Vitamin A intake requirements in women:

Evaluating the Estimated Average Requirement using stable isotope dilution

By

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Index of Abbreviations

At%, atom percent

$^{13}C_2$-RID, $^{13}C_2$-retinol isotope dilution

CIC, conjunctival impression cytology

DRD, deuterated-retinol-dilution

DRI, Dietary Reference Intakes

EAR, estimated average requirement

FFQ, food frequency questionnaire

IOM, Institute of Medicine

GCCIRMS, gas chromatography-combustion-isotope ratio mass spectrometer

OC, oral contraceptive

RAE, retinol activity equivalents

RBP, retinol binding protein

RDA, recommended dietary allowance

TBR, total body reserves

TLR, total liver reserves

UL, Tolerable Upper Intake Level

UW, University of Wisconsin

VA, vitamin A

3DDR, three-day diet record

ABSTRACT

Vitamin A (VA) is an essential nutrient that is important for visual, reproductive, and immunological health. VA status is difficult to measure because liver biopsy is the gold standard. The estimated average requirement (EAR) for VA is 500 μg for non-pregnant, non-lactating women aged 18 – 30 y. The recommended dietary allowance (RDA) is equal to the EAR plus two times a CV of 20%, *i.e.*, 700 μg per day. The EAR is a calculated intake to maintain a minimally-acceptable liver concentration of 20 μg VA/g. This concentration is assumed to prevent clinical signs of deficiency, maintain adequate plasma VA concentrations, allow biliary excretion of VA, and protect an individual consuming a VA-deficient diet against clinical signs of deficiency for four months. The supporting data came from three studies, two in animals and one in men with a sample size of eight, conducted in the late 1970s and early 1980s. Liver reserves have not been evaluated with respect to VA intake in women of any age group defined in the Dietary Reference Intakes (DRI).

The $^{13}C_2$-retinol isotope dilution ($^{13}C_2$-RID) test is a minimally-invasive method for measuring VA status. We collected dietary VA intake data and applied the $^{13}C_2$-RID test of VA status to evaluate the EAR in a series of studies of young adult women. Baseline VA intake was about 60% greater than recommended and baseline liver reserves were nearly 7 times higher than the minimally-acceptable liver concentration in our sample. A group of women, who underwent an intervention restricting VA intake to 25, 50, or 100% of the RDA for six weeks, did not change status. From this study, we estimated an EAR to maintain status of approximately 300 μg/day, which is lower than the current EAR. The EAR for VA may need to be reduced for healthy, well-nourished women, in order to maintain safe liver reserves. Similar studies could be conducted in all the life-stage and gender groups of the DRI to improve our current recommendations for daily VA consumption.

CHAPTER 1

INTRODUCTION: Evaluating the estimated average requirement for vitamin A in light of improved technology

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The recommended dietary allowance (RDA) for vitamin A is based on a limited number of animal and human studies conducted in the late 1970s and early 1980s. Since then, increasingly sophisticated techniques for determining vitamin A status in humans have been developed. These are the stable isotope dilution methods known as the deuterated-retinol dilution and $^{13}C_2$-retinol isotope dilution techniques. They are minimally-invasive and can quantitatively estimate the total body pool of vitamin A. Paired with vitamin A intake data these techniques are a powerful way to evaluate the current recommended intakes of human subjects. This chapter summarizes the basic functions of vitamin A and definitions of vitamin A status, examines how the current RDA was set, reviews indicators of vitamin A status, and discusses how isotope dilution methods can be used to evaluate the RDA.

Vitamin A: functions and status

Vitamin A is a fat-soluble micronutrient essential to humans for normal cell differentiation, vision, reproduction, growth, and a healthy immune system. It is found in the diet as both the "preformed" vitamin (*i.e.*, in the form of vitamin A esters) and in "provitamin A" carotenoids (predominantly β-carotene, α-carotene, and β-cryptoxanthin), which are cleaved *in vivo* to yield vitamin A. The provitamin A carotenoids provide varying amounts of vitamin A and their bioefficacy (*i.e.*, the total amount of vitamin A formed *in vivo* per carotenoid consumed) is dependent on multiple factors, including the nutritional status of the host (1). However, the Institute of Medicine (IOM) has proposed standard conversion factors that are independent of vitamin A status. The conversion factors describe vitamin A in terms of retinol activity equivalents (RAE) where 1 RAE is equivalent to 1 µg all-*trans*-retinol (*i.e.*, vitamin A), 2 µg supplemental all-*trans*-β-carotene, 12 µg dietary all-*trans*-β-carotene, 24 µg

of α-carotene, and 24 μg of β-cryptoxanthin (2). In a healthy, well-nourished individual, about 80 – 90% of total body vitamin A is stored in the liver, mainly in the form of the vitamin A ester, retinyl palmitate (3). Liver stores decrease as the total body pool of vitamin A decreases, and proportionally less vitamin A remains in the liver such that less than 50% of the total pool may be found in the liver during deficiency (2).

Vitamin A deficiency and toxicity are well-defined by their respective clinical manifestations. Clinical signs of deficiency include a spectrum of symptoms that are associated with xerophthalmia, or "dry-eye" syndrome. These include night-blindness, follicular hyperkeratosis, and Bitot's spots (build-up of keratin in hair follicles and the eye, respectively), corneal xerosis, corneal scar, and subsequent irreversible blindness (4). It has been proposed that clinical signs of chronic deficiency appear when liver reserves are < 10 μg retinol/g liver (5). Less specific consequences of vitamin A deficiency include immune dysfunction (6, 7) and increased mortality risk, especially among infants and children (8-10). Chronic toxicity is clinically evident by skin changes, liver abnormalities, such as cirrhosis, and birth defects in infants of mothers consuming excess vitamin A during pregnancy. Acute toxicity can result in increased intracranial pressure, blurred vision, nausea, and vomiting. Both vitamin A deficiency and toxicity (acute and chronic) can lead to death.

Less clear are the impact and definition of subclinical deficiency or toxicity. It is likely that immune function is compromised in subclinical deficiency resulting in increased morbidity and mortality risk (11). It has been proposed that liver reserves less than 30 μg retinol/g liver should be considered subclinical deficiency as an enhanced biological response to a challenge dose of vitamin A_2 is observed below this reserve in piglets (12). "Subclinical hypervitaminosis A" may have impact on bone health, causing an increased risk of

osteoporosis and fracture, although studies have shown mixed results (13, 14). A liver reserve of ≥ 200 μg retinol/g was defined as "excessive" and ≥ 300 as "toxic" by Olson in 1990 (15), but there are no data in humans to support these cut-offs or describe the clinical consequences (12).

Even less clear is "adequate vitamin A status" and the appropriate intake required to maintain it. Ideally, an appropriate daily intake would maintain the total body pool, and thus liver reserves, at a range that protects against deficiency and toxicity. It should also avoid subclinical insufficient and excessive states. Unfortunately, there are very few studies that examine long-term daily intake with regards to any measure of vitamin A status, making it challenging to set a recommendation. In 1987, Dr. Olson proposed 20 μg/g as a minimal "liver vitamin A concentration that prevents deficiency, provides a suitable reserve for periods of stress and/or low intake, and is fully consistent with good health (5)." This value was chosen because, purportedly, 1) no clinical signs of deficiency are observed, 2) plasma retinol is maintained, 3) induced biliary excretion is observed, and 4) there is approximately four months of protection against vitamin A deficiency on a vitamin A-free diet (2, 5). The assumption that 20 μg retinol/g liver maintains plasma vitamin A and induces biliary excretion of vitamin A is based on rat studies (16, 17). The assumption of four months protection against a vitamin A-deficient diet is from a study of eight "healthy" male prisoners published in 1974 (18). Of note, this liver concentration is less than the proposed cut-off for subclinical deficiency (< 30 μg/g) based on data from piglets (12).

Vitamin A intake requirements: the EAR and RDA

In the US, the recommendation for vitamin A intake is based on an estimated average requirement (EAR). The EAR is the "average daily nutrient intake that is estimated to meet the nutrient needs of half of the healthy individuals in a life stage or gender group" and is based on published data (2). The EAR for vitamin A is 625 and 500 μg RAE daily for men and women age 18 y and over, respectively. Surprisingly, there was a paucity of experimental data in humans that was useful for setting the EAR for vitamin A in 2001. In fact, the EAR for vitamin A was based on Olson's definition of minimal liver vitamin A concentration and his calculation for an appropriate intake to maintain it:

$$\text{Daily Intake} = A \times B \times C \times D \times E \times F$$

Where,

A = Percent body vitamin A stores lost daily when consuming a diet devoid of vitamin A (*i.e.*, 0.5%) (5, 18)

B = Minimum acceptable liver reserves (*i.e.*, 20 μg/g) (5)

C = Liver weight:body weight (*i.e.*, 1:33, or liver ≈ 3% of body weight)

D = Reference weight (kg) for age/gender (*i.e.*, 61 for adult women and 76 for adult men, IOM 2001 definition) (19)

E = Total body vitamin A:liver vitamin A (*i.e.*, 10:9 in well-nourished populations)

F = Efficiency of storage of orally consumed vitamin A (*i.e.*, 2.5, assuming 40% of an oral dose of vitamin A gets stored in the liver) (20)

The recommended dietary allowance (RDA) for a nutrient is an estimate of intake that should meet the needs of 97 – 98 percent of the population and is calculated based on the EAR. For vitamin A, the RDA is the EAR plus two times a coefficient of variance of 20% (*i.e.*, for vitamin A, the RDA = 1.4*EAR), rounded to the nearest 100 (2). The RDA for vitamin A is 900 and 700 μg RAE daily for adult men and women, respectively (2).

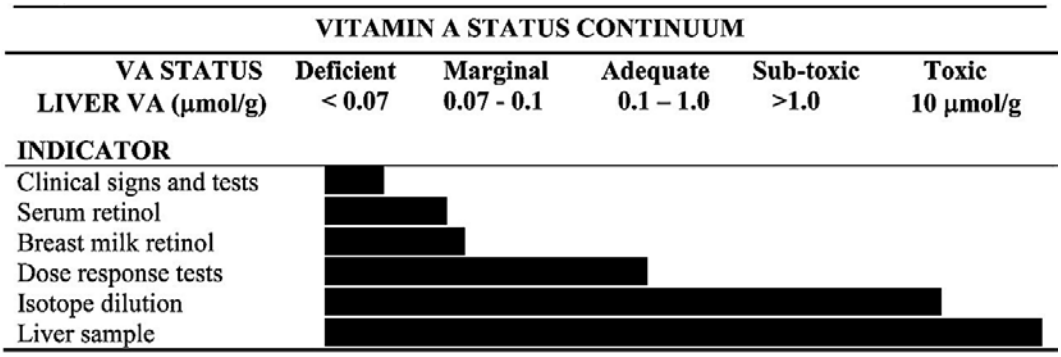
Determining appropriate vitamin A intake is challenging due to not only a lack of published studies that evaluate status with respect to intake and poorly defined subclinical deficiency and toxicity, but also because methods assessing vitamin A status have several limitations and require the use of numerous assumptions. Actual liver reserve (vitamin A per gram liver) is currently considered the "gold standard" for predicting the total body pool of vitamin A (21, 22). However, it is an imperfect indicator because the percentage of the total body pool stored in the liver varies and is dependent on the total body pool itself (*i.e.*, less is stored in the liver when the body pool is low). Furthermore, direct assessment of liver reserves in humans is rarely feasible or justifiable.

Vitamin A: status indicators

Most research on vitamin A status indicators has focused on the ability of the indicator to reflect liver stores (12, 21). These indicators include biological descriptions of deficiency such as xerophthalmia and night blindness, functional indicators of deficiency such as abnormal dark adaptometry, histological indicators of deficiency, such as abnormal conjunctival impression cytology (CIC), and biochemical methods for assessing liver and total body pools such as serum retinol, breast milk retinol, the relative and modified relative

dose response tests, and isotope dilution assays (21). A summary of the estimated relationships of status indicators to liver reserves is shown in **Figure 1**.

CHAPTER 1, FIGURE 1: Biomarkers of vitamin A status in relation to liver-reserve concentration (µmol vitamin A/g liver)



From: Tanumihardjo SA, Am J Clin Nutr 2011;94:658S-665S

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Dark adaptometry, a "clinical test" of vitamin A status, measures the ability to see dim light in the dark. It tests the functionality of an aldehyde form of vitamin A (11-*cis*-retinal) in rhodopsin of rod cells to maintain vision in dim light. Severe loss of this function is called "night blindness". The intake required to correct abnormal dark adaptometry and night-blindness has been studied extensively (2). However, by definition, an individual with abnormal dark adaptometry or night-blindness has clinical vitamin A deficiency and resolving the visual symptoms with vitamin A gives no guarantee that subclinical deficiency will also be avoided (2). Similarly, correcting abnormal CIC, which assesses the epithelial integrity of conjunctiva and can detect abnormalities before the clinical manifestations of xerophthalmia, does not guarantee protection against subclinical vitamin A deficiency.

Serum retinol, the predominant form of circulating vitamin A, is the most commonly used vitamin A status indicator. Unfortunately, serum retinol concentration is not a robust measure of vitamin A status because it does not correlate with liver stores (23, 24). It is usually maintained within a narrow range until liver reserves are clinically deficient or toxic (21), It can change during acute infection (25), due to the acute-phase response (26-28) even if status has not changed (26, 29). Serum retinol concentration can also decline during protein-energy malnutrition (30), zinc deficiency (31, 32), iron deficiency (33), and, interestingly, iron supplementation (34). Serum retinol rebounds after supplementation with zinc or iron, consumption of sufficient protein and energy, or recovery from illness even when no vitamin A is administered (29, 35-39).

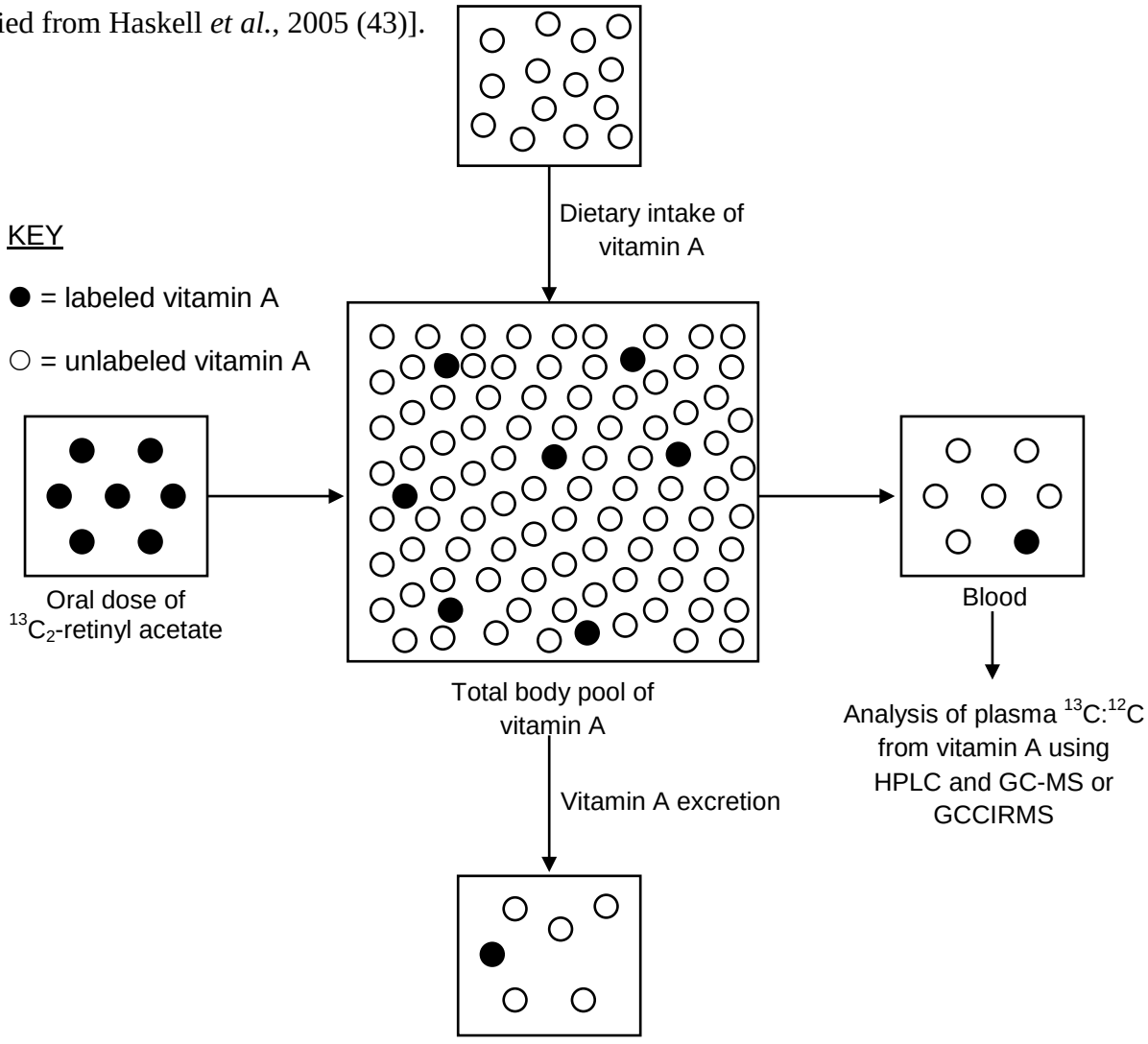
Both the modified relative dose response test and its precursor the relative dose response test are biochemical indicators of suboptimal vitamin A status. They are based on hepatic accumulation of retinol binding protein (RBP) during times of vitamin A depletion. RBP transports retinol from the liver to tissues. The *holo*-protein binds one molecule of retinol and usually circulates in the blood bound to transthyretin (*a.k.a.*, prealbumin). Work by Goodman and colleagues, both *in vitro* using rat tissue culture and *in vivo* in rats, indicates that hepatic RBP synthesis is independent of retinol status, while its release from the liver is highly dependent on retinol status (40-42). As a result, when vitamin A intake is inadequate and hepatic vitamin A is low, *apo*-RBP accumulates. Muto *et al.* (42) showed that in rats fed a vitamin A-deficient diet, *apo*-RBP starts accumulating in the liver before the liver is depleted of vitamin A. In response to newly ingested vitamin A, accumulated *apo*-RBP binds retinol and gets rapidly mobilized from the liver to the serum as the *holo*-complex (42). The accumulation of *apo*-RBP and rapid release of *holo*-RBP after ingestion of vitamin A

provides the biological framework for both of the dose-response tests. While seemingly good predictors of preclinical vitamin A deficiency, these indicators lack utility to identify excessive vitamin A status (21).

Stable isotope dilution tests are perhaps the most sensitive and desirable methods to measure the total body pool of vitamin A and estimate liver reserves in humans. There are currently two stable isotope dilution tests that have been used in the field of vitamin A, the deuterated-retinol-dilution (DRD) technique and the $^{13}C_2$-retinol isotope dilution ($^{13}C_2$-RID) test. Briefly, an oral dose of labeled vitamin A (deuterium, or ^{2}H, in the case of DRD technique and ^{13}C in the case of the $^{13}C_2$-RID test) is given. A blood sample is taken when the labeled dose has mixed with endogenous stores and equilibrium is achieved. The samples are analyzed for labeled and unlabeled vitamin A and the baseline TBP of vitamin A can be calculated from the resulting dilution of the labeled tracer (**Figure 2**). Hepatic vitamin A can be estimated assuming 50 - 90% of TBP is found in the liver, depending on baseline status. These minimally-invasive methods, coupled with assessment of dietary vitamin A intake may be the most effective way to evaluate the EAR and RDA for the different life-stage and gender groups.

CHAPTER 1, FIGURE 2: Basic principles of isotope dilution for determining total body vitamin A.

