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Use of electrochemical detection to quantify the effect of added fat on intestinal carotenoid absorption from fresh vegetables in humans

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Use of electrochemical detection to quantify the effect of added fat on intestinal carotenoid absorption from fresh vegetables in humans

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This is to certify that the Master's thesis of

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Has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

To my parents: Doug and Bonnie Brown

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ABSTRACT

Previously, high pressure liquid chromatography (HPLC) with coulometric electrochemical array detection (ECD) was shown to have enhanced sensitivity compared with HPLC with ultraviolet/visible light detection (UV/VIS) in carotenoid analysis. The objective of this study was to apply HPLC with electrochemical detection to quantify and compare the appearance of carotenoids in plasma chylomicrons after subjects ingested fresh vegetable salads with fat-free, reduced-fat, and regular fat salad dressing. Healthy, normolipidemic male and female subjects (n = 7) consumed a single salad that consisted of 48 g spinach, 48 g romaine lettuce, 66 g carrots, and 85 g cherry tomatoes. Salad dressings were prepared with varying amounts of canola oil to provide 0, 6, and 28 g fat. The salads with the three salad dressings were ingested in random order and separated by a washout period of at least two weeks. Blood samples were drawn at baseline and at hourly intervals for 12 hours after consumption of the test salad. The plasma chylomicron fraction was isolated by cumulative rate ultracentrifugation, and the carotenoids were extracted and quantified by HPLC-ECD. After ingestion of the salads with the fat-free salad dressing, the appearance of carotenoids in the plasma chylomicron fraction was negligible. After ingestion of the salads with reduced-fat as compared with fat-free dressing, the area under the curve (AUC) increased 20.9 nmol/L (P = 0.010), 97.5 nmol/L (P = 0.012), and 3.6 nmol/L (P = 0.016) for all *trans*-α-carotene, all *trans*-β-carotene, and all *trans*-lycopene, respectively. After ingestion of the salads with regular fat as compared with reduced-fat salad dressing, the AUC for all *trans*-α-carotene, all *trans*-β-carotene, and all *trans*-lycopene increased 2.5-fold (P = 0.025), 2.0-fold (P = 0.033), and 4.0-fold (P = 0.031), respectively. The use of HPLC-ECD to measure the postprandial carotenoid response in chylomicrons is a sensitive and

efficient approach to screen for dietary factors such as added fat that enhance β-carotene bioavailability. Consumption of a vegetable salad with fat-free salad dressing resulted in virtually no carotenoid absorption. Consumption of the salad with salad dressing containing dietary fat increased carotenoid absorption, and the increase in carotenoid absorption with regular fat dressing was higher than the absorption with reduced-fat dressing.

GENERAL INTRODUCTION

Thesis Organization

This thesis is organized into three chapters including a literature review, a manuscript to be submitted to the American Journal of Clinical Nutrition, and general conclusions. The literature review covers the provitamin A and non-provitamin A actions of carotenoids, the role of carotenoids in chronic disease, carotenoid absorption and bioavailability, and the use of electrochemical detection in carotenoid analysis. The manuscript describes a study carried out using electrochemical detection to quantify low concentrations of carotenoids in the chylomicron fraction of subjects who ingested a single test meal of fresh vegetables with a varying amount of dietary fat and to determine the amount of fat needed to optimize carotenoid absorption. The general conclusions chapter summarizes the findings of this study.

Literature Review

Introduction

Carotenoids are pigments found in yellow, orange, red, and green plants and fruits. More than 600 carotenoids have been identified (1). There are two major categories of carotenoids. Carotenes, the first category, consist of hydrocarbons such as α-carotene and β-carotene. The second category, xanthophylls, consist of oxycarotenoids (oxygenated hydrocarbons) such as lutein and zeaxanthin (2). In plants, carotenoids serve two functions, as accessory pigments in photosynthesis and in photoprotection (1). The structure of carotenoids allows them to absorb light and to quench, and thereby inactivate, singlet oxygen and to trap free radicals. The putative health protective effects of carotenoids are thought to be related to their antioxidant functions and provitamin A activity. Based on their structure,

approximately 50 of the known carotenoids have provitamin A activity (2). Preformed vitamin A can be obtained in the diet from animal foods, but in many developing countries, animal foods are scarce, and fruits and vegetables provide 70-90% of total vitamin A intake from provitamin A carotenoids (3).

Provitamin A Activity

Provitamin A carotenoids are converted enzymatically in the intestinal mucosa to produce retinal, retinoic acid, and retinol (1). Retinal functions in the retina in transduction of light into the neural signals necessary for vision (4). Retinoic acid (a derivative of retinal) also aids in vision by maintaining normal differentiation of the cells of the conjunctival membranes, cornea, and other ocular structures, thus preventing xerophthalmia (4). Vitamin A also plays a major role in fetal development. Retinol must be transported within the embryo and converted to retinoic acid locally for normal postgastulational development (4). Retinoic acid regulates a set of genes responsible for proper development of the spinal cord, vertebrae, limbs, heart, eyes, and ears. Additionally, vitamin A plays a role in immunity (4). In vitamin A deficiency, both cell-mediated immunity and antibody-mediated responses are generally decreased. Immune function is rapidly restored after vitamin A supplementation, suggesting that signaling pathways necessary for normal immune function are impaired during retinoid deficiency (4).

An enzyme to convert β-carotene to vitamin A was first described in the mid-1950's (5). Many attempts to fully purify and characterize this enzyme were made but were only partially successful. The mode of β-carotene cleavage was highly controversial. Two types of cleavage were proposed (6). If the enzyme catalyzed a central cleavage mechanism, β-carotene would be cleaved in the center at the 15,15' double bond resulting in two molecules

of retinal. By the eccentric cleavage mechanism, β-carotene would be cleaved asymmetrically producing one molecule of one of various apo-carotenals, which, via further processing, could result in one molecule of retinoic acid.

In 2000, von Lintig and Vogt (7) cloned and identified a β-carotene dioxygenase from *Drosophila melanogaster* by expressing it into a β-carotene synthesizing and accumulating strain of *Escherichia coli*. This newly discovered enzyme was found to only produce retinal, and thus supposed to function via the central cleavage mechanism. This enzyme was named β-carotene 15,15' dioxygenase based on the 15,15' double bond that it cleaves. The same year, Wyss et al. (5) cloned and expressed chicken β-carotene 15,15' dioxygenase that produced only retinal when incubated with β-carotene as a substrate, confirming that the enzyme specifically cleaves β-carotene at the 15,15' double bond. The same group that first identified β-carotene 15,15' dioxygenase in *D. melanogaster* also identified a murine cDNA encoding a second type of carotene dioxygenase that exclusively catalyzes the asymmetric oxidative cleavage of β-carotene at the 9'10' double bond resulting in β-apo-10'-carotenal and β-ionone (8). This enzyme is also able to oxidatively cleave lycopene.

Following the isolation and cloning of β-carotene 15,15' dioxygenase, much was learned about its activity. β-Carotene 15,15' dioxygenase is a soluble cytosolic enzyme whose activity is dependent upon an iron-containing cofactor (6). In mammals, the highest carotene dioxygenase activity was found in intestinal mucosa, with the activity decreasing from the duodenum to the jejunum to the colon. β-Carotene dioxygenase also has been found in the lung, kidney, and brain (6).

Current research is focused on the regulation of this enzyme. It is estimated that 60-70% of absorbed β-carotene is converted directly to retinal whereas the remainder is deposited in adipose or other tissues (6). It has been known for some time that high doses of β-carotene are not toxic. Long-term treatment for erythropoietic protoporphyria (EPP) involves high doses of β-carotene and does not result in vitamin A toxicity (6). However, the β-carotene supplementation trials indicate that supplementing smokers with high doses of β-carotene may have deleterious effects (9). Researchers have speculated that β-carotene 15,15' dioxygenase is regulated by retinoids or carotenoids (6). A study by Parvin et al. (10) showed that the nutritional status of rats affects the activity of β-carotene 15,15' dioxygenase. In vitamin A deficient animals supplemented with 50, 100, 200, or 300 μg β-carotene, the level of β-carotene 15,15' dioxygenase activity was more than twice as high as in vitamin A adequate control animals, and the area under the curve ($AUC_{0\text{-}12hr}$) of plasma vitamin A was correlated in a dose-dependent manner to the amount of β-carotene given as a supplement up to, but not including the 300 μg dose.

Non-provitamin A Activity

The major non-provitamin A activities of carotenoids are related to their antioxidant activities. A second activity, up-regulation of gap junctional communication, will be discussed briefly.

There are two ways in which carotenoids can act as antioxidants: quenching singlet oxygen and interacting with free radicals. Carotenoids can quench singlet oxygen by physical or chemical means. The predominant method, physical quenching involves transferring excitation energy from the singlet oxygen to the carotenoid producing a ground-

state oxygen and an excited carotenoid (11). The carotenoid dissipates this energy by releasing it as heat without transferring it to other molecules. Thus, the regenerated carotenoid functions as a catalyst, able to quench another singlet oxygen. The ability of a carotenoid to quench singlet oxygen increases with an increasing number of conjugated double bonds (12). It is for this reason that lycopene with 11 linearly conjugated double bonds has greater quenching activity compared with other carotenoids (11). The less common chemical quenching leads to the destruction of the carotenoid (12). In interactions of carotenoids with free radicals, excess energy is not lost as heat, the carotenoid donates the missing electron. Often the carotenoid molecule is destroyed by reactions with free radicals (12).

The antioxidant ability of carotenoids to quench singlet molecular oxygen and to inactivate free radicals has been well studied *in vitro* (12). Evidence from the literature on the antioxidant activity *in vivo* is inconsistent. In the case of erythropoietic protoporphyria (EPP), a rare genetic photosensitivity disease, carotenoids do show an antioxidant photoprotective effect *in vivo*. β-Carotene and canthaxanthin have been used for over 25 years to successfully treat EPP (1). Additionally, Stahl et al. (13) showed that oral β-carotene supplements decrease erythema formation in human subjects exposed to increasing doses of irradiation with UV light. The authors suggest that the protection that β-carotene supplements afford is due to the ability of β-carotene to scavenge reactive oxygen species generated during photooxidative stress. There is much interest in research to determine if antioxidant properties of carotenoids may protect against diseases which are believed to be initiated by free radicals such as cataracts, age-related macular degeneration, and atherosclerosis (11). However, there is no definitive evidence for an *in vivo* antioxidant role

of carotenoids in protecting against chronic disease. Riso et al. (14) showed that consumption of 60 g of tomato puree (which provided 16.5 mg lycopene and 0.6 mg β-carotene) daily for three weeks by female subjects decreased DNA damage in lymphocytes initiated *ex vivo* by hydrogen peroxide. Whether the protective effect was due solely to lycopene cannot be determined because the tomato puree contained other compounds with antioxidant properties such as vitamin C, other carotenoids, flavonoids, and other phytochemicals. These components could work alone or synergistically with lycopene to inhibit DNA damage. In a follow-up study, Porrini and Riso (15) repeated the previously mentioned study except with an amount of tomato puree consistent with a normal diet to determine if it would have the same protective effects. Female volunteers were fed 25 g of tomato puree (which provided 7 mg lycopene and 0.3 mg β-carotene) daily for two weeks. Carotenoid contents of plasma and lymphocytes were measured. DNA damage was inversely associated with lycopene content of both plasma and lymphocytes. They concluded that either lycopene contributes to the protection of DNA from reactive oxygen, or lycopene is a good marker of the antioxidant properties of tomato. In contrast, the following study does not support a role for carotenoids in fruits and vegetables in decreasing oxidative damage. Bianchini et al. (16) studied oxidative damage to DNA in lymphocytes by measuring 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) (a biomarker of oxidative damage in DNA) in two populations: women in Granada, Spain who followed a Mediterranean diet high in fruits and vegetables and women in Malmo, Sweden who followed a Northern European diet lower in fruits and vegetables. Though plasma concentrations of lycopene, zeaxanthin, and β-cryptoxanthin were significantly higher in

Granda compared with Malmo, plasma concentrations of carotenoids and 8-oxodGuo content in lymphocytes were positively correlated for both groups.

β-Carotene and other carotenoids such as canthaxanthin and zeaxanthin show increased anti-oxidant activity at low oxygen concentrations (12). At high oxygen pressures, β-carotene may act as either an anti-oxidant or a pro-oxidant, but these mechanisms are not well understood (12). Carotenoids also play a role in preventing lipid peroxidation. Dixon et al. (17) measured lipid peroxidation in two groups of women, one on a low carotenoid diet, and the other on the same diet with the addition of a β-carotene supplement. The group on a low carotenoid diet (without the supplement) had significantly higher concentrations of plasma malondialdehyde-thiobarbituric acid, a marker of lipid peroxidation, compared with the group taking the β-carotene supplement.

Carotenoids and retinoids are known to induce gap junctional communication (GJC) between cells (18). Gap junctions connect the cytosol of two neighboring cells and allow the cells to exchange nutrients and ions and facilitate the transfer of electrical signals between cells. Tumor promoters inhibit GJC; thus, carotenoids may serve an anti-carcinogenic role by stimulating GJC. Non-provitamin A carotenoids as well as provitamin A carotenoids and retinoids stimulate GJC. In a study by Krutovskikh et al. (19), rats were given daily doses of one of three carotenoids (α-carotene, β-carotene, or lycopene at doses ranging from 0.5 –50 mg/kg body weight) for 5 days following which, hepatocytes were tested for GJC. The highest dose of each of the three carotenoids inhibited GJC. The 5 mg/kg middle dose of each of the three carotenoids enhanced GJC, and the lowest dose of each of the three carotenoids had no effect on GJC in the liver. Carotenoids increase the expression of connexin 43, a gene that encodes a major gap junctional protein (20). Increased levels of this

protein result in an increased number of junctional channels, which results in increased cell-to-cell communication (20).

