1-1-2003

Bioefficacy of beta-carotene in lutein-free lut2 leaves compared with wild-type Arabidopsis leaves fed to gerbils

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Bioefficacy of beta-carotene in lutein-free *lut2* leaves compared with wild-type *Arabidopsis* leaves fed to gerbils

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Nutrition

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Iowa State University

Ames, Iowa

2003

Graduate College

Iowa State University

This is to certify that the master's thesis of

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has met the thesis requirements of Iowa State University

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ABSTRACT

The relatively constant carotenoid composition of leaves in higher plants suggests *Arabidopsis* leaves could model interactions of β-carotene and lutein ingested in vegetable leaves. We compared liver vitamin A stores in gerbils fed β-carotene in lutein-free (*lut2*) mutant or wild-type (WT) *Arabidopsis* leaves. Gerbils were fed a vitamin A-free diet for 4 weeks. They were then fed one of 4 diets for 6 weeks: 1) vitamin A-free diet ($n = 8$); 2) vitamin A-free diet supplemented with purified β-carotene (22.0 nmol β-carotene/g diet; $n = 8$); 3) vitamin A-free diet supplemented with *lut2* leaves (61.3 nmol β-carotene/g diet; $n = 3$); or 4) vitamin A-free diet supplemented with WT leaves (69.1 nmol β-carotene/g diet; $n = 3$). There were no group differences in body or liver weights. Liver vitamin A stores were 48% higher in gerbils fed *lut2* leaves (2.94 ± 0.14 μmol) than in those fed WT leaves (1.99 ± 0.10 μmol; $P = 0.005$). Liver vitamin A stores were higher in gerbils fed purified β-carotene (3.80 ± 0.27 μmol) than in those fed WT leaves ($P = 0.003$) or vitamin A-free diet (0.45 ± 0.08 μmol; $P < 0.001$). The difference in liver vitamin A stores in gerbils fed purified β-carotene or *lut2* leaves was not statistically significant. Although our finding may not extrapolate to humans, for the first time, we have shown carotenoid-carotenoid interactions when ingested within a plant matrix.

GENERAL INTRODUCTION

Thesis organization

There are three chapters in this thesis. The first chapter gives a general background on the protective associations between β-carotene and chronic diseases, as well as between vitamin A and diseases. There is also a review of the low bioavailability of β-carotene in plant matrices and the factors that may affect the bioavailability of β-carotene, particularly, the inhibitory effect of lutein on utilization of β-carotene ingested from dark green leafy vegetables. The lutein-deficient mutant of *Arabidopsis* plant is addressed. The relative merits of different animal models of human β-carotene metabolism are also discussed in this chapter.

The second chapter is a manuscript to be submitted for publication in the journal Nature. It describes an animal study using the Mongolian gerbil (*Meriones unguiculatus*) as a model and *Arabidopsis* wild-type (WT) and lutein-deficient *lut2* leaves to model interactions of β-carotene and lutein ingested in dark green leafy vegetable leaves. The findings and significance of this study are described in detail.

The third chapter gives the general conclusions derived from the study.

Literature Review

Carotenoids are red and yellow pigments synthesized by a variety of plants and abundant in most darkly colored fruits, vegetables, and red palm oil[1]. The carotenoids consist of more than 600 compounds. Lycopene is the major pigment of red-fleshed fruits and fruit vegetables, such as watermelon, red-fleshed papaya, and red or pink grapefruit and tomatoes. β-Carotene is the most common of all carotenoids in foods, such as apricot, carrot, mango, loquat, palm fruits. Lutein is the predominant carotenoid in leaves, green vegetables, and yellow flowers[2].

Dietary carotenoids are related to decreased risks of cancers, cardiovascular disease, age-related macular degeneration, and other diseases. Numerous of studies have been carried out to investigate the effects of low intake of vegetables, fruits and carotenoids on cancers[3]. Hydrocarbon carotenoids such as β-carotene and lycopene are related to risk of specific cancers and heart disease. A case-control study in New York City[4] showed that there was an association between the risk of breast cancer and the serum concentrations of β-carotene and other carotenoids. Those women with the lowest concentrations of total carotenoids had about a 2.3-fold increase in risk. Jialal's group[5] showed that β-carotene inhibited LDL oxidation, which indicated that β-carotene might play an important role in the prevention of atherosclerosis. Results from the Physicians' Health Study[6] showed that, in a large population of men, higher intake of vegetables rich in carotenoids was associated with lower risk of coronary heart disease. Age-related macular degeneration is the leading cause of blindness in the United States[7]. Oxygenated carotenoids, which are called xanthophylls, such as lutein and zeaxanthin, may be important in protecting eye tissues[8, 9]. A study[10] carried out in patients with cataracts and age-related macular degeneration (ARMD) showed that

subjects supplemented with lutein at dietary amount had increased serum lutein concentrations and improved visual function, which indicated an association between high intake of lutein and improved visual acuity. The Los Angeles Atherosclerosis Study[11] showed an inverse association between intake of lutein and progress of early atherosclerosis.

The inhibitory effects of carotenoids on cancers and heart disease have been suggested to be mediated through the antioxidant properties of certain carotenoids in scavenging free radicals and other oxidants[12, 13]. Liebler and McClure[14] detected some β-carotene-radical oxidation products which were associated with antioxidant reactions. The alternating double and single bonds form the C_{40} carbon skeleton, which is called the polyene chain of carotenoids. Carotenoids are not stable toward oxidation because of the highly reactive polyene chain which is easily attacked by electrophilic reagents, but that is also how carotenoids function as antioxidants[15]. The ability of carotenoids to quench singlet oxygen is related to the conjugated double-bond system, and maximum protection is given by those having nine or more double bonds[16].

Structurally, vitamin A (retinol) is one-half of the molecule of β-carotene with an added molecule of water at the end of the lateral polyene chain. Vitamin A is provided in the diet in two major forms. Preformed vitamin A as retinol is obtained from animal sources such as liver, butter, cheese, margarine, dried milk, cream, fortified milk, egg, and some seafood. Provitamin A is provided as β-carotene, α-carotene, and other provitamin A carotenoids and is found mainly in colored fruits and orange or green-colored vegetables[1]. Important sources of provitamin A carotenoids are sweet potatoes, pumpkin, squash, carrots, tomatoes, apricots, mangos and most greens, broccoli, brussels sprouts, and asparagus.

Of the approximately 600 identified naturally-occurring carotenoids, about 60 have provitamin A activity[17]. Provitamin A carotenoids are the primary dietary source of vitamin A for most of the world's population[18]. The major provitamin A carotenoid in fruits and vegetables is β-carotene, which has the greatest provitamin A activity. The amount and bioavailability of dietary provitamin A carotenoids determine whether vitamin A requirements are met in most populations, especially those in developing countries[19].

Vitamin A deficiency (VAD) is a public health problem in about 76 countries, especially in South-East Asia[20]. It is the leading cause of preventable blindness in children. 2.8 million children of preschool age are estimated to be clinically affected, and 258 million are subclinically affected[20]. Among the 2.8 million children, about 350,000 who have eye impairment caused by VAD go blind every year and up to 60% die within several months of becoming blind[20]. VAD is also associated with increased severity of infections, particularly measles and diarrheal disease[21]. Vitamin A also plays an important role in the immune system and sufficient vitamin A stores are associated with decreased risk of transmission of human immunodeficiency virus (HIV) from infected mothers to their infants[21]. Sudanese children with lower dietary vitamin A intakes were found to have much higher risk of mortality[22].

Vitamin A deficiency is endemic in areas of the world where dark green leafy vegetables, such as spinach, water spinach, and cassava leaves are a staple. The prevalence of xerophthalmia in the Republic of Kiribati is among the highest reported in the world although carotenoid-containing fruits and vegetables are consumed, among which the dark green leafy vegetables are the most common source of provitamin A carotenoids[23]. In Bangladesh, subclinical vitamin A deficiency is highly prevalent among adolescent female

garment factory workers whose vitamin A intakes are mainly from dark green leafy vegetables[24]. de Pee's group[25] found that consumption of dark green leafy vegetables (cassava leaves, water spinach, spinach, or carrots) in Indonesian women did not improve vitamin A status but a wafer enriched with the same amount of β-carotene produced a substantial improvement. The low efficiency of conversion of plant sources of provitamin A in humans, along with the problem of malnutrition and low-fat diets, is a major constraint to a sustainable, plant food-based solution to vitamin A deficiency[26].

There are many factors that affect the bioavailability and bioconversion of carotenoids, such as food matrix, interaction with fat and fiber, nutritional status, interaction with other carotenoids, aging and parasite infection. In nature carotenoids in a wide variety of plants, animals, and microorganisms are protein-bound. Release from the food matrix is an important step in the absorption process. Dietz et al[27] found that β-carotene bioavailability increased with heat treatments. Pure β-carotene dissolved in oil or aqueous dispersions is efficiently absorbed[28, 29], whereas carotenoids in uncooked vegetables such as β-carotene in carrot are poorly absorbed[29]. A fruit and vegetable dietary intervention study[30] was carried out in Indonesia schoolchildren. The changes in serum retinol and β-carotene concentrations in the fruit group were higher than those in the vegetable group. Consumption of mechanically homogenized spinach induces higher plasma concentration of lutein, which indicates that the bioavailability of lutein can be improved by disruption of the vegetable cell walls[31]. van het Hof et al[32] also found that heat treatment and mechanical homogenization enhanced the release and improved the bioavailability of lycopene from tomatoes. They concluded that disruption of the cellular matrix of tomatoes increased the bioavailability of carotenoids. A study[33] designed to examine the effect of variously

processed spinach products and dietary fiber on serum carotenoid concentrations found that the relative bioavailability of β-carotene in the group fed enzymatically liquefied spinach was almost twice as high as that in the group fed whole spinach leaf. Therefore, heat treatment helps to release carotenoids contained within the cell walls by softening the cell walls and hence increases the bioavailability of intestinal carotenoids.

The absorption of β-carotene requires dietary fat. Addition of fat to spinach resulted in greater β-carotene absorption and utilization in Indian children than the same amount of spinach without fat added[34]. Addition of 5 g or 10 g fat to the meal resulted in similar increases in serum vitamin A concentration, which indicated that as little as 5 g of fat might be enough to optimize carotene absorption from the diet. Prince and Frisoli[35] found that β-carotene administered without fat did not cause detectable accumulation in the serum, but there was an increase in serum β-carotene when β-carotene was administered with fat. Roodenburg et al[36] showed that the amount of dietary fat consumed (3 g or 36 g) did not affect plasma concentrations of α- and β-carotene after α- and β-carotene supplementation but did affect increase in plasma concentration of lutein after lutein ester supplementation. This study indicates that the amount of fat required for optimal intestinal uptake of specific carotenoids may be different and also there is limited amount of fat needed for uptake of β-carotene. Fiber may decrease the bioavailability of carotenoids by entrapping them and by interacting with bile acids to result in increased fecal excretion of carotenoids[37]. Dietary fiber also has an inhibitory effect on the utilization of β-carotene in humans. Pectin added to a meal decreased the plasma β-carotene concentration by one half[38]. Therefore, fiber and the amount of fat in the diet can affect the bioavailability of β-carotene.