An oral dose of labeled vitamin A (either $^{2}H_4$- or $^{13}C_2$-retinyl acetate, for the DRD technique or $^{13}C_2$-RID test, respectively) is administered. After a mixing period, the dose reaches equilibrium with the body pool (14 – 20 days). A blood sample is taken at baseline ($^{13}C_2$-RID test) and/or after equilibrium (DRD technique and $^{13}C_2$-RID test) and the serum is analyzed for the ratio of labeled to unlabeled species within the serum retinol pool. The total body pool can be calculated using mass-balance equations corrected for loss of tracer over time. [Figure modified from Haskell *et al.*, 2005 (43)].



Stable isotope dilution tests to assess vitamin A status

The DRD and $^{13}C_2$-RID techniques have been applied and validated against liver reserves in humans and animals, respectively. Prior to development of the stable isotope dilution tests, numerous studies in animals validated radioisotope dilution (using tritium and ^{14}C) as predictive of liver vitamin A (44-49). Launching from this work, Furr *et al.* (50) showed that predicted and actual liver reserves using the DRD technique were highly correlated in 11 American surgical patients (Spearman's rank correlation coefficient, 0.95, $P < 0.002$). Haskell *et al.* (20) also showed a linear correlation between predicted and actual liver values in 31 Bangladeshi surgical patients ($r = 0.75$, $P < 0.0001$). For the $^{13}C_2$-RID test, Tanumihardjo (51) showed a strong linear relationship between predicted and actual liver reserves in rats consuming low, medium, and high doses of vitamin A ($r = 0.98$, $P < 0.0001$). Escaron *et al.* (52) showed that the $^{13}C_2$-RID test correctly identified excessive liver reserves in rhesus monkeys with severe hypervitaminosis A, although the predicted values underestimated actual reserves.

For the DRD technique, hepatic reserves are estimated by an equation developed by Furr *et al.* (50): total liver reserves (TLR) = $F \times \text{dose} \times (S \times a \times [(1/D{:}H) - 1])$, where F is a factor for the efficiency of storage of an orally administered dose of vitamin A [*i.e.*, 0.5 (46)], the dose is the amount of isotope in mg, S is the ratio of [$^2H_4$]retinol to retinol in plasma to that in liver [*i.e.*, 0.65 (17)], $D{:}H$ is the isotopic ratio of $^2H_4$-retinol to retinol in plasma, and a corrects for the irreversible loss of vitamin A based on its half-life in the liver. Factor a is assumed to be independent of hepatic vitamin A and time-invariant and $a = e^{-kt}$, where $k = \ln(2)/140$ (53). The – 1 at the end of the equation corrects for the contribution of the dose to hepatic stores (20).

For the $^{13}C_2$-RID test, a baseline and follow-up blood sample are taken and a standard mass balance equation is solved for *b*, the TBP at baseline: $(F_a \times a) + (F_b \times b) = (F_c \times c)$ where:

$F_a = R_{dose}/(R_{dose} + 1)$ of the dose = 0.1, because the number of ^{13}C and ^{12}C atoms in the dose are 2 and 18, respectively

$F_b = R_b/(R_b + 1)$ of the serum at baseline

$F_c = R_c/(R_c + 1)$ of the serum after dosing

a = μmol vitamin A absorbed from the dose [assumed to be 100% for a small test dose given to a person with normal to excessive stores (52)]

b = μmol vitamin A in body pool at baseline

c = μmol vitamin A in body pool after dosing = a + b

and,

$R_x = {}^{13}C/{}^{12}C$ at time x

The calculated TBP should also be corrected for loss of the tracer by multiplying it by e^{-kt} where $k = \ln(2)/140$. Total liver reserves can be estimated by multiplying the total body pool (TBP) by the percent of vitamin A assumed to be stored in the liver, generally 40 – 90% depending on expected baseline status (2, 54). The $^{13}C_2$-RID test is not corrected for differences in distribution of the tracer between liver and serum because the tracer mixes equally between the two pools due to the small dose administered and assumed fasting blood sampling (51, 52). For both the DRD technique and $^{13}C_2$-RID test, vitamin A per gram liver is calculated assuming liver weight is 2.4 – 3% of body weight (2, 5).

An advantage of the DRD technique is the requirement for only one blood draw after the dose reaches equilibrium. On the other hand, while the $^{13}C_2$-RID test requires two blood samples, it has several advantages over the DRD technique. In the $^{13}C_2$-RID test carbon is labeled, as opposed to hydrogen, so there is less of a chance of loss of labeled atoms from the tracer molecule during spontaneous chemical reactions. Additionally, the instrumentation used to analyze ^{13}C is more sensitive than that used to analyze hydrogen isotopes; thus, smaller doses of ^{13}C tracer can be used, which decreases the degree to which the dose perturbs the endogenous vitamin A pool (43). For example, in a recent study using the DRD technique in men, a tracer dose size of 35 μmol was used (55) while in our studies using the $^{13}C_2$-RID test in women, a dose size of 2 μmol was used (Chapters 2 and 3). Another advantage of the smaller dose is greater absorption. In monkeys with excessive hepatic vitamin A, the unabsorbed $^{13}C_2$-retinyl was undetectable in feces up to two days after dosing (52). This suggests most of the oral dose gets absorbed. In contrast, it is assumed that only 50% of the oral tracer dose makes it to the liver in the DRD technique.

Drawbacks for both tests are the numerous assumptions required in the calculations. For the DRD technique, these include the efficiency of storage of the tracer dose and the ratio of tracer in plasma to that in liver. For the $^{13}C_2$-RID test, assumptions include 90-100% absorption of the tracer dose and 50 – 90% of body vitamin A located in the liver. For both tests it is assumed that the dose is in equilibrium with body stores and recent dietary intake at the time of blood sampling. Furthermore, mechanisms to absorb more dietary vitamin A, especially via increased cleavage and absorption of carotenoids, may get up-regulated when vitamin A status, and possibly intake, decreases (1, 56, 57). Another assumption for both the DRD technique and $^{13}C_2$-RID test is that the calculations apply to individuals regardless of

baseline status. The half-life correction assumes that vitamin A follows first-order kinetics and the half-life of the vitamin is also not affected by baseline status. A kinetic analysis by Cifelli *et al.* showed that vitamin A disposal rate was directly related to the vitamin A pool in well-nourished Chinese and American adults and appears to increase as the pool increases (58). The DRD technique and $^{13}C_2$-RID test may be improved by taking into consideration presumed baseline status and its impact on the assumptions of the tests. Additionally, fewer assumptions need to be made if vitamin A status is defined in terms of TBP as suggested by Olson (22). It may be time to consider redefining vitamin A status as a function of the TBP as opposed to hepatic vitamin A.

Despite their limitations, stable isotope dilution methods are currently the best indicators of vitamin A status, short of liver biopsy. A liver biopsy itself can be limited as vitamin A is not uniformly distributed throughout the liver (59). These minimally invasive methods can and should be used to evaluate the current EAR for vitamin A, including specific intake requirements for the life-stage and gender groups defined in the dietary reference intakes.

How stable isotope dilution methods can be used to evaluate the EAR

To date, there has been only one published study (55) in which stable isotope dilution has been applied *a priori* to assess status relative to intake and provide data for the EAR. This study was performed by Haskell *et al.* in Bangladeshi men with low and high baseline pools of vitamin A and extrapolated to US men. Briefly, participants were assigned to either a low- or high-vitamin A pool size, fed a diet low in vitamin A (~100 RAE/d), and given a controlled daily vitamin A supplement for 60 days, resulting in a total of eight different

supplementation levels within each pool size group. The change in whole body vitamin A over the supplementation period was plotted against daily intake and a regression line was calculated. The x-intercept (where change in the body pool of vitamin A = 0) is the intake required to maintain stores. Haskell *et al.* estimated approximately 500 μg [point estimates of 362 μg with supplements and 462 μg when diet is included] for the EAR for American men from data in Bangladeshi men, which is lower than the current EAR of 625 μg. However, their value for food retinol activity equivalents used the IOM conversion factor of 12:1 instead of the FAO retinol equivalents conversion factor of 6:1, which may be more appropriate for Bangladeshi men. Similarly designed studies could be conducted to confirm this finding and evaluate the EAR for the other life-stage and gender groups. In the subsequent chapters of this dissertation, we report our findings assessing the EAR of young adult women using the $^{13}C_2$-RID test.

An alternative approach would be to use stable isotope dilution methods to evaluate the variables in the equation used to set the EAR: Daily Intake = A x B x C x D x E x F. For example, variable A is the percent body vitamin A stores lost daily when consuming a diet devoid of vitamin A. A study in rats with various vitamin A statuses could be performed where stable isotope dilution is used to assess the baseline and follow-up vitamin A pools after consuming a diet without vitamin A and the percent lost calculated. Findings could be confirmed with liver analyses. Variable F also deserves further study. F is the efficiency of storage of orally consumed vitamin A, which assumes 40% of an oral dose is stored. In the study by Haskell *et al.* from which this value is derived, the range of retention of the tracer dose was 19 – 72% in Bangladeshi surgical patients (20). Similarly, storage observed by Rietz *et al.* (49) ranged from 6 – 63% in rats given radioactive vitamin A. In both studies,

storage increased as endogenous liver stores increased. Also in both studies, there was considerable vitamin A deficiency as determined by liver reserves, which may not be appropriate models of the US population. To that end, storage of an oral dose of vitamin A could be evaluated in a vitamin A-sufficient pig model using stable isotope dilution and liver analysis. Storage may be considerably higher than 40% in well-nourished Americans with consistent access to dietary vitamin A.

Summary

To summarize, vitamin A status is poorly defined in the subclinical but abnormal range and is difficult to measure. Very few studies have been designed *a priori* to evaluate vitamin A status with respect to intake. Finally, the EAR was based on few data from studies in rats and men conducted several decades ago. Stable isotope dilution tests, despite their limitations, are the best methods currently available to revisit and define the EAR appropriately for all life-stage and gender groups.

Explanation of dissertation format

After this introduction (Chapter 1) there are three chapters and an appendix, which includes preliminary data associated with this dissertation and three published papers on work that I did as a graduate student. Chapter 2 is a study of daily vitamin A intake and status assessment in young adult women and has been re-submitted for potential publication in the *American Journal of Clinical Nutrition*. Chapter 3 is a longitudinal study of vitamin A status in young adult women assigned to daily vitamin A intakes of 25, 50, or 100% of the vitamin A RDA for 6 weeks. Chapter 4 includes conclusions and future directions.

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CHAPTER 2

Vitamin A isotope dilution predicts liver stores in line with long-term vitamin A intake above the current Recommended Dietary Allowance for young adult women

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ABSTRACT

Background: The Estimated Average Requirement (EAR) and Recommended Dietary Allowance (RDA) for vitamin A are 1.7 and 2.4 μmol/d (500 and 700 μg retinol activity equivalents/d), respectively, for non-pregnant, non-lactating women >19 y. This intake is presumed to maintain a minimally-acceptable liver concentration of 0.07 μmol (20 μg) retinol per g, but liver reserves have not been evaluated with respect to vitamin A intake in women of any age group defined in the Dietary Reference Intakes.

Objective: This cross-sectional study examined vitamin A intake and liver reserves estimated by stable isotope dilution testing.

Design: Forty non-pregnant, non-lactating women (22.4 ± 2.3 y) completed a Harvard Food Frequency Questionnaire (FFQ) and 3-day diet record (3DDR) prior to undergoing vitamin A status assessment using a $^{13}C_2$-retinol stable isotope dilution test.

Results: Vitamin A intake was 70% higher than the RDA by both dietary assessment methods ($P < 0.001$). The mean liver concentration of vitamin A was 0.45 ± 0.31 μmol/g (129 ± 89 μg/g) and ranged from 0.09 μmol/g (26 μg/g) to 1.79 μmol/g (513 μg/g). Liver and total body vitamin A were highly correlated with intake measured by FFQ ($P \leq 0.009$) but not 3DDR ($P \geq 0.22$). Prediction equations were developed for 3- and 7-d data.

Conclusions: In this well-nourished population, vitamin A consumption was considerably higher than recommended and liver reserves were consistent with intake. Because of their sensitivity, stable isotope techniques can help to describe the vitamin A status and better characterize intake needs of all groups defined in the Dietary Reference Intakes.

INTRODUCTION

Vitamin A is an essential nutrient that is important for visual, reproductive, and immunological health (1). In the US, an estimated average requirement (EAR) is the "average daily nutrient intake that is estimated to meet the nutrient needs of half of the healthy individuals in a life stage or gender group" and is calculated from published data (2). The recommended dietary allowance (RDA) for a nutrient is a mathematically derived estimate that should meet the needs of 97 – 98 percent of the population and is calculated from the EAR.

For vitamin A, the EAR is the estimated daily intake required to meet physiological needs and maintain "minimal acceptable liver reserves," (1, 3) which are estimated to be 0.07 μmol (20 μg) vitamin A (retinol) per g liver. This concentration is assumed to prevent clinical signs of deficiency, maintain adequate plasma vitamin A concentrations, allow biliary excretion of vitamin A, and protect an individual consuming a vitamin A-deficient diet against clinical signs of deficiency for four months (1). The RDA for vitamin A is the EAR plus two times a coefficient of variation of 20%. For women ≥ 19 y, the EAR and RDA for vitamin A are estimated to be 500 and 700 μg retinol activity equivalents (RAE)/d (1.7 and 2.4 μmol retinol/d), respectively (1). Liver reserves have not been assessed with respect to vitamin A intake in women of any age group defined in the Dietary Reference Intakes. Thus it is unknown if the EAR and RDA yield the intended liver reserves.

Measuring liver reserves of vitamin A is difficult because liver biopsy, the gold standard, is rarely justifiable or feasible. Stable isotope dilution methods are a less invasive alternative. Simply put, a small dose of vitamin A tracer labeled with stable deuterium (^{2}H) or carbon (^{13}C) can be administered orally and after mixing with the endogenous vitamin A

pool, blood samples are obtained and analyzed for ^{2}H- or ^{13}C-retinol. The dilution of tracer can be used to calculate the total body pool (4). The amount of vitamin A stored in the liver is estimated to be 40 – 90% of the total body pool depending on the size of the pool, which is positively correlated with hepatic storage (5, 6).

Previous researchers have shown that liver reserves calculated using tetradeuterated vitamin A were highly correlated with actual hepatic reserves measured from liver biopsy obtained during abdominal surgery in Bangladeshi and American patients (r = 0.75 and 0.88, respectively) (7, 8). Liver reserves calculated with ^{13}C-retinol isotope dilution were also highly correlated to liver reserves (r = 0.98) in rats given low and moderate doses of vitamin A (9) and in non-human primates with hypervitaminosis A (10). Our goal in this study was to evaluate the relationship between self-reported vitamin A intake in young adult women (age 19 – 30 y) with liver stores, using a minimally-invasive and safe $^{13}C_2$-retinol isotope dilution test.

SUBJECTS AND METHODS

All aspects of the study were approved by the Health Sciences Institutional Review Board of the University of Wisconsin School of Medicine and Public Health prior to participant recruitment. This was a cross-sectional study of vitamin A intake and vitamin A status in women aged 19 – 30 y old. The primary variables were vitamin A intake as estimated by a 3-day diet record (3DDR) and Harvard food frequency questionnaire (FFQ), and total and hepatic vitamin A as assessed by the $^{13}C_2$-retinol isotope dilution test. Secondary variables included BMI, body composition, and serum retinol. The study design is shown in **Figure 1** and described in detail subsequently.

Women 19 – 30 y old who had a normal BMI (18.5 – 24.9 kg/m^2) by self-report and were non-smokers, not pregnant or trying to become pregnant, not lactating, and living in the greater Madison, WI area were invited to participate via posted flyers. An email advertisement was also sent to female students at the University of WI, Madison. Recruitment occurred during February and March of 2008. Exclusion criteria included weight loss of 4.5 kg (10 lbs) or more during the three months prior to recruitment, actively trying to lose weight, inability to refrain from drinking alcohol when requested, inability to follow the study diet, amenorrhea, acute or chronic illness including hepatitis, and current or previous history of anorexia or bulimia. Participants were invited to participate consecutively until the study was full. Participants gave written informed consent after receiving verbal and written information about the study and having all questions answered by a study investigator.

Baseline nutrient intakes were quantified using a 3DDR and the Harvard FFQ 2007 Booklet, which are standard methods for estimating both short (11) and long-term (over a one- to four-y period) (12, 13) nutrient intakes, respectively. Harvard FFQs were completed privately and collected by the study investigator immediately after completion. The Harvard School of Public Health quantified the FFQs and results were not shared with participants. For the 3DDR, participants were asked to record all foods and beverages, including vitamins and supplements, for two weekdays and one weekend day before starting the study diet. As an incentive to complete the 3DDR, study participants were given copies of the results of their dietary analysis.

3DDR were analyzed using Nutritionist Pro™ (Axxya Systems, Stafford, TX, Version 4.3.0), which uses nutrient analysis from the USDA food composition database

among others. For any foods not found in Nutritionist Pro™, nutrient composition was estimated by nutritional information provided on food labels, by restaurants, or by recipe analysis using Nutritionist Pro™. Vitamin A intake was quantified in RAE, which include contributions from preformed vitamin A and provitamin A carotenoids using the Institute of Medicine 2001 conversion factors (1), where 1 RAE = 1 μg all-*trans*-retinol, 2 μg supplemental all-*trans*-β-carotene, 12 μg dietary all-*trans*-β-carotene, or 24 μg of all other provitamin A carotenoids (i.e., α-carotene and β-cryptoxanthin).

Fat and fat-free body mass were estimated using air-displacement plethsmography and whole body densitometry in a BOD POD® (Life Measurements, Inc., Concord, CA), an FDA-approved medical device for estimating body composition. The Siri equation, where % body fat = (4.95/body density – 4.5)*100 was used (14). One certified BOD POD® user (ARV) performed all measurements. Body weight was measured on the BOD POD® electronic scale, which was calibrated prior to each participant. Height was measured using a stadiometer and BMI was calculated [(wt in kg)/(ht in m)^2]. After baseline measurements, participants were weighed weekly using a floor scale to monitor weight while consuming the study diet.

Study diet

Participants were instructed to avoid all vitamin A-containing foods and supplements for the duration of the 21-d study. The study diet, provided free-of-charge, contained 1900-2200 kcal daily with 15-30% of kcal from protein, 20-30% from fat, and the remainder from carbohydrates. The diet supplied approximately 100% of the Adequate Intake for calcium

(1000 mg) and 25% of the RDA for vitamin A. Participants started the study diet 7 days before the isotope dilution test was performed.

We provided all foods and limited vitamin A intake throughout the study to maintain a consistent intake of vitamin A. We instructed all participants to discontinue any supplements during the study. The contents of multivitamins and supplements are not FDA regulated and therefore contain unknown and variable amounts of vitamin A despite labeling claims. An inconsistent influx of vitamin A would affect isotope dilution kinetics. Participants were given daily food logs, which listed the calorie, fat, protein, and carbohydrate content of all study foods. The logs also indicated which foods contained vitamin A. Participants were instructed to eat the full portion of all foods containing vitamin A, and as much or as little of any other foods they wished. Participants were asked to indicate the amount of the vitamin A-containing foods they consumed in their food logs, and record any non-study foods they added, so that vitamin A intake during the study could be more accurately estimated.

Sample collection

Prior to baseline (d -7), participants provided one venous blood sample in the morning after an eight-hour fast in order to quantify serum retinol. Immediately after the blood draw, they started the study diet. A fasting blood sample was collected again seven days later (baseline, d 0) immediately after which participants were given an oral dose of 2 μmol $^{13}C_2$-retinyl acetate (15) dissolved in corn oil measured in a medical 1 cc syringe. Participants were required to eat peanut butter on either a cracker or banana immediately after dosing to provide a fat source for adequate dose absorption. A fasting blood sample was

collected again 14 days after dosing (d 14) to estimate total body and liver retinol reserves. The equilibrium period was chosen based on animal studies. In rats with medium to high vitamin A stores, equilibrium of labeled vitamin A between serum and liver was observed after eight days (9), while in rhesus monkeys with high vitamin A stores it was achieved between 7 and 14 d (10). Blood samples were also collected at three and seven days post-dosing (d 3 and 7, respectively) to determine if these earlier time points could predict the 14-d value. Participants were asked to abstain from drinking alcohol for two days prior to each blood draw and on the day of $^{13}C_2$-retinyl acetate dosing. Participants were paid $25 for completion of paperwork and blood draws.