Carotenoids and Chronic Disease

Cancer

It has been estimated that 70% of all cancer can be attributed to diet (21). Numerous epidemiologic studies (both prospective cohort and case-control studies) have found an association between high fruit and vegetable intake and a decreased risk of cancer. Steinmetz (21) reviewed 206 human epidemiologic studies and found statistically significant inverse associations for fruit and vegetable intake in 80% or more of the studies of cancers of the stomach, esophagus, lung, oral cavity, pharynx, rectum, bladder, cervix, endometrium, and larynx. Giovannucci (22) reviewed epidemiological studies that measured tomato intake or plasma lycopene concentrations. Of 72 studies, 57 showed inverse associations between either tomato consumption or plasma lycopene concentrations and the risk of cancer; 35 of these inverse associations were statistically significant. The remaining 15 studies indicated a slight direct association (relative risks ranged from 1.0-1.2) or were inconclusive; however, none of the direct associations between tomato or lycopene consumption and cancer risk were significant. Thus, consumption of tomatoes, which contain lycopene, and other fruits and vegetables that contain a variety of carotenoids is associated with a decreased risk of various types of cancer.

As mentioned previously, β-carotene is an antioxidant and may protect against free-radical damage. Additional activities of β-carotene that may be protective against cancer include: inhibiting cell proliferation and increasing cell-to-cell communication (21). Cancer cells are characterized by a lack of differentiation. β-Carotene can be converted to retinal

and its derivative retinoic acid, which can act as a nuclear receptor ligand and induce cell differentiation (23). Other carotenoids may also play a role in cancer prevention. Lutein and lycopene are antioxidants and may protect against cancer by blocking damage initiated by free radicals (21). α-Carotene is also an antioxidant and a vitamin A precursor, although less potent than β-carotene. Thus, it could have the same cancer preventative effects as β-carotene.

Lung Cancer Lung cancer is the most common cause of cancer death in the United States. One hundred percent of the lung cancer studies reviewed by Steinmetz (21) showed that fruit and vegetable intake was protective against this disease. Because of the link between carotenoid intake and decreased cancer risk, β-carotene supplementation trials were begun with populations that would be expected to benefit most from supplementation – cigarette smokers and asbestos workers. Compared with other carotenoids, conversion of β-carotene to vitamin A is the most efficient. The conversion efficiency of β-carotene coupled with the fact that β-carotene had been studied more extensively led researchers to choose β-carotene as the carotenoid supplement for these trials.

The Alpha-Tocopherol-Beta-Carotene (ATBC) Trial (9) involved 29,000 males over age 50 in Finland who were heavy smokers. Participants received either 50 mg/day α-tocopherol, 20 mg/day β-carotene, a combination of the two supplements, or a placebo for five to eight years. Surprisingly, subjects receiving the β-carotene supplement (either alone or with vitamin E) had a significantly higher incidence of lung cancer and mortality compared with participants receiving the placebo. Subjects receiving supplemental β-carotene did not exhibit increased incidence of other cancers (prostate, bladder, colon/rectum,

or stomach cancers). In the placebo group, those who had higher β-carotene intake and serum β-carotene concentrations (as measured at the onset of the study) had a lower incidence of lung cancer.

The increased incidence of lung cancer in smokers supplemented with β-carotene was replicated in the United States in the Carotene and Retinol Efficacy Trial (CARET) (24). The subjects of the CARET study, heavy smokers and asbestos-exposed workers, both populations at high risk for lung cancer, were split into two groups. One group received 8000 retinol equivalents (RE) vitamin A and 30 mg β-carotene daily whereas the other group received a placebo. An evaluation completed after supplementation for an average of four years showed that lung cancer incidence had increased by 28% in the supplemented subjects (significantly more cancers than in the placebo group). The study was ended nearly two years early, because it seemed unlikely that the supplementation would prove to be beneficial and likely that it would prove harmful in this population.

In a third supplementation study, the Physicians' Health Study (25), 22,000 male physicians were given either a 50 mg β-carotene supplement or a placebo to be taken every other day for 12 years. The supplement had no effect on lung cancer incidence. It is important to note in relation to the previous studies that only 11% of the physicians were smokers upon entry to the study.

Many researchers have proposed hypotheses as to how β-carotene could stimulate cancer. It is possible that the doses given in these studies, 20-30 mg/day, were too high. Typical intakes of β-carotene in most populations are less than 5 mg/day (23). The serum β-carotene concentrations found to be protective against cancer were much lower than those of

the subjects participating in the supplementation trials. Serum β-carotene concentrations were 3.0 mg/L, 2.1 mg/L, and 1.2 mg/L for the ATBC, CARET, and Physicians' Health Study trials, respectively (23). According to the National Health and Nutrition Examination Survey (NHANES III), the 95th percentile of serum concentration in the U.S. population is only 0.5 mg/L (23). It is possible that beneficial effects of β-carotene might only be seen at physiological and not pharmacological concentrations. Additional explanations for this unexpected detrimental effect linked to β-carotene include the hypothesis that large doses of β-carotene inhibit the intestinal absorption of other nutrients or that β-carotene acts as a pro-oxidant in the lungs of heavy smokers (1). It has been shown that β-carotene has pro-oxidant activity at high partial pressures of oxygen, and the partial pressure of oxygen is highest in the outermost cells of the lung (26). Thus, these cells may be susceptible to pro-oxidant activity of β-carotene. Paolini et al. (26) proposed that β-carotene may have a cooperative role in activating latent tumors rather than initiating new ones. After supplementing rats with high doses of β-carotene, these researchers found a significant increase in the carcinogen-metabolizing cytochrome P450 enzyme CYP1A1/2 which could act together with β-carotene to produce a co-carcinogenic response. This phenomenon may be accentuated in the environment of a smoker's lung which contains numerous bioactivated tobacco-smoke procarcinogens.

To further determine the link between β-carotene, cigarette smoking, and lung cancer, Wang et al. (27) studied the effects of β-carotene and cigarette smoking *in vivo* in an animal model. Ferrets were used because ferrets absorb a proportion of β-carotene intact in the intestinal mucosa similarly to humans. The three treatment groups included one given 2.4

mg β-carotene daily (intended to be equivalent to the 30 mg dose of β-carotene given to subjects in supplementation trials), one exposed to cigarette smoke, and a third both given the β-carotene supplement and exposed to cigarette smoke. A control group was not given a supplement or exposed to cigarette smoke. The investigators found an increase in eccentric cleavage products of β-carotene in the lung tissue of ferrets exposed to cigarette smoke, and a strong proliferative response in lung tissue and squamous metaplasia in all β-carotene supplemented animals, with exposure to cigarette smoke increasing the latter two responses. All three treatment groups showed a decrease in retinoid signaling resulting from an inhibition of retinoic acid receptor-β (RARβ) (thought to be a tumor suppressor), gene expression, and over expression of activator protein 1 which causes cell proliferation. Thus, Wang et al. proposed the following possible mechanism for the harmful effect of β-carotene supplementation in smokers: Free-radicals in the lungs of smokers increase β-carotene oxidation and the formation of eccentric cleavage products, which could decrease retinoid signaling by down regulating RARβ expression and decreasing retinoic acid concentrations in lung tissue and up-regulating activator protein 1. The overall predicted effect would be accelerated tumorigenesis.

From an epidemiological perspective, it is possible that the presence of high concentrations of β-carotene in the diets and/or plasma of persons with a low incidence of cancer is not indicative of the functional compound that prevents cancer, but simply of a marker for other protective factors. For example, diets high in fruits and vegetables also tend to be low in saturated fat and cholesterol and high in fiber (21).

Cardiovascular Disease

Atherosclerosis is thought to begin when low-density lipoproteins (LDL) are oxidized (28). Oxidized LDL accumulates in macrophages, which form foam cells that deposit lipids in fatty streaks leading to the thickening of the endothelial wall and cardiovascular disease (CVD). Rupture of these plaques can cause thrombosis or complete arterial occlusion. A proposed protective mechanism for β-carotene (and other carotenoids) is to interfere in this process by preventing LDL oxidation.

Numerous epidemiological studies have shown a decreased risk of cardiovascular disease with increased dietary intake of fruits and vegetables or increased serum/tissue levels of β-carotene (27-30). In a study of over 12,000 U.S. men and women aged 45-64 years, those consuming the greatest amounts of carotenoid-containing foods had the lowest prevalence of arterial plaques (28). This difference was only statistically significant in women. After the results were adjusted for possible confounders, only women in the second quintile of intake had a significantly lower prevalence of plaques. Smoking did affect the association between carotenoid intake and plaque prevention; women smokers in the lowest intake quintile had a 41.4% prevalence of plaques, whereas those in the highest intake quintile had a 31.5% prevalence, both of which were higher than the 26.2% and 22.8% prevalence of plaques in former smokers and nonsmokers. There was no statistically significant evidence of an interaction between smoking, carotenoid intake, and plaque prevalence for men. Street et al. (29) studied the association between serum concentrations of antioxidants (β-carotene, lycopene, lutein, zeaxanthin, α-tocopherol) and myocardial infarction (MI) in a nested case-control study. Blood samples were collected 7-14 years before the onset of MI. They found that the risk for subsequent myocardial infarction

increased with decreasing levels of β-carotene, but this trend was only significant for smokers. The data for lutein was suggestive of a similar but nonsignificant trend. In the European EURAMIC study (30), a case control study which measured carotenoid concentrations in adipose biopsies of men with recent MI, smokers and former smokers with acute MI had lower adipose tissue β-carotene than controls which may be indicative of a lower long-term β-carotene intake. Additionally, lycopene in adipose was significantly associated with a lower risk of MI. The effect of lycopene was strongest among smokers (31).

Not all of the epidemiological literature points to a link between increased carotenoid concentrations and a decreased risk of cardiovascular disease as illustrated in the following study. In a nested-case control study that used participants in the Multiple Risk Factor Intervention Trial (MRFIT), carotenoid, retinol, and α-tocopherol concentrations in serum were compared with incidence of nonfatal myocardial infarction or death from coronary heart disease (32). There were no significant odds ratios showing an inverse relation between any of the antioxidants measured and either nonfatal myocardial infarction or death from coronary heart disease. There were also no significant differences between smokers and non-smokers for the link between any of the measured antioxidants and the cardiovascular outcomes measured.

Because the results of epidemiological studies were encouraging, trials with β-carotene were initiated. The associations between β-carotene supplementation and cardiovascular disease were similar to the results with lung cancer. In the ATBC trial (9), an increase in coronary artery disease (CAD) was found in the group supplemented with β-

carotene. Results of the CARET study (24) also showed an increased risk of cardiovascular endpoints with β-carotene supplementation. In the Physicians' Health Study (25), no significant differences in cardiovascular disease risk existed between the β-carotene supplemented group and the placebo group.

Diseases of the Eye

Age-Related Macular Degeneration Age-related macular degeneration (ARMD) is the leading cause of blindness in people over the age of 65 in Western countries. An estimated 13 million people in the United States have evidence of ARMD and the disease causes visual impairment in 1.2 million (33). There is no effective treatment for the majority of people who suffer from ARMD (34).

The macular pigment (MP) of the human retina is visible as a yellow spot (macula lutea) in the central retina. The macular pigment is composed of three carotenoids, lutein, zeaxanthin, and meso-zeaxanthin, which represent approximately 36%, 18%, and 18% of the total carotenoid content of the retina (35). The macular pigment is able to absorb and attenuate high energy blue light striking the central region of the retina, and reduce chromatic aberration in the eye (35). Degeneration of the macula leads to ARMD. Females, subjects with light iris color, and smokers have decreased macular pigmentation compared with males, subjects with dark iris color, and non-smokers, respectively (35). These same factors have been identified as risk factors for age-related macular degeneration. Other risk factors include age and lifetime exposure to sunlight. Age is a primary risk factor as evidenced by the Beaver Dam Eye study (36), in which the prevalence of ARMD increased from 16.8% in patients 55-64 years of age to 25.6% in patients 65-74 years of age, and to 42% in patients over 75.

The visual sensitivity of subjects older than 60 years who have high MP density is not significantly different from that of younger subjects; however, older subjects with low MP density had lower visual sensitivity than younger subjects (37). Thus, high MP density is associated with the retention of youthful visual sensitivity. How does the MP protect the retina? Two protective roles have been proposed (33, 35). First, the MP filters blue light, which is particularly damaging to the photoreceptors and the retinal pigment epithelium (RPE). The fact that lutein and zeaxanthin absorb blue light supports this theory. Second, carotenoids act as antioxidants to limit oxidative stress that occurs in the high oxygen and high light environment within the eye. Lutein and zeaxanthin are known to be good antioxidants, which lends credence to this theory.

It is possible to modify the density of the MP by increasing the dietary consumption of lutein and zeaxanthin. Human subjects eating diets with increased amounts of lutein and zeaxanthin in the form of corn and spinach had increases in macular pigmentation at the end of 15 weeks (38). Landrum et al. (39) supplemented two subjects with 30 mg/day lutein for 140 days. The subjects showed a 20-40% increase in macular pigment optical density (MPOD) at the end of the supplementation period. A high dietary intake of lutein- and zeaxanthin-containing foods and elevated concentrations of lutein and zeaxanthin in serum are associated with lower risk for ARMD (35).

Age-Related Cataract Age-related cataract is the leading cause of blindness in the world (34). In the United States, cataract extraction is the most frequently performed surgical procedure in the elderly (1), making it the most expensive item in the US Medicare budget (34). The exact etiology of age-related cataract is not known. Cataracts are thought

to result from photooxidation of lens proteins resulting in protein damage, accumulation, aggregation, and precipitation in the lens (1).