Nutritional status also affects vitamin A status. The link between vitamin A and iron deficiency anemia is of interest intense. One study[39] was designed to determine the effects of vitamin A and iron supplementation on vitamin A and iron status in pregnant Indonesian women. There were greater improvements in vitamin A status when vitamin A and iron were administered together than when they were given alone.

The low bioavailability of β-carotene in leafy vegetables is the focus of many researchers. The bioavailability of β-carotene in yellow-orange fruits (mango and papaya) was more than twice that in leafy green vegetables (cassava leaves, water spinach, and spinach) and the conversion efficiency of the β-carotene in green vegetables to vitamin A is only 26:1, wt:wt[30]. The bioavailability of β-carotene in spinach leaves was only 5% compared with purified β-carotene in a supplement[33]. Tomato paste caused a higher retinyl palmitate response in the triacylglycerol-rich lipoprotein fraction than spinach although spinach had higher β-carotene content[40]. Another study[41] designed to assess interactions between vegetable-borne carotenoids also found that tomato puree resulted in a higher chylomicron β-carotene response than a spinach meal although the spinach had more than 5 times the β-carotene content of the tomato puree. Even among the vegetables, green leafy vegetables have lower bioavailability compared with others. van het Hof et al[31] found that spinach, a dark green leafy vegetable, is not as efficient as broccoli and green peas, which are the flower and seed parts of vegetable plants, respectively, in increasing plasma β-carotene concentration although spinach has about 10 times higher β-carotene content than broccoli and green peas.

van het Hof et al[42] investigated the relative bioavailability of β-carotene and lutein from mixed vegetables compared with purified β-carotene and lutein supplements. The bioavailability of β-carotene from a mixed-vegetable diet was 14% while that of lutein was 67%. The bioavailability of lutein from vegetables was 5 times higher than that of β-carotene. Johnson et al[43] also found that concentrations of β-carotene did not change significantly after subjects were fed spinach and corn for 15 weeks but lutein from the same food source caused significant increases in serum and tissue lutein concentrations.

Carotenoids are highly hydrophobic molecules and tend to aggregate and crystallize in aqueous circumstance[15]. In the chromoplasts of higher plants, aggregation of carotenoids is common[44], which changes their physical properties such as light absorption, and chemical reactivity, such as solubilization, and may affect their absorption and bioavailability in vivo[15].

After release from the food matrix, ingested carotenoids need to be dissolved in lipid droplets and then incorporated into mixed micelles, which are a mixture of bile acids, free fatty acids, monoglycerides, and phospholipids, before they are taken up by the mucosa of the small intestine and incorporated into triacylglycerol-rich chylomicrons[45, 46, 47, 48]. The amount of carotenoid incorporated into micelles depends on the polarity of the carotenoid[49]. The more polar oxygenated carotenoids may be more easily incorporated into micelles, and therefore may more easily be taken up into the enterocyte and incorporated into chylomicrons, which may increase their bioavailability compared with the less polar hydrocarbon carotenoids[50]. Carotenoids also distribute differently in chylomicrons because of their polarity. The more polar carotenoids are preferentially accumulated at the surface of the chylomicron particles while the less polar ones are accumulated in the core. Thus, during

postabsorptive transport, the oxygenated carotenoids are more easily transferred to or exchanged with other lipoproteins[49], which may also increase their bioavailability. Since lutein is an oxygenated carotenoid which is more polar than β-carotene, a hydrocarbon carotenoid, the mechanisms described above may be one explanation of why the bioavailability of β-carotene is usually lower than that of lutein.

Lutein is a predominant and nonprovitamin A carotenoid in photosynthetic tissues. It is the most abundant carotenoid in green leaves[51]. Typically, lutein represents about 45% of the total carotenoid content of leaves, whereas β-carotene and lesser amounts of other hydrocarbon carotenes contribute only about 25%[52]. β-Carotene 15,15′-monooxygenase is the enzyme that cleaves provitamin A carotenoids in the intestinal mucosa. As a nonprovitamin A carotenoid, lutein could serve as a pseudosubstrate for that enzyme and hence may affect the bioconversion of β-carotene to vitamin A[47].

Interactions of lutein and β-carotene during absorption and in postabsorptive metabolism have been observed in both animal and human studies, as well as *in vitro* studies. Studies in rats showed that relatively large amounts of lutein impaired the utilization of β-carotene whereas small amounts enhanced utilization[53]. When the animals were dosed with a carotene: lutein ratio of 1.2 or greater, the total retinol stores in liver and kidneys were higher compared with those of when same amount of carotene was given alone. When the ratio was as low as 0.06, total retinol stores were markedly decreased. This study showed that in rats lutein is an antagonist of vitamin A storage from carotene when lutein is the dominant carotenoid in the diet.

A human study designed to study the effect of a single, combined dose of β-carotene and lutein showed that the β-carotene response in the triacylglycerol-rich lipoprotein (TRL) fraction of men was significantly decreased when they ingested β-carotene and lutein in combined doses and equal amounts, as compared with that when β-carotene was given alone[40]. A subsequent study of the effect of β-carotene/lutein ratio showed that the β-carotene TRL response was decreased by 40% when β-carotene/lutein ratio was 1:2 compared with that when a single dose of the same amount of β-carotene was given[54]. Another human study found that β-carotene and lutein interact with each other during intestinal absorption, metabolism and serum clearance[55]. Lutein decreased the area under the curve (AUC) values for serum β-carotene when given in combined equimolar doses compared with that when a single equimolar dose of β-carotene was given. One study designed to study β-carotene cleavage activity *in vitro* found retinal formation was decreased when lutein was added to the incubation with β-carotene at the ratio of 3:1 lutein to β-carotene[56].

The above studies showed that purified lutein interacts with purified β-carotene and, specifically, lutein inhibits the utilization of β-carotene when lutein is the dominant carotenoid ingested. The same results were observed when lutein was given with vegetable sources of β-carotene. The chylomicron response to β-carotene was lower when purified lutein was added to tomato puree which was provided as a β-carotene source by Tyssandier's group[41]. These results show that there are interactions between added lutein and β-carotene in vegetables sources. The study[33] mentioned earlier found that the relative bioavailability of lutein in spinach was more than 5 times higher than that of β-carotene. The inhibitory effect

lutein has on utilization of β-carotene might explain the prevalence of vitamin A deficiency in developing countries despite high intakes of β-carotene from leafy vegetables.

In leaves, the carotenoids are found in the chloroplasts of green tissues and their color blends into that of the chlorophylls. Leaves of all species have a strikingly constant carotenoid pattern, often referred to as the chloroplast carotenoid pattern, the main carotenoids being lutein (about 45%), β-carotene (usually 25-30%), violaxanthin (15%) and neoxanthin (15%). There are also small amounts of α-carotene, α- and β-cryptoxanthin, zeaxanthin and antheraxanthin[57]. As predicted by the known carotenoid composition in photosynthetic tissues, lutein is the dominant carotenoid in dark green leafy vegetables.

Arabidopsis thaliana is a small mustard plant and a model organism for plant molecular genetics. In December 2000, it was reported that the genome of *Arabidopsis* had been completely sequenced, which was the first sequence of a plant genome to be completed. *Arabidopsis* is a major model system for plant genetics with a wide array of tools for its genetic and molecular characterization and recent availability of its complete genome sequence. The relatively constant carotenoid composition of leaves in higher plants suggests *Arabidopsis* leaves could model interactions of β-carotene and lutein ingested in vegetable leaves. The lutein/β-carotene ratio in the wild-type Arabidopsis leaves is about 2.33:1[51]. We used *Arabidopsis* as a model for dark green leafy vegetables due to the existence of *lut2*, which is a well-characterized lutein-deficient mutant of *Arabidopsis* isolated in Dr. Dean DellaPenna's laboratory at Michigan State University (East Lansing, MI, USA). The biochemical phenotype is consistent with a disruption of ε ring cyclization. There are no

known hazards associated with the consumption of *Arabidopsis thaliana*. Although *Arabidopsis thaliana* are weeds, the leaves are edible and have a mild flavor[58].

In plants, cyclization of the two end groups of lycopene produces β-carotene, which contains 2 β rings, and α-carotene, which contains a β and an ε ring. Lycopene is cyclized twice by the enzyme lycopene β-cyclase to form β-carotene. The hydroxylation of both β rings of β-carotene yields zeaxanthin. One end group of zeaxanthin is then epoxidated to form antheraxanthin. Epoxidations of both end groups of zeaxanthin forms violaxanthin. Neoxanthin is derived from violaxanthin by an additional rearrangement. The cyclization of the two end groups of lycopene by lycopene β-cyclase and lycopene ε-cyclase, respectively, forms α-carotene. Zeinoxanthin and lutein are each derived from α-carotene. Hydroxylation of the β ring of α-carotene forms zeinoxanthin. Subsequent hydroxylation of the ε ring produces lutein[51, 59, 60]. The *lut2* mutation eliminates lutein production because the enzyme lycopene ε-cyclase is disrupted. This enzyme is required for biosynthesis of lutein but not of β-carotene and other β,β-carotenoids[51].

At moderate light intensities, growth and development of *lut2* mutants are not visibly affected by the absence of lutein, despite its presumed significant role in photosynthesis. Chlorophyll content and Chl a-to-Chl b ratio are unaltered[51]. The absence of lutein doesn't impair the photosynthesis in mature plants at moderate light intensities. However, nonphotochemical quenching is defective in *lut2* mutants under high-light stress[59]. *Lut2* mutant viability is possibly caused by partial or total functional compensation by other xanthophylls, violaxanthin and antheraxanthin, which increase markedly in the absence of lutein[51]. When plants absorb more light intensity than needed (high-light stress), there is

conversion between violaxanthin and zeaxanthin via antheraxanthin, which is called the xanthophyll cycle[61]. The angle of the epsilon ring of lutein is the same as that of the epoxy ring(s) of antheraxanthin and violaxanthin, which may explain the functional compensation of those xanthophylls. Although zeaxanthin and lutein are similar molecules, the differences in their three-dimensional shapes make zeaxanthin function less effectively than violaxanthin and antheraxanthin in the absence of lutein[59].

The aims for our study are: 1) to evaluate the utility of *Arabidopsis thaliana*, a genetically-facile higher plant, as a model to investigate food matrix effects that limit the bioavailability of β-carotene and other nutrients; and 2) to test our hypothesis, which is lutein is a naturally-occuring antagonist of the utilization of β-carotene in green leafy vegetables for conversion to vitamin A, in vivo in a rodent model by determining the extent to which the utilization of β-carotene for conversion to vitamin A is enhanced in lutein-deficient *lut2 Arabidopsis* leaves as compared with wild-type leaves.