Trained phlebotomists collected blood samples into serum separator tubes with clot activator (Becton-Dickinson, USA). After clotting at room temperature in the dark to avoid photolysis of vitamin A, samples were centrifuged at 2200 X *g* for 10 min at 4ºC. Serum was collected and placed into vials of 1 or 2 mL aliquots. Nitrogen was blown into the vials to displace oxygen before storage at –70ºC until analysis.

Carbon isotope composition of serum retinol and estimation of vitamin A stores

Retinol was purified from 1 – 2 mL serum samples as described previously (16) with minor modifications: a GraceSmart RP18 (150 x 4.6 mm, 5 µm, 120 Å) column was used for the first purification and a GraceSmart RP18 (250 x 4.6 mm, 5 µm, 120 Å) column for the second. Reconstituted purified retinol from the serum was injected into the same gas chromatograph-combustion-isotope ratio mass spectrometer (GCCIRMS) system as previously published (16) and run under the same conditions used by Escaron et al. (10). The

GCCIRMS generates a value based on the enrichment of the carbon pool with ^{13}C, or atom percent ^{13}C (At% ^{13}C).

Total body reserves (TBR) of vitamin A were estimated by applying the standard mass-balance equation (17) to ^{13}C in retinol:

Equation 1: $(F_a \times a) + (F_b \times b) = (F_c \times c)$

Where a is μmol retinol absorbed from the dose, estimated to be 100% of the administered dose for those with normal to excessive stores (10); b is the TBR in μmol vitamin A at baseline; and c is the TBR in μmol of vitamin A after dosing ($c = a + b$). F_a is the fraction of the dose that is labeled with ^{13}C, which is equal to 0.100 because $^{13}C_2$-retinyl acetate is converted to $^{13}C_2$-retinol *in vivo* at the time of absorption, two of the 20 carbons were labeled, and F = R/(R + 1) where R is $^{13}C/^{12}C$. F_b is the At% ^{13}C in retinol at baseline in decimal form, and F_c is the decimal form of At% ^{13}C after dosing.

Equation 1 is solved for b, the TBR of retinol at baseline. The baseline TBR of vitamin A can then be corrected for catabolism of the dose of labeled vitamin A, which is assumed to be equivalent to the half-life of unlabeled vitamin A (140 d in adults) and independent of vitamin A status and time (8, 18) using equation 2.

Equation 2: Corrected TBR of retinol = $b \times e^{-kt}$; where b = TBR (from equation 1), k = ln(2)/140 and t = time in days since dosing.

Previous studies have shown that liver storage is positively correlated with total body status and can range from 40 – 90% (1, 19) of total body vitamin A. In rhesus monkeys with hypervitaminosis A, a liver reserve of 80% of TBR of vitamin A underestimated actual liver storage (10). Thus, to estimate hepatic stores of vitamin A, we assumed we were studying a well-nourished population and estimated 80% of TBR was contained in the liver (i.e., Total hepatic vitamin A = corrected TBR × 0.8). Finally, liver vitamin A was expressed per gram assuming liver weight is 2.4% of body weight in adults (3). Serum was analyzed for retinol by HPLC as published previously (16).

Statistics

Two-tailed *t*-tests, paired and unpaired, were applied to compare means as appropriate. Pearson correlation, simple linear regression, and multiple linear regression were used to evaluate associations between variables and for prediction equations. Data are presented as means with measurement uncertainty described with SD, unless otherwise indicated. Participants with missing data were included in the analyses. All analyses were performed with SPSS version 20 (IBM, Ireland, ©2011). $P < 0.05$ is considered statistically significant unless a Bonferroni correction was applied (noted in text).

RESULTS

Forty women participated in the study. Baseline characteristics of study participants are presented in **Table 1**. All women completed FFQs, 33 returned 3DDR, and 39 were successfully analyzed by the $^{13}C_2$-RID test to assess vitamin A status. Twenty-five women (64%) reported using oral contraceptives. *Post-hoc* analyses were performed to compare

baseline characteristics between women who reported the use of oral contraceptives (Table 1). One woman did not disclose her use or nonuse of oral contraceptives so $n = 39$. A more conservative *P*-value ($P < 0.01$) was used for the *post-hoc* analyses to account for multiple comparisons. There were no differences in baseline characteristics between groups.

Baseline daily intake of vitamin A was significantly higher than the RDA as estimated by both the FFQ and 3DDR ($P < 0.001$). Approximately 33% of daily vitamin A intake over the prior year was from supplements or multivitamins, as assessed by FFQ. Using FFQ data, bivariate correlations revealed that vitamin A intake including supplements was not correlated to calorie intake (Pearson Correlation Coefficient = 0.29, $P = 0.066$). However, when supplements were excluded, vitamin A intake was positively correlated to calories (Pearson Correlation Coefficient = 0.74, $P < 0.001$). Vitamin A intake including supplements by FFQ was not correlated to intake by 3DDR (Pearson Correlation Coefficient = 0.31, $P = 0.084$), but when supplements were excluded from the FFQ analysis there was a significant correlation with the 3DDR (Pearson Correlation Coefficient = 0.48, $P = 0.005$). Daily vitamin A intake on the study diet was 155 ± 29 μg RAE (~22% of the RDA) and body weight did not change ($P > 0.05$, data not shown).

Mean liver concentration (Table 1) was higher than 0.07, the current cut-off for deficiency ($P < 0.0001$, *t*-test). None of the women had deficient stores. However a higher cutoff for deficiency has recently been proposed at 0.1 μmol/g (20) and one woman had liver stores below this cutoff. Two women had stores above the proposed upper limit of normal (1.05 μmol/g liver) (4).

Simple linear regression showed that liver and TBR vitamin A were dependent on intake as assessed by FFQ when supplements were included (Pearson Correlation 0.41, *P* =

0.009, **Figure 2** and Pearson Correlation 0.40, $P = 0.011$, respectively), but there was no association when supplements were excluded ($P \geq 0.48$). TBR and hepatic vitamin A were not correlated to intake by 3DDR ($P \geq 0.22$). TBR and liver vitamin A were not correlated with FFQ total calorie, fat, protein or carbohydrate intake ($P \geq 0.86$). TBR and liver reserves were negatively correlated to percent fat mass (Pearson Correlation -0.34, $P \leq 0.034$), but not correlated to BMI or body weight ($P \geq 0.24$). Fat mass was not correlated to vitamin A intake by FFQ ($P = 0.13$). The linear regression model improved when both vitamin A intake by FFQ and percent fat mass were included as independent predictors of hepatic liver reserves ($R^2 = 0.25$, $P = 0.005$). Finally, removal of an outlier made only a marginal improvement in the model ($R^2 = 0.27$, $P = 0.004$). There was no association between serum retinol and liver reserves ($P = 0.18$).

Liver stores of vitamin A calculated using the 3- and 7-d %At ^{13}C were positively correlated to ($P < 0.0001$ for all), but significantly different from ($P < 0.0001$, paired *t*-tests with Bonferroni correction) the 14-d estimate (Pearson Correlation Coefficient = 0.62 and 0.87, for 3- and 7-d data respectively). Linear regression equations were calculated for the 14-d value as a function of the 3- or 7-d data. For the 3-d data, the slope was 3.53 ($P < 0.0001$) and intercept was 0.009 ($P = 0.93$). For the 7-d data, the slope and intercept were 1.72 ($P < 0.0001$) and –0.083 ($P = 0.22$), respectively. For simplicity, the prediction equations were rounded as follows: 14-d predicted liver reserves = 3.5*(3-d estimate) and = 1.7*(7-d estimate). Using these equations, predicted liver reserves were calculated and plotted against the actual 14-d liver reserve in **Figure 3 (panels A and B)**. Bland-Altman plots are shown in **Figure 4 (panels A and B)**. The difference between actual 14-d values and predicted 14-d values using the 3-d equation was not different from zero ($P = 0.76$).

There was a significant difference between actual 14-d values and predicted 14-d values using the 7-d prediction equation where the 7-d prediction equation overestimated liver reserves by 0.07 μmol/g ($P = 0.026$). Thus, an intercept was included in the final 7-d prediction equation such that 14-d predicted liver reserves = 1.7*(7-d estimate) – 0.07 (Pearson Correlation Coefficient = 0.81, $P < 0.0001$). Figure 3 (panel C) shows the relationship between the final 7-d prediction equation and actual 14-d values and Figure 4 (panel C) shows the respective Bland-Altman plot.

Because mean vitamin A intake was considerably higher than the EAR and RDA, we performed two *post hoc* analyses to examine liver reserves among women who consumed vitamin A in amounts closer to the EAR and/or RDA. We arbitrarily chose a range of vitamin A consumption of 400 – 1000 RAE (i.e., the RDA ± 300) and 300 – 700 RAE (i.e., the EAR ± 200) by FFQ, and calculated the average liver reserves of women whose intake was within these ranges. Nineteen women consumed between 400 and 1000 RAE daily (mean = 709 ± 167 RAE/d) and had mean liver reserves of 0.32 ± 0.11 μmol/g. Twelve women consumed 300 – 700 RAE/d (mean = 521 ± 119 RAE/d) and had mean liver reserves of 0.30 ± 0.10 μmol/g.

DISCUSSION

Our study contributes several new findings on vitamin A intake and status in young adult women in the US. We provide evidence that vitamin A intake among this group is higher than the RDA and show a correlation between reported intakes using FFQ and estimated hepatic concentration of vitamin A using a stable isotope dilution technique. Further, our data suggest that at these higher-than-recommended intakes, liver reserves

remain within normal limits for most women. Additionally, our study is the first to provide rudimentary evidence that the current RDA results in higher liver storage than expected. Finally, these data suggest that using earlier time points to predict liver reserves is likely feasible with appropriate corrections, as has been suggested in previous literature on vitamin A status assessment using isotope dilution techniques (21, 22).

In this study, vitamin A intake in women with a mean age of 22.4 y was approximately 70% higher than the RDA for their age/gender group. Nearly one-third of dietary vitamin A was obtained from supplements over the long-term. Vitamin A intake as assessed by FFQ when supplements were included was highly correlated to liver concentration as assessed by the $^{13}C_2$-retinol isotope dilution test. High intake in this age group is not surprising given the low cost of supplements, availability of vitamin A-fortified foods, and general encouragement by health care professionals to take a daily multivitamin. Concerns have been raised about the ease of inadvertent overconsumption of vitamin A (23) and a possible association between high intake as an older adult and increased risk of osteoporosis (24). It is possible that a chronic high intake of vitamin A, starting at a young age, may have a greater impact on osteoporosis risk than high intakes for only a few years at an older age. Although intake in this study was higher than recommended, it was less than the Tolerable Upper Intake Level (UL) and liver reserves reflected an adequate but not excessive vitamin A status in most women.

The results of this study allow for a rudimentary evaluation of the RDA for women aged 19 – 30 y. The EAR, from which the RDA is derived, was set by calculating the amount of daily vitamin A intake that would maintain normal physiologic function plus four months of protection during times of deficient vitamin A intake using the following equation:

Equation 3: $A \times B \times C \times D \times E \times F$

Where, A = % of body vitamin A stores lost per day when ingesting a vitamin A-free diet = 0.5%; B = minimum acceptable liver vitamin A reserve = 0.07 μmol/g liver (i.e., g 20 μg/g); C = liver:body weight = 0.03; D = reference weight of a specific age group and gender (i.e., 61 kg for adult women); E = ratio of total body:liver vitamin A reserves = 1.1; and F = efficiency of storage of ingested vitamin A = 0.5 (1, 3).

We examined a subset of women with a mean daily vitamin A intake similar to the EAR (mean = 521 ± 119 RAE/d), and found an average liver reserve of 0.30 ± 0.10 μmol/g, which is over 4 times the minimum acceptable reserve. There are many possible explanations for this finding. First, variable F in Equation 3 may underestimate efficiency of hepatic vitamin A storage when total body vitamin A is high. Alternatively, variable E may also vary with the total body pool of vitamin A. Finally, an additional factor or factors that account for duration of adequate intake or status may be required to adjust the EAR for alterations in vitamin A metabolism and storage during periods of adequacy or excess.

Our study also contributes to the literature on isotope dilution testing for vitamin A status. Using the standard mass-balance equation, liver reserves calculated using the 3- and 7-d %At ^{13}C were different from but highly correlated to those calculated using the 14-d value. Using linear regression, we were able to calculate a simple prediction equation for liver reserves for each of the earlier time points. There is mounting evidence that a prediction equation could be developed to estimate liver reserves based on a 3-d blood sample (21, 22, 25 – 27). For our study, the Bland-Altman plot shows that as liver reserves increase, the liver reserves estimated by the 3-d prediction equation become less similar to the 14-d calculated values. Prior research has shown that 3 days is too short for the test dose to equilibrate with

the body pool of vitamin A (7, 9, 10, 26). The 3-d sampling may be best suited for a qualitative, rather than quantitative, description of vitamin A status. Our final prediction equation using the 7-d data was highly correlated to and without any systematic deviation from the 14-d calculated liver reserves, and appears to be a fairly good predictor of liver reserves in our study population. We caution against the routine use of either of the prediction equations we calculated in this study, since this is preliminary data based on a homogenous study population. Using earlier time points to predict liver reserves must be investigated across a wider range of vitamin A statuses, as well as after an intervention in order to be confident of their accuracy.

Our study is limited by the homogenous sample that was primarily white university students in their early twenties. This group of women likely represents a higher socioeconomic demographic than all women age 19 – 30 y living in the US. Thus, our study may be confounded by a higher nutritional and educational status contributing to greater intake of vitamin A. We do not know the magnitude of this potential sampling bias although our results are consistent with other studies that show high intakes are correlated with normal to high liver stores in both animals and humans. Given our limited sample, this study may not be generalizable to all American women age 19 – 30 y. However, we believe our findings offer a foundation from which to base and compare future investigations.

Additional limitations of this, and other isotope dilution tests, are the multiple assumptions that are applied when calculating vitamin A liver reserves. We assumed a liver storage of 80% TBR based on prior research. This storage percentage may be highly variable both among different populations and individuals, and there are no data to suggest 80% is the best value to use in this group of women. Furthermore, we estimate liver size. Perhaps

vitamin A status should be redefined in terms of TBR, as opposed to hepatic storage, to avoid multiple assumptions. A difficulty of defining vitamin A status in terms of TBR is the effect of body habitus. We found that vitamin A status was negatively correlated to body fat in our population of normal-weight women. Were vitamin A status to be defined in terms of TBR, a correction for body fat, weight, or BMI may be necessary.

In summary, vitamin A intake is higher than recommended among our sample of American women in their early twenties, who are a defined subgroup for the RDA. While liver storage is generally within normal limits, two women had liver reserves considered hypervitaminotic by some researchers, although the clinical significance of this definition is currently unknown. In a subset of women consuming an average EAR for vitamin A, liver reserves were four times greater than the minimally acceptable cutoff from which the EAR was originally calculated. It is unknown if liver stores of this size in young adulthood pose any clinical concerns. The EAR for well-nourished populations needs to be reevaluated in regards to vitamin A balance and healthy vitamin A status needs to be better defined to avoid potential long-term consequences of high intake. Stable isotope technology is a promising approach for addressing these issues (20).

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ARV and SAT designed research, ARV and CRD conducted research, ARV analyzed data, ARV and SAT wrote the paper, SAT had primary responsibility for final content. The authors have no conflicts of interest with the content of this manuscript.

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TABLE 1. Baseline characteristics of study participants and subgroup analysis of oral contraceptive (OC) usage[1]

	All participants	OC user *n* = 25	No OC *n* = 14	*P* – value
Age (y)	22.4 ± 2.3 [19, 27]	22.2 ± 2.2	23.0 ± 2.5	0.29
Weight (kg)	61.2 ± 7.2 [45.6, 77.3]	60.4 ± 6.4	62.7 ± 8.3	0.35
BMI (kg/m^2)	22.3 ± 2.2 [18.6, 26.0]	22.0 ± 2.1	22.6 ± 2.3	0.39
% Fat Mass	26.6 ± 6.1 [13.2, 41.9]	26.3 ± 4.9	26.8 ± 8.1	0.84
Kcal intake/d[2]	1768 ± 578 [555, 3600]	1648 ± 486	2034 ± 650	0.042
Protein (g) intake/d[2]	73 ± 26 [30, 151]	67 ± 21	85 ± 30	0.031
Total fat (g) intake/d[2]	57 ± 23 [19, 123]	54 ± 20	64 ± 27	0.22
Vitamin A intake including supplements by FFQ (RAE/d)[2]	1213 ± 778 [378, 3890]	1035 ± 588	1479 ± 999	0.088
Vitamin A intake excluding supplements by FFQ (RAE/d)[2]	811 ± 405 [259, 2190]	706 ± 281	1026 ± 514	0.046
Vitamin A intake by 3DDR (RAE/d)[3]	1180 ± 705 [78, 3020]	1009 ± 591	1478 ± 850	0.077
% Reporting supplement use on FFQ (*n*)	40% (16)	40% (10)	43% (6)	0.67

% Reporting supplement use on 3DDR (*n*) [4]	9% (3)	5% (1)	18% (2)	0.43
Serum retinol (μmol/L)[4]	2.68 ± 0.88 [0.81, 5.89]	2.68 ± .69	2.69 ± 1.3	0.96
Total-body vitamin A (μmol)[5]	816.5 ± 537.4 [141.5, 3116]	854.8 ± 639.5	731.2 ± 289.7	0.51
Hepatic vitamin A (μmol/g)[6]	0.45 ± 0.31[0.09, 1.79]	0.48 ± 0.37	0.40 ± 0.18	0.48

[1]Data are presented as mean ± SD [Min, Max] and $n = 40$ except where indicated. $P < 0.01$ was considered significant due to multiple *post-hoc* comparisons; t-tests or chi-square tests were used to compare groups. FFQ = food frequency questionnaire; OC = oral contraceptive; RAE = retinol activity equivalents where 1 RAE = 1 μg all-*trans*-retinol, 2 μg supplemental all-*trans*-β-carotene, 12 μg dietary all-*trans*-β-carotene, or 24 μg of all other provitamin A carotenoids (i.e., α-carotene and β-cryptoxanthin); 3DDR = three-day diet record.

[2]Baseline dietary intake evaluated by Harvard FFQ.

[3]Baseline dietary intake evaluated by 3DDR, $n = 33$. Includes supplements/vitamin pills.

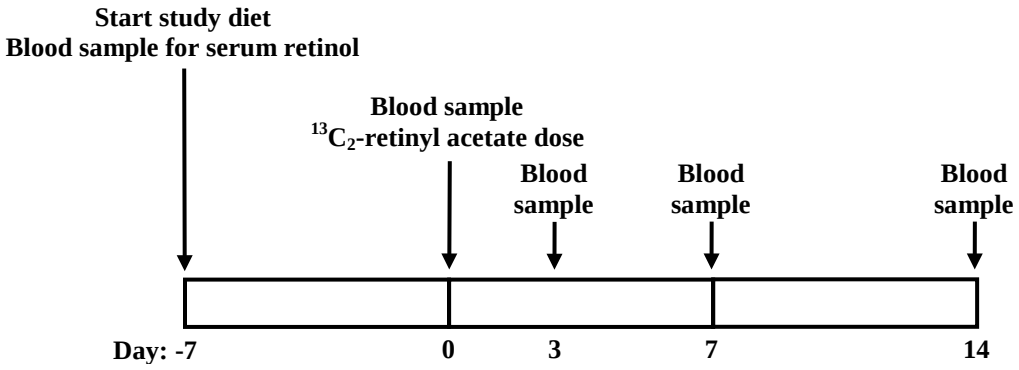
[4]$n = 33$.