Small amounts of lutein and zeaxanthin have been detected in the human lens (40). There is much interest in determining whether these carotenoids have a functional role in the lens, and whether there is an association between lutein and zeaxanthin and cataract incidence. Hammond et al. (41) studied the relation between MP density (long-term measure of tissue carotenoids) and lens optical density (indicator of lens health). Lens density increased with age as expected. In the oldest group of subjects, a significant inverse relation was found between macular pigment density and lens density. This inverse relationship implies that lutein and zeaxanthin may be protective against cataract formation. A prospective study of over 77,000 female registered nurses ages 45-71, found (after controlling for confounding risk factors such as age and smoking) that subjects with the highest intake of lutein and zeaxanthin (measured by food frequency questionnaires administered at regular intervals) had a 22% decreased risk of cataract extraction compared with those in the lowest quintile (40). Other carotenoids (α-carotene, β-carotene, lycopene, and β-cryptoxanthin) and vitamin A showed no significant association with incidence of cataract. Additionally, increased intake of spinach and kale (foods rich in lutein) was associated with a moderate decrease in cataract risk. The Beaver Dam Eye Study compared nuclear opacity and antioxidant intake in a cohort study with over 1,300 subjects (42). In this study, lutein + zeaxanthin were the only carotenoids associated with nuclear cataract. Persons in the highest quintile of lutein + zeaxanthin intake were half as likely to have an incident cataract. These three studies show that there is a link between lutein and zeaxanthin

intake and density in the macula and cataract incidence, but more study is needed to further elucidate the mechanism(s).

Carotenoid Bioavailability

Bioavailability has been defined as the fraction of an ingested nutrient that is available for utilization in normal physiologic functions or for storage (43). de Pee (44) expands the definition of the bioavailability of carotenoids to include the freeing of carotenoids from food, their absorption, and their subsequent circulation in serum. There are many factors that affect the bioavailability of carotenoids during carotenoid absorption. These factors are explored below following an overview of the mechanisms of carotenoid absorption.

Overview of Carotenoid Absorption

Carotenoid absorption can be divided into four steps: release of carotenoids from the food matrix, formation of lipid-mixed micelles, uptake of carotenoids into intestinal mucosa cells, and packaging of carotenoids into chylomicrons for transport in the general circulation. It is important to note that fat is required at each of the three latter steps (45).

Before absorption can occur, carotenoids must be released from the food matrix. Carotenoids are not free in food; they are associated with proteins and other cell structures (46). Physical alteration (cooking, chopping, and blending) as well as chemical alteration in the stomach (gastrointestinal hydrolysis of lipids and proteins) release carotenoids from the food matrix (46). Once released, carotenoids dissolve in an oily phase of lipid droplets which become mixed with other gastric contents to form emulsified particles (46). Normal movements of the digestive tract cause the formation of a fine lipid emulsion as the contents of the stomach pass into the duodenum. This emulsion is composed of a triacylglycerol

(TAG) core surrounded by a layer of partially digested proteins, polysaccharides and lipids (phospholipids and fatty acids) (46). The hydrocarbon carotenes are incorporated into the layer surrounding the TAG core (47), and the polar xanthophylls are distributed along the surface of the emulsion (46). The surface components of the emulsion transfer from lipid droplets to mixed micelles unassisted whereas the carotenoids associated with the core of the emulsion require digestion of TAG before transfer (46). Dietary fat stimulates bile secretion to aid in the formation of lipid micelles in the intestine. Carotenoids are incorporated into micelles and transported to intestinal mucosal cells where carotenoids are taken up intact (45). In the intestinal mucosa, some of the provitamin A carotenoids are cleaved by β-carotene-15,15'-dioxygenase and other dioxygenase enzymes discussed above to form vitamin A and related retinoids (45). The xanthophyll carotenoids, uncleaved hydrocarbon carotenoids and retinyl esters are then packaged into chylomicrons with phospholipids and triacylglycerols and travel through the lymphatic system to the systemic circulation, and ultimately reach the liver (45). In the liver, the contents of what are now called chylomicron remnants (because they are TAG-depleted) are repackaged into other lipoproteins. The distributions of β-carotene, α-carotene and lycopene are similar to cholesterol. Approximately 75% of these hydrocarbon carotenoids are associated with LDL, and the remaining 25% are associated with high-density lipoprotein (HDL) and very-low-density lipoprotein (VLDL) (48). Lutein and zeaxanthin are distributed equally between HDL and LDL (46). Percent absorption of a single dose measured over a wide range of 45 μg to 39 mg β-carotene (using isotope tracers) is reported to range from 9 to 22 percent, but the absorption efficiency decreases as the amount of dietary carotenoids increases in the diet (49).

It is likely that adipose tissue, liver, and plasma contain most of the body's carotenoid content (50). Based on his study of carotenoid content of adipose tissue and the studies of others, Parker (50) estimated that a 70 kg individual with 24% of body weight as fat would have ~10 mg β-carotene in adipose, ~ 8.6 mg in the liver, and 0.8 mg in plasma. Certain carotenoids are selectively taken up into specific tissues. For example, lutein and zeaxanthin are selectively taken up into the macular pigment of the retina (46), lycopene is taken up into prostate (46), and β-carotene is selectively taken up in the corpus luteum (51).

DePee and West (52) originally grouped what they considered to be the main factors affecting carotenoid absorption and bioconversion into the mnemonic "SLAMENGHI". West and Castenmiller (53, 54) reviewed these nine factors: **S**pecies of carotenoids, molecular **L**inkage, **A**mount of carotenoids consumed in a meal, **M**atrix in which the carotenoid is incorporated, **E**ffectors of absorption and bioconversion, **N**utrient status of the host, **G**enetic factors, **H**ost-related factors, and mathematical **I**nteractions. These nine factors are expanded on below.

Species of carotenoid: The all-*trans* isomers of carotenoids occur naturally in plant foods; and are absorbed more readily than *cis* isomers (54).

Molecular **L**inkage: Esters of carotenoids, which are common in fruits and vegetables, seem to have greater bioavailability do than free carotenoids (55), but this point is controversial.

Amount of carotenoids consumed in a meal: Serum β-carotene levels vary depending on the amount of β-carotene in a meal. A meta-analysis (54) performed on 31 studies that gave subjects β-carotene supplements of less than 50 mg daily for less than one year showed a serum response per mg of supplemental β-carotene that ranged from 0.0317 to 0.0388

μmol/L. The duration of supplementation was a significant predictor of β-carotene response. In a separate study, when subjects took β-carotene daily in three divided doses with self-selected meals, the serum β-carotene concentration increased three-fold compared with the same total dose administered once a day (56). Additionally, carotenoids interact with one another and can increase or decrease the absorption of one another. This will be discussed in depth later in this review.

Matrix in which the carotenoid is incorporated: β-Carotene dissolved in oil is absorbed far more readily than β-carotene in foods (53). Carotenoids, which exist in foods as crystals such as α- and β-carotene in carrots, are less bioavailable than carotenoids that are dissolved in oil droplets in foods such as carotenoids in mango, papaya, pumpkin, and sweet potato (53). Cooking increases the bioavailability of carotenoids perhaps because it disrupts plant cell walls (57, 58). This topic will be covered in more detail later in this review.

Effectors of absorption and bioconversion: Certain nutrients when consumed with carotenoids may affect carotenoid absorption and/or bioconversion. Protein in the small intestine stabilizes fat emulsions and enhances micelle formation and carotenoid uptake (53). Lecithin may increase TAG absorption by facilitating micelle formation, and long-chain fatty acids increase cholesterol absorption; thus, both increase absorption of fat-soluble substances such as carotenoids (54). Interactions in the GI tract with drugs may decrease absorption (53). When a β-carotene supplement was consumed without any fat, no detectable increase of serum β-carotene was found indicating that no β-carotene was absorbed (56). Dietary fiber interacts with bile acids and may interfere with micelle formation necessary for

absorption of β-carotene (59). Sucrose polyester, a nonabsorbable fat substitute, markedly decreases plasma carotenoid concentrations (54).

Nutrient status of the host: Absorption of carotenoids is likely to be dependent on vitamin A status as well as the health status of the host, which may affect nutrient status. Diets low in vitamin A have been shown to increase dioxygenase activity, which would produce more vitamin A (10). Zinc deficiency may affect the synthesis of retinol-binding protein and bioconversion of β-carotene to vitamin A (60). Additionally, intestinal parasites, malabsorption diseases, and liver or kidney diseases could decrease carotenoid absorption (45).

Genetic factors: Genetic determination of carotenoid absorption is linked to individual fat absorption (53). An inability to cleave β-carotene is a rare condition that can lead to carotenemia or vitamin A deficiency if retinol intake is low (54).

Host-related factors: Initial serum concentration of β-carotene has been proposed as the key predictor of the response to an oral dose of β-carotene, but this is controversial (53). The initial β-carotene serum concentration is dependent on long-term β-carotene intake, genetic factors, and post-absorptive metabolism of β-carotene.

Mathematical **I**nteractions: According to West and Castenmiller (53), "Mathematical interactions refers to the difference observed when two factors play a role together compared with the sum of the effects observed separately. However, data are not yet available to allow any estimate of mathematical interactions to be made."

In the past several years, much research has been performed on the dietary effectors of carotenoid absorption and the food matrix in which carotenoids are embedded. A further review of these subjects is provided here.

Amount of Dietary Fat

As mentioned previously, fat is needed in the latter three steps of carotenoid absorption (45). When subjects were given 51 mg of β-carotene in the absence of dietary fat, there was no detectable change in serum β-carotene (56). But how much fat is needed for optimal carotenoid absorption? The same β-carotene dose administered with 200 g of fat increased serum β-carotene 2.5-fold from baseline at 40 hours following ingestion of the dose (56).

Several studies found optimal absorption of carotenoids from foods ingested with much smaller amounts of fat (61-65). In a study by Jalal (61), two groups of Indonesian boys aged 3 to 6 years were fed red sweet potatoes; one group received 15 g supplemental fat (coconut oil and coconut milk) and the other group received only 3 g fat. The 15 g fat added to the β-carotene rich sweet potato increased serum retinol concentrations compared with ingestion of the sweet potato with 3 g fat, but this increase was not statistically significant. In a study by Roels et al. (62), in Indonesian children aged 3 to 13 years, supplementation with 1 g palm oil per kg body weight (which contained 410 μg β-carotene/g) produced a similar serum vitamin A response as supplementation with 2000 I.U. vitamin A acetate. Supplementation of another group of children in the same study with 1 g coconut oil per kg body weight caused a small increase in serum carotene. Eighty percent of the subjects' protein intake was from vegetables, and 90% of their vitamin A intake came from

carotenoids, so although this oil supplement was not given with a source of β-carotene, it may have increased the absorption of carotenoids already present in the subjects' diet. In another study by Roels et al. (63), 9 to 16 year old boys in Rwanda receiving 200 g carrots and 18 g olive oil per day absorbed 25% of the carotene from carrots whereas those receiving 200 g carrots without any oil, absorbed only 5% of the carotene from carrots. In a study involving 2-6 year old Indian children who were given 40 g spinach along with 0, 5, or 10 g ground nut oil, Jayarajan et al. (64) found a significant increase in mean serum concentrations of vitamin A in all three treatment groups. The mean increase in serum vitamin A was higher in the groups who received additional fat in their diet; however, there was no difference in serum vitamin A between the groups who received 5 and 10 g oil. Another study that showed that large amounts of fat are not needed to maximize β-carotene absorption was performed by Roodenburg et al (65). In this study, human subjects were given either 8 mg lutein esters or 8 mg of a combination of α-carotene and β-carotene incorporated into a low-fat (3 g) or a high-fat (36 g) spread which was included as part of a larger meal. Unexpectedly, the increases in serum α- and β-carotene concentrations were slightly, but not significantly larger after consumption of the low-fat meal compared with the high-fat meal; however, the plasma lutein response was significantly higher when lutein esters were consumed as part of the high-fat meal compared with the low-fat meal. The difference in absorption between the carotenoids may be because carotenoid esters are more lipid-soluble than free carotenoids (55).

The limited available data shows that the addition of fat to a carotenoid supplement increases serum β-carotene and/or retinol concentrations compared with the carotenoid supplement alone (56, 61, 63-65). And, when different amounts of fat are given, it has been

suggested that only a minimum amount of fat is needed for maximal absorption (3-5 g) (64, 65). However, this is highly dependent on several factors such as the carotenoid being absorbed, the type of dietary fat that accompanies the carotenoid supplement and the food matrix in which the carotenoid is packaged. The latter two variables will be described in the following sections.

Type of Dietary Fat

Few studies have focused on the type of dietary fat ingested with carotenoid-containing foods, but as the following studies show this can have a major impact on carotenoid absorption. When subjects were given a pharmacological dose of β-carotene with a meal containing medium-chain triacylglycerols (MCT) or long-chain triacylglycerols (LCT), the chylomicron β-carotene response was markedly diminished in the group who consumed the MCT compared with those who consumed LCT (66). Medium chain fatty acids are primarily absorbed via the portal vein. Thus, chylomicron formation is low after a meal containing primarily MCT, which may explain why the chylomicron β-carotene response was low (66). Our group showed that in women who each ingested two meals containing β-carotene (47 μmol) and 60 g of either sunflower oil or beef tallow, the appearance of β-carotene in chylomicrons and VLDL was lower after consumption of the meal containing sunflower oil compared with the meal containing beef tallow (67).

Food Matrix

Several studies have compared the effect of carotenoid supplements and food sources of carotenoids in increasing plasma carotenoid concentrations. Brown et al. (68) measured plasma carotenoid concentrations in subjects who ingested a single dose of β-carotene (12 or

30 mg), or cooked carrots containing 29 mg β-carotene. The 30 mg dose of β-carotene produced a response that was significantly higher than the 12 mg dose which itself produced a response that was significantly higher than that of the cooked carrots. The 30 mg dose of β-carotene produced a peak response 1.6 times greater than that of the 12 mg dose, which may suggest a reduced absorption efficiency at the higher intake. The cooked carrots produced a plasma response that was one-fifth to one-seventh the response produced by the 30 mg β-carotene supplement. In a similar study (69), subjects were given the above test meals daily for 6 weeks (instead of a single meal). Again, the 30 mg β-carotene supplement produced the highest response, which was significantly higher than the 12 mg β-carotene supplement, and the 12 mg β-carotene supplement produced a response that was significantly higher than that of the cooked carrots. The cooked carrots produced an average maximal change in plasma β-carotene that was only 18% of that produced by the 30 mg β-carotene supplement. In these two studies, a purified form of β-carotene was more bioavailable than the food form. Carotenoids in mature carrots are thought to be present in the form of carotene bodies, carotenoids in crystalline form surrounded by sheets of membranes within the carrot chromoplast (70). It is hypothesized that β-carotene in this crystalline form is less bioavailable than purified β-carotene (68, 69).