There are obvious advantages using animals instead of humans in studies. Tissue collection is mostly impossible in human subjects but accessible in animals, which gives researchers more details and insight about the mechanisms of the interactions. Since no one animal can absolutely mimic human absorption and metabolism of carotenoids, it is important to choose the appropriate animal model for the specific application[62]. Different animals have been used to model the absorption and metabolism of carotenoids in humans. Studies have been conducted to estimate the utilization of vitamin A and the ability to utilize β-carotene as a source of vitamin A in rats and mice, ferrets and gerbils, which are all small-sized animals and can be easily managed in the laboratory.

Rats can absorb β-carotene and other carotenoids when high doses of carotenoids are fed[63] and rat intestinal mucosal cells cleave β-carotene to vitamin A as humans do[64]. Rats have been used in many studies designed to evaluate the efficiency of β-carotene conversion to vitamin A[65, 66, 67]. Rats are a good model to study the effects of vitamin A deficiency because of the rapid development of vitamin A deficiency in this species. Rats on vitamin A-free diets show symptoms of vitamin A deficiency rapidly (33 days)[68]. However, this species converts β-carotene to vitamin A too efficiently[63] and it does not absorb physiologic doses of β-carotene intact[69]. Because humans both cleave β-carotene to form vitamin A and absorb a variety of carotenoids (including β-carotene) intact, the rat is not the appropriate model for β-carotene utilization studies[62].

Ferrets, like humans, can absorb β-carotene intact in the lymph[70] and accumulate β-carotene in tissues and sera[71, 72]. Ferrets are able to absorb other carotenoids, such as lycopene and canthaxanthin intact[73, 74]. Also, ferrets convert β-carotene to vitamin A in the small intestine as humans do[64]. However, ferrets don't convert β-carotene as efficiently as humans. Lederman et al[75] showed that ferrets didn't utilize β-carotene efficiently enough to improve liver vitamin A stores. The efficiency of conversion of a diet β-carotene to vitamin A was poorer than 15:1 under vitamin A-deficient status, much lower than that in humans, which is about 12:1. Also, the serum retinyl ester concentrations of ferrets are high unlike humans[75]. Therefore, the ferret is not a good model for studying dietary β-carotene conversion to vitamin A.

Gerbils absorb β-carotene when fed in large amounts[76]. Other studies[77, 78] showed that Mongolian gerbils store β-carotene in both serum and tissues, such as liver, kidney,

spleen and adrenals. Gerbils also absorb β-carotene intact at low dietary levels and β-carotene is converted to vitamin A with similar efficiency as in humans. Pollack et al[79] used Mongolian gerbils to determine if the animals would absorb β-carotene intact from a test meal. They found that gerbils, like humans, were able to absorb β-carotene intact when β-carotene was given as a physiological dose. It was accumulated in serum and liver. Lee et al[78] found that gerbils have similar conversion efficiency as humans. The same study also found that, when gerbils became marginally vitamin A deficient or deficient, the efficiency of absorption and conversion of β-carotene to vitamin A increased, which is similar to humans. Thus the gerbil is a good animal model for evaluation of β-carotene bioavailability and metabolism. Unlike ferrets, gerbils are both inexpensive and commercially available. However, it is difficult to deplete hepatic vitamin A stores in gerbils. Lee et al[78] found gerbils had lower liver vitamin A stores after 10 weeks of feeding a vitamin A-free diet than those fed the same diet for 8 weeks. The 10-week liver vitamin A content approached deficiency, although it was not significantly different from the level reached after 8 weeks of feeding. Thatcher et al[77] also showed that gerbils approached vitamin A deficiency after being fed a vitamin A-free diet for 84 days. Since it is difficult to deplete the vitamin A stores in gerbils, it is necessary to maintain those animals on a vitamin A-free diet for a period of time to lower baseline liver vitamin A stores before the experimental period starts. Above all, we predicted that gerbils could model humans for us to investigate the bioefficacy of β-carotene when the β-carotene in their diet is from the *Arabidopsis* leaves, which is a similar plant matrix from which most populations of the world ingest carotenoids.

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THE BIOEFFICACY OF BETA-CAROTENE IN LUTEIN-FREE LUT2 LEAVES IS HIGHER THAN IN WILD-TYPE ARABIDOPSIS LEAVES FED TO GERBILS

A paper to be submitted for publication in the journal Nature

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Abstract

The relatively constant carotenoid composition of leaves in higher plants suggests *Arabidopsis* leaves could model interactions of β-carotene and lutein ingested in vegetable leaves. We compared liver vitamin A stores in gerbils fed β-carotene in lutein-free (*lut2*) mutant or wild-type (WT) *Arabidopsis* leaves. Gerbils were fed a vitamin A-free diet for 4 weeks. They were then fed one of 4 diets for 6 weeks: 1) vitamin A-free diet ($n = 8$); 2) vitamin A-free diet supplemented with purified β-carotene (22.0 nmol β-carotene/g diet; $n = 8$); 3) vitamin A-free diet supplemented with *lut2* leaves (61.3 nmol β-carotene/g diet; $n = 3$); or 4) vitamin A-free diet supplemented with WT leaves (69.1 nmol β-carotene/g diet; $n = 3$). There were no group differences in body or liver weights. Liver vitamin A stores were 48% higher in gerbils fed *lut2* leaves (2.94 ± 0.14 μmol) than in those fed WT leaves (1.99 ± 0.10 μmol; $P = 0.005$). Liver vitamin A stores were higher in gerbils fed purified β-carotene (3.80 ± 0.27 μmol) than in those fed WT leaves ($P = 0.003$) or vitamin A-free diet (0.45 ± 0.08 μmol; $P < 0.001$). The difference in liver vitamin A stores in gerbils fed purified β-carotene or *lut2* leaves was not statistically significant. Although our finding may not extrapolate to humans, for the first time, we have shown carotenoid-carotenoid interactions when ingested within a plant matrix.

Introduction

Bioavailability is defined as the fraction of an ingested nutrient available for use in normal physiologic functions and storage. The amount and bioavailability of dietary provitamin A carotenoids determine whether vitamin A requirements are met in most populations, especially those in developing countries[1]. β-carotene is the major provitamin A carotenoid in fruits and vegetables, and it has the greatest provitamin A activity. Vitamin A deficiency is a severe problem in developing countries, where dark green leafy vegetables are widely consumed. In vegetables, the bioavailability of β-carotene is remarkably low. The bioavailability of β-carotene in yellow-orange fruits is more than twice that in green vegetables[2]. In spinach leaves, the bioavailability of β-carotene was only 5% compared to that in a β-carotene supplement[3]. The low bioavailability of β-carotene in plant-based foods is therefore a major constraint to a sustainable, plant-based solution to vitamin A deficiency[4].

Lutein is the most abundant carotenoid in green leaves. β-Carotene-15,15′-monooxygenase is the enzyme that cleaves provitamin A carotenoids in the intestinal mucosa, liver, and other tissues. As a nonprovitamin A carotenoid, lutein may serve as pseudosubstrate for that enzyme and hence may affect the bioconversion of β-carotene to vitamin A[5]. The bioavailability of β-carotene from a mixed-vegetable diet is 14% while that of lutein is 67%[6]. Thus the bioavailability of lutein from vegetables is 5 times higher than that of β-carotene. Ingestion of lutein results in significantly more accumulation of lutein in serum and tissues compared with ingestion of β-carotene ingested from the same food sources, spinach and corn[7]. In rats, lutein inhibits utilization of β-carotene when the β-carotene/lutein ratio is low[8]. The same effect was observed in humans in that lutein inhibits

β-carotene absorption[9], especially at a lower β-carotene/lutein ratio[10]. The limitation of previous studies designed to investigate interactions between β-carotene and lutein is that pure carotenoids were used instead of carotenoids in a plant matrix.

The leaves of higher plants have relatively constant carotenoid composition. Wild-type *Arabidopsis* leaves have similar carotenoid content and composition as that in green leaves of other higher plants[11], which suggests they could model interactions of β-carotene and lutein ingested in dark green leafy vegetable leaves. We used *Arabidopsis thaliana* also due to the existence of well-characterized *lut2*, which is a lutein-deficient mutant of *Arabidopsis* lacking ε-cyclase activity. The *lut2* mutants do not accumulate lutein but have normal β-carotene concentrations similar to those in wild-type *Arabidopsis*[12].

Gerbils were used in this study because they absorb β-carotene intact when ingested at a physiological level[13] and store β-carotene in both serum and tissues[14, 15]. Gerbils also convert β-carotene to vitamin A with efficiency similar to humans[15].

The objective of this study was to compare the bioavailability of β-carotene from WT and *lut2 Arabidopsis* leaves fed to gerbils. We hypothesized that the bioavailability of β-carotene in *lut2* leaves would be higher than that in WT leaves. In that case, genetic modification to modulate the high lutein content in green leafy vegetables would be useful in combating vitamin A deficiency in developing countries.

Materials and Methods

Animals

The animals were 30-day old male weanling Mongolian gerbils (n = 36). They were purchased from Charles River Laboratories (Kingston, NY, USA). Their body weights

ranged from 19.6 – 36.5 g. They were individually housed in standard polycarbonate tubs (conventional caging) under room temperature and 12 hour light/dark cycle. They had free access to food and water. Sawdust was used as contact bedding.

Each animal's body weight was recorded daily throughout the study.

All animal care procedures were approved by the Iowa State University Committee on Animal Care (COAC).

Arabidopsis thaliana

Lut2 Arabidopsis thaliana seeds were kindly donated by Dr. Dean DellaPenna, Department of Biochemistry and Molecular Biology, Michigan State University. Wild-type *Arabidopsis thaliana* seeds were kindly donated by Dr. Steven Rodermel's laboratory at Iowa State University. Wild-type and *lut2 Arabidopsis thaliana* plants were grown in a greenhouse at Iowa State University. The temperature was controlled by a computer and set at 72°. During summer, the plants were shielded from direct sunlight by a shield on the roof. The green leaf tissue of 8-week-old plants was harvested. The leaves were harvested by mowing using scissors. Leaf stems were avoided and the integrity of the leaf was maintained in order to avoid activating lipoxygenase enzymes, which might cause the carotenoids in the leaves to degrade. The leaves were washed and air dried under yellow light. Each aliquot of 100 g of leaves was stored in a Bitran series "PE" 9 x 12" Zip-loc storage bag (Fisher Scientific, Carbondale, IL, USA) and then 5 bags of leaves were stored together in a 172-oz multipurpose container (Fisher Scientific, Hanover Park, IL, USA). The containers were then stored at -70°C until leaves were freeze-dried.