[5]Calculated using equations 1 and 2 (please refer to methods for details.), $n = 39$.

[6]Calculated by assuming 80% Total-body vitamin A is stored in the liver and a liver weight of 2.4% body weight (please refer to methods for additional details), $n = 3$.

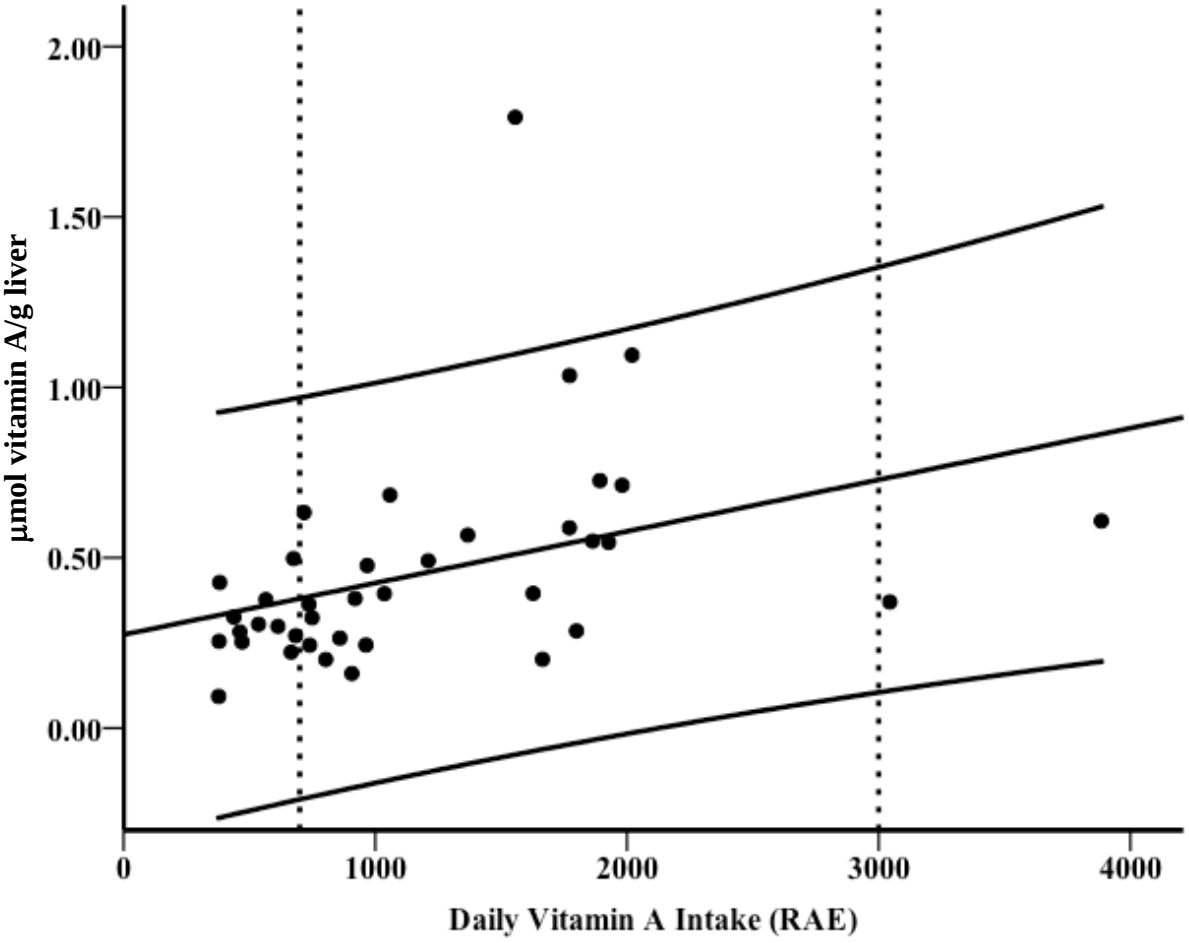
CHAPTER 2, FIGURE 1: Study timeline.

Rectangles represent 1 week. Study days are enumerated underneath the rectangles and study events are denoted by an arrow.



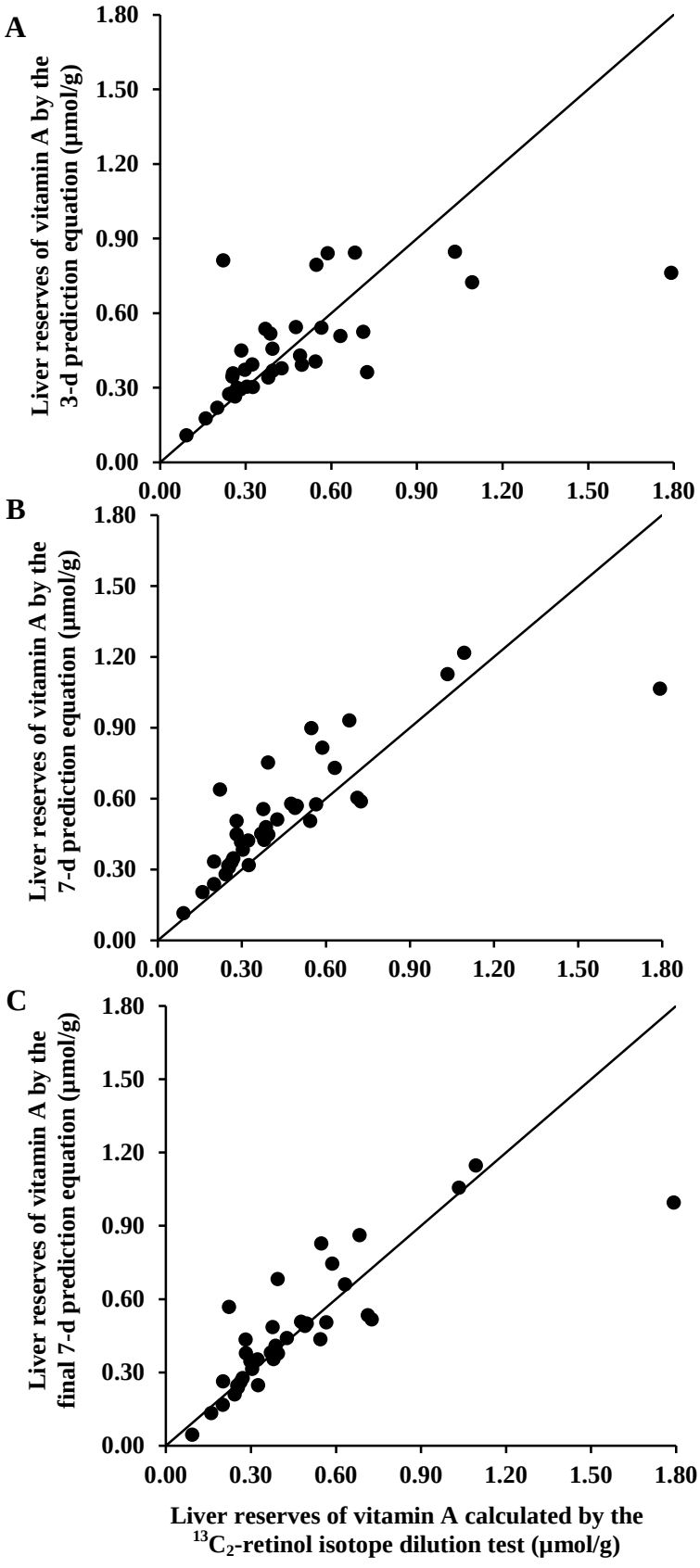
CHAPTER 2, FIGURE 2: Liver reserves of vitamin A in young adult women vs. vitamin A intake.

Calculated liver reserves of vitamin A (μmol/g) in young adult women (*n* = 39) using the $^{13}C_2$-retinol isotope dilution test were correlated with vitamin A intake, including supplements and vitamins, as assessed by Harvard Food Frequency Questionnaire in retinol activity equivalents (RAE) (Pearson Correlation Coefficient = 0.41, *P* = 0.009). 1 RAE = 1 μg all-*trans*-retinol, 2 μg supplemental all-*trans*-β-carotene, 12 μg dietary all-*trans*-β-carotene, or 24 μg of all other provitamin A carotenoids (i.e., α-carotene and β-cryptoxanthin). The center solid line is the linear regression line (liver reserves = 0.0001*daily intake + 0.23, *P* = 0.009 and 0.017 for the slope and intercept, respectively) with 95% confidence intervals for the individual data. Vertical dashed lines represent the Recommended Dietary Allowance (700 μg/d) and the Tolerable Upper Intake Level (3,000 μg/d) for women ≥ 19 y old. ***Figure is shown on the following page.***


2.00
1.50
1.00
0.50
0.00
µmol vitamin A/g liver
0
1000
2000
3000
4000
Daily Vitamin A Intake (RAE)

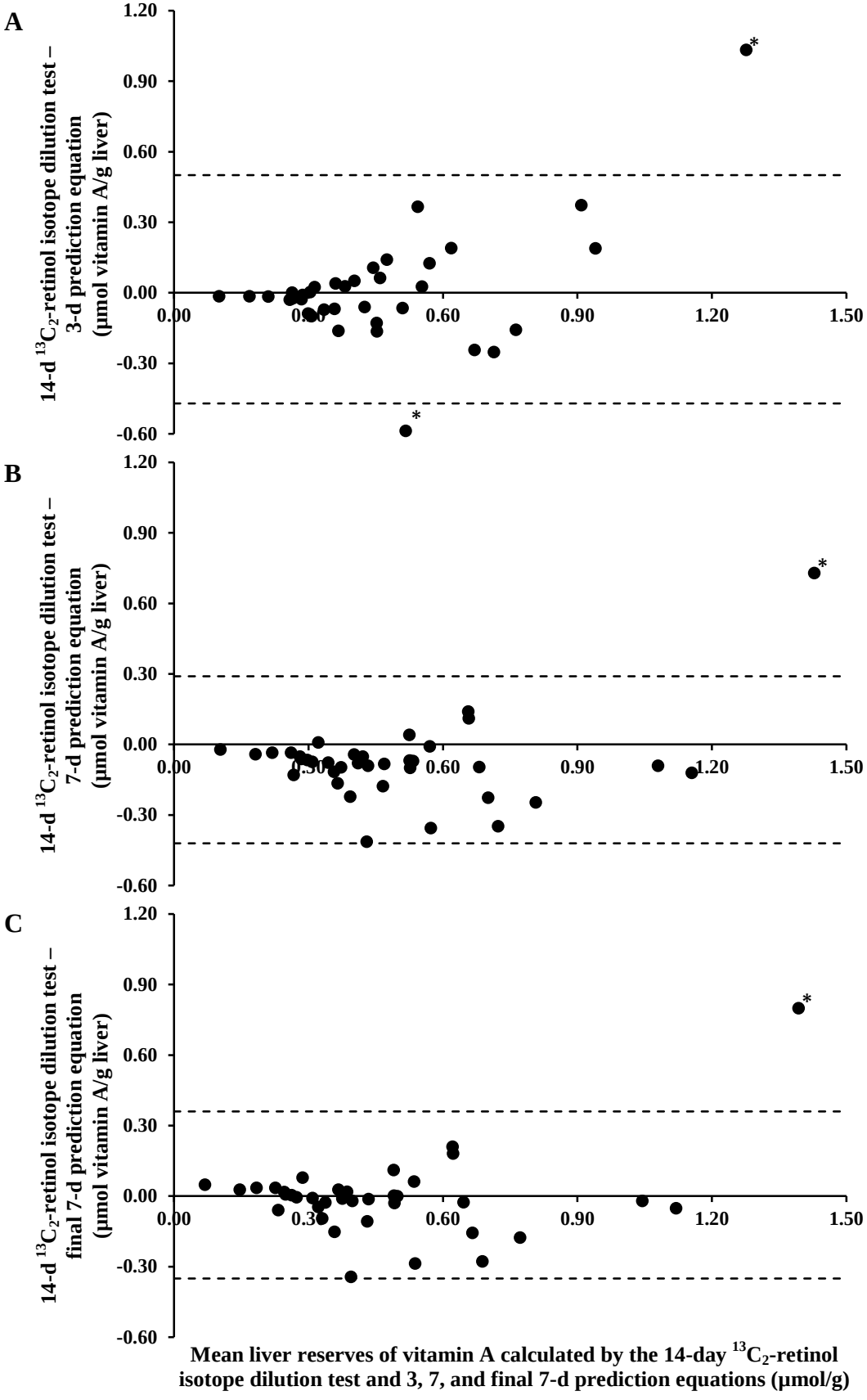
CHAPTER 2, FIGURE 3: Liver reserves in young adult women were calculated by the 3- (panel A), 7- (panel B), and final 7-d (panel C) prediction equations versus liver reserves by the 14-d $^{13}C_2$-retinol isotope dilution test.

The prediction equations were: 3-d prediction equation, liver reserves (µmol/g) = 3.5∗(3-d $^{13}C_2$-retinol isotope dilution test value); 7-d prediction equation, liver reserves (µmol/g) = 1.7∗(7-d $^{13}C_2$-retinol isotope dilution test value); and final 7-d prediction equation, liver reserves (µmol/g) = 1.7∗(7-d $^{13}C_2$-retinol isotope dilution test value) - 0.07. The $^{13}C_2$-retinol isotope dilution test value was obtained for each time point by solving the following equation for *b*: (F_a x *a*) + (F_b x *b*) = (F_c x *c*). Where *a* is µmol retinol absorbed from the 2 µmol dose, estimated to be 100%; *b* is the total body reserves (TBR) in µmol vitamin A at baseline; and $c = a + b$. F_a is the fraction of the dose that is labeled with ^{13}C (0.100 because two of the 20 carbons were labeled with ^{13}C). F_b is the atom percent ^{13}C (At% ^{13}C) in serum retinol at baseline in decimal form, and F_c is the decimal form of At% ^{13}C in serum retinol at d 3, 7, or 14 after dosing. Variable *b* was corrected for catabolism of the dose [$b \times e^{-kt}$; where k = ln(2)/140 and t = time in days since dosing]. n = 36, 38, and 38 for panels A, B, and C, respectively. ***Figure is shown on the following page.***


A
Liver reserves of vitamin A by the 3-d prediction equation (µmol/g)
B
Liver reserves of vitamin A by the 7-d prediction equation (µmol/g)
C
Liver reserves of vitamin A by the final 7-d prediction equation (µmol/g)
0.00
0.30
0.60
0.90
1.20
1.50
1.80
Liver reserves of vitamin A calculated by the $^{13}C_2$-retinol isotope dilution test (µmol/g)

CHAPTER 2, FIGURE 4: Bland-Altman plots of the 14-d $^{13}C_2$-retinol isotope dilution test and 3- (panel A), 7-d (panel B), and final 7-d (panel C) prediction equations in young adult women.

Liver reserves were calculated once using the 14-d $^{13}C_2$-retinol isotope dilution test and the 3-, 7-, and final 7-d prediction equations where estimated liver reserves = 3.5*(3-d $^{13}C_2$-retinol isotope dilution test value), = 1.7*(7-d $^{13}C_2$-retinol isotope dilution test value), or 1.7*(7-d $^{13}C_2$-retinol isotope dilution test value) - 0.07, respectively. The difference and mean of the 14-d $^{13}C_2$-retinol isotope dilution test and each prediction equation was calculated and plotted. Limits of agreement (mean difference ± 2SD) are shown by the dashed lines (- - -). Possible outliers are denoted with an asterisk (*****). The 3-d prediction equation appears to have increasing variability from the 14-d $^{13}C_2$-retinol isotope dilution test as liver reserves increase. The 7-d prediction equation systematically overestimated liver reserves compared to the 14-d $^{13}C_2$-retinol isotope dilution test by 0.07 µmol/g (t-test, P = 0.026). The mean difference between the final 7-d prediction equation and the 14-d $^{13}C_2$-retinol isotope dilution test was not different from zero (t-test, P = 0.96). n = 36, 38, and 38 for panels A, B, and C, respectively. ***Figure is shown on the following page.***


A
B
C
14-d $^{13}C_2$-retinol isotope dilution test – 3-d prediction equation (µmol vitamin A/g liver)
14-d $^{13}C_2$-retinol isotope dilution test – 7-d prediction equation (µmol vitamin A/g liver)
14-d $^{13}C_2$-retinol isotope dilution test – final 7-d prediction equation (µmol vitamin A/g liver)
1.20
0.90
0.60
0.30
0.00
-0.30
-0.60
0.00
0.30
0.60
0.90
1.20
1.50
Mean liver reserves of vitamin A calculated by the 14-day $^{13}C_2$-retinol isotope dilution test and 3, 7, and final 7-d prediction equations (µmol/g)

CHAPTER 3

Retinol Stable Isotope Dilution Predicts an Estimated Average Requirement of 300 Retinol Activity Equivalents in 19 to 30 y Old Women[1-3]

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[1]ARV has no conflicts of interest (COI); CRD, no COI; AKH, no COI; SAT, no COI.

[2]This research was supported by NIHNIDDK 61973 and the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2003-35200-05377.

[3]Registration was not required for this trial.

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[5]Current affiliation: Oregon Health and Sciences University (ARV).

To be submitted for peer-review

Abstract

The estimated average requirement (EAR) for vitamin A was calculated from studies in rats and a small sample of adult males several decades ago. Isotope dilution testing for vitamin A status is a sensitive tool for characterizing vitamin A status and intake needs in humans. This study evaluated the daily intake requirement for vitamin A status maintenance for women 19 – 30 y old using the $^{13}C_2$-retinol isotope dilution ($^{13}C_2$-RID) test. No prior research has evaluated this age and gender group as defined in the Dietary Reference Intakes of the US. Women consumed food containing 175 μg (0.6 μmol) retinol activity equivalents (RAE) daily for 12 wk. For the middle 6 wk, women (n = 41) were randomized to take a daily supplement of 0, 175 μg, or 525 μg (1.8 μmol) retinol as retinyl palmitate. Liver and total body vitamin A were measured using the $^{13}C_2$-RID test at baseline and follow-up. Dietary vitamin A intake decreased from baseline in the groups given supplements with 0 and 175 μg retinol (P = 0.005 and 0.018, respectively) but not in the group given 525 μg (P = 0.25). Mean baseline liver reserves 132 ± 92 μg (0.46 ± 0.32 μmol) retinol/g liver were significantly greater than 20 μg/g (0.07 μmol/g), the cut-off for deficiency (P < 0.0001). Liver reserves and total body vitamin A did not change in any group during the intervention (P > 0.05). An estimate for daily RAE intake to maintain the total body vitamin A pool and liver concentrations was 300 μg RAE/d after rounding. The EAR (500 μg RAE) for vitamin A for well-nourished women aged 19 – 30 y provides robust liver stores and may be higher than necessary.

Key Words: dietary requirements, EAR, retinol, stable isotope, vitamin A

Introduction

The estimated average requirement (EAR) for vitamin A for adults aged 19 y and older is calculated to maintain a minimal liver concentration (0.07 μmol or 20 μg/g) that protects individuals from clinical deficiency during times of stress or low intake. Specifically, individuals with this concentration should have no clinical signs of deficiency, have plasma vitamin A concentrations within normal limits, excrete vitamin A in bile, and be protected from deficiency for 4 mo if they stop consuming vitamin A. The supporting data come from studies in rats (1, 2) and a small study of eight male volunteers, aged 31 y and over (3). The recommended dietary allowance (RDA) is equal to the EAR plus two times a CV of 20% (i.e., RDA for vitamin A = 1.4*EAR) (4).

Increasingly sophisticated techniques for measuring vitamin A status and metabolism in humans, such as stable-isotope dilution methods, have been developed in the time since the studies used in the calculation of the EAR. The $^{13}C_2$-retinol isotope dilution ($^{13}C_2$-RID) test is among the most sensitive isotope dilution methods. Briefly, a small physiological oral dose of vitamin A tracer labeled with stable carbon (^{13}C) mixes with the endogenous vitamin A pool, and a blood sample is analyzed for ^{13}C-retinol. The dilution of tracer can be used to calculate the total body pool (5). Repeat testing after an intervention allows calculation of the change in the total body pool. Determining the change in total body pool over several levels of intake yields a line, and the x-intercept (where there is no change) is an estimate of the intake required to maintain the baseline body pool (6). Isotope dilution techniques are promising for reevaluating the EAR using human subjects (6).