In a study by Paetau et al. (71), subjects were fed lycopene-rich tomato juice, tomato oleoresin, and lycopene beadlets, each with a similar amount of lycopene, daily for four weeks. Plasma lycopene concentrations were significantly higher in the tomato juice, oleoresin, and lycopene beadlet consumption groups compared with placebo consumption group. The plasma lycopene response of the three lycopene treatments were not significantly

different from one another. In this study, absorption of lycopene from purified lycopene was as effective as absorption of lycopene from tomato juice.

Recently, studies have focused on determining differences in bioavailability of carotenoids from foods with different matrices. Dark green leafy vegetables have been emphasized as a good source of provitamin A carotenoids to decrease incidence of vitamin A deficiency in developing countries because they are generally available and easily sustainable and because they add additional, important nutrients to the diet (3). A study by de Pee et al. (3) raised doubts as to the efficacy of dark green leafy vegetables as a good vitamin A source. In this study, lactating women received either stir-fried vegetables (cassava leaves, water spinach, spinach, or carrots) containing 3.5 mg β-carotene along with 7.8 g fat, or a wafer enriched with 3.5 mg β-carotene, iron, vitamin C, and folic acid that contained 4.4 g fat everyday for 12 weeks. The enriched wafer increased serum retinol significantly (38% from baseline), whereas the stir-fried vegetables did not. Serum β-carotene concentration increased by 390% in the enriched wafer group and by 17% in the stir-fried vegetable group. According to de Pee et al., the increase in serum β-carotene concentration caused by vegetable intake was too small to have much impact on nutritional status.

Why was the bioavailability of β-carotene from dark green leafy vegetables so low? In green leaves, β-carotene is found in pigment-protein complexes located in chloroplasts, and it is difficult to free β-carotene from this complex matrix (3). In yellow and orange fruits on the other hand, β-carotene is dissolved in lipid droplets inside of chromoplasts, which may be more bioavailable (3). To investigate the difference in bioavailability of β-carotene from orange fruit and dark green leafy vegetables, and the effect of each food on vitamin A status,

de Pee et al. (44) supplemented Indonesian school children with either orange fruits (papaya, mango, and squash pumpkin) containing an average of 2.3 mg β-carotene per day or dark green leafy vegetables (cassava leaves, water spinach, and spinach) and carrots containing an average of 3.5 mg β-carotene per day. These foods were split into two meals and were given to the children six days a week for nine weeks. The increase in serum retinol was larger in the fruit group, but not significantly larger than the increase in serum retinol in the vegetable group. Taking into account the differences in the amount of β-carotene that the fruit and vegetables supplied resulted in an increase in serum β-carotene that was 5-6 times higher in the fruit group than in the vegetable group. Thus, de Pee et al. concluded that β-carotene is less available from dark green leafy vegetables than from orange fruits. In the vegetable group, the increase in serum carotenoid compared with the amount of carotenoid provided was higher for lutein than for β-carotene.

These studies by de Pee et al. challenged the then current system of assigning carotenoid- containing foods a retinol equivalency value which assumed that 6 μg dietary β-carotene is equivalent to 1 μg retinol equivalent (RE). According to de Pee et al. (44), the mean apparent effectiveness of fruit in improving vitamin A status in the Indonesian population studied was only 50%, and for dark green leafy vegetables and carrots only 23% of what had been assumed. de Pee et al. (74) further examined the relation between the effect of plant and animal foods on vitamin A status in a population of mothers with young children in Indonesia. Vitamin A intake was recorded with a semi-quantitative 24-hour food recall that distinguished between plant and animal foods, and blood samples were drawn to assess serum retinol status. Vegetables and fruits provided 89% of the subjects' dietary

vitamin A while animal foods provided only 11% of dietary vitamin A intake. They found that serum retinol concentration was related to vitamin A intake in a dose-response manner. Indonesian food composition tables based on RE of plant foods overestimated the vitamin A contribution of plant foods (3, 44, 72), but when the vitamin A activity of plant foods was corrected for the lower bioavailability of the provitamin A carotenoids they contain, the relation with serum retinol improved.

As a result of the findings of de Pee et al. (3,44) and others (69, 73), the retinol activity equivalency (RAE) system (46) was developed. RAE is the system currently used to estimate conversion of carotenoids to vitamin A. The RAE system assumes that 12 μg of dietary β-carotene must be ingested to yield 1 μg retinol in the body.

In one of the studies that provided a basis for the RAE system, Castenmiller et al. (73) examined the effects of multiple food matrix factors on the bioavailability of β-carotene and lutein from spinach. Human subjects were fed a control diet with supplemental carotenoids or whole leaf spinach, minced spinach (mechanical homogenization), enzymatically liquefied spinach (to remove the effect of dietary fiber), or liquefied spinach and added dietary fiber. Consumption of all types of spinach significantly increased serum concentrations of β-carotene, lutein, and α-carotene. The increase in serum β-carotene was significantly higher in both liquefied spinach groups compared with the whole leaf and minced spinach groups. Adding 10 g fiber to the liquefied spinach had no effect on carotenoid response. The various methods of spinach processing did not affect lutein bioavailability. The relative bioavailability of β-carotene from whole leaf, minced, liquefied, and liquefied spinach plus dietary fiber compared with that of the β-carotene supplement, was 5.1, 6.4, 9.5, and 9.3%,

respectively. Bioavailability of lutein from whole leaf, minced, liquefied, and liquefied spinach plus dietary fiber compared with that of the lutein supplement was 45, 52, 55, and 54%, respectively. Thus, the bioavailability of lutein from spinach was higher than that of β-carotene, and enzymatic disruption of the matrix increased the bioavailability of β-carotene, but had no effect on lutein bioavailability.

Persson et al. (74) carried out a supplementation trial comparing the effects of dark green leafy vegetables and sweet pumpkin on serum β-carotene and retinol concentration in children in Bangladesh. Children were fed six days a week for six weeks, one meal that contained either 4.4 mg β-carotene from dark green leafy vegetables, 1.5 mg β-carotene from sweet pumpkin, or vegetables containing virtually no β-carotene. The serum β-carotene concentrations in all three groups increased. The serum β-carotene increase compared with the carotenoid free vegetable control group was only significant in the dark green leafy vegetable group. The increase in serum retinol concentration (from baseline to endpoint) was statistically significant in the dark green leafy vegetable group, but the mean change in serum retinol in the dark green leafy vegetable group was not significantly different from the mean change in serum retinol in the other two groups. No serum retinol changes were significant for the sweet pumpkin group.

The results of this study contradict the previously mentioned study by de Pee et al. (44) which showed that β-carotene was more bioavailable from orange fruits than from dark green leafy vegetables; however, in the present study, the amount of sweet pumpkin fed to subjects only contained 34% of the β-carotene fed to subjects consuming dark green leafy vegetables. In the study by de Pee et al., the orange fruits served to subjects contained 66%

of the β-carotene in the dark green leafy vegetables served to subjects. Persson also noted that in the present study all subjects were dewormed, whereas approximately 80% of the children in the study by de Pee et al. had at least one parasitic infection that may interfere with carotenoid absorption.

In a human feeding study to evaluate β-carotene bioavailability from plant foods (75), 10 men consumed stir-fried shredded carrots, a stir-fried dark green leafy vegetable (water convolvuvus), deep-fried sweet potato ball, purified β-carotene, or purified β-carotene plus β-carotene-free oriental radish. All treatments were consumed with approximately the same amount of fat (55 g). They found that purified β-carotene raised serum concentrations of β-carotene significantly higher than did the other four meals. The changes in serum β-carotene for purified β-carotene plus radish were significantly higher than those for carrot, dark green leafy vegetables, and sweet potato. Increases in serum β-carotene for sweet potato were not significantly higher than those for dark green leafy vegetables. The investigators determined that the relative bioavailability of β-carotene from stir-fried and deep-fried vegetables was approximately one-third to one-fourth that of the purified β-carotene.

Food Processing

In a study by Porrini et al. (76) female subjects were given a single portion of either tomato puree (homogenized and heated) or fresh, raw tomato containing 16.5 mg total lycopene along with 10 g olive oil. In a second study (76) subjects were given one of the above tomato products daily for 7 days. In both studies, the tomato puree produced a significantly greater increase in total lycopene compared with raw tomato. A similar study by Gartner et al. (77) shows that lycopene is more bioavailable from tomato paste than from

fresh tomatoes. In this study, a single dose of fresh tomatoes or tomato paste (each contained 23 mg lycopene) was given to subjects along with 15 mg corn oil, and carotenoid concentrations in the chylomicron fraction were measured. Ingestion of tomato paste produced 2.5-fold higher total and all-*trans*-lycopene peak concentrations and a 3.8-fold higher AUC response compared with the ingestion of fresh tomatoes. For *cis* isomers, only the AUC response was significantly higher after ingestion of tomato paste. Heat processing of tomato products is thought to increase the content of *cis*-lycopene isomers. According to analysis of several types of commercially heat processed tomato products (76), less than 10% of the all-*trans*-lycopene isomer (raw tomatoes contain 95.4% all-*trans*) was isomerized to *cis* meaning that the all-*trans* isomer of lycopene is relatively resistant to isomerization induced by heat.

The hypothesis that homogenization and heat treatment improve carotenoid bioavailability was tested by Zhou et al. (79) who studied the bioavailability of β-carotene and α-carotene from three days of supplementation with β-carotene beadlets, heated and unheated carrot juice, and heated or unheated isolated carrot chromoplasts in ferrets. Following supplementation, β-carotene beadlet supplemented animals had a significantly higher β-carotene serum concentration than all the other groups. Carrot chromoplast-supplemented animals had significantly higher β-carotene and α-carotene serum concentrations than did carrot juice-supplemented animals. The carrot chromoplasts fed to the animals contained carotene bodies and the inner and outer membranes of the chromoplasts, whereas the carrot juice contained carrot chromoplasts plus the rest of the food matrix including carrot fiber. It can be concluded from these results that mechanical

breakdown of the food matrix (isolation of chromoplasts) increased β-carotene bioavailability. Heat treatment decreased the relative bioavailability of β-carotene and α-carotene, but the differences between heated and unheated juices or isolated carrot chromoplasts were not significant. Thus, there is less evidence for the effect of heat treatment improving bioavailability of carotenoids other than lycopene. Rock et al. (57) examined the plasma β-carotene response to raw versus processed carrots and spinach. In this study, subjects consumed approximately 9.3 mg β-carotene daily for four weeks from either raw or thermally processed and pureed vegetables. Total and all-*trans* (but not *cis*) plasma β-carotene concentrations were significantly greater than baseline concentrations following consumption of processed vegetables. Daily consumption of processed carrots and spinach increased plasma β-carotene concentrations three times more than consumption of the same amount of β-carotene from these vegetables in the raw form; however, this increase was not statistically significant ($P = 0.09$) in this study which included eight subjects. This study suggests that a combination of heat treatment and mechanical processing was effective in increasing bioavailability of β-carotene.

Carotenoid-carotenoid Interactions

White et al. (80) proposed that carotenoid-carotenoid interactions during intestinal absorption are limited to interactions of β-carotene (or possibly other hydrocarbon carotenes) and oxycarotenoids. The following three studies support this hypothesis. In a study done by our group (81), subjects were given β-carotene (a hydrocarbon carotenoid) alone, canthaxanthin (an oxycarotenoid) alone, and a combined dose. Ingestion of the combined dose significantly inhibited the appearance of canthaxanthin in plasma, chylomicrons, and

each of the four VLDL subfractions but did not significantly affect the rapid accumulation of canthaxanthin in LDL within ten hours of ingestion. Ingestion of the combined dose did not significantly affect the appearance of β-carotene in plasma or plasma lipoproteins. Another study which supports this hypothesis was performed by Kostic et al. (82). Kostic et al. gave subjects single equimolar doses (0.5 μmol/kg body weight) of lutein and β-carotene in true solution in oil and a combined dose of the two carotenoids. When the two carotenoids were consumed together, the mean serum AUC value for lutein was reduced to 54% of its AUC value when given alone ($P < 0.025$); the AUC value for β-carotene decreased in five subjects and increased in three subjects. van den Berg and van Vliet (83) measured the carotenoid response in the triacylglycerol-rich lipoprotein (TRL) fraction after giving subjects 15 mg β-carotene, and 15 mg β-carotene combined with 15 mg lycopene or lutein. Their results showed that lutein, but not lycopene negatively affected β-carotene absorption. Not all studies showed an interaction between hydrocarbon and oxycarotenoids. Clark et al. (84) infused equal concentrations of lycopene, canthaxanthin, and a combined dose of the two carotenoids into the duodenums of rats. The combined dose did not significantly affect the absorption of either carotenoid compared to its absorption alone.

Though the literature is inconsistent regarding carotenoid-carotenoid interactions, and mechanisms of these interactions are unclear, it is clear that some carotenoids do affect absorption of other carotenoids. Some possible mechanisms for carotenoid-carotenoid interactions include competition for incorporation into micelles, inhibition of β-carotene cleavage, and carotenoid exchange between lipoproteins in the postprandial state (85). Many meals and even individual foods contain more than one carotenoid; thus, it is of value to

further elucidate the interactions and interaction mechanisms of carotenoids in order to further improve the bioavailability of carotenoids.

Other Factors Affecting Carotenoid Bioavailability

There are several other factors not previously mentioned that affect carotenoid absorption. Using rats as an experimental model, Grolier et al. (86) determined that reduction of gut microflora resulted in increased utilization of α- and β-carotene by rats. In humans, increasing gastric acidity increases β-carotene absorption; however, below a pH of 4.5 there is a marked decrease in β-carotene incorporation into micelles (46).