Diets

There were four diet groups: β-carotene-free, purified β-carotene, WT *Arabidopsis* leaves and *lut2 Arabidopsis* leaves. The purified β-carotene diet was identical to the vitamin A-free control diet except purified β-carotene (Sigma-Aldrich, St Louis, Mo, USA) was added to give 22.0 nmol β-carotene/g diet. The ground WT and *lut2 Arabidopsis* leaf powders were shipped overnight on dry ice to Research Diets, Inc. (New Brunswick, NJ, USA) where they were combined and pelleted with the vitamin A-free control diet. There was 69.1 nmol β-carotene/g diet and 61.3 nmol β-carotene/g diet in the WT *Arabidopsis* leaf diet (WT diet) and *lut2 Arabidopsis* leaf diet (*lut2* diet), respectively. Diets were adjusted with corn starch, casein, inulin, and cellulose to have similar contents of carbohydrate, protein, soluble and insoluble fiber (Table 4). The added fat content was the same in the 4 diets. The diets were stored at -70°C until use.

Study design

After 15 hours of food deprivation, 5 animals were killed on the second day after their arrival to determine baseline liver retinol stores. Their livers, kidneys and serum were collected and stored at -70°C until analysis. The remaining 31 animals were fed a standard laboratory animal diet during the initial 5-day adaptation period. At the end of the adaptation period, one gerbil died from an unknown cause, which may have been starvation because it had the lowest body weight among all the animals when they arrived. For the remaining animals, the diet was changed to a purified β-carotene-free diet (vitamin A-free AIN-93G diet) for 4 weeks to deplete their liver vitamin A stores. At the end of the 4-week depletion period and after 15 hours of food deprivation, 8 animals were killed to determine plasma

retinol concentrations and liver vitamin A stores. Liver, kidney and plasma were collected and stored at -70°C until analysis. The remaining gerbils were randomly assigned to 4 experimental groups of balanced body weight distribution. In the 4 experimental groups, animals were fed WT, *lut2*, purified β-carotene and β-carotene-free diets, respectively, for 6 weeks. Because growing and harvesting the *Arabidopsis* leaves was so labor-intensive, the WT group and *lut2* group each had only 3 animals. The purified β-carotene and β-carotene-free groups each had 8 animals. At the end of the 6-week experimental period and after 15 hours of food deprivation, the animals were killed. Livers, kidneys and serum were collected and stored at -70°C until analysis.

Blood samples were collected via cardiac puncture under CO_2 anesthesia. Blood samples were protected from light and held at room temperature for 30 minutes to facilitate clotting. Then the samples were kept on ice until serum was separated by centrifugation in a 4°C cold room. The animals were killed by asphyxiation. Livers and kidneys were collected and weighed. Individual samples were stored in Whirl-pak write-on style 2-oz plastic bags (Fisher Scientific, Hanover Park, IL, USA).

Serum and tissue samples were stored at -70°C until analysis. Samples were analyzed for vitamin A, β-carotene, and lutein contents. Vitamin A and carotenoids were extracted from liver (0.2 g), kidney (0.1 g) and serum (400-600 μL) samples. Ethyl β-apo-8′-carotenoate (trans) (Sigma-Aldrich, St Louis, MO, USA) was added as an internal standard for analysis of β-carotene. N-(3-Hydroxyphenyl)-all-trans-retinamide purchased from Midwest Research Institute (Kansas City, MO, USA) was added to the liver samples as an internal standard for analysis of vitamin A. Retinyl acetate was added to the kidney and

serum samples as an internal standard for analysis of total vitamin A and retinol, respectively. Blood and tissue samples were handled either under yellow light or protected from ambient light.

Freeze drying

A VIRTIS Genesis series 25LE freeze drier (The VirTis company, Gardiner, NY, USA) was used for freeze-drying the leaves. After "POWER" was switched on, "FREEZE" was switched on to begin refrigerating the shelves. The shelves were allowed to cool to -40°C. The open plastic bags containing the frozen leaves were then placed on their sides on the shelves and the leaves were spread in a thin layer to maximize contact between the leaves and the shelves. Then the door was closed and shut tightly. The "CONDENSER" was switched on to begin cooling the condenser. The condenser was allowed to chill to -40°C, which required about 20 minutes. The temperature reading on the display was C1. Before starting the vacuum, trapped water was removed from the condenser drain hose by pulling out and down gently. The drain hose was then plugged. The "RELEASE" button was switched off and the condenser door and chamber door were checked to be sure they were closed and air tight. Then "VACUUM" was turned on until it reached < 100 microns, which was the V1 reading on the display. The "HEAT" switch was turned on and the "FREEZE" switch remained on. The shelf temperature was then adjusted to -30°C. When the temperature reached -30°C, it was held for 2 hours. Then the temperature was increased by 5°C each hour until it reached 0°C. Beginning at 8 hours, the leaves were weighed every 2 hours until the weight was stable. The leaves were then allowed to remain in the freeze drier for an additional 2 hours. The freeze-dried leaves were brittle and crumbled when touched. The water content of the *Arabidopsis thaliana* leaves was determined to be 90%.

The freeze-dried leaves were stored in bell jar dessicators to keep them from absorbing moisture until they were ground.

Grinding leaves

Freeze-dried leaves were mixed well in plastic barrels before they were ground. The UDY cyclone sample mill (UDY Corporation, Port Collins, CO, USA) was located in the laboratory of Dr. Trish Patrick in Agronomy Hall, Iowa State University. The powder was passed through a 20-mesh screen during grinding. The powder was collected in airtight glass jars and shipped overnight on dry ice to Research Diets, Inc. (New Brunswick, NJ, USA) to be pelleted with the purified diets.

High performance liquid chromatography (HPLC)

The HPLC system included a 717Plus Autosampler with temperature control set at 5°C, two 510 HPLC pumps and a 996 Photodiode Array Detector, all of which were purchased from Waters Instruments (Milford, MA, USA). The system was operated with Millennium^{32} Software version 3.05.01 (Waters Corporation). A 5-μm C_{30} 4.6 × 250 mm analytical column (Carotenoid Column, Waters Instruments, Milford, MA, USA) was used to separate the carotenoids. A linear mobile phase gradient was used to elute the carotenoids from the C_{30} column. A linear gradient of 100% solvent A (90:10 methanol:acetonitrile, v/v) to 100% solvent B (methyl-tert butyl ether) over 60 minutes at a flow rate of 1 mL/min was used for analysis of the Arabidopsis leaves. A linear gradient of 100% solvent A (methanol) to 100% solvent B (methyl-tert butyl ether) over 60 minutes at a flow rate of 0.9 mL/min was used for analysis of serum and tissue samples.

Analysis of carotenoid profile of *Arabidopsis* leaves

WT or *lut2* leaves were chopped finely using a food processor. A 10-g sample of the chopped and mixed leaves was mixed in a beaker with 4 g diatomaceous earth (Sigma-Aldrich, St. Louis, MO, USA) and 1 g solid calcium carbonate (Sigma-Aldrich). 50 mL of methanol and tetrahydrofuran (THF) (1:1, v/v) containing 0.01% (w/v) butylated hydroxytuluene (BHT) was added with the internal standard (IS). The IS was ethyl β-apo-8′-carotenoate (trans), which was dissolved in THF containing 0.01% (w/v) BHT. The absorbance was measured at 445 nm and the concentration was calculated using the absorption coefficient ($E^{1\%}_{1cm}$) of 2500. The formula used to calculate concentration was Absorbance/$E^{1\%}_{1cm}$ × 10000 = Concentration (ng/μL). The same IS was used for analyses of β-carotene and lutein, and also for analyses of chlorophyll a and chlorophyll b. The mixture was homogenized for 1 minute using a Brinkmann (Brinkmann Instruments, Inc, Westbury, NY, USA) Polytron PT 3000 homogenizer and then was filtered through a 100-mm porcelain buchner funnel using vacuum created by a Vacuubrand ME2C vacuum pump (GMBH+CO, Wertheim, Germany). The filter papers used were #1 qualitative 90 mmØ on the top and #42 ashless 90 mmØ on the bottom, both of which were purchased from Whatman International Ltd (Maidstone, UK). The filtrate was saved. The filter cake was then transferred to a beaker by using a spatula, mixed with 50 mL THF (0.01% BHT)/methanol (1:1, v/v), and homogenized again. The extraction, homogenization and filtration steps were repeated 3 times. The filtrates were combined in a 500 mL separatory funnel. 50 mL petroleum ether containing 0.01% (w/v) BHT and 50 mL of an aqueous 10% (w/v) NaCl were added. The mixture was shaken quickly and then allowed to stand to facilitate phase separation. The upper petroleum ether phase was collected. The lower aqueous/THF/methanol phase was re-

extracted with another 50 mL of petroleum ether (0.01% BHT). This step was repeated a total of 3 times. The upper petroleum ether layers were combined in a 200-mL volumetric flask and were brought to volume with petroleum ether (0.01% BHT). A 10-mL aliquot of the extract was dried under vacuum using a SpeedVac AS160 (Savant Instruments, Inc, Farmingdale, NY, USA). The dried residue was immediately reconstituted with 400 μL of methyl-tert butyl ether (MTBE) and then with 1600 μL of methanol. The reconstituted extracts were filtered through a 13-mm 0.2 μm Nylon syringe filter (Alltech Assoc. Inc, Deerfield, IL, USA). A 25-μL aliquot was injected into the HPLC system. Each sample was analyzed in duplicate. The protocol used in analyzing leaves was based on that of Hart and Scott[16].

The same procedures were used for analysis of the ground leaf powders except that 1 g of powder was used for each assay.

The concentration of each internal standard was measured once a week.

Analysis of carotenoid and tocopherol contents in experimental diets

A 10-g sample of pelleted diet was mixed and finely ground using a porcelain mortar and pestle. A 1-g aliquot of the ground powder was extracted with 3 mL of THF containing 0.01% (w/v) BHT in a Kimble culture tube (25 × 150 mm). Echinenone in THF containing 0.01% (w/v) BHT was added as IS for analysis of β-carotene and lutein. The absorbance of the IS solution was measured at 458 nm and an absorption coefficient of 2158 was used to calculate concentration. Rac-tocol (Matreya, Inc, State College, PA, USA) in ethanol was added as IS for α-tocopherol. The absorbance was measured at 292 nm and an absorption coefficient of 87.99 was used to calculate the concentration. The mixture was vortexed using Vortex Genie (Fisher Scientific, Hanover Park, IL, USA) for 1 minute. Then 2.7 mL of

methanol was added and the mixture was vortexed for 1 minute. With constant stirring, 1 mL of 40% methanolic KOH solution was added to saponify at 40°C for 30 minutes, during which the tube was uncapped but wrapped with aluminum foil and protected from light. After saponification, the mixture was cooled. Then approximately 1 g of ascorbic acid was added to neutralize the KOH. The pH was tested with pH paper until it reached neutral pH 7.0. Then 12 mL of 10% (w/v) aqueous NaCl solution was added followed by 18 mL of diethyl ether/hexane (1:1, v/v) containing 0.01% (w/v) BHT. The mixture was vortexed for 1 minute and then centrifuged for 15 minutes. The extraction step was repeated 3 times. The combined upper phases were washed with 15 mL of water 3 times. Each time the tube was centrifuged for 5 minutes to separate organic and aqueous phases. A 3-mL aliquot of extract was dried under vacuum using a Savant SpeedVac AS160. The dried extract was immediately reconstituted with 400 μL of MTBE and then with 1600 μL of methanol. The reconstituted extract was filtered through a 13-mm 0.2 μm Nylon syringe filter and then a 20-μL aliquot was injected into the HPLC system. The protocol used was based on that of Sharpless et al[17]. Each sample was analyzed in duplicate.