In this study, we sought to determine the daily intake requirement for vitamin A status maintenance for women 19 – 30 y old using the $^{13}C_2$-RID test. Our primary outcomes

of interest were change in body pool and liver reserves. Our intervention restricted vitamin A intake to 175 μg (0.6 μmol), 350 μg (1.2 μmol), or 700 μg (2.4 μmol) retinol activity equivalents (RAE) (35, 70, 140% of the EAR or 25, 50, or 100% of the RDA, respectively) for 6 wk. For women over 19 y of age, the EAR and RDA for vitamin A are 500 μg RAE/d (1.7 μmol) and 700 μg RAE/d (2.4 μmol), respectively (4). We hypothesized that women consuming less than the EAR would decrease stores during the study, while those consuming more than the EAR would maintain stores. We predicted the x-intercept would correspond with an intake of 100% of the EAR.

Materials and Methods

Study design and vitamin A supplements

This was a double-blinded, parallel-arm study where participants were randomly assigned, with restrictions on group size, to one of three vitamin A intake groups. Each group took a daily vitamin A supplement containing 0, 175 μg (0.6 μmol), or 525 μg (1.8 μmol) retinol as retinyl palmitate while consuming a study menu containing approximately 175 μg (0.6 μmol) RAE. The intended total daily vitamin A intake was therefore approximately 175 μg (0.6 μmol), 350 μg (1.2 μmol), or 700 μg (2.4 μmol) RAE during the intervention; groups will be referred to as 1, 2, and 3, respectively, hereafter. The study duration was 12 wk, participants took supplements for the middle 6 wk, and vitamin A status was assessed at baseline and at follow-up using the $^{13}C_2$-RID test as described below. A timeline is shown in **Fig. 1**.

We enrolled 51 participants for three groups of 17. At the time of enrollment, there

was no similar prior work on which to base a power calculation for group size and thus the *n* per group was based on expert opinion of a minimum of 15/group for supplementation studies (7) and increased to 17 to allow for missing data. At the beginning of wk 4, an individual who was not associated with the study assigned participants to groups, in the order of enrollment, by drawing letters out of a bowl. The bowl contained 42 slips of equal-sized paper squares with the letter A, B, or C written on each (14 of each letter, to limit group size). At the time of randomization, only 41 participants remained in the study (see results for details). The same individual who randomized participants to groups assigned a vitamin A intake level to each group by drawing the supplement dose size out of a hat. Group assignments were revealed to study investigators at the time of data analysis, but the supplement dose key was kept in a sealed envelope in a locked file cabinet that was not opened until the end of data analysis.

Tischon Corporation, a supplement manufacturer registered with the US FDA as a Drug Establishment, custom-manufactured supplements (retinyl palmitate in corn oil) and placebos (corn oil only). All soft-gel capsules looked identical. Tischon provided a certificate of analysis verifying the supplement contents and quality, which we confirmed in our lab. Two individuals not involved in data analysis prepared supplement packets. Participants received 3 wk of supplements (21 pills) at a time in UV-blocking bags. Participants were instructed to swallow one pill daily with a meal, and store the supplements in a cool, dark place as recommended by the supplier. To verify supplement consumption, participants were asked to return unconsumed capsules at the end of each 3-wk period.

The Health Sciences Institutional Review Board of the University of Wisconsin (UW) School of Medicine and Public Health approved this study. Participants gave written consent

after receiving written and verbal explanations of the study and having their questions answered. All study events took place at the Nutritional Sciences building on the UW-Madison campus.

Participants

We invited women living in and around Madison, WI to participate via posted flyers. An email advertisement was sent to female students at UW-Madison. Inclusion criteria were age 19 – 30 y, normal BMI (18.5 – 24.9 kg/m^2) by self-report, non-smoking, not pregnant or trying to become pregnant, and not lactating. Exclusion criteria included weight loss ≥ 10 pounds (4.5 kg) during 3 mo prior to recruitment, actively trying to lose weight, inability to refrain from drinking alcohol when requested, amenorrhea, acute or chronic illness including hepatitis, current or previous history of anorexia or bulimia, inability to pick up the study food from the research facility weekly, planned vacation of >1 wk duration during the study, known scheduling conflict with a blood draw, inability to prepare light meals as requested, major food allergies/intolerances, inability to safely store a 7-d allotment of food, unwillingness to discontinue personal nutritional supplements/vitamins, concurrent participation in other studies, social circumstances that would make it difficult to consume the study food for 12 wk, or a family member already enrolled in the study.

A study investigator (ARV) discussed the purpose and basic study design as well as the menu and compensation with potential participants during an initial phone conversation. If the potential participant expressed interest in continuing, she was assessed for study eligibility (with the exception of medical history questions) over the phone and was invited for an in-person visit if criteria were met. At the in-person visit, potential participants were

given further details about the study and all questions were answered. After obtaining consent, a brief medical history was elicited to evaluate exclusion criteria. Participants were also asked about food preferences so the study menu could be individualized.

Baseline dietary habits and body composition

Baseline vitamin A intake was quantified in RAE using the Harvard Food Frequency Questionnaire (FFQ) 2007 Booklet. This tool has been validated for estimating long-term nutrient intake (8, 9). One RAE = 1 µg all-*trans*-retinol, 2 µg supplemental all-*trans*-β-carotene, 12 µg dietary all-*trans*-β-carotene, or 24 µg of all other provitamin A carotenoids (i.e., α-carotene and β-cryptoxanthin) (4). The Harvard School of Public Health quantified FFQs and individual results were not shared with participants.

Study diet

All study foods were provided and contained 1900-2200 kcal daily with 15-30% kcal from protein, 20-30% from fat, and the rest from carbohydrates. It also contained approximately 25% of the RDA for vitamin A and approximately 100% of the Adequate Intake for calcium (1000 mg). Food was packaged weekly for participants and they picked it up from the study kitchen every Tuesday during the study.

Each week, participants received daily food logs, which contained a detailed menu of breakfast, lunch, dinner, and snack foods for a given day and showed the calorie, fat, protein, carbohydrate, and calcium content of each item. Study foods containing vitamin A were clearly indicated on the logs, although the specific amount of vitamin A in the food was not disclosed. We instructed participants to eat the full portion of all study foods containing

vitamin A. Participants could consume other study foods *ad libitum* to account for individual energy requirements.

Participants were allowed to eat non-study foods as long as the food did not contain vitamin A. Participants were given instructions on how to read food labels in order to avoid foods containing vitamin A. They were also given lists of common foods that usually do not have labels (e.g. produce, bulk foods) detailing which were okay to eat and which contained vitamin A and therefore should be avoided. The lists were discussed with each participant individually, and it was stated at least twice during the educational session that participants should avoid adding fruits and vegetables with color (e.g. dark-green, orange, yellow, red) to the diet. Vitamin A-free skim milk was produced by and purchased from Babcock Hall Dairy Plant at UW-Madison specifically for this study.

Participants started the study menu 7 d prior to the baseline isotope dilution test (study d -7, Fig. 1). To estimate actual vitamin A intake during the study, participants recorded the amount of the vitamin A-containing foods they consumed as well as any non-study foods they added in the daily logs, which they returned weekly. Participants had email and phone access to a study investigator (ARV) at all times in case of questions regarding the menu or other study logistics.

$^{13}C_2$-retinyl acetate isotope dilution testing and blood sample collection

$^{13}C_2$-retinyl acetate was synthesized using published methods (10). Trained phlebotomists collected venous blood samples into serum separator tubes with clot activator (Becton-Dickinson, USA). Blood samples were collected in the morning, after a fast of at least 8 h. Participants were asked to refrain from drinking alcohol for two days prior to blood

draws and on the days of $^{13}C_2$-retinyl acetate dosing. Seven d prior to baseline $^{13}C_2$-RID testing, participants provided a blood sample for baseline serum retinol. On the morning of study d 0 (Fig. 1), the baseline blood sample for the $^{13}C_2$-RID test was drawn. Immediately after sample collection, participants received an oral dose of 2 µmol $^{13}C_2$-retinyl acetate dissolved in corn oil measured in a 1-cc medical syringe. Participants ate ~2 tablespoons of peanut butter on either a cracker or banana immediately after dosing to provide a fat source to enhance dose absorption. Samples clotted at room temperature in the dark to avoid photolysis of vitamin A and were centrifuged at 2200 X *g* for 10 min at 4ºC to separate serum. Serum was collected and placed into vials of 1 or 2 mL aliquots and purged with nitrogen before storage at –70ºC.

Blood samples were collected again on d 14 after dosing (Fig. 1) in the same manner as described, but without $^{13}C_2$-retinyl acetate dosing. This timing was based on published evidence that in animals with normal to high vitamin A stores equilibrium between the test dose and endogenous stores occurs between 7 and 14 d (11, 12). The follow-up $^{13}C_2$-RID testing occurred 7 d after stopping supplementation. The protocol for follow-up $^{13}C_2$-RID testing was the same as at baseline. The washout period (1 wk) between stopping supplementation and retesting allowed the most recent vitamin A intake to mix with endogenous stores. Participants were paid $100 upon completion of the follow-up blood draws.

Carbon isotope composition of serum retinol and estimation of vitamin A stores

Retinol was purified from 1-2 mL serum as described previously (13) with minor modifications. A GraceSmart RP18 (150 x 4.6 mm, 5 µm, 120 Å) and a GraceSmart RP18

(250 x 4.6 mm, 5 µm, 120 Å) column were used for the first and second purifications, respectively. Reconstituted retinol was injected into the same gas chromatography-combustion-isotope ratio mass spectrometer (GCCIRMS) system as previously published (13) and run under the same conditions (12). The GCCIRMS determines the atom percent ^{13}C (At% ^{13}C) based on the enrichment of CO_2 with ^{13}C.

The standard mass-balance equation was used to estimate the total body pool (TBP) of vitamin A (14): $(F_a \times a) + (F_b \times b) = (F_c \times c)$. Where a is µmol retinol absorbed from the dose, estimated to be 100% of the administered dose for healthy individuals with normal to excessive stores (12); b is the TBP in µmol vitamin A at d 0 of $^{13}C_2$-RID testing; and c is the TBP in µmol of vitamin A after dosing ($c = a + b$). F_a is the fraction of the dose that is labeled with ^{13}C, which is equal to 0.1 because $^{13}C_2$-retinyl acetate is converted to $^{13}C_2$-retinol *in vivo* at the time of absorption, and 2 of the 20 carbons were labeled. F_b is the At% ^{13}C in serum retinol at baseline in decimal form, and F_c is the decimal form of At% ^{13}C in serum retinol at follow-up (d 14). For any F, $F_x = R_x/(R_x + 1)$ where R_x is $^{13}C_x/^{12}C_x$. Equation 1 is solved for b, the TBP of retinol at d 0 of testing.

TBP of vitamin A was corrected for catabolism of the dose of labeled vitamin A, which is assumed to be equivalent to the half-life of unlabeled vitamin A (140 d in adults) and independent of vitamin A status and time (3, 15) using the following equation: Corrected TBP of retinol = $b \times e^{-kt}$. Where b = TBP (from equation 1), k = ln(2)/140 and t = time in days since dosing. Liver storage of vitamin A is positively correlated with total body status and can range from 40 – 90% (4, 16) of total body vitamin A. An 80% storage of TBP in the liver was assumed, given the presumed adequate stores prior to enrollment in our well-nourished study sample. A study of rhesus monkeys with hypervitaminosis A showed that an

estimate of 80% of the TBP storage in the liver underestimated actual liver storage (12). To calculate liver vitamin A it was assumed that liver weight is 2.4% of body weight in adults (17). Serum retinol concentration was analyzed as published (13).

To estimate the daily dietary vitamin A intake required to maintain the TBP and liver reserves, the change in body pool or liver reserves was plotted against the mean group intake. A regression line was calculated and *y* was set to 0 (no change in TBP or liver reserves) and the *x*-intercept obtained was considered a point-estimate for daily maintenance intake.

Statistical analysis

Primary outcomes of interest were change in body pool, change in liver reserves, and daily intake. To compare means across groups, the Kruskal Wallis Test was used. For differences between groups, Wilcoxon rank-sum tests were used. For paired data, Wilcoxon signed ranks tests were used. Pearson correlation was used to evaluate associations between variables. Data are presented as means $\pm$ SD, unless otherwise noted. $P < 0.05$ was considered significant. Analyses were performed with SPSS version 20.

Results

Participant flow is shown in **Fig. 2**. We did not have IRB approval to inquire why participants chose to discontinue the study. Baseline characteristics of those completing the study did not differ among supplementation groups (**Table 1**). The mean baseline TBP for all participants was 234 ± 158 mg (817 ± 550 μmol) retinol. Baseline liver reserves were 132 ± 92 μg (0.46 ± 0.32 μmol) retinol/g liver. This is significantly greater than the cut-off for deficiency (0.07 μmol; two-sided *t*-test, $P < 0.0001$) and lower than the proposed cut-off for

excess (1.05 μmol; two-sided *t*-test, $P < 0.0001$). No woman had serum retinol <0.7 μmol/L, the current cut-off for clinical deficiency. Serum retinol was not correlated to baseline TBP or liver reserves ($P \geq 0.26$). The baseline vitamin A intake for all groups combined (797 ± 399 RAE) was not different from the current RDA when supplements were excluded from the FFQ analysis (two-sided *t*-test, $P = 0.065$). However, when supplements were included, the mean baseline intake (1148 ± 782 RAE) was significantly greater than the RDA (two-sided *t*-test, $P < 0.0001$). Twenty women reported using supplements (63%).

The study vitamin A supplement intake was assumed to be 100% because no unused pills were returned. Analysis of the daily food logs (excluding vitamin A from study supplements) showed a daily dietary intake of 154 ± 59, 150 ± 15, and 141 ± 16 RAE for groups 1, 2, and 3, respectively ($P = 0.80$). With the study supplements added in, daily intake was approximately 154 ± 59, 325 ± 15, and 666 ± 16 RAE for groups 1, 2, and 3, respectively ($P \leq 0.001$). Groups 1 and 2 decreased their daily intake from baseline ($P =$ 0.005 and 0.018, respectively) while group 3's intake did not change. For group 1, the reduction in vitamin A intake from baseline for the entire course of the study (12 wk) was approximately 77%. Body weight did not change from baseline to follow-up (data not shown).

The change in TBP and liver reserves from baseline to follow-up was plotted (**Supplemental Fig. 1)**. The mean difference in total body pool from baseline was -13.4 $\pm$ 401.5, -15.3 $\pm$ 302.8, and +121.5 $\pm$ 178.5 μmol for groups 1, 2, and 3, respectively. No group significantly changed TBP or liver reserves and there was no difference among groups, even when two outliers were excluded ($P \geq 0.29$). The change in TBP and liver reserves were plotted against the mean daily intake of the group (**Fig. 3)**. For the liver reserves, the

regression equation is (Δ liver reserves) = 0.0002 (daily intake) – 0.0573 (r = 0.21, P = 0.26). For the TBP, the regression equation is (Δ TBP) = 0.282 (daily intake) – 77.987 (r = 0.19, P = 0.31). These gave point estimates for the daily maintenance intake of 287 and 277 μg RAE, respectively; the median of these is 282 μg RAE.

Discussion

The EAR for vitamin A is the intake required to maintain liver stores of 20 μg/g in 50% of the population. This was chosen to provide 4-mo protection from clinical signs of vitamin A deficiency with no vitamin A intake. At baseline, the women in this study consumed more than double the EAR (or 1.6 times the RDA) with supplements included and had baseline liver reserves nearly 7-times greater than the maintenance value used in the EAR calculations. Linear regression equations derived from the change in liver reserves and change in TBP of vitamin A versus group mean intake gave point estimates of approximately 300 μg RAE for daily maintenance, which is less than the current EAR of 500 μg RAE for this sex and age group.

Liver stores did not appreciably change from baseline for any group during the 6-wk intervention. Group 1 experienced the greatest reduction in intake (77% decrease) for the longest duration (12 wk) and still had no change in vitamin A status. There are several possible explanations for these findings. Mechanisms to absorb more dietary vitamin A, especially via increased cleavage and absorption of provitamin A carotenoids, are up-regulated when vitamin A status or intake decrease (18-21). Thus, group 1 may have been cleaving provitamin A carotenoids at a bioconversion value lower than 12 μg β-carotene

equivalents to 1 μg retinol (21). Lastly, our trial was likely underpowered and too short to detect a difference among the groups.

We based our group enrollment on expert opinion of at least 15 per group for intervention studies (5) given there is no previous research in the population on which to base a power calculation. Unfortunately, we had a high dropout rate, which we believe was due to the highly controlled menu and food choices. The foods were calorically adequate and designed to be palatable and account for individual preferences, but likely differed substantially from the participants' normal foods, and therefore could be difficult to maintain for 12 wk. In terms of the intervention length, it likely should have been longer to cause an overall change in vitamin A status. A paper published by Haskell et al. (6) after our study was conducted also showed no change in vitamin A pool size after a 60-d intervention in Bangladeshi men with small and large initial vitamin A pools. These findings further support the fact that the body regulates mechanisms to maintain vitamin A status (e.g., vitamin A absorption, absorption/cleavage of carotenoids, greater retinol recycling) despite flux in dietary availability. The results of these trials suggest that intervention studies using retinol isotope dilution tests need $n > 15$/group and study durations longer than 60 d on treatments.

The finding that vitamin A status is stable in a consistently well-nourished population is also in line with a study of 13 well-nourished English adults who consumed vitamin A-free foods for 6 – 24 mo and only 3 of whom showed clinical signs of deficiency as measured by dark adaptation and serum retinol concentrations beginning after 13 mo (22). In addition to regulation of mechanisms for maintaining status, we surmise that in a population of well-nourished individuals, a life-long consistently sufficient intake of vitamin A with no periods of stress allows vitamin A stores to grow considerably. A remaining question is whether

having such robust stores could be detrimental. It has been suggested that excess vitamin A intake may interfere with vitamin D signaling and normal calcium metabolism increasing the risk for fracture (21, 23), although this connection is still controversial. Perhaps the higher than anticipated liver reserves of vitamin A in our study suggest that the EAR and therefore RDA are higher than necessary for VA status maintenance in a well-nourished population of young adult women.

The goal of this study was to estimate the daily intake of vitamin A required to maintain total body stores in young adult women who are healthy and not pregnant or lactating. The study design relied on mobilization of and change in vitamin A stores so that change could be plotted against daily intake and the x-intercept of the regression line could be used to estimate maintenance intake. Even though the intervention was not long enough to observe a statistical change in status in this well-nourished population, the point-estimate of approximately 300 μg RAE/d to maintain status is of value considering the dearth of information and studies on the EAR. Had the intervention been longer, status would change and variability decreased in the groups consuming the lowest amounts of vitamin A. Although our point-estimate may not be exact for setting policy, we believe it suggests that the EAR may need to be lowered and that it could be used to design future studies. Nonetheless, this study is limited by our population, which was college-attending white women living in Wisconsin with a normal BMI who may be more educated and healthier than most 19 – 30 y old women in the US. Status and requirements may differ for those who are undernourished, overweight, or obese. Future studies should include broader population representation to evaluate vitamin A requirements by sex, age, activity, and body size.

In conclusion, we show that women who consume at least the RDA have sufficient stores of vitamin A and adaptive regulation to maintain the vitamin A pool, such that their vitamin A status is unaffected when intake is reduced significantly for several weeks to months. These data suggest that the current intake recommendations are higher than necessary to maintain adequate stores of vitamin A in young adult women. Considering that multivitamin supplements typically contain 100% of the Daily Value, which is based on 1968 RDAs of 1500 μg retinol equivalents (5000 IU) (23), and 63% of our study participants reported taking supplements, decreasing the amount in supplements would be a strategic move in the US to lower intakes.