HPLC with Electrochemical Detection for Carotenoid Analysis

Overview of Electrochemical Detection

Electrochemical detection (ECD) for high-performance liquid chromatography (HPLC) was first reported in the 1950's but initially received little attention (87). McClintock and Purdy (88) eloquently state that ECD was originally developed because "many compounds function *in vivo* as a result of electron transfer processes; it is therefore only a logical extension to assume that these same processes can be monitored by utilizing the electron transfer process, i.e. measurement by electrochemical means." Electrochemical detection has benefits over ultraviolet/visual light detection as will be detailed below.

HPLC is the method of choice for separating and quantifying carotenoids in a variety of samples. The current state-of-the-art method for detecting carotenoids utilizes a multi-wavelength ultraviolet/visible light (UV/VIS) detector or photodiode array detector (PDA). These detectors pass a specific wavelength of light through a cell and measure the absorbance in real time. Compounds that absorb light (chromophores) will increase the absorbance measured by the detector as they pass through the cell creating an absorbance

peak that has an area directly related to the concentration of the chromophores (89). Each compound has a specific wavelength at which it exhibits maximal absorption, thus the amount of light absorbed by any chromophore depends on the wavelength of light used (89). The absorbance of each chromophore is plotted against wavelength to produce a spectrum unique to each compound. Electrochemical detection (ECD) uses an analogous process (89). Instead of passing light through a cell, electrochemical detectors apply a voltage at an electrode surface over which the HPLC eluent flows. Electroactive compounds eluting from the column are oxidized or reduced, which generates a current peak in real time. The amount of current generated depends on both the concentration of the analyte and the voltage applied. Each electroactive compound has a specific voltage at which it begins to oxidize. Just as chromophores in UV/VIS detectors pass through the detector and absorb light creating a spectrum in a plot of absorbance versus wavelength, in electrochemical detectors, current (signal) is plotted as a function of the applied potential (voltage) to the working electrode. This produces a sigmoidal graph called a hydrodynamic voltammogram (HDV) unique to each compound. Faraday's law, $Q = nFN$, relates the current produced by each compound to the quantity of the compound being analyzed, where Q = charge transferred in coulombs; n = number of electrons transferred in equivalents/mole; F = Faraday's constant (coulombs/equivalent); and N = moles of reactant (mole) (90). The current peak produced is measured in charge (current over time). Therefore, if the amount injected into the HPLC is known, the peak area (charge) can be predicted, or if the peak area is known, the exact amount of compound can be determined accurately, if using coulometric electrodes.

There are two types of electrochemical detectors. Flat-plate amperometric detectors have a glassy carbon surface that oxidizes or reduces approximately 5% of the compounds

flowing over it (89). The second, more advanced type is a porous, flow-through amperometric detector that has a larger surface area allowing oxidation or reduction of close to 100% of the compounds flowing through it (89). When the efficiency of detection is 100%, this is referred to as coulometry, and these specialized amperometric detectors are termed "coulometric" (87). The terms amperometric and coulometric mean different things to different people; so a definition is required here. Amperometry is the measurement of current in a general sense; thus, all electrochemical detectors can be considered amperometric (90). When measuring signal from 100% of the analyte, the peak area represents total current as a function of time or charge, which is measured in coulombs (90). In this paper, electrodes that approach 100% oxidation of the electrochemically active compounds flowing through them will be considered "coulometric," and the measure of the charge produced will be called coulometry. Those electrodes that are less efficient will be referred to as "noncoulometric," and the measure of current flowing over them will be referred to as amperometry.

Oxidization of 100% of a compound increases sensitivity and selectivity as explained in the following example. Generally in ECD, a series of electrodes set at incrementally higher voltages are used to maximize the voltage range over which compounds can be detected. When using a series of non-coulometric electrodes set at incrementally higher voltages, each compound has a specific potential and thus an electrode at which it will oxidize. After initial oxidation, each compound will continue to be detected at all subsequent electrodes because only 5% of the analyte is oxidized at each point in the array (89). This greatly reduces the resolution of these compounds and the analytical sensitivity of the method. When using a series of coulometric electrodes set at incrementally higher voltages,

which oxidize close to 100% of the analyte passing through them, compounds will be detected at a certain electrode depending on their individual oxidation potentials and once fully oxidized, will not be detected by downstream electrodes with higher voltages (87). In addition, coulometric electrodes are more stable and require less maintenance than do non-coulometric electrodes (87). Coulometric electrodes are resistant to temperature changes and stray electromagnetic radiation (87). Additionally, the surface of a coulometric electrode has a much greater surface area than is required to react with normal concentrations of the analyte passing through them, such that up to 95% of the surface can be contaminated with no loss of response (87). Non-coulometric electrodes, on the other hand, are sensitive to temperature changes and stray electromagnetic radiation and require frequent cleaning followed by long stabilization periods (87).

In the 1980's, Matson et al. (91) developed the voltammetric equivalent of the photodiode array (PDA) detector in which a series of 17 coulometric sensors resolve compounds based on their redox potential. Currently, this is the type of electrochemical detector most commonly used.

Advantages of HPLC–ECD Compared with HPLC–UV/VIS

There are several advantages of HPLC with ECD compared with UV/VIS detection. The most important advantages are improvements in sensitivity and resolution. These and other advantages are explained in detail below.

Unlike a UV/VIS detector, coulometric electrochemical detectors are able to distinguish between compounds that have the same retention time but different oxidation potentials (89). Coulometric electrochemical detectors are able to resolve chromatographically co-eluting compounds if their half-wave potentials (potential at half

signal maximum) differ by at least 60 mV or more (87). Electrochemical array detectors can confirm compound purity by the following method (89). Compounds are detected on three contiguous electrodes. The first electrode will oxidize a small portion of the compound; the second or dominant electrode oxidizes a large portion of the compound, and the third electrode oxidizes the remainder of the compound. Pure standards will give a predictable response at all three electrodes. This enables the calculation of a ratio of oxidation at the dominant electrode versus the other two electrodes. This ratio remains constant independent of concentration. When assaying samples, the ratio of the sample can be compared to the ratio of the standard. If the ratios match, this confirms the identity of the sample. A difference in the ratio indicates either that this is not the same compound as the standard or that there is another compound co-eluting with the sample compound of interest.

This method of measuring purity, which requires oxidation at three consecutive electrodes, is confusing because as previously mentioned, the advantage of coulometric electrodes is their capability to oxide 100% of a specific compound at one electrode closest to that compound's oxidation potential thus allowing resolution of compounds with the same retention time. So why would it be preferable to set the voltages to allow each compound to be oxidized at three electrodes instead of one? If the major objective is to maximize the signal from a particular compound, then the oxidation potential of a specific coulometric electrode should match the oxidation potential maximum of that compound (90). However, this gain in sensitivity results in a loss of resolution (90). To maximize qualitative data, incrementally increasing potentials should be applied over the range of the analyte's oxidation potential, allowing the signal at consecutive electrodes to be used to determine purity (90).

The wide range of voltages in a coulometric array increases sensitivity (89). The increased sensitivity of ECD allows for the analysis of smaller samples with accurate results (92). Typical lower limits of detection for electrochemical detection are in the subfemtomole ($<10^{-15}$) range (five orders of magnitude more sensitive than the best UV/VIS detector) (87). Other researchers estimated the sensitivity of electrochemical detection to be 100 to 1000 times more sensitive than UV/VIS detection based on the detection limits for β-carotene (93). Some compounds will only oxidize at high voltages, but working at high oxidation potentials results in high background currents and decreased sensitivity. Electrochemical detection with several coulometric electrodes of varying voltage connected in series solves this problem by allowing the lower voltage electrodes to buffer the higher voltage electrodes resulting in reduced background current with increased sensitivity. As mentioned previously, compounds will oxidize at the electrode or electrodes closest to their oxidation potential, and then their signal is effectively removed from the system. Additionally, this selectivity allows electrochemical detectors to detect trace levels of analytes against a background that includes numerous other compounds (94). Many samples such as plasma and urine require extensive extraction and cleaning procedures in order to remove interfering compounds before analyzing the sample with HPLC. Though it is always important to remove protein and other contaminants, the added resolving power of electrochemical array detectors can often separate interfering compounds from the compound of interest (89). Thus, it may be possible to simplify sample preparation procedures.

Use of HPLC-ECD for Carotenoid and Retinoid Analysis

There has been limited application of electrochemical detection for carotenoid or retinoid analysis. A brief review of the literature regarding carotenoid analysis with HPLC-

ECD follows. Motchnik et al. (95) measured lycopene and β-carotene in human plasma using an ECD system consisting of two noncoulometric electrodes. They were not able to completely resolve lycopene and β-carotene from other electrochemically active compounds in plasma using this method. This is consistent with the problems with noncoulometric detectors detailed above. MacCrehan and Schonberger (96) were more successful in resolving carotenoids using noncoulometric electrochemical detection. They determined the retinol, α-tocopherol and β-carotene content of serum using liquid chromatography with UV/VIS detection and with electrochemical detection. The one amperometric electrode of the detector was set at a potential between the oxidation potentials of the three compounds, resulting in a loss of sensitivity. The UV/VIS detector had the capability to measure absorbance over multiple wavelengths. The detection limits for the electrochemical detector were superior to UV/VIS for all analytes. The selectivity of the UV/VIS absorbance detector was slightly better than the ECD for retinal and β-carotene. Serum components such as retinal palmitate were detected electrochemically, but not by UV/VIS. The responses of retinal palmitate and β-carotene interfered with each other in electrochemical detection because the one electrode was set at a potential at which both compounds oxidize. This interference could be prevented by using a lower potential setting or having several electrodes in series, each at increasing potentials (similar to the principle used by the multiwave length UV/VIS detector).

Using an isocratic HPLC method, Sakhi et al. (97) utilized a coulometric electrochemical detector with two channels in series to quantify retinoids in mouse embryos. The limits of detection were approximately 10 picograms (pg) for the retinoic acids (9-*cis*-retinoic acid, 13-*cis*-retinoic acid, all *trans*-retinoic acid) and 25 pg for all *trans*-retinol. The

intra-assay precision (relative standard deviation) was better than 4% (n = 6) for the four retinoids, and inter-assay precision was in the range of 3-4% (n = 10). Recoveries were in the range of 86-103%. Using a combination of electrochemical detection and column switching resulted in lowered detection limits compared with UV/VIS detection. Only 10 mg of embryonic sample was necessary using this method, whereas 350 mg is required for UV/VIS detection. Finckh et al. (92) measured β-carotene and β-cryptoxanthin in 5 or 10 μl of neonatal plasma by coulometric electrochemical detection with two electrodes in series. Detection limits for the two carotenoids were between 21 and 60 femtomole (fmol), corresponding to 0.004-0.012 μmol/L plasma. The with-in day precision (coefficient of variation) was between 3 and 14%. Ferruzzi et al. (93) developed methods to measure carotenoids in vegetables, and in microsamples of human serum and tissues using coulometric ECD with 8 channels in series. The detection limit for β-carotene was 10 fmol, representing approximately a 100 to 1000-fold increase over conventional HPLC-UV/VIS techniques. Bocchi et al. (98) compared UV/VIS, coulometric ECD and particle beam mass spectrometric detection methods for HPLC determination of phenolic acids. The electrochemical detector used was comprised of two serially connected cells. Electrochemical detection had detection limits ranging from 1-5 pg injected. Coulometric detection provided advantages in comparison with either UV/VIS or mass spectrometric detection. Eight phenolic acids were detected and quantified by ECD in the low nanogram (ng) range. UV detection of the phenolic acids was not possible because of interference from numerous peaks. The particle beam mass spectrometric HPLC technique was only able to determine four of eight phenolic compounds.

In conclusion, researchers have confirmed the enhanced sensitivity of coulometric array electrochemical detection compared with UV/VIS detection. ECD has allowed for analysis with smaller sample sizes and has proved to be a reliable method that gives reproducible results.

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USE OF ELECTROCHEMICAL DETECTION TO QUANTIFY THE EFFECT OF ADDED FAT ON INTESTINAL CAROTENOID ABSORPTION FROM FRESH VEGETABLES IN HUMANS

A paper to be submitted to the American Journal of Clinical Nutrition

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Abstract

Background: Previously, HPLC with coulometric array electrochemical detection (ECD) showed enhanced sensitivity compared with HPLC with ultraviolet visible light detection (UV/VIS) in carotenoid analysis.

Objective: The objective of this study was to apply HPLC-ECD to compare the appearance of carotenoids in plasma chylomicrons after subjects ingested vegetable salads with fat-free, reduced-fat, and regular fat salad dressings.

Design: Healthy men and women (n = 7) consumed a single salad containing fresh spinach, romaine lettuce, carrots, and cherry tomatoes. The salads with three salad dressings containing 0, 6, and 28 g fat were ingested in random order and separated by a washout period of at least two weeks. Blood samples were drawn immediately before ingestion of the salad and at hourly intervals for the following 12 hours. The chylomicron fraction was isolated by ultracentrifugation.

Results: After ingestion of the salad with the fat-free dressing, appearance of carotenoids in the chylomicron fraction was negligible. After ingestion of the salad with reduced-fat as

compared with fat-free dressing, the area under the curve (AUC) increased 20.9 nmol/L (P = 0.010), 97.5 nmol/L (P = 0.012), and 3.6 nmol/L (P = 0.016), and with regular fat as compared with reduced-fat dressing, the AUC increased 2.5-fold (P = 0.025), 2.0-fold (P = 0.033), and 4.0-fold (P = 0.031), for all *trans*-α-carotene, all *trans*-β-carotene, and all *trans*-lycopene, respectively.

Conclusions: The use of HPLC-ECD is a sensitive and efficient approach to measure the postprandial carotenoid response in chylomicrons. Carotenoid absorption was negligible when a vegetable salad was consumed with fat-free salad dressing; carotenoid absorption was higher with regular than with reduced-fat salad dressing.