Analysis of liver vitamin A and carotenoids

The frozen liver samples were thawed at room temperature. The entire liver sample was finely minced using two scalpels. A 0.2-g aliquot of liver tissue was mixed with 1 mL of freshly prepared 10% (w/v) ethanolic potassium hydroxide (KOH) containing 1% (w/v) pyrogallol. Then the tube was flushed with argon and wrapped with aluminum foil and capped. The tissue was saponified at 40°C for 30 minutes with constant stirring. After saponification, the mixture was cooled and 1 mL of deionized water was added. N-(3-

Hydroxyphenyl)-all-trans-retinamide and ethyl β-apo-8′-carotenoate (trans) were added as IS for retinol and β-carotene, respectively. N-(3-Hydroxyphenyl)-all-trans-retinamide was dissolved in methanol (0.01% BHT). The molar absorption coefficient at 362 nm used to calculate concentration was 59049. The concentration was calculated using Absorbance/59049 = Concentration in mol. The molecular weight of N-(3-Hydroxyphenyl)-all-trans-retinamide is 391.53 g. The absorbance used for ethyl β-apo-8′-carotenoate (trans) was 2500 at 445 nm with ethanol (0.01% BHT) as solvent. Then 10 mL of diethyl ether/hexane (1:1, v/v) containing 0.01% (w/v) BHT was added to extract carotenoids and vitamin A. The mixture was vortexed for 1 minute and then centrifuged for 10 minutes. The upper organic phase was saved and the extraction step was repeated. The upper organic phases were combined and washed twice with 15 mL of water. The mixture was centrifuged for 5 minutes each time to separate aqueous and organic phases. A 5-mL aliquot of extract was dried under vacuum. The residue was reconstituted first with 80 μL of MTBE and then with 320 μL of methanol. A 20-μL aliquot was injected into the HPLC system. The protocol used was based on that of Olson and Frolik[18]. Each sample was analyzed in duplicate.

Analysis of kidney vitamin A and carotenoids

Kidney samples were thawed at room temperature. The two kidneys from the same animal were finely minced together using two scalpels. A 0.1-g aliquot of kidney tissue was mixed with 1 mL of freshly prepared 10% (w/v) ethanolic KOH containing 1% (w/v) pyrogallol. Then the tube was flushed with argon and wrapped with aluminum foil and capped. The tissue was saponified at 40°C for 30 minutes with constant stirring. After

saponification, the mixture was cooled and 1 mL of deionized water was added. Then 10 mL of diethyl ether/hexane (1:1, v/v) containing 0.01% (w/v) BHT was added to extract the carotenoids and vitamin A. The mixture was vortexed for 1 minute and then centrifuged for 10 minutes. The upper organic phase was saved and the extraction step was repeated. The upper organic phases were combined and washed twice with 15 mL of water. The mixture was centrifuged for 5 minutes each time. Then retinyl acetate was added as IS for retinol. Retinyl acetate was dissolved in ethanol (0.01% BHT); the absorbance was measured at 328 nm and the absorption coefficient of 1565 was used in calculating the concentration. Ethyl β-apo-8′-carotenoate (trans) in ethanol (0.01% BHT) was added as IS for β-carotene. The entire 20 mL of extract was dried under the vacuum and the residue was reconstituted with 40 μL of MTBE and then with 160 μL of methanol. A 80-μL aliquot was injected into the HPLC system. Each sample was analyzed in duplicate.

For the kidney assays, retinyl acetate was added as IS instead of N-(3-Hydroxyphenyl)-all-trans-retinamide which was used for liver assays. Because there was co-elution with N-(3-Hydroxyphenyl)-all-trans-retinamide and retinol in the kidney samples and there was better resolution between retinyl acetate and retinol on the HPLC column, retinyl acetate was chosen to be used as IS for the kidney assays.

Analysis of serum vitamin A and carotenoids

The frozen serum was thawed at room temperature. A 400-600 μL aliquot was mixed with 400-600 μL of enzyme reagent (see Appendix), and incubated at room temperature for 1 hour[19]. A 600 μL-volume of ethanol containing 0.01% BHT was added to deproteinate the serum. Ethyl β-apo-8′-carotenoate (trans) in ethanol (0.01% BHT) and retinyl acetate in

ethanol (0.01% BHT) were added as IS for β-carotene and retinol, respectively. The mixture was vortexed 30 seconds. A 5-8 mL-volume of hexane (0.01% BHT) was added to extract the carotenoids and vitamin A. The mixture was vortexed 1 minute and then centrifuged for 10 minutes. The upper phase was removed and saved. The extraction step was repeated. The upper organic phases were combined and dried under vacuum. The residue was reconstituted with 50 μL of MTBE and then with 150 μL of methanol. A 160-μl or 190-μL aliquot was injected into the HPLC system. Serum assays were not duplicated because of the limited available volume of sample.

Statistical analyse

Daily body weight data during the 6 week feeding period were analyzed by repeated-measures analysis of variance (ANOVA) with day as a covariate. Liver weight data were analyzed using one-way analysis of variance (ANOVA). Liver, kidney, and serum vitamin A and β-carotene data were analyzed using one-way analysis of variance (ANOVA) followed by the independent t-test (SPSS11.0) when there was a significant difference among groups. A P value < 0.05 was considered significant.

Results

Animals' growth

Each animal's body weight was recorded daily in order to monitor for signs of vitamin A deficiency. The animals gained weight steadily throughout the experimental period (Figure 1). According to the repeated-measures ANOVA, there was no evidence of a difference in weight gain among the 4 treatment groups. There were no other clinical signs of vitamin A deficiency.

Carotenoid content in the leaves

The *Arabidopsis* plants were grown under moderate light intensity. Under these conditions, the growth and development of *lut2* mutants were not visibly affected by the absence of lutein. There was no detectable difference in the appearance of the wild-type and *lut2* plants (Figure 2).

Our WT and *lut2 Arabidopsis* leaves were analyzed for carotenoid content (Table 1). The ratio of lutein (103.6 μg/g fresh leaves) to β-carotene (64.8 μg/g fresh leaves) content was 1.67:1 in WT leaves (Table 1). As seen in the chromatograms (Figure 3), in *lut2* leaves, there was no lutein detected and there was an increase in the concentrations of the xanthophyll cycle pigments, zeaxanthin, violaxanthin, and antheraxanthin, as compared with those in WT leaves, respectively (Table 1). There was also a higher content of β-carotene in *lut2* leaves than in WT leaves. According to Pogson et al[12], the contents of lutein, β-carotene, zeaxanthin, violaxanthin, and antheraxanthin in *lut2* leaves was 0 μg/g, 71 μg/g, 7 μg/g, 90 μg/g, and 36 μg/g, respectively, as compared with 133 μg/g, 57 μg/g, 0 μg/g, 30 μg/g, and 0 μg/g in WT leaves, respectively. The content of carotenoids in our leaves was remarkably similar to that of Pogson's group.

Carotenoid content in powdered, lyophilized leaves

After freeze drying and grinding, the powdered WT and *lut2* leaves were analyzed to determine whether freeze drying caused loss of carotenoids. Table 2 shows the carotenoid contents in the powdered leaves. Based on a comparison of the carotenoid content in fresh leaves, there was no degradation of β-carotene during the freeze drying and grinding processes. β-Carotene content in the powdered WT leaves was 752.6 μg/g powder, which

was roughly equivalent to 752.6 μg/10 g fresh leaf tissue or 75.26 μg/g fresh leaf tissue based on a water content of fresh Arabidopsis leaves of approximately 90%. In the fresh WT leaves, the β-carotene content was 64.8 μg/g fresh leaf tissue. The lutein content in powdered WT leaves was roughly equivalent to 124.6 μg/g leaf tissue as compared with 103.6 μg/g fresh leaf tissue. In powdered *lut2* leaves, β-carotene content was roughly equivalent to 94.7 μg/g fresh leaf tissue as compared with 88.2 μg/g fresh leaf tissue. Based on estimated water content of the fresh leaves, there were also increases in neoxanthin, violaxanthin and antheraxanthin in WT and *lut2* leaves powder, respectively, compared with the WT and *lut2* leaves, respectively. Those might be explained by further drying during the grinding process, during which the heat was generated, so the contents were more concentrated than those in the leaves before grinding. Thus, the actual water content in the leaves would be expected to have been higher than 90%, which was estimated from the freeze drying process. Because we used the leaf powders in the experimental diets and analyzed carotenoid content in the leaf powders and in the diets, the changed water content did not affect the accuracy of the data.

Experimental diets

The β-carotene and lutein contents in the WT and *lut2* diet (Table 3) were lower than expected based on the added amount of powdered leaves. The WT leaf powder was expected to contribute 145.4 nmol β-carotene/g diet. The amount of the *lut2* powder added to the *lut2* diet was adjusted to provide β-carotene content equivalent to that in WT diet. But analysis of the WT, *lut2* and purified β-carotene diets in our laboratory showed that there was only 69.2 nmol β-carotene/g WT diet and 61.3 nmol β-carotene/g *lut2* diet. The amount of β-carotene

added to the purified β-carotene diet was calculated to be 6-fold less (by wt) than the β-carotene content in the WT and *lut2* diets, and should have been 24.2 nmol β-carotene/g purified β-carotene diet. Analysis in our laboratory showed there was 22.0 nmol β-carotene/g purified β-carotene diet, which was approximately consistent with what we expected.

WT and *lut2* diets were also analyzed for the contents of xanthophyll cycle pigments and neoxanthin (Table 3). There were also some losses in those carotenoids.

Because heat was used during the pelleting of the diet and carotenoids are not stable under heat, some destruction of carotenoids may have occurred.

The composition of the experimental diets is shown in Table 4. Diets were adjusted with corn starch, casein, inulin, and cellulose to have similar contents of carbohydrate, protein, soluble and insoluble fiber, as well as the same amount of fat.