Acknowledgments and Author Contributions

ARV and AKH conducted the study. CRD conducted the GCCIRMS analyses. ARV and SAT analyzed data, designed research, and wrote the manuscript. All authors have read and approved the final version of the paper. None of the authors had any financial interest in the work or a conflict of interest with the sponsors of this study.

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TABLE 1. Baseline Characteristics of Study Participants (n = 32)[1]

Characteristic	Group 1 (n = 11)	Group 2 (n = 12)	Group 3 (n = 9)
Age (y)	23.3 ± 2.4	21.7 ± 2.3	22.3 ± 2.3
Weight (kg)	59.3 ± 7.8	59.6 ± 6.5	61.9 ±7.6
BMI (kg/m^2)	21.7 ± 2.1	21.5 ± 1.9	22.9 ± 2.2
RAE/d[2]	1340 ± 904	1160 ± 571	1370 ± 1070
RAE/d excluding supplements[2]	903 ± 560	766 ± 266	777 ± 365
Total body pool vitamin A (μmol)[3]	893 ± 750	901 ± 484	611 ± 277
Liver reserves vitamin A (μmol/g)[4]	0.52 ± 0.44	0.51 ± 0.27	0.33 ± 0.15
Serum retinol (μmol/L)	2.58 ± 0.46	2.73 ± 0.60	3.5 ± 1.34

[1]RAE = retinol activity equivalents where 1 RAE = 1 μg all-*trans*-retinol, 2 μg supplemental all-*trans*-β-carotene, 12 μg dietary all-*trans*-β-carotene, or 24 μg of all other provitamin A carotenoids (i.e., α-carotene and β-cryptoxanthin). Data are presented as mean ± SD. No differences existed among groups $P \geq 0.11$, Kruskal Wallis Test.

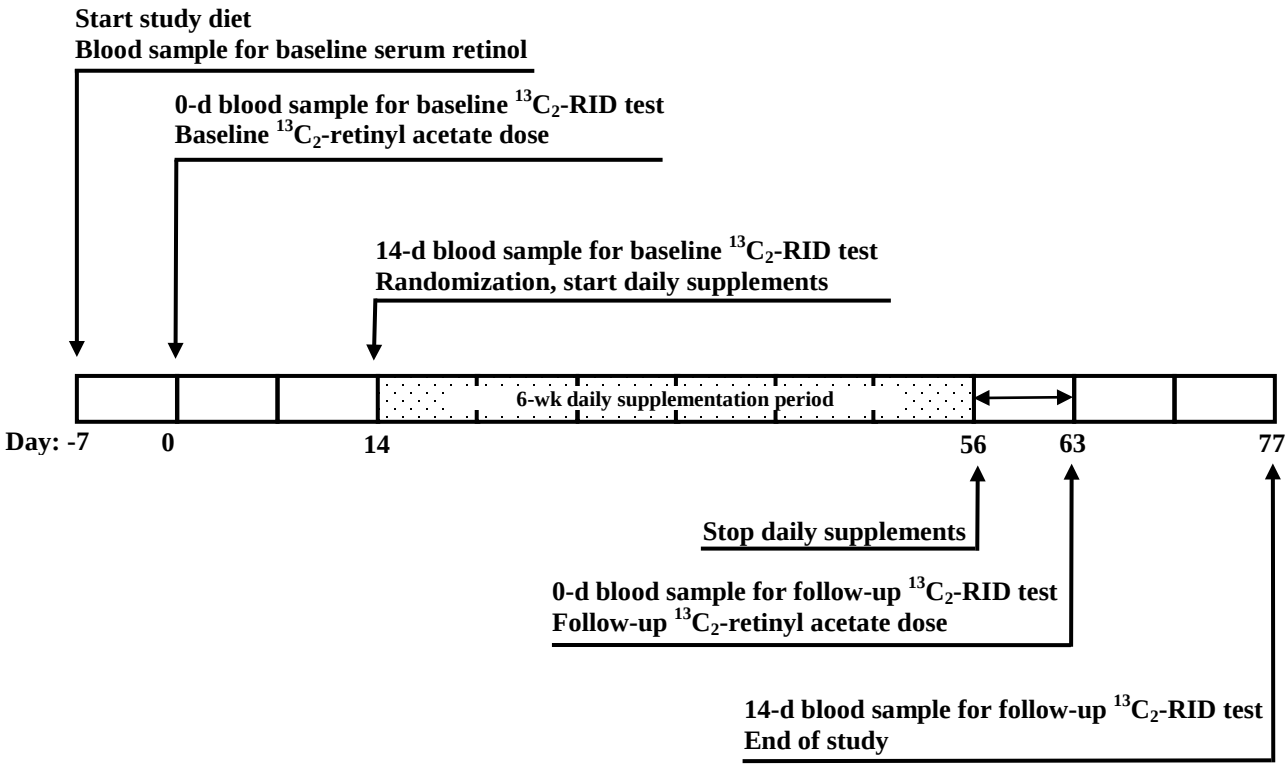
[2]Dietary intake reported from Harvard Food Frequency Questionnaires.

[3]Calculated using corrected standard mass-balance equation (please refer to methods for details.)

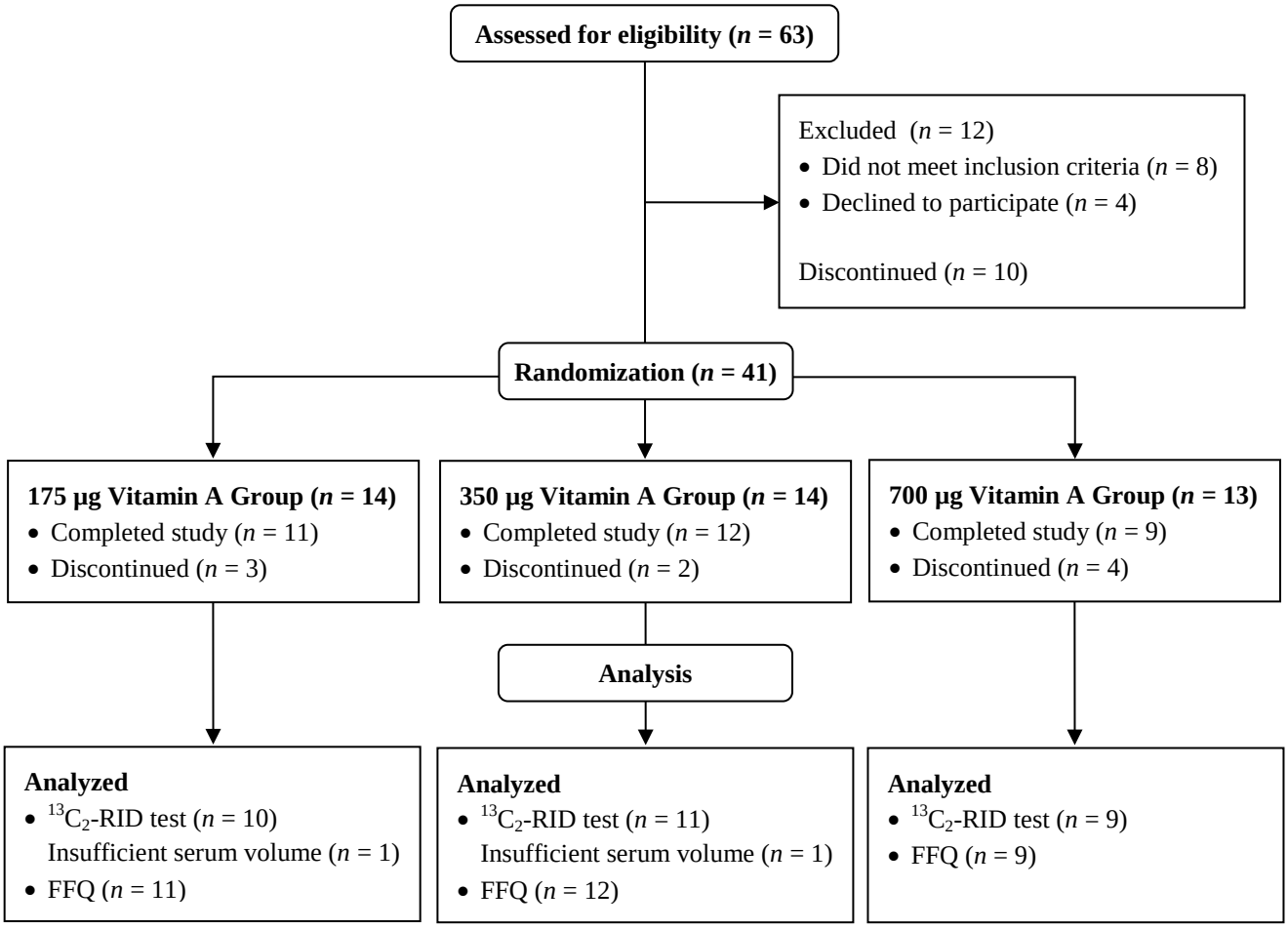
[4]Assumes 80% of total body vitamin A is stored in the liver and liver weight is 2.4% of body weight (please refer to methods for details).

CHAPTER 3, FIGURE 1: Study timeline.

The $^{13}C_2$-retinyl acetate isotope dilution ($^{13}C_2$-RID) test was performed at baseline and after a 6-wk period of vitamin A supplementation. Each rectangle represents 1 week, and white rectangles show when participants were consuming the study diet without taking supplements. ⟷ = 7-day mixing period.

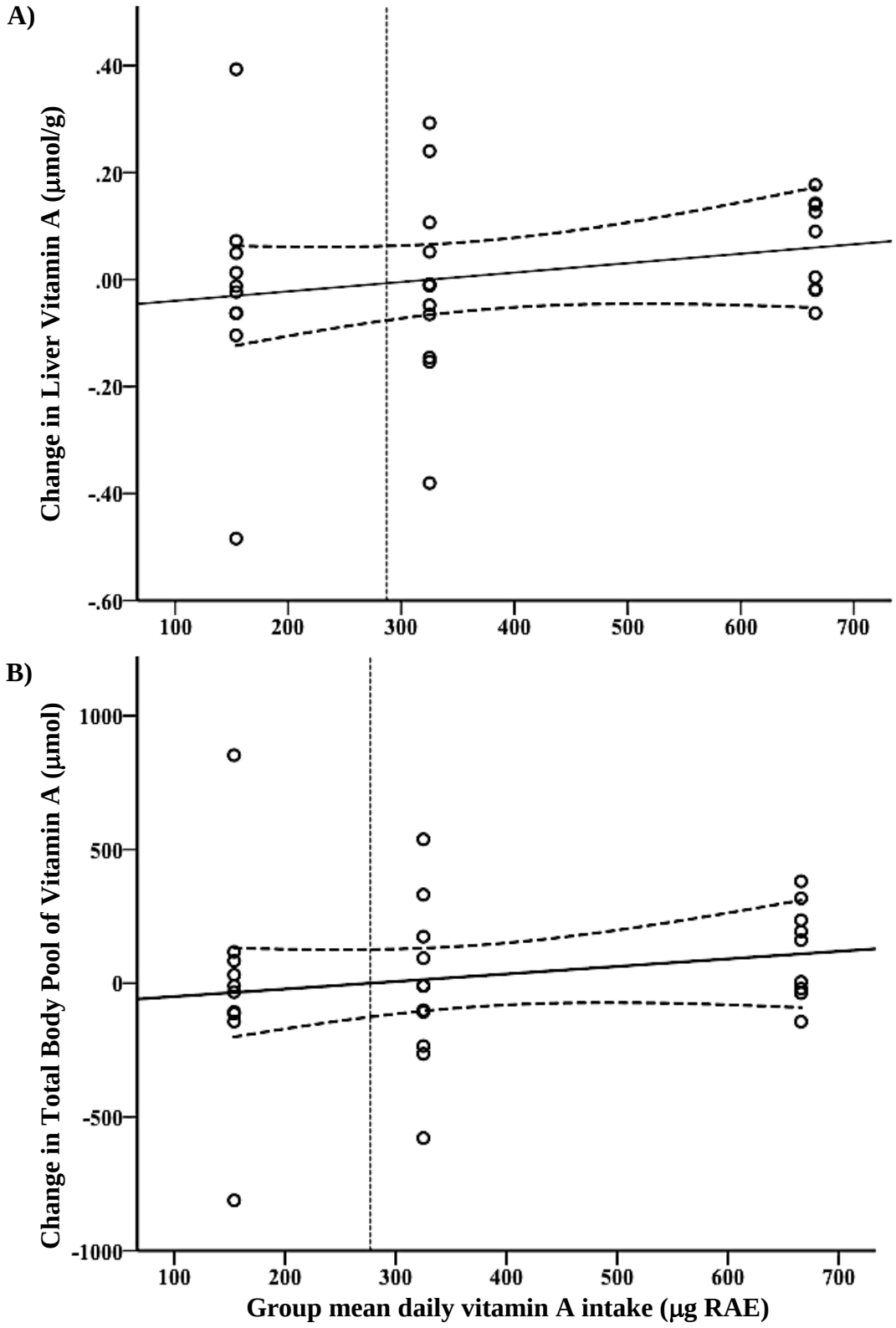


CHAPTER 3, FIGURE 2: Participant flow through a study that evaluated the current estimated average requirements and recommended dietary allowances for vitamin A in young adult women aged 19-30 y.



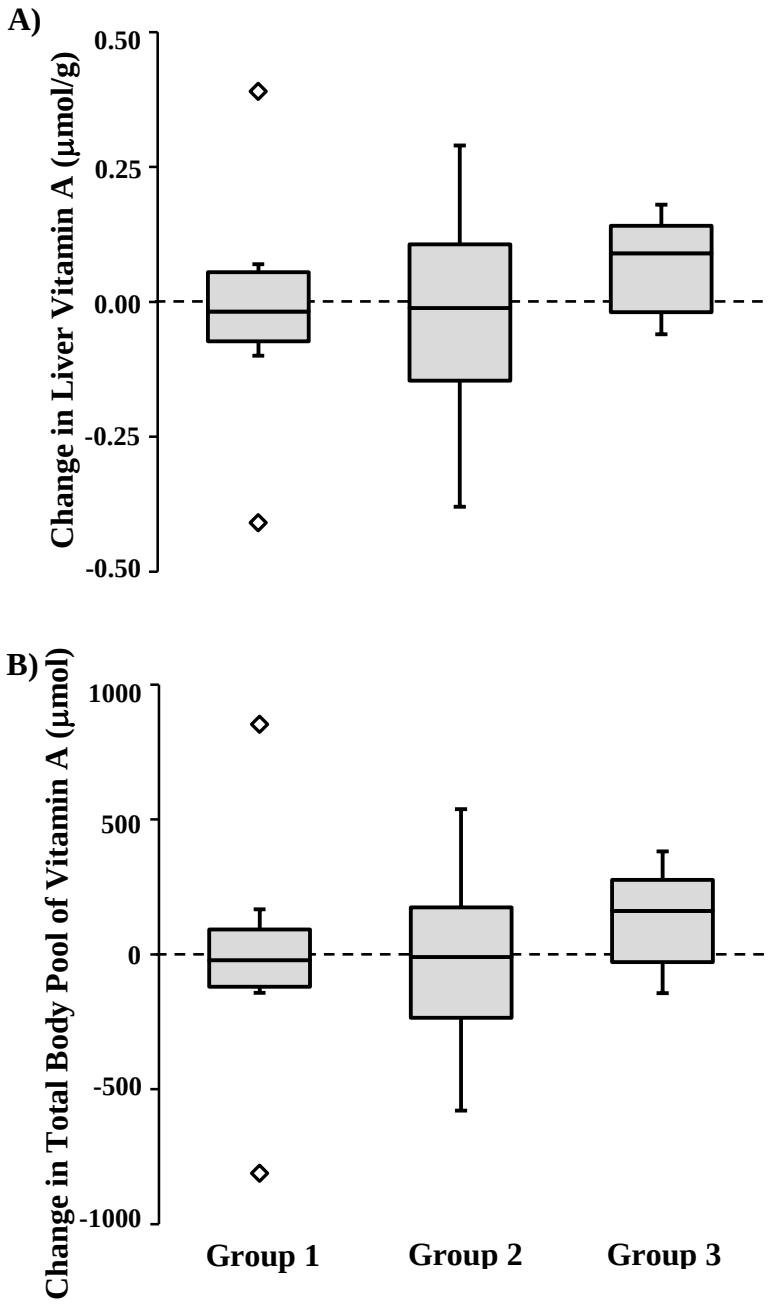
CHAPTER 3, FIGURE 3: Change in vitamin A per g liver (panel A) and change in total body pool (TBP) of vitamin A (panel B) versus mean group intake.

Participants were assigned to consume approximately 25, 50, or 100 % of the RDA (175, 350, or 700 μg retinol groups, respectively) over a 6-wk period. Actual intake was estimate from returned dietary logs (please see methods) and averaged within each group. Liver reserves are calculated assuming 80% of total body vitamin A is stored in the liver and liver weight is 2.4% of body weight. For panel A, (change in liver reserves) = 0.0002(daily intake) – 0.0573 (r = 0.21, P = 0.26). For panel B, (change in TBP) = 0.282(daily intake) – 77.987 (r = 0.19, P = 0.31). The regression lines are shown as solid lines and the 95% CI for the regression line are shown as dashed lines. The dotted vertical lines represent the x-intercepts (287 and 277 μg RAE for the panels A and B, respectively). ***Figure is shown on the following page.***


A)
Change in Liver Vitamin A (μmol/g)
.40
.20
.00
-.20
-.40
-.60
100
200
300
400
500
600
700
B)
Change in Total Body Pool of Vitamin A (μmol)
1000
500
0
-500
-1000
100
200
300
400
500
600
700
Group mean daily vitamin A intake (μg RAE)

CHAPTER 3, SUPPLEMENTAL FIGURE. Box plots of change in vitamin A per g liver (panel A) and change in total body vitamin A (panel B) after a 6-week consuming approximately 25, 50, or 100 % of the RDA (175, 350, or 700 μg retinol activity equivalent groups, respectively).

Liver reserves are calculated assuming 80% of total body vitamin A is stored in the liver and liver weight is 2.4% of body weight. There were no significant differences between groups (Kruskal Wallis Test, $P \geq 0.29$). Possible outliers are denoted with diamonds (◇).



CHAPTER 4

Conclusions and future directions

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The research in this dissertation focused on the evaluation of the estimated average requirement (EAR) for vitamin A for young adult women, who are not pregnant or lactating, using stable isotope dilution technology. Two different experimental approaches were applied to study the daily intake required to maintain vitamin A status. Prior to this research, only one other study had been conducted to assess vitamin A intake requirements, and that was in Bangladeshi men (1).

In the first study, daily intake was assessed using a food frequency questionnaire and baseline status was determined using the $^{13}C_2$-retinol isotope dilution ($^{13}C_2$-RID) test. An average daily intake of approximately 1200 μg retinol activity equivalents (RAE) was associated with hepatic liver reserves of 0.45 μmol vitamin A/g liver. This concentration is over six times the proposed minimally-acceptable liver reserves (0.07 μmol/g) originally used to calculate the EAR. The daily intake was also ~70% greater than the current recommended daily allowance (RDA). This study showed that liver vitamin A is consistent with intake, but raised the question whether this level of hepatic reserves could have any clinical implications.

It is known that cleavage and absorption of provitamin A carotenoids is dependent on host nutritional status and vitamin A intake (2, 3). However, absorption of preformed vitamin A is not saturable (4) and the efficiency of absorption can be as high as 90% (5). Given that 40% of women in this study reported taking supplements, which comprised 33% of dietary vitamin A, it is possible these women are chronically exposed to substantial boluses of preformed vitamin A. This fat-soluble vitamin can then accumulate in the liver over time. At a mean age of 22 y, there are several decades for vitamin A to accumulate and possibly impact bone health well before the onset of osteoporosis (2). Cohort studies of vitamin A

intake, status, and osteoporosis starting at young-adult years in women are needed to clarify the relationship between elevated vitamin A and bone health. Such studies would help determine if liver reserves that are six times the minimally-acceptable level in the 20th decade contribute to osteoporosis later in life. If so, the EAR should be reduced.