Introduction

Health-conscious U.S. consumers are buying a myriad of fat-free products (1), including fat-free salad dressings (2). Particularly if consumed in the absence of other sources of dietary fat, fat-free salad dressing may prevent the absorption of carotenoids from salad vegetables, which are fat-soluble and have putative health protective effects. Consumption of fruits and vegetables containing carotenoids has been correlated with decreased risks of cancer (3, 4) and cardiovascular disease (5, 6). Consumption of β-carotene supplements in the absence of fat results in no detectable increase in plasma β-carotene (7). The bioavailability of carotenoids from fresh vegetables is much lower than that of purified carotenoid supplements (8, 9). Ingestion of daily supplements of dark green leafy vegetables and carrots resulted in small improvements in serum β-carotene and retinol concentrations compared with those resulting from ingestion of purified carotenoids or yellow fruits and tubers (8, 10-13).

It is critical to determine dietary factors that modulate carotenoid absorption in order to maximize absorption. Carotenoid absorption can be divided into four steps: release of carotenoids from the food matrix, formation of lipid-mixed micelles, uptake of carotenoids into intestinal mucosal cells, and packaging of carotenoids into lipoproteins for transport in the general circulation (14). Fat is required in each of the latter three steps (14). Consumption of fat together with carotenoid-rich vegetables increases serum carotenoid and/or retinol concentrations (15-17). Previous studies suggest that the amount of fat required per meal for optimal carotenoid absorption is relatively small (3-5 g) (15, 17-18).

Few carotenoid absorption studies involve ingestion of a single serving of fresh vegetables; most are carried out with weeks of daily dietary supplementation with cooked vegetables. Gärtner et al (19) fed subjects a single serving of 400 g fresh tomatoes and 40 g tomato paste (both containing 23 mg lycopene) with 15 g corn oil. When chylomicron lycopene content was analyzed by high performance liquid chromatography (HPLC) with conventional ultraviolet/visible light (UV/VIS) detection, lycopene from both fresh and processed tomatoes was detectable. The tomato paste yielded 2.5-fold higher total lycopene concentrations compared with fresh tomatoes. The amount of fresh tomatoes fed to subjects in this experiment was larger than would normally be considered a single serving. van den Berg and van Vliet (20) also gave subjects a large single serving of individual carotenoid-containing vegetables (395 g carrots, 255 g spinach, 80 g tomato paste). When analyzed by HPLC-UV/VIS, carotenoid appearance in the triacylglycerol-rich lipoprotein fraction (TRL) could not be reliably quantified, except for lycopene from tomato paste, which showed a measurable increase. A more sensitive analytical method is needed to quantify the small amounts of carotenoids absorbed in the intestine from a realistic single serving of vegetables.

Ferruzzi et al. (21) developed protocols to measure carotenoids in microsamples of human tissue and plasma by using HPLC with coulometric array electrochemical detection (ECD). They found electrochemical detection to be 100 to 1000 times more sensitive than conventional UV-VIS detection (21).

The objective of this study was to compare carotenoid absorption from a single mixed fresh vegetable salad when ingested with salad dressing containing 0, 6 and 28 g fat. The salad was intended to simulate a typical dinner salad eaten in the United States with a representative amount of fat-free, reduced-fat, or regular fat salad dressing. HPLC-ECD was applied to quantify carotenoids in the chylomicron fraction of subjects who consumed a single salad of fresh vegetables.

Materials and Methods

Subjects

Ten healthy, nonsmoking, normolipidemic men and women 19-28 years of age were enrolled in the study; of these, two were found to have veins incompatible with phlebotomy. A third subject was found to have high triacylglycerol and carotenoid content in the plasma chylomicron fraction collected at baseline after a 12-hour (overnight) fast. We suspected noncompliance and excluded data from this subject in our analyses.

Subjects were screened first by interview using a standardized questionnaire that addressed diet, health, lifestyle, and anthropometric data (height and weight). Those who met initial criteria for inclusion (see list of exclusion criteria below) underwent a complete blood count, blood chemistry profile, physical examination, and for female subjects, a pregnancy test. Criteria for exclusion included lactose intolerance, psychological aversion to phlebotomy, history of eating disorders, vegetarian diet, unusual food habits (such as severe

reducing diets, avoidance of any one food group), history of chronic disease, lipid malabsorption or intestinal disorders, light sensitivity disorder, hypo- or hyper-thyroidism indicated by measured serum thyroxine (T_4) and thyroid stimulating hormone (TSH), history of anemia or excessive bleeding, high triacylglycerol or cholesterol concentrations indicated by plasma lipid and lipoprotein profile, current use of medications that may affect lipid absorption or transport (including antibiotics), menstrual cycle irregularities, current or planned pregnancy, use of oral contraceptives or contraceptive implants, current or recent (last six months) use of vitamin or mineral supplements, current or recent (last 12 months) cigarette smoking, frequent consumption of alcoholic beverages (> 1 drink/day), recent significant change in weight (> 4.5 kg in a month). Informed consent was obtained from all subjects, and the study protocol was approved by the Human Subjects Research Review Committee of Iowa State University.

Experimental Diet

Subjects were given a list of carotenoid-rich foods and instructed to avoid those foods for the four days preceding each experimental period. During the experimental periods, subjects consumed a controlled, low-carotenoid diet of conventional foods on the day preceding the test meal. The macronutrient content of the diet was analyzed by Nutritionist V software (N-Squared Computing Inc., Salem, OR). For male subjects, the diet provided 12.02 MJ, 107 g protein, 90 g fat, and 417 g carbohydrate. For female subjects, the diet provided 9.29 MJ, 84 g protein, 66 g fat, and 329 g carbohydrate. Following consumption of the test salad in the morning of the second day, two additional, low-fat meals were provided. These meals were the same for male and female subjects. The low-fat lunch provided 2.22 MJ, 30.0 g protein, 5.5 g fat, and 88.0 g carbohydrate. The supper provided 2.11 MJ, 37.5 g

protein, 5.7 g fat, and 75.5 g carbohydrate. All foods were weighed, prepared, and consumed in the Human Metabolic Unit of the Center for Designing Foods to Improve Nutrition, Iowa State University. The only exceptions were the lunch, and afternoon and evening snacks, which were carried out on the day preceding ingestion of the test salad. Duplicate aliquots of composites of the diet were analyzed by HPLC for carotenoid content according to the protocol used by White et al. (22). The low carotenoid diet on the day preceding the test meal provided no detectable α-carotene, or lycopene; the diet for the male subjects contained 15 μg β-carotene, 290 μg lutein, and 58 μg zeaxanthin whereas the diet for the female subjects contained 15 μg β-carotene, 217 μg lutein, and 50 μg zeaxanthin. Together, the two low-fat meals given to both male and female subjects on the day of test salad consumption contained a total of 28 μg β-carotene, 83 μg lutein, 34 μg zeaxanthin and no detectable α-carotene or lycopene.

Test Meals

The test salad consisted of 48 g fresh spinach (Popeye Flat Leaf Spinach, River Ranch Fresh Foods, Salinas, CA), 48 g romaine lettuce (Romano Prepackaged Salad Mix, Dole Fresh Vegetables, Salinas, CA), 66 g raw, shredded carrots (Dole Prepackaged Shredded Carrots, Salinas, CA), and 85 g of raw cherry tomato (Capitol City Fruit Co., Norwalk, IA) (≈ 6.5 cherry tomatoes). The combined weight (247 g) was chosen as the 90th percentile of the quantity of salad (lettuce and other vegetables) eaten by adults as part of a meal in the 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII) (23). The Romano prepackaged salad mix was sorted to exclude the radicchio and to include only romaine lettuce; then, the romaine lettuce and spinach leaves were sorted to ensure that their

green color, and therefore their carotenoid content, was consistent across study periods (24). The salad dressing was prepared from a commercial salad dressing mix containing dry ingredients (Good Seasons Italian Dressing Mix, Kraft Foods, Glenview, IL). To make the three salad dressings, 59 g white vinegar (HJ Heinz Co, Pittsburgh, PA) and 44 g water were added to the dry mix along with canola oil (Hunt-Wesson, Inc., Fullerton, CA), and/or additional water substituted on a weight/weight basis to create regular fat (112 g canola oil), reduced-fat (24 g canola oil, 88 g water), and fat-free (112 g water) salad dressings. Prepared salad dressings were analyzed in duplicate by Covance Laboratories Inc. (Madison, WI) for fat content using total fat acid hydrolysis (25). The fat-free, reduced-fat, and regular fat dressings contained 0, 6, and 28 g fat, respectively. The serving size and corresponding fat contents of the salad dressings were chosen to approximate the 90th percentile of the quantity of low calorie and full fat salad dressings reported by adults eating salad components as part of a meal in the 1994-1996 CSFII (23). The intent was to simulate the typical intake of salad dressing for a person eating a salad as all or most of a meal.

The salad was served with the dressing on the side in a small dish. Subjects were given a spatula and instructed to scrape the dressing out of the dish and onto the salad. Additionally, subjects were instructed to wipe the dish clean with a lettuce leaf to insure that all of the dressing was ingested. The salad was served with 500 mL water.

Experimental Design

Each subject ingested three test salads identical in vegetable composition, but with 60 g of Italian salad dressing containing either 0, 6, or 28 g of canola oil, which will be referred to as the fat-free, reduced-fat, and regular fat salad dressings, respectively. The salads were ingested in random order separated by washout periods of two or more weeks.

Blood samples were collected via a catheter with a disposable obturator placed in a forearm vein by a registered nurse. A 12-mL blood sample was drawn at baseline after an overnight fast and immediately preceding the ingestion of the test salad. Additional 12-mL blood samples were collected at hourly intervals for 12 hours after ingestion of the test salad. Blood collection tubes, which contained EDTA as an anticoagulant, were placed on ice immediately after blood collection, protected from light, and centrifuged (1380 × g, 4° C, 30 minutes) to separate plasma. Between blood draws, 3-mL of sterile physiological saline (0.9% sodium chloride) were injected into the catheter via the obturator to prevent clotting. Subjects were fed a low-carotenoid, low-fat (5.5 g fat) lunch after the fifth hourly blood draw and a low-carotenoid, low-fat (5.7 g fat) supper after the tenth blood draw.

Chylomicron Isolation

Chylomicrons were used as a vehicle to distinguish newly absorbed from endogenous carotenoids. Cumulative rate ultracentrifugation (26-28) was used to isolate the chylomicron fraction. Cumulative rate ultracentrifugation affords clean separation of chylomicrons from VLDL (27). All procedures were carried out under yellow light. Solutions were prepared (1.006 g/mL, 1.065 g/mL, 1.020 g/mL) using sodium chloride, potassium bromide, and sodium ethylenediametetraacetic acid (EDTA) in order to form a density gradient. The densities were confirmed using a digital density meter (Anton-Paar USA, DMA-48, Ashland, VA). Plasma was transferred to a centrifuge tube (Ultra Clear, Beckman Instruments, Inc., Spinco Division, Palo Alto, CA), and the density was adjusted to 1.10 g/mL by adding potassium bromide (23). 4-(Chloromercuri)benzenesulfonic acid was added as a preservative. The plasma was overlayed with the prepared density solutions in order of most to least dense. Plasma samples were centrifuged in an ultracentrifuge (Beckman Instruments

Inc., L8-70M, Palo Alto, CA) using a swinging bucket rotor (SW 40i) at 28.3K for 43 minutes. The chylomicron fraction was removed from the centrifuge tube and stored at –80° C until analyzed.

Extraction of Carotenoids from Chylomicron Fraction with HPLC-ECD

Carotenoids were extracted from a 2.0-mL aliquot of the plasma chylomicron fraction by deproteination with ethanol and three extractions with hexane containing 1.0 g butylated hydroxytoluene (BHT)/L. The combined hexane layers were dried under nitrogen, redissolved in 50:50 (by vol.) methanol:methyl-tert-butyl-ether (MTBE), and analyzed immediately. The carotenoids were separated by HPLC using a C_{30} Carotenoid Column (Waters, Milford, MA). The components included a Hewlett Packard (Wilmington, DE) model 1050 pump, autosampler, and solvent prep station. The pump was a quartinary gradient-capable system, and solvents were degassed by helium sparge. A gradient elution system that consisted of different proportions of ethanol:water:MTBE:1.0 M ammonium acetate buffer (pH 4.5) in reservoirs A (88:5:5:2) and B (25:0:73:2) was used. Initial conditions were 100% solvent A followed by a linear gradient to 15% solvent A, 85% solvent B from 0 to 40 minutes. Flow rate was 1 mL/min. An ESA Model 5600 Coularray electrochemical detector (Chelmsford, MA) with eight channels in series was used for the analysis (21). The potential of the eight channels were set from 200 to 680 mV in 60-mV increments with major carotenoids responding dominantly between 380 and 500 mV. The ESA Coularray software and data management system were used to collect and integrate the chromatographic data.