α-Tocopherol and chlorophyll contents in powdered, lyophilized leaves

α-Tocopherol is the predominant tocopherol in *Arabidopsis* leaves[20]. The WT and *lut2* diets were analyzed for α-tocopherol contents, which were 56.53 μg/g diet (equivalent to 56.53 μg/g leaves) and 43.23 μg/g diet (equivalent to 43.23 μg/g leaves), respectively. Because α-tocopherol was also added as a dietary component, the α-tocopherol content in the WT diet was higher compared with 19.1 pmol/mg (8.23 μg/g) α-tocopherol in the WT *Arabidopsis* leaves which was reported by Callakova and DellaPenna[20].

Chlorophyll contents and chlorophyll a-to-chlorophyll b (Chl a-to-Chl b) ratio were measured in the WT and *lut2* leaves (Table 5). There was a difference in the chlorophyll a content between the WT and *lut2* leaves. There was also a difference in the chlorophyll b

contents between the WT and *lut2* leaves. There was no apparent difference in the Chl a-to-Chl b ratios between the WT and *lut2* leaves. According to Pogson et al[12], there were no significant differences between *lut2* and wild-type *Arabidopsis* plants in Chl a, Chl b, or the Chl a-to-Chl b ratio. We also analyzed the chlorophyll a and chlorophyll b contents in the powders (Table 6). The chlorophyll a and chlorophyll b contents in the powdered WT and powdered *lut2* leaves were similar. The Chl a-to-Chl b ratios between the powdered WT and powdered *lut2* leaves were also very similar. Those results are consistent with those of Pogson et al[12]. There were apparent increases in the chlorophyll contents in the leaf powders after grinding, which were similar to those observed in the carotenoid contents. The discrepancy between the chlorophyll contents in leaves and powdered leaves may again be explained by further drying during grinding. Also, we did not perform measurements on multiple samples so sampling error may be another explanation for the discrepancy.

Liver vitamin A and β-carotene contents

At the end of the 6-wk feeding period on the experimental diets, the purified β-carotene group had the highest liver vitamin A stores (Table 7). The liver vitamin A stores were higher in the WT, *lut2*, and purified β-carotene groups than in the β-carotene-free group which had the lowest liver vitamin A stores ($P < 0.05$). The difference in liver vitamin A stores in gerbils fed purified β-carotene or *lut2* leaves was not statistically significant ($P > 0.05$). Thus only 7 animals in that group were included in the statistical analysis.

There was also a difference in liver vitamin A stores between *lut2* and WT groups ($P = 0.005$). Liver vitamin A stores were 48% higher in gerbils fed *lut2* leaves than in those fed WT leaves. Liver vitamin A stores were 2-fold higher in gerbils fed purified β-carotene than

in those fed WT leaves ($P = 0.003$). Given the 3-fold higher β-carotene content in the WT diet, our results are consistent with the 6:1 retinol activity equivalency (RAE) ratio of β-carotene in plant matrices and in purified form in humans.

There was no significant change in the liver vitamin A stores in the baseline group and 4-week depletion group, which was 1.41 ± 0.27 μmol, and 1.21 ± 0.11 μmol, respectively. When these data were compared with the liver vitamin A stores in the β-carotene-free group, after 6 additional weeks on the depletion diet, the animals' liver vitamin A stores decreased as expected.

As shown in Table 7, there were no significant differences in liver β-carotene content among the treatment groups. At the end of the study, no β-carotene was detected in the livers of the gerbils in the β-carotene-free group. No lutein was detected in liver tissues in any of the groups.

Like humans, gerbils accumulate intact β-carotene in tissues[13, 15], which was confirmed in this study. β-Carotene concentrations in normal human livers range from 5.8 to 25 nmol/g. In this study, the range of liver weights and liver β-carotene content in gerbils was 1.6 to 2.4 g and 29.6 to 74.3 nmol, respectively. Thus the liver β-carotene concentrations ranged from 16.1 to 37.8 nmol/g, which were similar to those reported in humans.

At the end of the study, the liver weights ranged from 1.96 to 2.13 g in the WT diet group; from 1.8 to 2.37 g in the *lut2* diet group; from 1.62 to 2.18 g in the purified β-carotene diet group; and from 1.65 to 2.41 g in the vitamin A-free diet group. There were no

significant differences in the liver weights among the 4 treatment groups using one-way ANOVA.

Kidney vitamin A and β-carotene contents

There were no significant differences in the kidney vitamin A stores among the 4 treatment groups (Table 8), which can be explained by adequate liver vitamin A stores to maintain vitamin A homeostasis. There was no β-carotene or lutein detected in kidneys in the animals in any of the groups.

Serum retinol and β-carotene contents

Serum retinol and β-carotene concentrations are shown in Table 9. There were no significant differences in the serum retinol concentrations among the 4 treatment groups. Similarly, there were no significant differences in the serum β-carotene concentrations among the 4 treatment groups. There was no lutein detected in any of the groups.

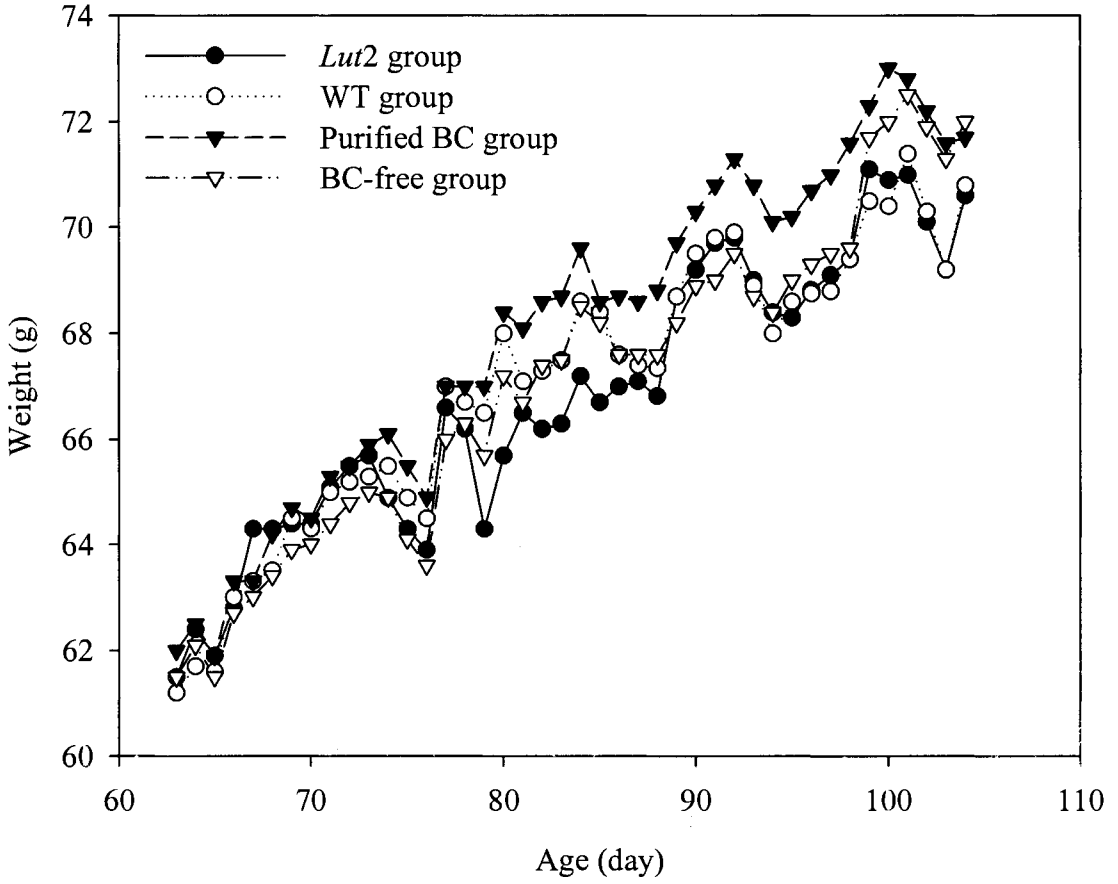

Figure 1. The body weights of gerbils in the *lut2*, wild-type (WT), purified beta-carotene, and beta-carotene-free diet groups measured daily during the 6-week feeding period on the experimental diets. Each point represents the average weight of the animals in each group. There was no significant difference in weight gain analyzed by repeated measures ANOVA with day as covariate.

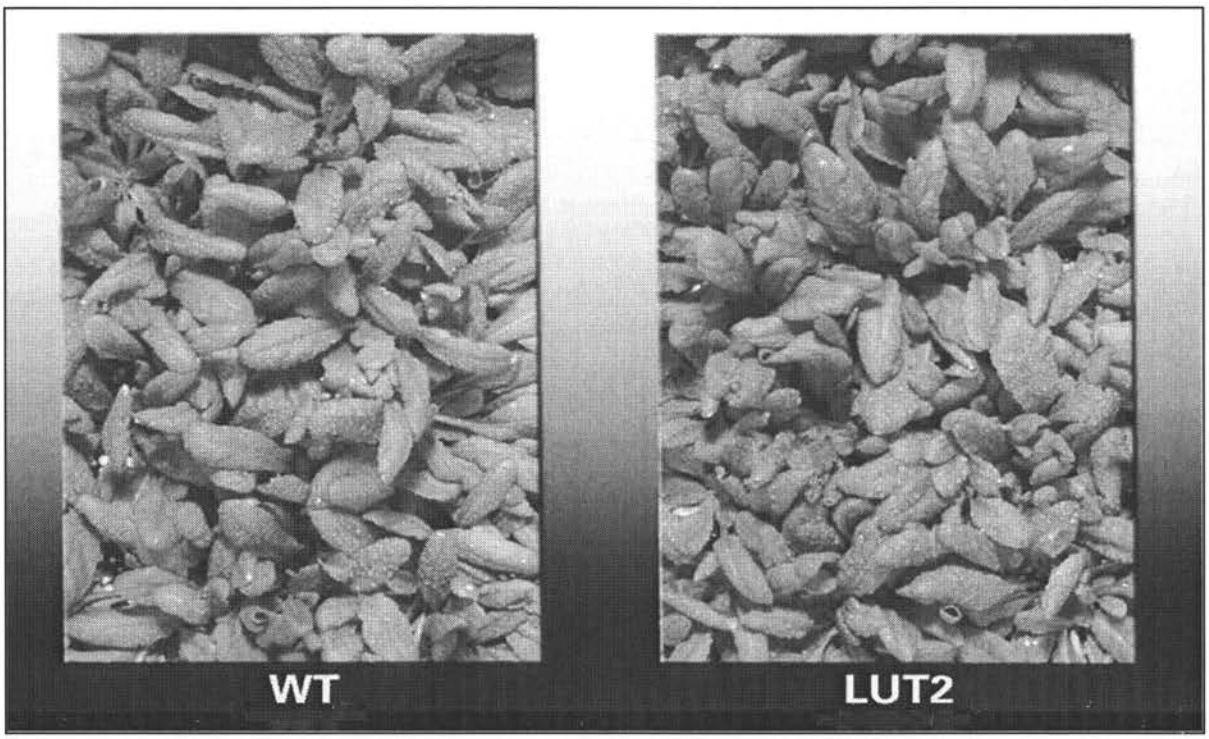

Figure 2. There were no phenotypic differences in the wild-type (WT) and *lut2 Arabidopsis* plants.