In the second study, vitamin A status was assessed before and after a 12-wk dietary intervention of three different levels of vitamin A intake. The baseline liver reserve of the women in this study was 0.46 μmol retinol per g liver. This estimate of baseline status is nearly identical to that in our first study. Status did not change significantly across groups during the intervention. A regression line was plotted and a point-estimates of 277 and 287 μg RAE were obtained for the daily intake required to maintain total body and liver vitamin A. Averaged together, 282 μg RAE is the estimated intake required for maintenance of a liver reserve of 0.46 μmol retinol per g liver. This is almost half the current EAR. This low maintenance intake further suggests that the women in study 1 are in positive vitamin A balance (*i.e.,* increasing their liver reserves over time).

As discussed in Chapter 1, vitamin A status is poorly defined in the subclinical but abnormal range and is difficult to measure. Even “normal” vitamin A status is difficult to describe, most notably because of the numerous assumptions applied to describe status in terms of liver reserves. We (Chapter 2), and others (1, 6), have proposed that the total body pool is the best way to describe vitamin A status. Difficulties arise in redefining status in terms of the total body pool due to variations in body-habitus and storage of vitamin A in adipose tissue. Stable isotope dilution testing with body composition assessment and kinetic modeling of the vitamin A pool would be useful for determining the impact of body habitus on vitamin A kinetics.

Finally, the two studies in this dissertation provide evidence that the EAR for vitamin A may need to be reduced for healthy, well-nourished populations, in order to maintain safe liver reserves. Further, we have shown that stable isotope dilution can be used to assess vitamin A status in young women in the US. Similar studies could be conducted in all the life-stage and gender groups of the Daily Reference Intakes to improve our current recommendations for daily vitamin A consumption.

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APPENDIX

The Kinetics of a Small Test Dose of $^{13}C_2$-Retinyl Acetate in Young Adult Women

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ARV has no conflicts of interest (COI); BG, no COI; CRD, no COI; SAT, no COI.

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Registration was not required for this trial.

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Abbreviations: At% ^{13}C, atom percent ^{13}C; $^{13}C_2$-retinol isotope dilution, $^{13}C_2$-RID; EAR, estimated average requirement; FFQ, food frequency questionnaire; GCCIRMS, gas chromatography-combustion-isotope ratio mass spectrometer; RAE, retinol activity equivalents; RDA, recommended dietary allowance; TBP, total body pool; VA, vitamin A.

Materials and Methods

Study design and sample collection. The Health Sciences Institutional Review Board of the University of Wisconsin (UW) School of Medicine and Public Health approved this study. Participants gave written consent after receiving written and verbal explanations of the study and having their questions answered. All study events took place at the Nutritional Sciences building on the UW-Madison campus.

Eight female participants were recruited for this study. In the morning of the first day of the study (d 0) study phlebotomists obtained baseline blood samples after an overnight fast of at least 10 h. Immediately after the baseline sample was taken, each participant was given an oral dose of 2 μmol $^{13}C_2$-retinyl acetate in corn oil. Participants ate peanut butter on either a cracker or banana immediately after dosing to provide a fat source for adequate dose absorption and were offered a breakfast containing no vitamin A. Participants were asked to refrain from drinking alcohol for three days before and four days after dosing to avoid altered vitamin A metabolism. $^{13}C_2$-retinyl acetate was synthesized according to previously published methods (1).

To minimize the total number of blood draws per individual during the first 24 h of the study, we divided participants into two groups of four. Group 1 had blood samples taken at 4, 7, 11 h after dosing and Group 2 had blood samples taken at 5, 9, and 13 h after dosing. Participants stayed at the Nutritional Sciences building on the UW-Madison campus for the 11 – 13 h after dosing so we could assure blood collection timing and provide vitamin A-free meals and snacks so as not to perturb the labeled dose. Meals were offered after obtaining blood samples. Both groups had blood samples taken at 24, 33, 48, and 57 h and 3, 4, 7, 10, 14, 17, 21, 27, 34, 42, and 52 d after dosing. Upon completion of the 52-day blood collection,

participants were paid an honorarium of $200. Participants were asked to also give blood at 62, 77, 97, 122, and 152 d after dosing and were paid an honorarium of $20/sample. After the first day of the study, all but two blood draws occurred in the mornings after a 10-h food and alcohol fast and participants were offered breakfast immediately after the sample was collected. The exceptions were the 33- and 57-h draws.

Experienced phlebotomists collected venous blood samples into serum separator tubes with clot activator (Becton-Dickinson, USA). After collection, samples clotted at room temperature in the dark to avoid photolysis of vitamin A. Study investigators centrifuged clotted samples at 2200 X *g* for 10 min at 4ºC to separate serum. Serum was placed into vials of 1 or 2 mL aliquots and oxygen was displaced with nitrogen before the samples were stored at –70º C.

Participants. We sent an email advertisement to female students at the UW-Madison to recruit participants. Inclusion criteria were age 19 – 30 y, normal BMI (18.5 – 24.9 kg/m^2) by self-report, non-smoking, not pregnant or trying to become pregnant, and not lactating. Exclusion criteria included weight loss ≥ 10 pounds (4.5 kg) during 3 mo prior to recruitment, actively trying to lose weight, inability to refrain from drinking alcohol when requested, amenorrhea, acute or chronic illness including hepatitis, current or previous history of anorexia or bulimia, concurrent participation in other studies, or a family member already enrolled in the study. Recruitment occurred during March, 2008.

A study investigator (ARV) discussed the purpose and study design with potential participants during an initial phone conversation. If the potential participant expressed interest in continuing, ARV assessed her for study eligibility, except for medical history, over

the phone and invited her for an in-person meeting if criteria were met. At the in-person meeting, ARV gave potential participants further details about the study and answered all questions. After obtaining consent, ARV took a brief medical history to evaluate exclusionary conditions.

Baseline dietary habits and body composition. To quantify baseline vitamin A intake, participants completed a 2007 Harvard Food Frequency Questionnaire (FFQ). The FFQ has been validated for estimating long-term nutrient intake (2, 3). The Harvard School of Public Health quantified FFQs and individual results were not shared with participants. The vitamin A intake estimates were given in retinol activity equivalents (RAE) where 1 RAE = 1 μg all-*trans*-retinol, 2 μg supplemental all-*trans*-β-carotene, 12 μg dietary all-*trans*-β-carotene, or 24 μg of all other provitamin A carotenoids (i.e., α-carotene and β-cryptoxanthin) (4).

Body composition (fat and fat-free mass) and body weight were measured using a BOD POD® (Life Measurements, Inc., Concord, CA), which is an FDA-approved medical device that uses air-displacement plethsmography and whole body densitometry. For this population, the Siri equation was used to estimate body composition from body density where % body fat = (4.95/body density – 4.5)*100 (5). Percent fat-free body mass = 100 – % body fat. ARV, a certified BOD POD® user, performed all BOD POD® measurements and calibrated the electronic scale prior to each participant. ARV measured height using a wall-mounted stadiometer.

Carbon isotope composition of serum retinol and estimation of vitamin A stores. One study investigator (CRD) performed all serum analyses. CRD purified retinol from 1-2 mL serum

as described previously (6) with minor modifications. For the first and second purifications, a GraceSmart RP18 (150 x 4.6 mm, 5 µm, 120 Å) and a GraceSmart RP18 (250 x 4.6 mm, 5 µm, 120 Å) column were used, respectively. CRD injected reconstituted purified retinol into the same gas chromatography-combustion-isotope ratio mass spectrometer (GCCIRMS) system as previously published (6) and run under the same conditions used by Escaron et al. (7). The GCCIRMS determines the atom percent ^{13}C (At% ^{13}C) based on the enrichment of the carbon pool with ^{13}C and the ratio of ^{13}C to ^{12}C.

To evaluate the disappearance and calculate the half-life of the ^{13}C dose from the serum retinol pool, the At% ^{13}C was used. First-order kinetics, a stable pool of ^{12}C, and the $^{13}C_2$-retinyl acetate dose making the only significant contribution of ^{13}C over time, were assumed.

The total body pool (TBP) of vitamin A was calculated at baseline using the standard mass-balance equation after the dose reached equilibrium (8): $(F_a \times a) + (F_b \times b) = (F_c \times c)$. Where a is µmol retinol absorbed from the dose, estimated to be 100% of the administered dose for those with normal to excessive stores (7); b is the TBP in µmol vitamin A at day 0 of $^{13}C_2$-RID testing; and c is the TBP in µmol of vitamin A after dosing ($c = a + b$). F_a is the fraction of the dose that is labeled with ^{13}C, which is equal to 0.1 since $^{13}C_2$-retinyl acetate is converted to $^{13}C_2$-retinol *in vivo* at the time of absorption, and two of the 20 carbons were labeled. F_b is the At% ^{13}C in serum retinol at baseline in decimal form, and F_c is the decimal form of At% ^{13}C in serum retinol at equilibrium. For any F, $F_x = R_x/(R_x + 1)$ where R_x is $^{13}C_x/^{12}C_x$. Equation 1 is solved for b, the TBP of retinol at baseline.

TBP of vitamin A was corrected for catabolism of the dose of labeled vitamin A, which is assumed to be equivalent to the half-life of unlabeled vitamin A using the following

equation: Corrected TBP of retinol = b x e^{-kt}. Where b = TBP (from equation 1), k = ln(2)/half-life and t = time in d since dosing. We used both a half-life from the literature [140 d assumed to be independent of vitamin A status and time (9, 10)] and the half-life calculated from these data.

Liver stores of vitamin A were estimated assuming 80% of TBP is in the liver. We assumed our sample was from a well-nourished population and based our storage assumption on a study of rhesus monkeys with hypervitaminosis A showed that an estimate of 80% of the TBP storage in the liver underestimated actual liver storage (7). Liver vitamin A assumed that liver weight is 2.4% of body weight in adults (4).

Model-based compartmental analysis. We followed the protocol used previously by Escaron *et al.* (7). Simulation, Analysis, and Modeling software for Windows (WinSAAM) was used to model the kinetics of the fraction of oral dose in serum. We estimated blood volume using the Nadler formula (11) where blood volume = $0.3561 \times (\text{height in meters})^3 \times 0.03308 \times (\text{weight in kg}) + 0.1833$. Serum volume was estimated assuming plasma is 55% of blood volume and contains 8% proteins (i.e., serum volume = blood volume $\times$ 0.55 $\times$ 0.92). Baseline serum retinol concentration was multiplied by estimated serum volume to estimate total serum retinol pool (μmol). This was multiplied by atom % ^{13}C to give μmol ^{13}C, and this was divided by 2 to obtain μmol ^{13}C-retinol because there are two ^{13}C atoms per labeled retinol molecules. The μmol ^{13}C-retinol was divided by the oral dose of ^{13}C-retinol given (2 μmol) to obtain the fraction of the dose in the serum at each time point.

Statistical analysis. The primary outcome of interest was the change in At% ^{13}C over time. To compare means over time, we performed a nonparametric analysis based on ranks that accounted for repeated measures within the same subject as well as unequal gaps in time between data points (Proc mixed ry with sp(pow) in SAS). Data are presented as means ± SD, unless otherwise noted. $P < 0.05$ is considered statistically significant. SPSS, version 20 (Ireland), was also used for some comparisons.

RESULTS

Baseline characteristics of the study participants are shown in **Table 1**. Baseline vitamin A intake was no different from the current RDA when supplements were included and excluded from the FFQ analysis (two-sided *t*-test, $P \geq 0.468$). Three (38%) women reported using supplements. Baseline liver vitamin A is shown in Table 1 and is greater than 0.07 µg/g, the cut-off for deficiency, and less than 1.05 µg/g, the cut-off for excess ($P < 0.024$ and < 0.0001, respectively, two-tailed t-tests).

The disappearance of ^{13}C from the serum retinol pool of carbon is shown in **Figure 1** as a function of AT% ^{13}C.

Acknowledgments

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TABLE 1. Baseline Characteristics of Study Participants (n = 8)[1]

Characteristic	Mean ± SD	[min, max]
Age (y)	22 ± 2	[19, 27]
Weight (kg)	59.2 ± 6.4	[50.4, 70.2]
BMI (kg/m^2)	24.3 ± 5.0	[19.5, 32.3]
Daily intake (RAE/d)[2]	1069 ± 1063	[354, 3154]
Daily intake excluding supplements (RAE/d)[2]	761 ± 513	[340, 1654]
Total body pool of vitamin A (μg)	724 ± 602	[197, 2036]
Liver reserves of vitamin A (μg/g)	0.40 ± 0.30	[0.13, 1.04]

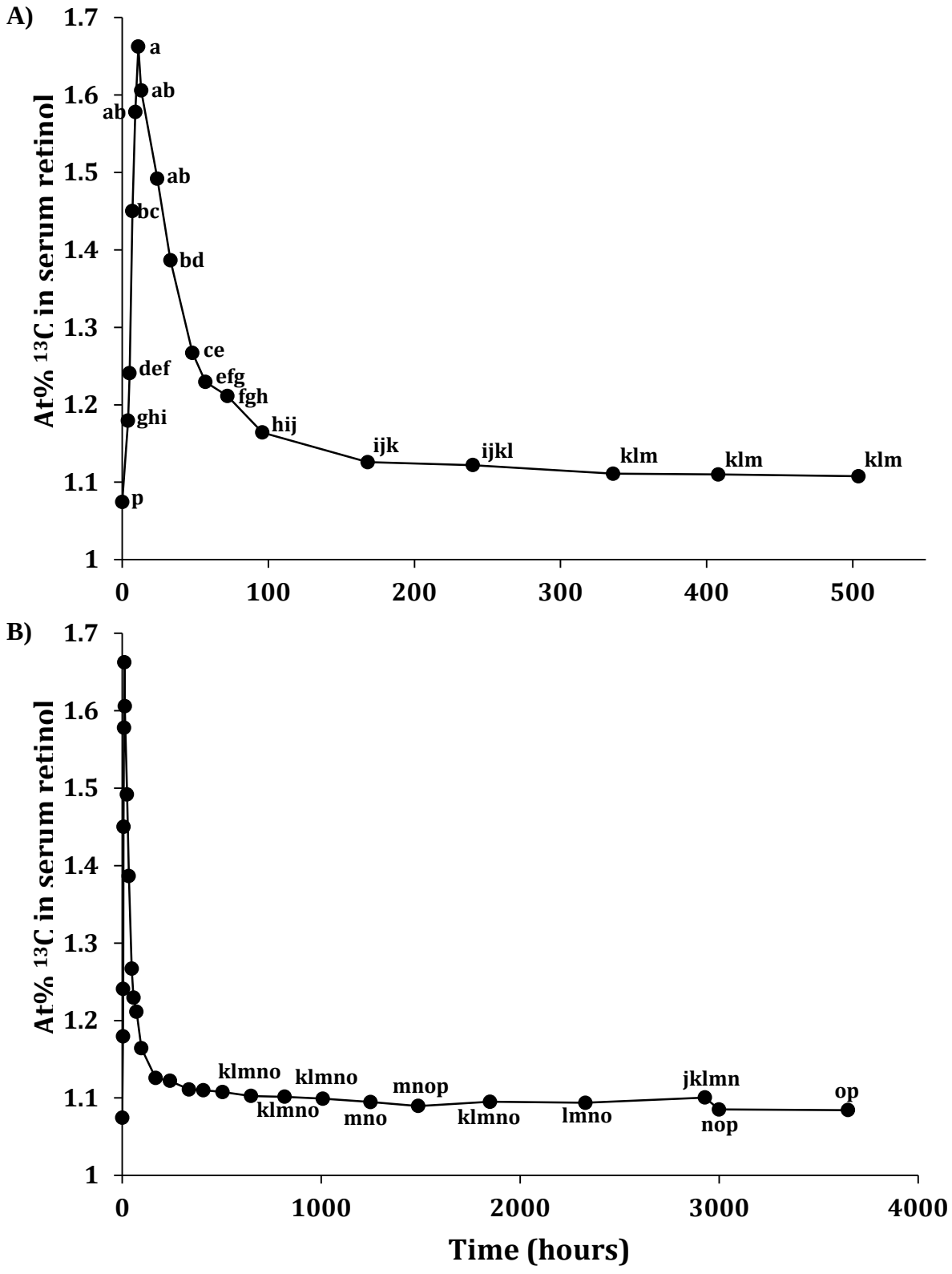
[1]RAE = retinol activity equivalents where 1 RAE = 1 μg all-*trans*-retinol, 2 μg supplemental all-*trans*-β-carotene, 12 μg dietary all-*trans*-β-carotene, or 24 μg of all other provitamin A carotenoids (i.e., α-carotene and β-cryptoxanthin).

[2]Dietary intake reported from Harvard Food Frequency Questionnaires.

FIGURE LEGEND.

At% ^{13}C in serum retinol as a function of time. Eight female volunteers took an oral dose of 2 μmol $^{13}C_2$-retinyl acetate dissolved in corn oil. Blood samples were analyzed using GCCIRMS for $^{13}C_2$ in serum retinol. There was an overall significant effect of time on At% ^{13}C. In panel A, the %At of ^{13}C is shown time 0 through hour 504 (day 21). In panel B, the entire study is shown. Time points with different superscripts are significantly different ($P < 0.05$), including across panels. Due to crowding of the data points, the superscripts for times > 504 h are only marked in panel A. ***Figure is shown on the following page.***

FIGURE 1.


A)
At% 13C in serum retinol
1.7
1.6
1.5
1.4
1.3
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1
0
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At% 13C in serum retinol
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Time (hours)
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mno
mnop
klmno
lmno
jklmn
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Strategies to Increase Vegetable or Reduce Energy and Fat Intake Induce Weight Loss in Adults

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For obese individuals seeking to optimize health and well-being, healthy dietary strategies are important. Vegetables and fruits contribute to a healthy diet, and increased consumption may cause weight reduction by displacing foods high in energy and fat. The objective of this study was to determine if advising high vegetable (8 servings) and moderate fruit (2–3 servings) consumption would result in weight reduction in obese individuals. We compared this to advising a more traditional strategy of reducing daily energy intake by 500 kcal (2.1 MJ)/d and limiting energy from fat to ≤25%. A randomized study design was used. Subjects (age 21–50 y, *n* = 30/group) received food (2 meals + 1 snack/d, 5 d/wk) and education (2 group lessons/wk plus individual consultations as requested) for the first 3 mo. Weight and body composition were measured at baseline and after 3, 12, and 18 mo. Fasting serum lipid panel, insulin, glucose, hematocrit, and C-reactive protein were measured at baseline, 3, and 12 mo. Both groups lost weight after 3 mo ($P = 0.0087$ for high vegetable diet and $P < 0.0001$ for energy reduction diet), and the energy and fat reduction diet resulted in lower weight over time ($P < 0.0001$, treatment effect). Total cholesterol and cholesterol:HDL decreased after 3 mo in both groups ($P \leq 0.0061$). Both strategies produced initial weight loss at 3 mo, but only the group following the caloric and fat reduction advice maintained weight loss at the 12- and 18-mo follow-up assessments. Nonetheless, the group following the high vegetable advice did not regain weight above baseline. In conclusion, traditional messages to reduce calories and fat are important, and increasing vegetable intake can assist individuals to maintain weight. Exp Biol Med 234:542–552, 2009

Key words: body composition; caloric restriction; obesity; vegetables; reducing diet; weight loss

Introduction

Due to concerns of negative health effects of obesity, strategies for optimizing health in obese individuals are needed. A potential dietary strategy is consumption of high amounts of vegetables and fruits (1, 2). Not only is it known that a diet high in vegetables and fruits can reduce the risk for cancer (3) and cardiovascular disease (4), improve bone health (5, 6), and reduce age-related cognitive decline (7), it may also result in weight loss (8–11). This may be due to a reduction in the energy density (kcal/g food) of the diet resulting in less energy consumed daily (11–14). Long-term maintenance of modest weight loss, as little as 10% of body weight, has been shown to be sufficient for reducing risk of multiple obesity-related medical complications, such as diabetes and cardiovascular disease (15, 16), sleep-disordered breathing (i.e., a condition characterized by either pauses in or shallow breathing during sleep) (17), and for improving overall physical and psychological health (18).