Extraction and HPLC Analysis of Carotenoids from Salad Vegetables

Representative samples of spinach, romaine lettuce, carrots, and cherry tomatoes from each of the three study periods were stored at –70°C and analyzed separately in duplicate according to a modification of the method of Hart and Scott (29). Vegetable tissue was processed in a food processor (Black & Decker Corp., Handy Chopper Plus HC3000, Towson, MD). A 10-g sample of processed vegetable tissue was combined with 4 g celite, 1 g solid $CaCO_3$ to neutralize organic acids, and 50-mL methanol and tetrahydrofuran (1:1 by vol) containing 0.1 g BHT/L and ethyl β-apo-8'-carotenoate (Fluka, Milwaukee, WI) as an internal standard. Carotenoids were extracted from the plant tissue by homogenizing for 1 min using a Brinkman (Westbury, NY) homogenizer. The resulting suspension was filtered through No. 1 and 42 Whatman filter papers in a Buchner funnel under vacuum (30). The filter cake was resuspended with 50-mL methanol and tetrahydrofuran (1:1 by vol), homogenized for 1 min and filtered through the same filter papers. The extraction of the filter cake with methanol and tetrahydrofuran was then repeated a third time. The combined methanol and tetrahydrofuran filtrates were transferred to a separatory funnel. The carotenoids were extracted by adding 50-mL petroleum ether (boiling range 41.5 – 56.5 °C, containing 0.1 g BHT/L) and 50-mL of NaCl solution (100 g/L) followed by careful shaking (29). The lower aqueous/methanol/THF phase was collected and the upper petroleum ether phase was transferred to a 200-mL volumetric flask. The aqueous/methanol/THF phase was extracted two more times with 50-mL aliquots of petroleum ether. The petroleum ether layers were transferred to the same 200-mL volumetric flask, which was then brought to volume with additional petroleum ether. For cherry tomatoes, a 4-mL aliquot was evaporated to dryness under vacuum; the dried lipid extract was reconstituted in 1-mL of

methyl-*tert*-butyl ether (MTBE) followed by 1-mL of methanol. For the other three vegetables, a 10-mL aliquot was evaporated to dryness under vacuum, and the dried lipid extract was reconstituted with 400 µL of methyl-*tert*-butyl ether (MTBE) and 1.6-mL of methanol. The reconstituted extract was filtered through a 0.2 µm nylon syringe filter (Alltech, Deerfield, IL), and a 25-µL aliquot was injected into the HPLC system. The components included a 717 Plus autosampler with temperature control set at 5°C, two 510 solvent-delivery systems, and the 996 photodiode array detector (Waters Corporation, Milford, MA). The system operated with Millennium^{32} Software Version 3.05.01 (Waters Corporation). Carotenoids were separated on a 5-µm C_{30} Carotenoid Column (4.6 × 250 mm; Waters Corporation) eluted by using a linear mobile-phase gradient from 100% methanol (containing 1 g ammonium acetate/L) to 100% MTBE over 55 min. The flow rate was 2.0 mL/min. Solvents were HPLC grade; the methanol, MTBE, and ammonium acetate were purchased from Fisher Scientific (Chicago, IL). The petroleum ether and tetrahydrofuran were purchased from VWR (Boston, MA). Internal standard calibration curves were generated for β-carotene (Fluka), lutein (Kemin Foods, Des Moines, IA), zeaxanthin (Indofine Chemicals, Belle Mead, NJ), lycopene (Sigma, St. Louis, MO), and α-carotene (Carolina Chemical Purities, Cary, NC).

Analysis of the Triacylglycerol Content of the Chylomicron Fraction

Sigma Diagnostics procedure number 339 was modified and carried out as follows: The GPO-Trinder reagent (Sigma Diagnostics, St. Louis, MO) was reconstituted with 25% of the amount of water specified in the instructions. A calibration curve consisting of increasing amounts of commercial glycerol standard (Sigma Diagnostics) was used to

quantify triacylglycerol in the samples. A 63-μL aliquot of chylomicron sample, glycerol standard (for calibration curve) or water (for reagent blank) was added to individual wells of 96-well microplates (Corning Inc., Corning, NY) followed by the addition of 192 μL of under-reconstituted reagent to each well with a multi-channel pipettor. After a 45 min incubation at room temperature, samples were analyzed using a microplate reader (El_x 808, Bio-Tek Instruments, Inc., Winooski, VT) which shook the microplate to mix the contents and then measured the absorbance of the color change reaction at 562 nm. The system operated with KC junior software version 1.14, 1998 (Bio-Tek Instruments, Inc.). Each chylomicron sample was run twice, and the results were averaged.

Statistical Analyses

Baseline-adjusted areas under the curve (AUC) were calculated by trapezoidal approximation for each carotenoid after ingestion of the test salad (31). In the case of missing data for one treatment, the corresponding data point was dropped from the other treatments for the purposes of comparison. Though blood samples were taken for 12 hours following ingestion of the test salad, carotenoid responses returned to baseline by nine hours so only data for hours 0-9 were included in the AUC analyses. AUC values were analyzed by one-tailed paired *t*-test. A P-value of less than 0.05 was considered significant.

Results

Subject Characteristics

The average age of the three male and four female subjects was 22 ± 1.1 years with a range of 19-28 years. The subjects' average body mass index (kg/m^2) was 23.4 ± 1.5, with a range of 19.1-29.0. All subjects were healthy based on the strict exclusionary criteria listed above, the physical examination, and the blood biochemical profile.

Test Salad Carotenoid Content

The carotenoid composition of the test salads is shown in Table 1. The total carotenoid content of the test salad was 31.319 mg, with α-carotene contributing only 4.444 mg of carotenoid to this total. Carotenoid contents of the vegetables were similar to the reported values in the USDA-NCC Carotenoid Database for U.S. Foods-1998 (32), except for lycopene values from cherry tomatoes, which were higher compared with the database, which contains data for tomatoes, but not cherry tomatoes.

Fresh vegetables are infrequently used in studies of carotenoid absorption because the carotenoid content of fresh vegetables can fluctuate widely. In this study, measures were taken to ensure a consistent carotenoid content of the test salads. Romaine lettuce and spinach were sorted to ensure that the color of the leaves, and thus the carotenoid content, was uniform. Zeaxanthin (found in the romaine lettuce and spinach) was the most variable carotenoid. The content of zeaxanthin in photosynthetic tissues is dependent on their exposure to light during growth (33).

Change in Chylomicron Carotenoid Content after Ingestion of the Test Salad

All *trans*-α-carotene, all *trans*-β-carotene, and all *trans*-lycopene were extracted from the chylomicron fraction and quantified (Figure 1). Several *cis* isomers of lycopene were detected, but not quantified. A high tocopherol content in the chylomicron samples resulting from the ingestion of canola oil in the salad dressing precluded measurement of lutein and zeaxanthin as these co-eluted with tocopherol.

The areas under the curve for all *trans*-α-carotene, all *trans*-β-carotene, and all *trans*-lycopene were negligible after ingestion of the test salad with fat-free dressing (Table 2). After ingestion of the test salad with reduced-fat salad dressing compared with fat-free salad

dressing, AUC values increased for all *trans*-α-carotene (P = 0.01), all *trans*-β-carotene (P = 0.012), and all *trans*-lycopene (P = 0.016) (Figure 2). After consumption of the test salad with regular dressing compared with reduced-fat dressing, AUC values increased 2.5-fold for α-carotene (P = 0.025), 2.0-fold for all *trans*-β-carotene (P = 0.033), and 4.0-fold for all *trans*-lycopene (P = 0.031). The added fat in the reduced-fat and regular fat salad dressings significantly increased absorption of carotenoids, and the increase in carotenoid absorption found with the regular fat dressing was significantly higher than the increase with reduced-fat dressing.

Absorption of lycopene was proportionally lower than that of the other carotenoids in the salad based on the high lycopene content of the cherry tomatoes in the salad. Other researchers have found lower bioavailability of lycopene from fresh tomatoes compared with processed tomatoes (34). To our knowledge, this is the first study to address lycopene absorption from cherry tomatoes.

Change in Chylomicron Triacylglycerol Content After Ingestion of the Test Salad

The appearance of triacylglycerol in chylomicrons peaked at 3 hours following consumption of the test salad with regular fat dressing with a smaller peak at 6 hours (Figure 2). The carotenoids peaked at 4 hours with a second, smaller peak at 6 hours after ingestion of the test salad with regular fat salad dressing. A low-fat lunch (5.5 g fat) was given after the 5-hour blood draw. Thus, the second peak of triacylgylcerol at six hours may correspond to the chylomicrons released from the intestine during the subsequent postprandial period. Borel et al. (35) supplemented subjects with 120 g β-carotene in a meal containing 40 g fat. Chylomicron triacylglycerol content peaked 3 hours after ingestion of the test meal with a second peak at 7 hours following consumption of a second meal at hour 6, which contained

40 fat, but no β-carotene. Additionally, Borel et al. reported a more rapid rate of appearance of triacylglycerol in chylomicrons compared with β-carotene. In the present study, we also found the rate of appearance of triacylglycerol in chylomicrons to be more rapid than the rate of appearance of each of the carotenoids quantified.

After consumption of the test salad with reduced-fat salad dressing, chylomicron triacylglycerol peaked at 3 hours. All *trans*-α-carotene and all *trans*-β-carotene peaked at 3-4 hours with a second, smaller peak at 6 hours, whereas lycopene peaked at 3-4 hours with no second peak after ingestion of the test salad with reduced-fat dressing. There was no peak in chylomicron triacylglycerol content following ingestion of the test salad with fat-free dressing, similar to the response of the carotenoids. The mean AUC values for triacylglycerol content in chylomicrons were 359.46 ± 59.01, 67.67 ± 29.40, and –0.51 ± 8.79 μmol/L for the regular fat, reduced-fat, and fat-free treatments, respectively. The mean AUC for triacylglycerol was increased by 68.18 μmol·h/L following ingestion of the test salad with reduced-fat dressing compared with fat-free dressing ($P = 0.018$). There was a 5.3-fold increase in the mean AUC for triacylglycerol following ingestion of the test salad with regular fat dressing compared with reduced-fat dressing ($P = 0.001$). In summary, the chylomicron triacylglycerol contents were correlated positively with amounts of dietary fat similar to the stepwise increase in postprandial chylomicron triacylglycerol observed by Dubois et al. (36) when subjects were given meals containing 0, 15, 20, 40, and 50 g fat.

Discussion

By using HPLC with coulometric array electrochemical detection, we were able to reliably measure the intestinal absorption of carotenoids as reflected in the carotenoid content

of chylomicrons after the ingestion of a single fresh vegetable salad. Most studies of β-carotene absorption and bioavailability use the increment in plasma β-carotene after chronic daily ingestion to measure β-carotene absorbed from vegetables (8, 9, 10, 13). There is a need for a rapid screening approach to accurately and acutely quantify the postprandial appearance of carotenoids in the triacylglycerol-rich lipoprotein (TRL) fraction following a single serving of vegetables. More sensitive detection would enable acute absorption studies to replace more time consuming and expensive chronic feeding studies. Other investigators were not able to reliably detect carotenoids after ingestion of a single serving of fresh vegetables in total plasma (37) or in the TRL fraction (20) using HPLC with UV/VIS detection. Typical lower limits of detection for electrochemical detection are in the subfemtomole ($<10^{-15}$) range, which is five orders of magnitude more sensitive than the best UV detector (38). We showed that coulometric array electrochemical detection is a sensitive method that enables detection of the small amounts of carotenoids absorbed from a single serving of fresh vegetables.

We measured β-carotene in the chylomicron fraction because circulating β-carotene in plasma has a half-life of ≈ 5 days (39), whereas chylomicrons contain only recently absorbed β-carotene and its cleavage products. van Vliet et al. (40) used the TRL fraction (includes chylomicrons, chylomicron remnants, and VLDL) as a vehicle to study absorption of a 15 mg β-carotene supplement. Inter-individual variation in the β-carotene response was smaller in the TRL fraction than in total plasma. van het Hof et al. (41) found the postprandial lycopene content of the TRL fraction to be a more sensitive measure of lycopene absorption from processed tomatoes than the total plasma content of lycopene. We

chose to use only the chylomicron fraction to avoid contamination with hepatic VLDL, which contains endogenous carotenoids. Previous studies from our group (28, 42) have found early postprandial carotenoid increments in the plasma VLDL fraction. The combination of carotenoid measurement in the chylomicron fraction and analysis with HPLC using electrochemical detection presents a powerful new approach for carotenoid analysis.

In the present study, following ingestion of the test salad with no fat, the carotenoid response in chylomicrons was negligible, but following ingestion with 6 g fat the increase in all *trans*-α-carotene, all *trans*-β-carotene, and all *trans*-lycopene was significantly higher. The response of each carotenoid was significantly higher following ingestion of the test salad with 28 g fat compared with 6 g fat. Relative bioavailability refers to the serum response following the ingestion of a given amount of carotenoid in a food form compared with the serum response following the ingestion of the same amount of purified carotenoid (43). The relative bioavailability of carotenoids from vegetables is low (8, 44). van het Hof et al. (8) reported that the relative bioavailability of β-carotene and lutein from mixed vegetables was only 15% and 67%. Castenmiller et al. (44) found the relative bioavailability of β-carotene and lutein from whole leaf spinach to be 5.1% and 45%, respectively.

Data on the amount of fat necessary for optimal intestinal carotenoid absorption is limited. Previous investigators suggested that the amount of fat required for optimal absorption of carotenoids is low (15, 17-18). Roodenburg et al. (18) found that increases in plasma concentrations of α- or β-carotene were similar when subjects consumed purified α- and β-carotene with either 3 or 36 g fat daily for seven days. However, the plasma lutein response was significantly higher after ingestion of purified lutein esters with 36 g fat compared with 3 g fat. They concluded that 3 g fat was sufficient for intestinal uptake of

purified α- and β-carotene, but insufficient to ensure absorption of lutein esters compared with 36 g fat. In a study by Roels et al. (16) 9- to 16-year-old boys in Rwanda receiving 200 g raw carrots and 18 g olive oil per day absorbed 25% of the carotene from carrots whereas those receiving 200 g carrots without any oil absorbed only 5% of the carotene from carrots. This study had high within group variations in response. Jalal et al. (15) found no difference in the increase in serum retinol when children consumed sweet potatoes with either 3 or 15 g dietary fat daily for three weeks. Jayarajan et al. (17) reported no difference in the serum vitamin A status of Indonesian children when 40 g cooked spinach was ingested with 5 or 10 g dietary fat. The studies by Jalal et al. and Jayarajan et al., which were conducted with populations at risk of vitamin A deficiency, indicate that the cut-off for enhanced absorption of carotenoids lies between 3 and 5 g fat. The lower fat threshold necessary for carotenoid absorption may reflect smaller vegetable servings as compared with the present study. Even with higher amounts of dietary fat, no differences in absorption were found. Takyi (45) found no difference in serum vitamin A when homogenized dark, green leafy vegetables were consumed with a meal containing 26 or 101 g fat. The homogenization of the plant tissues would increase carotenoid bioavailability compared with dark green leafy vegetables given in the present study. It is evident that the literature regarding the effect of fat on absorption of carotenoids is conflicting. Several of the older studies have design flaws (46) such as use of spectrophotometric methods much less precise than HPLC techniques to quantify α- and β-carotene (16), lack of a negative control group (17), and comparison of the mean value of each treatment group at baseline with that of the same treatment group at follow-up instead of using a more statistically powerful within-subject comparison (17). Lower dietary fat thresholds for optimal carotenoid absorption than those found in the

present study may reflect differences in study design because vegetables were homogenized (45), carotenoids were given in purified rather than food form (18), and carotenoid-containing foods were given as a chronic supplement (15-18). Additionally, compared with the present study, larger amounts of fat were given relative to the weight of the vegetables ingested (15, 17, 18, 45). Dietary intake of carotenoids from fruits and vegetables is poorly correlated with plasma carotenoid concentration (47). Based on the results of the present study, potentially this correlation could be strengthened by controlling for dietary fat intake.