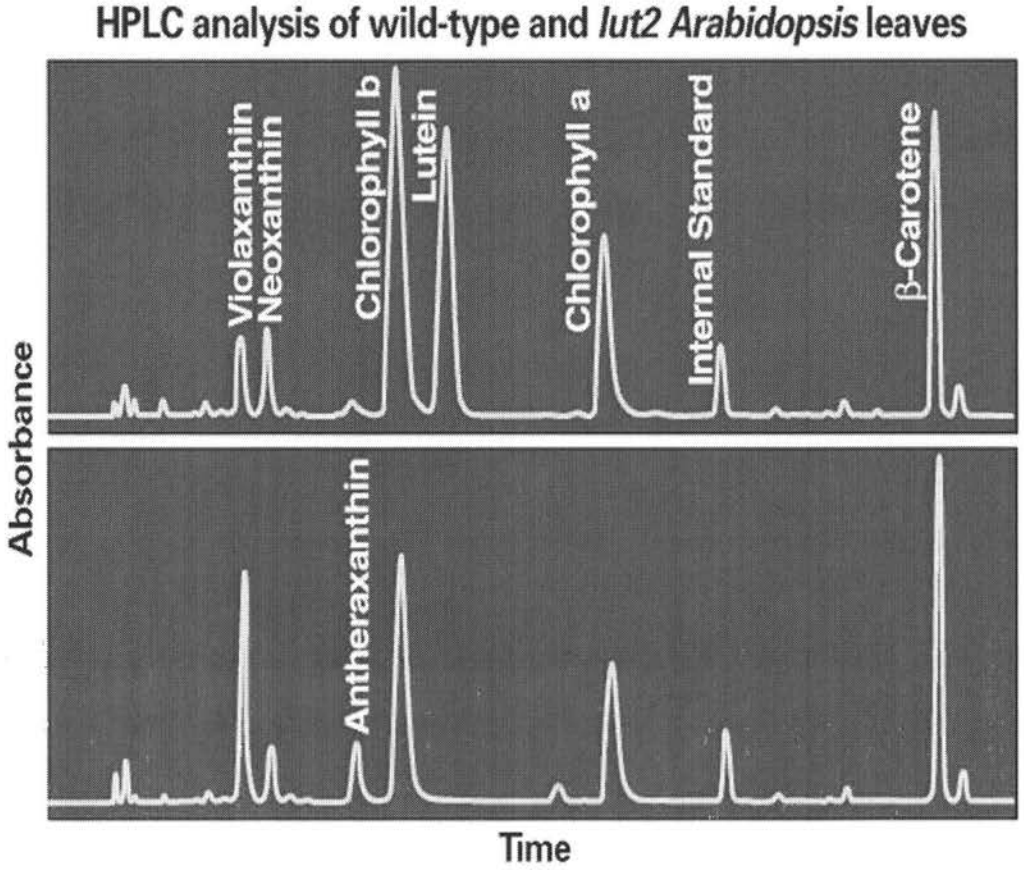

Figure 3. HPLC chromatograms of wild-type (upper) and *lut2* (lower) *Arabidopsis* leaves. No lutein was detected in *lut2* leaves.

Table 1. Carotenoid content in fresh WT and *lut2* leaves

Genotype	Neoxanthin	Violaxanthin	Antheraxanthin	Zeaxanthin	β-Carotene	Lutein
			μg/g leaves			
WT	32.5	24.0	5.1	-	64.8	103.6
lut2	20.8	72.0	18.9	6.0	88.2	-

Table 2. Carotenoid content in powdered WT and *lut2* leaves

Genotype	Neoxanthin	Violaxanthin	Antheraxanthin	Zeaxanthin	β-Carotene	Lutein
			μg/g powder			
WT	394.9	347.6	72.2	-	752.6	1246.2
lut2	320.6	983.3	282.2	93.5	946.6	-

Table 3. Carotenoid content in wild-type and *lut2* diets

Genotype	Neoxanthin	Violaxanthin	Antheraxanthin	Zeaxanthin	β-Carotene	Lutein
			μg/g diet			
WT	14.7	-	2.2	-	37.1	75.2
lut2	-	2.5	1.2	3.3	32.9	-

Table 4. Macronutrient composition of the experimental diets

	Protein	Carbohydrate	Fat	Fiber, soluble	Fiber, insoluble
			% (wt/wt)		
WT	16.5	54.6	15.6	1.0	4.9
lut2	16.6	54.9	15.7	1.0	4.9
Purified β-carotene	16.9	55.9	16.0	1.0	5.0
β-Carotene-free	16.9	55.9	16.0	1.0	5.0

Table 5. Chlorophyll a and chlorophyll b contents in fresh WT and *lut2* leaves

Genotype	Chl a	Chl b	Chl a/Chl b
	μg/g	μg/g	
WT	744.88	298.59	2.50
lut2	596.12	218.54	2.73

Table 6. Chlorophyll a and chlorophyll b contents in powdered WT and *lut2* leaves

Genotype	Chl a	Chl b	Chl a/Chl b
	μg/0.1g	μg/0.1g	
WT	910.28	335.54	2.71
lut2	847.12	307.87	2.75

Table 7. Liver vitamin A and β-carotene stores in the treatment groups at the end of the 6-week feeding period

Diet Group	Vitamin A[1]	β-Carotene[1]
	μmol	nmol
WT	1.99 ± 0.10[a]	48.66 ± 9.32
lut2	2.94 ± 0.14[b]	46.05 ± 11.00
Purified β-carotene	3.80 ± 0.27[b]	54.38 ± 5.31
β-Carotene-free[2]	0.45 ± 0.08[c]	Not detected

[1]Values represent group means ± SEM. Values with different superscripts are significantly different (independent t-test), $P < 0.05$.

[2]One animal was dropped from the β-carotene-free group as an outlier because it had abnormally high liver vitamin A store (2.11 μmol) compared with the mean of the other animals.

Table 8. Kidney vitamin A stores in the treatment groups at the end of the 6-week feeding period

Diet Group	Vitamin A[1]
	nmol
WT	1.55 ± 0.06
lut2	1.60 ± 0.12
Purified β-carotene	1.46 ± 0.09
β-Carotene-free	1.42 ± 0.07

[1]Values represent group means ± SEM.

Table 9. Serum retinol and β-carotene concentrations in the treatment groups at the end of the 6-week feeding period

Diet Group	Retinol[1]	β-Carotene[1]
	μmol/L	nmol/L
WT	2.57 ± 0.16	7.61 ± 1.38
lut2	2.49 ± 0.17	6.89 ± 2.30
Purified β-carotene[2]	2.60 ± 0.11	5.18 ± 1.17
β-Carotene-free	2.22 ± 0.14	not detected

[1]Values represent group means ± SEM.

[2]One animal was dropped from the purified β-carotene group due to sample loss.

Discussion

Vitamin A deficiency is a severe problem in many developing countries, and especially in South-East Asia[21]. It is the leading cause of preventable blindness in children[21] and is associated with increased severity of infections[22] and higher risk of mortality[23]. β-Carotene is the major provitamin A carotenoid in fruits and vegetables, and it has the greatest provitamin A activity. But vitamin A deficiency is still endemic in areas of world where dark green leafy vegetables, such as spinach and cassava leaves are widely consumed. The low bioavailability of β-carotene in vegetables, especially in dark green leafy vegetables has been observed in many studies[2, 3, 9, 24, 25]. Low dietary fat intake[26, 27], dietary fiber[28], complex food matrix[2, 3, 29], nutritional status[30] and interactions with other carotenoids[5] can contribute to the poor bioavailability and bioconversion of β-carotene to vitamin A. Particularly, the interactions between β-carotene and lutein have been studied[8, 9, 10, 25, 31, 32], which showed the inhibitory effects of lutein on the utilization of β-carotene when lutein is the dominant carotenoid ingested. Because lutein is the predominant and most abundant carotenoid in green leaves[12], an interaction between β-carotene and lutein might explain the prevalence of vitamin A deficiency in developing countries despite high intakes of β-carotene from leafy vegetables. The limitation of previous studies designed to investigate interactions between β-carotene and lutein is the use of purified β-carotene and lutein instead of the carotenoids in leafy vegetable matrix form, which is the major provitamin A source in developing countries.

This study was designed to compare the bioavailability of β-carotene in a leaf matrix, which is the *Arabidopsis thaliana* plant, fed to a rodent model. We used *Arabidopsis* to model dark green leafy vegetables in this study because: 1) the leaves of all species of higher

plants have a strikingly constant carotenoid pattern[33], which implies the carotenoid profile of *Arabidopsis* is similar to that in dark green leafy vegetables; 2) we took advantage of the existence of *lut2*, which is a well-characterized lutein-deficient mutant of *Arabidopsis*. By comparing the bioavailability of β-carotene from WT and *lut2 Arabidopsis* leaves fed to an animal model, we were able to assess the inhibitory effect of lutein on utilization of β-carotene.

Mongolian gerbils were used for this study. Gerbils absorb β-carotene intact when ingested at a physiological level[13], and store β-carotene in both serum and tissues, such as liver, kidney, spleen, and adrenals[14, 15]. Particularly, gerbils convert β-carotene to vitamin A with similar conversion efficiency to humans[15]. Thus the gerbil is a good model to evaluate the bioavailability and metabolism of β-carotene. In this study, we didn't detect β-carotene in the animals' kidneys, which may be explained by either the β-carotene contents in the experimental diets were not high enough to accumulate in the animals' kidneys or the β-carotene concentrations in the kidneys were too low to be detected by HPLC. Because it is difficult to deplete hepatic vitamin A stores in gerbils[14, 15], the gerbils in our study were fed a β-carotene-free vitamin A-free diet for 4 weeks to lower their liver vitamin A stores before they were fed the experimental diets. The liver vitamin A stores at the end of the 4-week depletion period was lower than those in the baseline groups. The liver vitamin A stores in the β-carotene-free group at the end of the 6-week feeding period was lower compared with those in the 4-week depletion group. Thus the animals' liver vitamin A stores were decreased when fed a β-carotene-free vitamin A-free diet for 10 weeks.

