.00156	0.00423	0.00474	0.00176	0.00135
	4	0	lost	0.00684	0.00422	0.01074	0.00713	0.03845	0.0258	0.02401	0.01899
	6	0.02217	lost	0.0254	0.01606	0.04051	0.02735	0.10789	0.11763	0.08655	0.06679
	8	0.05827	lost	0.05085	0.02481	0.06627	0.03707	0.1888	0.14917	0.13085	0.10646
	10	0.05968	lost	0.05391	0.02982	0.07353	0.0369	0.18656	0.12763	0.08793	0.08345
LDL	0	lost	lost	0	0	0	0	0	0	0	0
	2	lost	lost	0.0029	0.00457	0.00324	0.00273	0.00589	0.00435	0.00167	-0.00059
	4	lost	lost	0.05232	0.05536	0.03501	0.03922	0.09233	0.08275	0.09252	0.0823
	6	lost	lost	0.21183	0.17007	0.1429	0.14165	0.26683	0.25502	0.36032	0.35705
	8	lost	lost	0.37538	0.24691	0.20303	0.19473	0.39343	0.37367	0.53638	0.53516
	10	lost	lost	0.4574	0.29067	0.22971	0.21707	0.49309	0.44448	0.60114	0.61735