The low bioavailability of carotenoids from vegetables presents a challenge in improving the vitamin A status of populations at risk of vitamin A deficiency in developing countries given these populations' dietary dependence on pro-vitamin versus preformed vitamin A. Recent studies show that adding fat to the diets of populations low in dietary fat and vitamin A can improve vitamin A status without intervention to change carotenoid or vitamin A intake (48, 49). Alam et al. (48) gave pregnant Bangladeshi women daily supplements of 20 ml soybean oil for six months and found that serum retinol from pregnancy to three months post-partum was greater in supplemented women compared with controls. Serum β-carotene concentrations decreased after parturition in both groups, but the decrease from pregnancy to three months post-partum was less in the supplemented group compared with controls. In northern Thailand, Nimsakal et al. (49) supplemented children 1-12 years of age and at risk for vitamin A deficiency with vegetable oil or soy sauce. Increasing dietary fat significantly improved vitamin A status as measured by conjunctival impression cytology (CIC). Therefore, increasing dietary fat alone may be an effective strategy to improve vitamin A status.

In the United States, intake of dietary fat among the general population is sufficient to excessive (50). Our findings support the U. S. Department of Agriculture's Dietary Guideline "Choose a diet that is low in saturated fat and cholesterol and moderate in total fat" (51). Some health conscious Americans go to extremes in lowering the fat content of their diets. Diets that are extremely low in fat may be counterproductive in terms of deriving the full benefit of cancer, vision, and heart protective substances from fresh vegetables. We have shown negligible carotenoid absorption after ingestion of a fresh vegetable salad with fat free dressing, despite the high carotenoid content. Ingestion of dietary fat from other meal components, may supply fat needed for carotenoid absorption allowing for the use of fat-free and reduced-fat salad dressings as part of a balanced moderate-fat diet.

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Table 1. Carotenoid content of test salad[1]

Ingredient	Weight (g)	α-Carotene	β-Carotene	Lutein	Lycopene	Zeaxanthin
				mg		
Carrot, grated	66	4.444 ± 0.388	6.956 ± 0.343	0.050 ± 0.006	0	0
Lettuce, romaine	48	0	1.639 ± 0.236	1.650 ± 0.181	0	0.052 ± 0.005
Spinach, leaf	48	0	2.830 ± 0.196	4.060 ± 0.264	0	0.111 ± 0.024
Tomato, cherry	85	0	0.799 ± 0.101	0.115 ± 0.011	8.613 ± 0.293	0
Totals:	247	4.444 ± 0.388	12.224 ± 0.038	5.875 ± 0.444	8.613 ± 0.293	0.163 ± 0.024

[1] $\bar{x}$ ± SEM, n = 3.

Table 2. Areas under the baseline corrected concentration versus time curves (AUC) for chylomicron all *trans*-α-carotene, all *trans*-β-carotene, and all *trans*-lycopene after ingestion of the test salad with each of three salad dressings[1, 2]

Carotenoid	Fat-free (0 g fat)	Reduced-fat (6 g fat)	Regular fat (28 g fat)
		nmol·h/L plasma	
α-Carotene	1.76 ± 0.81	22.66 ± 6.33	57.94 ± 11.28
β-Carotene	5.98 ± 15.92	103.49 ± 28.07	220.46 ± 37.83
Lycopene	-0.29 ± 0.60	3.31 ± 1.16	4.00 ± 4.02

[1] $\bar{x}$ ± SEM, n = 7.
[2] Each mean is significantly different from the others in the same row, $P < 0.05$ (one-tailed paired *t*-test).

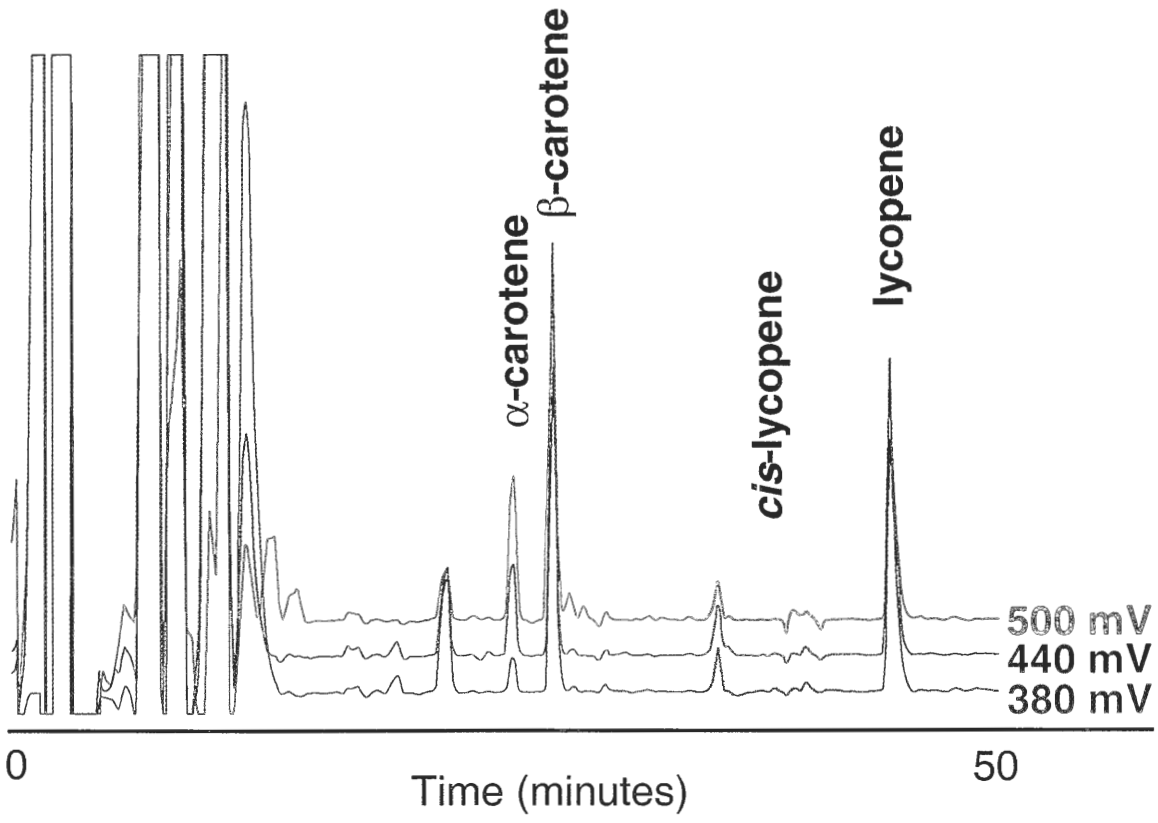

Figure 1. HPLC-ECD chromatogram showing the current produced by all *trans*-α-carotene, all *trans*-β-carotene, *cis*-lycopene isomers, and all *trans*-lycopene extracted from the chylomicron fraction.

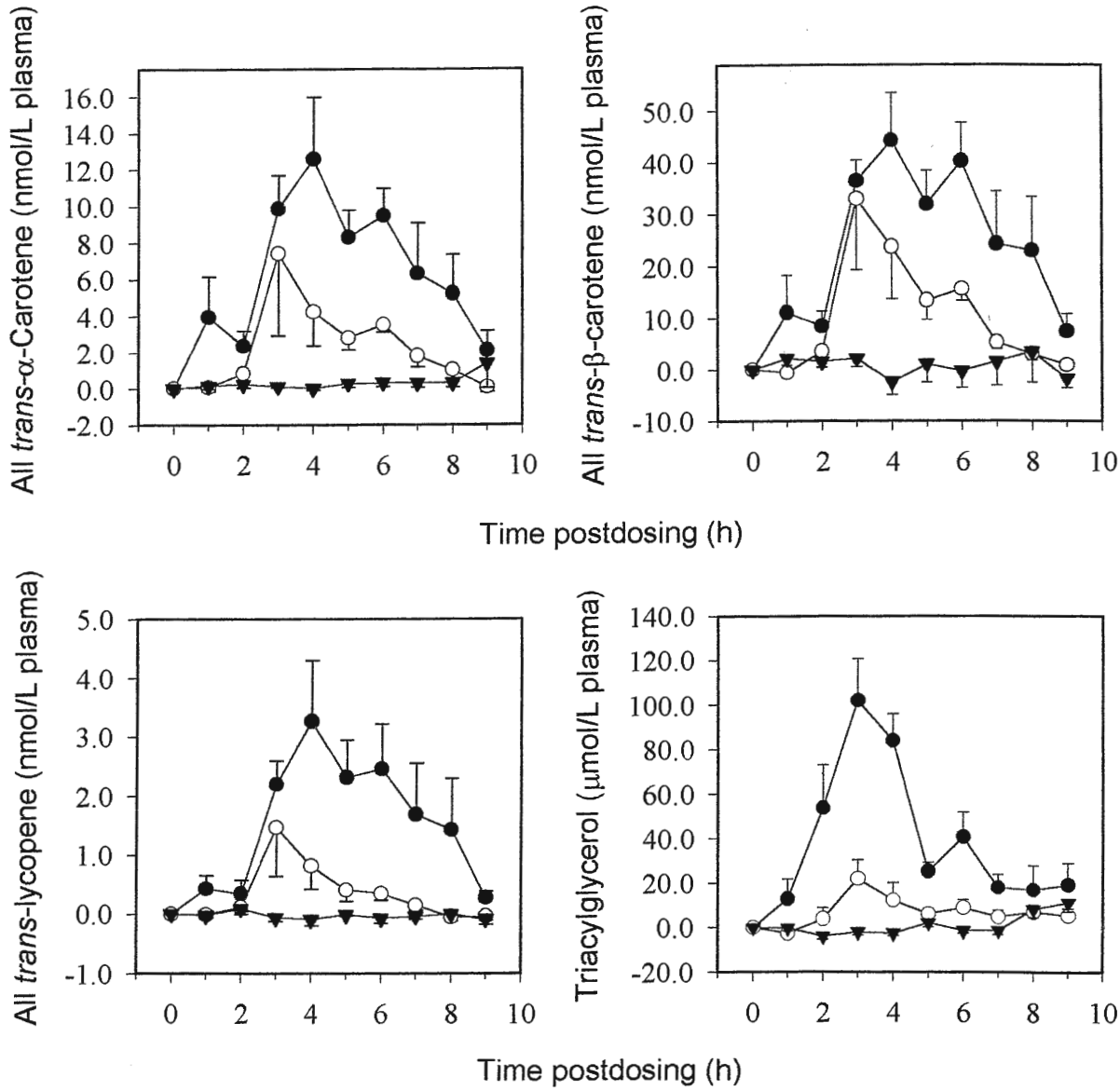

FIGURE 2. Change in all *trans*-α-carotene, all *trans*-β-carotene, all *trans*-lycopene and triacylglycerol in the plasma chylomicron fraction after subjects ingested the test salad with regular fat —●— , reduced-fat —○— , and fat-free —▼— salad dressings separated by a washout period of at least 2 weeks. $\bar{x} \pm$ SEM, n = 7.

GENERAL CONCLUSIONS

We applied high sensitivity high pressure liquid chromatography with coulometric electrochemical detection (HPLC-ECD) to quantify carotenoids in the plasma chylomicron fraction as a measure of intestinal absorption from a single serving of fresh vegetables. Use of electrochemical detection will allow acute feeding studies to replace more expensive and laborious chronic feeding studies. This approach can be used as a quick screening method to study factors that affect carotenoid absorption. Determination of factors that increase carotenoid absorption is important because dietary consumption of carotenoids has been linked to decreased risks of cancer, cardiovascular disease, and chronic degenerative diseases of the eye (age-related macular degeneration, cataracts). The use of HPLC-ECD to measure the postprandial carotenoid response in chylomicrons is a sensitive and efficient approach to detect the small amounts of carotenoids absorbed from a single serving of fresh vegetables. The ability to detect small amounts of analyte creates new possibilities in carotenoid analysis. Electrochemical detection will enable carotenoid researchers to further elucidate the intestinal absorption and metabolism of carotenoids.

This study also adds to the body of literature regarding the amount of dietary fat necessary for the optimal absorption of carotenoids from vegetables. Fat has been shown to enhance the absorption of carotenoids from vegetables, but studies differ on the amount of fat needed for optimal absorption. The limited literature on this subject suggests that optimal absorption of carotenoids occurs with only 3-5 g fat. We found absorption of carotenoids from a salad of fresh vegetables to be negligible when the salad was ingested with fat-free salad dressing (0 g fat). However, ingestion of the test salad with both regular fat (28 g fat) and reduced-fat (6 g fat) salad dressing significantly increased absorption of all *trans*-α-

carotene, all *trans*-β-carotene, and all *trans*-lycopene. Furthermore, the absorption of each carotenoid was significantly higher when the test salad was consumed with regular fat salad dressing compared with reduced-fat dressing. Thus it is possible to optimize carotenoid absorption from fresh vegetables by increasing the amount of fat consumed with the vegetables.

These results have significance for both U.S. consumers and populations of developing countries. Some health conscious Americans go to extremes in limiting fat in their diets. In many developing countries, fat is often limiting as part of the diet. Diets that are extremely low in fat may decrease the potential protective effects of carotenoids ingested from fruits and vegetables. Thus, it is important to include a moderate amount of fat in the diet. In developing countries where the majority of vitamin A intake comes from carotenoid-containing fruits and vegetables, the addition of fat to the diet may be adequate to improve vitamin A status.