Liver vitamin A stores were highest in the purified β-carotene diet group which is consistent with the higher bioavailability of purified β-carotene compared with β-carotene in vegetable matrices. Liver vitamin A stores were 2-fold higher in gerbils fed purified β-carotene than in those fed WT leaves. The liver vitamin A stores in the *lut2* diet group were 1.5-fold higher than in the WT diet group. Comparing the macronutrient contents (Table 4) and carotenoid contents (Table 3) between WT and *lut2* diets, we can see that the two diets were similar in composition except for the large difference in lutein content. As seen in Table 3, there was a higher β-carotene content in the WT diet than in the *lut2* diet, but the *lut2* group had higher liver vitamin A stores at the end of the study. Also, although there were differences in the content of xanthophyll cycle pigments in the WT and *lut2* diets (Table 3), these differences were less marked than the substantial difference in lutein content. Thus the data suggest there were interactions between β-carotene and lutein ingested in *Arabidopsis* leaves. The bioavailability of β-carotene from lutein-free *lut2 Arabidopsis* leaves is higher than from WT *Arabidopsis* leaves in Mongolian gerbils. The data suggest lutein is the factor that caused the treatment effect.

There was no lutein detected in serum or tissues in any of the treatment groups. Thus we concluded that lutein was not absorbed by the gerbils. There is the possibility that lutein could inhibit β-carotene being taken up into the mucosa of the small intestine or β-carotene being cleaved to vitamin A in the enterocyte. In this study, lutein most likely interacted with β-carotene before β-carotene was absorbed. Based on the rough calculation in each group of the conversion of absorbed β-carotene to vitamin A using the liver vitamin A and β-carotene content data, and assuming a molar conversion efficiency of 2:1. The liver vitamin A store ÷

2/(liver vitamin A store ÷ 2 + liver β-carotene store), the conversion was almost the same in WT (1.99 ÷ 2/(1.99 ÷ 2 + 0.04866)= 95.34%), *lut2* (2.94 ÷ 2 /(2.94 ÷ 2 + 0.04605) = 96.96%) and purified β-carotene (3.8 ÷ 2/(3.8 ÷ 2 + 0.05438) = 97.22%) diet groups. So it seems that lutein inhibited β-carotene absorption instead of β-carotene cleavage to vitamin A. Because the small intestines of the gerbils were not collected in this study, we could not determine whether lutein was taken up into the enterocyte. Further work needs to be done to better understand the mechanism of interaction in the small intestines of this species.

β-Carotene content in the WT diet was 3-fold higher than in the purified β-carotene diet and liver vitamin A stores were 2-fold higher in gerbils fed purified β-carotene than in those fed WT leaves. Our results are consistent with the 6:1 retinol activity equivalency (RAE) ratio of β-carotene in plant matrices and in purified form in human diets[34]. Thus our study supports the conclusion that conversion efficiency in gerbils is similar to that in humans[15]. The accumulation of β-carotene in serum and tissues in this species is also consistent with previous reports[14, 15].

Throughout the study, there was no sign of vitamin A deficiency in any of the animals, which is consistent with the reported difficulty in depleting liver vitamin A stores in this species[14, 15] (Figure 1). This could explain the similar kidney and serum vitamin A stores in the 4 treatment groups because the animals' liver vitamin A stores were not exhausted and thus they were able to maintain vitamin A homeostasis.

We found in this study that lutein is not absorbed or accumulated in serum or tissues in this species. Unlike most species, humans are indiscriminate accumulators and accumulate both carotenes and xanthophylls[35]. Thus the gerbil is not an appropriate model

for extrapolation to humans when studying lutein metabolism. Our findings may have application for production of animal feeds, such as cattle feed. Like gerbils, some cattle absorb significant amounts of β-carotene from the diet but do not efficiently absorb xanthophylls[36]. That which is not converted to vitamin A is deposited intact in tissues. Genetic modification such as to eliminate or decrease the amount of lutein in, e.g., feed corn, may be beneficial in improving the bioavailability of β-carotene in cattle. β-Carotene levels are related to luteal function[37, 38] and bovine fertility[39]. Although our finding does not extrapolate to humans, for the first time, we have shown carotenoid-carotenoid interactions when ingested within a plant matrix. Our study shows that genetic modification may be a good approach to improve the bioavailability of certain carotenoids and may be useful in combating vitamin deficiency in developing countries.

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GENERAL CONCLUSIONS

Vitamin A deficiency is a public health problem in about 76 countries, especially in South-East Asia[1]. The amount and bioavailability of dietary provitamin A carotenoids determine whether vitamin A requirements are met in developing countries[2]. β-Carotene is the major provitamin A carotenoid in fruits and vegetables. Dark green leafy vegetables are widely consumed in developing countries where vitamin A deficiency is prevalent thus the low bioavailability of β-carotene in these vegetables has been concern. The food matrix[3, 4, 5], interactions with fat and fiber[6, 7, 8], nutritional status[9], and interactions with other carotenoids[10] can affect the bioavailability and bioconversion of carotenoids. In particular, lutein has inhibitory effects on utilization of β-carotene[11, 12, 13, 14, 15, 16] when lutein is the dominant carotenoid ingested. Lutein is the predominant carotenoid in dark green leaves[17], which might explain the prevalence of vitamin A deficiency in developing countries despite high consumption of leafy vegetables.

The interactions between lutein and β-carotene in previous studies were investigated when carotenoids were ingested in purified form instead of in the plant matrix from which most of the world's populations ingest carotenoids. In our study, we used *Arabidopsis* leaves as a model for dark green leafy vegetables due to the remarkably constant carotenoid composition in leaves of higher plants and the availability of a well-characterized lutein-deficient mutant of *Arabidopsis*, *lut2*. We investigated the interactions of lutein and β-carotene in a rodent model, the gerbil, which β-carotene metabolism similar to humans[18, 19, 20]. We found that the β-carotene is more bioavailable in *lut2* leaves than WT leaves fed to gerbils. Our data suggest lutein has an inhibitory effect on the bioavailability of β-carotene in dark green leaves of higher plants in this rodent model. We also found that lutein was not

absorbed in gerbils so this study may not extrapolate to humans. However, for the first time, we have shown carotenoid-carotenoid interactions when ingested within a plant matrix. This study has also shown the potential for genetic modification to modulate the bioavailability of β-carotene to combat vitamin A deficiency in developing countries.

Further study should be conducted to investigate whether lutein is taken up into the enterocyte to better understand lutein-β-carotene interactions in the small intestine of this species.

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APPENDIX A

ENZYME REAGENT FOR SERUM RETINOL ASSAY

1) Prepare 0.1 M monobasic solution. Monobasic is $NaH_2PO_4 \cdot H_2O$ (molecular weight 137.99 g).

0.1 M = 0.1 mol/L = 0.1 × 137.99 g/L = 13.799 g/L = 1.3799 g/100 mL.

1.3799 g of $NaH_2PO_4 \cdot H_2O$ is dissolved in 100 mL of deionized water to prepare 0.1 M monobasic solution.

2) Prepare 0.1 M dibasic solution. Dibasic is $Na_2HPO_4 \cdot 12H_2O$ and the molecular weight is 358.14 g.

0.1 M = 0.1 mol/L = 0.1 × 358.14 g/L = 35.814 g/L = 3.5814 g/100mL.

3.5814 g of $Na_2HPO_4 \cdot 12H_2O$ is dissolved in 100 mL of deionized water to prepare the 0.1 M dibasic solution.

3) The buffer is prepared by mixing 39 mL of 0.1 M monobasic ($NaH_2PO_4 \cdot H_2O$) and 61 mL of 0.1 M dibasic ($Na_2HPO_4 \cdot 12H_2O$). The pH is measured using a pH meter. The pH of the buffer is 7.0. Then 0.1 mL of Triton-X-100 (Sigma-Aldrich, St. Louis, MO, USA) is added to the buffer.

4) 1000 U cholesterol esterase (Calbiochem-Novabiochem Corp., San Diego, CA, USA) and 100,000 U triacylglycerol lipase (Sigma-Aldrich) were stored at -20°C until they were added to the buffer. The powder residues in the bottles were rinsed into the buffer using buffer. The enzyme solution was stored in 500-μL aliquots in 1.5-mL microcentrifuge tubes at -20°C. Each aliquot was used once to avoid detrimental effects of freeze-thaw cycles on enzyme activity.

The protocol used in preparing the enzyme reagent was based on that of Handelman et al (1999), in which for 100 μL of plasma samples, 1 U cholesterol esterase and 100 U triacylglycerol lipase were added. For the 400 μL, 500 μL or 600 μL plasma samples we used for our study, 400 μL, 500 μL and 600 μL of enzyme reagent were used, respectively. At the time of the serum assay, the frozen serum was thawed at room temperature. Then the available volume of serum was transferred to a culture tube (16 × 100 mm, Fisher Scientific). An equal volume of enzyme reagent was added and the mixture was allowed to incubate at room temperature for 1 hour during which light was avoided. Then the same procedure described in the Materials and Methods Section was followed.

APPENDIX B

LIVER WEIGHT OF EACH ANIMAL IN BASELINE, 4-WEEK DEPLETION, VITAMIN A-FREE, WILD-TYPE, LUT2, AND PURIFIED BETA-CAROTENE GROUPS

Baseline	4-Week depletion	Vitamin A-free	WT	*Lut2*	β-Carotene
		g			
0.85	2.19	1.67	2.1	2.37	1.96
0.87	2.1	1.96	2.13	1.8	2.18
1.19	1.51	2.41	1.96	1.84	2.09
1	1.92	1.82			1.82
1.06	1.67	1.65			2.04
	1.7	1.71			1.86
	2	1.78			1.62
	1.95	1.68			1.9

ACKNOWLEDGMENTS

First, I would like to thank my major professor, Dr. Wendy White, for her expertise and guidance in this project and for her tremendous help during my study and work. Also I would like to thank her for the knowledge and everything I learned from her.

I would like to thank Dr. Steven Rodermel, my co-major professor, for his generous help with this project and for providing greenhouse and growth chamber space for growing the *Arabidopsis* plants.

I also would like to thank Cody Sanderson, a graduate student working under the direction of Dr. Rodermel. He invested considerable time and effort in growing *Arabidopsis* plants, which was a significant contribution to this study. Also, I learned from Cody how to grow the plants.

I would like to thank Dr. Dean DellaPenna from Department of Biochemistry and Molecular Biology, Michigan State University who kindly donated the lut2 seeds.

I would like to give thanks to Dr. Ed Ulman, Research Diets, Inc (New Brunswick, NJ) for his assistance in formulating the purified diets.

I greatly appreciate the assistance of Dr. Kathleen Mullin, Veterinarian, who collected the blood samples by cardiac puncture.

Trish Patrick, Research Associate, kindly allowed us to use the grinder in her laboratory in Agronomy Hall.

I also appreciate the help from my lab mates and those who ever kindly helped me with my work, and even my friends who supported me when I needed them.

Of course, I need to give thanks to my parents and my sister for their unconditional love and support during my study.