Several weight-loss studies of obese individuals have examined dietary strategies that include a focus on increasing vegetable and fruit consumption in addition to other goals such as a reduction in energy intake (8) or fat (11) or an increase in grains (19) or low-fat dairy products (20, 21). This study evaluated the impact of advising high vegetable consumption (≥8 servings/d) and moderate fruit consumption (2–3 servings/d), in the absence of other goals, on weight and body composition after 3, 12, and 18 mo and on serum chemistry values after 3 and 12 mo in obese individuals. The hypothesis of this study was that including high amounts of vegetables and moderate amounts of fruit would induce weight loss by causing a reduction in total

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energy and fat consumed daily, presumably by displacing foods higher in energy and fat from the diet. We predicted that subjects following this dietary advice would continue to lose weight long-term, because once incorporated as a routine part of the diet, high vegetable and fruit consumption would be more sustainable than counting calories and grams of fat from food labels.

Materials and Methods

Subjects. Subjects were recruited from the greater Madison, WI, area using posted flyers and newspaper and website advertisements seeking obese volunteers for a weight-loss study. Respondents were screened by telephone interview for major eligibility and then via medical history, physical activity questionnaire, and physical exam by the study medical doctors.

To be considered for the study, subjects had to be 19–50 y of age, have a BMI $\geq$30 but $\leq$40 kg/m^2, and be willing to visit the study kitchen twice per week during the first 16 wk of the study for food pick-up and group lessons. Exclusion criteria comprised aerobic exercise >90 min/wk, consumption of $\geq$5 servings vegetables and/or fruits per day, history of insulin treatment, history of drug or alcohol abuse, participation in other research studies that could confound results, plans to move away from the study area within 12 mo of the study start, pregnancy or lactation, serious medical or psychiatric illness, unwillingness or inability to discontinue use of supplements containing carotenoids, use of drugs that might affect weight loss, and weight change >3% of body weight during the 3 mo prior to recruitment. Only 1 member per household was eligible to participate.

Of the 77 people who completed screening (Fig. 1), 14 declined participation, and 3 were excluded, resulting in 60 subjects (16 males, 44 females). These 60 subjects were stratified by gender and BMI and randomized to either the high vegetable and modest fruit consumption (HiVeg) group or the energy- and fat-reduction diet (Reduction) group (n = 30/group; 8 males/group). Of the randomized subjects, 78.3% were Caucasian, 8.3% were black or African American, 5% considered themselves Hispanic or Latino, 3.3% were Asian, and 5% were other or did not report their race or ethnicity. The majority of the subjects were married (51.7%), and 74.2% of those married had children; 45% were single, and 18.5% of single subjects had children. The remaining 2 subjects considered themselves partnered (e.g., engaged) with 1 of them having children. Subjects gave written informed consent. The Health Sciences Institutional Review Board at the University of Wisconsin School of Medicine and Public Health approved all aspects of this study.

Body composition was measured by air displacement plethysmography (BOD POD®, Life Measurements, Inc., Concord, CA) (22, 23). Weight was measured using the BOD POD® scale, which was tested with calibration weights each day of use. Height was measured at baseline using a wall-mounted stadiometer. All body composition and weight assessments were conducted by BOD POD® certified users.

Blood samples were drawn into sterile Vacutainer® tubes containing 5.4 mg K_2EDTA or serum separator gel (Becton Dickinson, NJ) after an overnight fast (at least 8 h). Samples sat for 10 min and then were centrifuged for 10 min at 4°C and 2410 $\times$ g. Serum was transferred to transportation tubes provided by the contract laboratory and stored on ice until analysis within 18 h for fasting serum triacylglycerols, total cholesterol, HDL cholesterol, VLDL cholesterol, LDL cholesterol (calculated by difference), cholesterol:HDL ratio, insulin, glucose, hematocrit, and C-reactive protein at the contract laboratory (Consultants Laboratory, Fond du Lac, WI).

Experimental Design. A randomized study design was used to compare the effects of the dietary strategies on weight, body composition, and serum chemistry profile. Primary outcomes of interest were change in weight, fat mass, fat-free mass, and absolute BMI. These were measured at baseline, 3, 12, and 18 mo. Secondary outcomes included fasting serum lipid panel (cholesterol, triacylglycerol, HDL, LDL, VLDL, cholesterol:HDL), insulin, glucose, hematocrit, and C-reactive protein, which were measured at baseline, 3, and 12 mo. Subjects were asked to report any adverse events throughout the trial.

Subjects randomized to the HiVeg group were educated about counting vegetable and fruit intake using the Food Guide Pyramid (24), where intake is described by the number of servings. The Food Guide Pyramid is a free and publicly available resource based on the *Dietary Guidelines for Americans, 5th Ed* (25), and was used because this study was initiated prior to, but concluded after, the release of the *Dietary Guidelines for Americans 2005, 6th Ed* (26) and *MyPyramid* (27). According to the Food Guide Pyramid, ½ cup raw or cooked vegetables, 1 cup raw leafy greens, or ¾ cup vegetable juice is equivalent to 1 serving of vegetables. For fruits, ½ cup of raw or cooked fruit and ¾ cup fruit juice equal 1 serving. HiVeg subjects had a daily goal of consuming 8 servings of vegetables and 2–3 servings of fruits. The HiVeg group was asked not to consume potato chips, fried vegetables, or fruit or vegetable juices to meet their goal. In the *post hoc* dietary analysis, however, fruits and vegetables were defined according to the updated *Dietary Guidelines for Americans 2005, 6th Ed* (26) and *MyPyramid* (27). Thus, potato chips, french-fried potatoes, 100% fruit juice, and 100% vegetable juice counted toward goals.

Subjects randomized to the Reduction group had two daily goals: reduce caloric intake by 500 kcal (2.1 MJ) from the estimated kcal needed for weight maintenance each day and consume <25% energy from fat. Daily kilocalories for weight maintenance were estimated by multiplying an individual's estimated resting energy expenditure (REE) by an individually appropriate activity factor. REE was

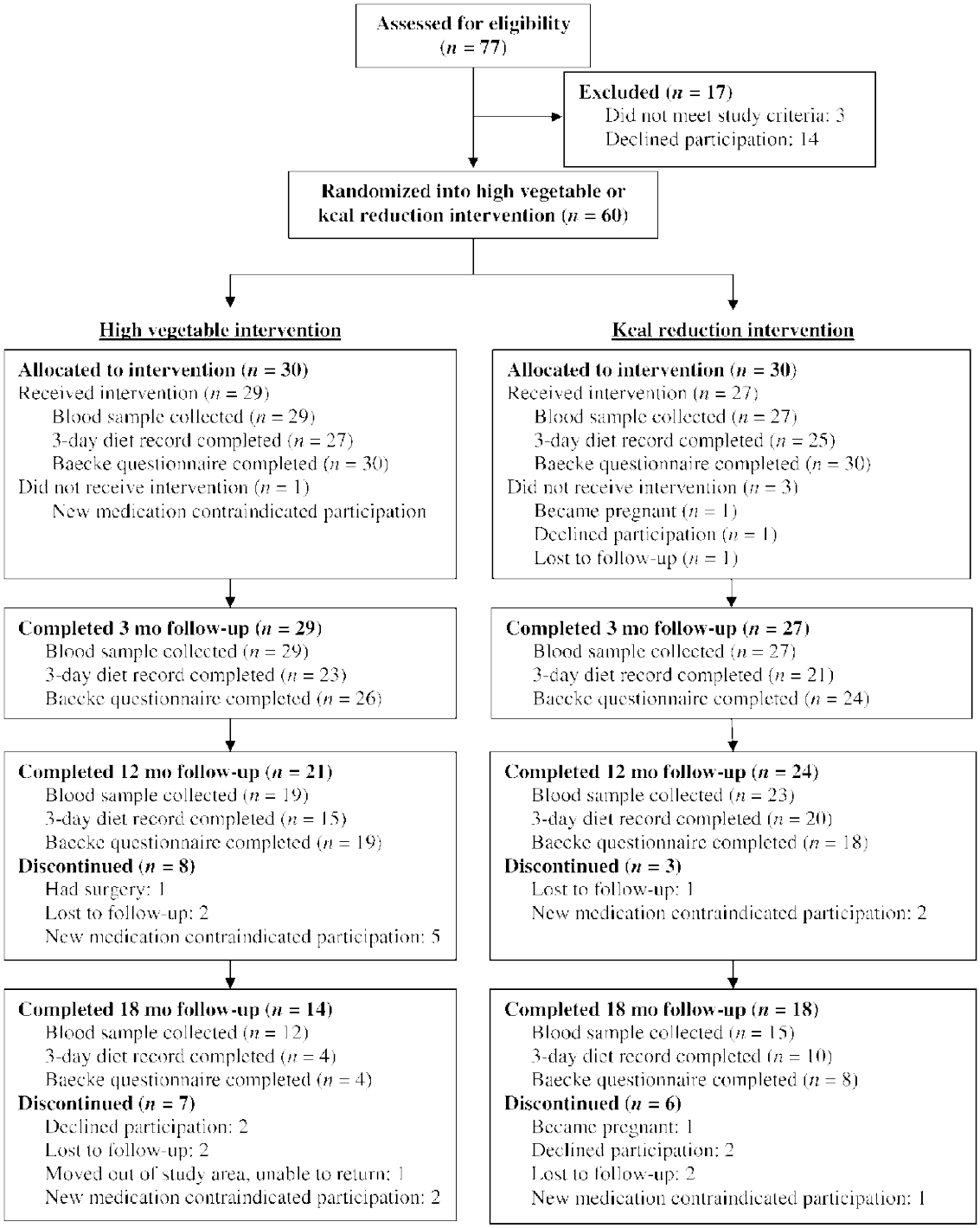

Figure 1. Subject progression through the trial. Obese individuals were recruited from Madison, WI, and surrounding areas. At baseline, male (n ¼16) and female subjects (n ¼44) were randomly assigned, stratified by gender and BMI, to a weight-loss intervention focused on including high amounts of vegetables and moderate amounts of fruit or restricting kilocalories and fat intake. Follow-up data were collected at 3, 12, and 18 mo. Subjects who were counted as completing a follow-up assessment had their weight and body composition measured. The number of subjects who also gave blood samples, completed 3-d diet records, and completed Baecke Physical Activity Questionnaires at each follow-up is shown. Reasons for discontinuation, when known, are listed.

Table 1. List of Foods Provided to Obese Subjects from Baseline to 4 mo[a]

Meal and foods provided
Breakfast
Fat-free milk, 8 oz (0.24 L)
Fruit,[b] 1 serving
Cereal,[b] 1 cup (0.24 L)
Muffin
Carrot muffin (3 d/wk)[c]
Pumpkin muffin (2 d/wk)[d]
Lunch
Lean Cuisine® entrée[b,e]
Fat-free milk, 8 oz (0.24 L)
Low fat or fat-free salad dressing,[b] 2 tablespoons (30 mL)
Vegetables
Raw vegetables[b,f,g] (Mondays)
Salad[h] + raw vegetables[b,f,i] (Tuesdays–Fridays)
Snack
Fat-free milk, 8 oz (0.24 L)
~60 kcal (0.25 MJ) snack[b,j]

[a] For the first 3 mo of the study, the menu was provided on weekdays (5 d/wk). For month 4 of the study, the menu was provided 2 weekdays per week.
[b] Indicates that subjects selected from offered options.
[c] Carrot muffins provided 1 or 0.5 serving vegetables to the HiVeg and Reduction groups, respectively.
[d] Pumpkin muffins provided 0.6 or 0.3 serving vegetables to the HiVeg and Reduction groups, respectively.
[e] Lean Cuisine® entrées provided 0.5–3 serving of vegetables; HiVeg subjects were always offered entrées with twice as many servings of vegetables as the entrées offered to Reduction subjects.
[f] Raw vegetable options included baby carrots, cauliflower, broccoli, snow peas, and tomatoes.
[g] On Mondays, 6 or 3 servings of raw vegetables were provided to the HiVeg and Reduction groups, respectively.
[h] On Tuesdays–Fridays, 2 or 1 servings of vegetables as a mixed-greens salad were provided to the HiVeg and Reduction groups, respectively.
[i] On Tuesdays–Fridays, 4 or 2 servings raw vegetables were provided to the HiVeg and Reduction groups, respectively.
[j] Snack options included 28 g soy nuts, 1 piece of fruit, or 14.5 g Baked Whole Grain Wheat Reduced Fat Triscuit® crackers, among others.

estimated using the equations by Mifflin et al. (28) which include height, weight, age, and gender. The activity factors used (1.3, 1.5, and 1.6 for very light, light, and moderate activity, respectively) were those recommended by the Institute of Medicine (29). A 500 kcal/d reduction was chosen to achieve a safe weight-loss rate of approximately 0.5 kg/wk. Caloric goals were modified weekly according to weight change for each subject.

Food, described in Table 1, was provided to subjects during the first 4 mo of the study to facilitate compliance. Although the food was intended for breakfast and lunch, subjects were free to consume it at anytime. Each day, 7–8 and 3.5–4 servings of vegetables were given to the HiVeg and Reduction groups, respectively. Both groups received 2 servings of fruit daily. Most vegetables were provided raw and subjects were encouraged to prepare them as they chose (e.g., consume raw, cooked, or use them in a recipe). To accommodate food preferences, subjects were allowed some choices. In general, choices were offered for frozen-meal entrées, vegetables, fruits, and snacks. When options were offered, the choices were nearly equivalent in type of food or macronutrients (e.g., contained the same number of vegetable servings, total energy, and/or fat). Food packaging and meal choices were similar between groups with the main difference being that the HiVeg group was given twice as many servings of vegetables as the Reduction group.

The trial comprised 4 phases. During Phase I (week 1–3), subjects were advised to transition from their usual eating habits to their assigned dietary strategy. Subjects were required to attend the morning educational sessions with members from the same treatment group 2 d/wk. Food was provided for weekdays (5 d/wk). Subjects were encouraged to ask for individual consultations if they desired additional assistance in achieving their dietary goals. For Phase II (week 4–12), subjects were asked to consistently meet their dietary goals. Subjects continued to have regular in-person education and support in the form of group education sessions (2 d/wk) and the optional individual consultations. Food was provided on weekdays (5 d/wk), and subjects were expected to adhere to their assigned dietary strategy on evenings and weekends. Phase III (month 4) was designed to transition subjects to following their dietary strategy independently. Food was provided only 2 d/wk. Group lessons were not offered, but subjects had the option to speak with researchers about any questions or concerns twice weekly when they picked up their study food. Individual consultations were still available upon request. Most individual consultations were accommodated informally after breakfast sessions in the mornings. During Phase IV (months 5–18), subjects were asked to follow their dietary strategy independently using the skills and knowledge obtained in the previous phases, but subjects could still request individual consultations. The coordinator provided support calls by telephone with gradually decreasing frequency (from weekly to monthly) from months 5 to 12.

It was not possible to blind subjects to their own treatment. However, the subjects were not aware of the other treatment by holding the morning educational sessions on different days, offering similar foods and education, and asking subjects not to discuss their diets with members of the other group. Similarly, it was not possible to blind researchers assisting with food distribution, giving dietary advice, or measuring body weight and composition. However, researchers and consultants who analyzed blood samples were unaware of the treatment assignments.

All subjects completed 3-d diet records (2 weekdays and 1 weekend day) at baseline, 3, 12, and 18 mo. These were analyzed for energy, fat, protein, fiber, vegetables, and fruit using Nutritionist Pro™ Version 3.1.0 (Axxya Systems; Stafford, TX, 2007).

Education. At the educational sessions, subjects were

Table 2. Baseline Characteristics by Treatment Group ($n = 30$/Group, Except Where Indicated)

	HiVeg[a]	Reduction[b]
Age (y)	30.7 ± 6.6[c,d]	36.4 ± 9.4
Male (*n*)	8	8
Body wt (kg)	94.2 ± 14.9	96.0 ± 17.1
Fat mass (kg)	40.0 ± 10.0[e]	39.8 ± 10.2[f]
Fat-free mass (kg)	54.0 ± 10.4[e]	56.4 ± 12.8[f]
BMI (kg/m^2)	33.7 ± 3.8	33.3 ± 3.5
Lifetime maximum body wt (kg)[g]	98.1 ± 17.0	103.2 ± 22.9
Calculated lifetime maximum BMI[h]	35.2 ± 4.5[e]	35.9 ± 5.9[f]
Age first overweight (y)[g]	12.8 ± 7.6[e]	15.2 ± 8.9
Maximum weight lost previously (kg)[g]	12.5 ± 7.9	16.6 ± 9.4[e]
No. of times lost 9.07 kg (20 lbs) or more[g]	1.4 ± 1.4[e]	1.8 ± 1.7[e]
No. of different weight-loss programs tried[g]	2.1 ± 1.3[e]	1.8 ± 1.4[e]
C-reactive protein (mg/L)	4.5 ± 3.2	8.0 ± 15.7
Insulin (pM)	79.0 ± 60.6	66.9 ± 31.9
Glucose (mM)	5.17 + 0.52	5.29 + 0.45
Hematocrit (vol/vol)	0.42 + 0.025	0.42 + 0.032

[a] HiVeg subjects followed a weight-loss strategy focused on including high amounts of vegetables and moderate amounts of fruit in the diet.
[b] Reduction subjects followed a weight-loss strategy focused on reducing energy and fat intake.
[c] Data are presented as means ± SD, for all such values. $P \leq 0.050$ is considered significant.
[d] Mean ages between groups are significantly different ($P = 0.0081$, *t* test).
[e] $n = 29$.
[f] $n = 27$.
[g] Self-reported answers were not verified by medical records.
[h] Calculated using reported lifetime maximum weight and assuming that height at time of maximum weight was not different from height measured at baseline.

taught strategies for meeting the goals of their diet, participated in lessons on nutrition and health, and received handouts. Both groups were taught to follow a healthy eating plan as described by the Food Guide Pyramid (24). All educational sessions and handouts were identical, except for those focused on how to count servings of vegetables and fruits (HiVeg) or kilocalories and fat grams (Reduction). Both groups were taught the same lessons about increasing physical activity. Recommendations included achieving 180 min/wk of aerobic activity and 270 weight-resistance exercise repetitions (e.g., arm curls) per week. All subjects were provided with pedometers and instructed on proper use (30), but pedometer use was optional. Subjects were asked to complete Baecke Physical Activity Questionnaires (31) at baseline, 3, 12, and 18 mo as a measure of changes in physical activity.

Statistical Analyses. Statistical analyses were performed using SAS software (version 9.1.3; SAS Institute, Cary, NC). Data are presented as means ± SD, and $P \leq 0.050$ is considered significant. The analysis was intention-to-treat. We chose not to use analytic approaches to reduce the effect of withdrawal (e.g., last-observation-carried-forward or multiple imputation for missing values); therefore, missing values were ignored by the statistical program.

Primary outcomes, secondary outcomes, 3-d diet records, and Baecke Physical Activity Scores were examined for main effects of treatment, time, and treatment × time using PROC MIXED to account for repeated measures. Age at baseline and age of overweight onset were included as fixed effects in all models. Baseline BMI was included as a fixed effect as appropriate. Gender was included as a fixed effect in models evaluating measures of physical activity. Changes within treatment groups were examined using PROC MIXED or adjusted *t* tests controlling for age at baseline, age of overweight onset, and baseline BMI (as appropriate).

Between-group differences in primary outcomes from baseline to 3 mo only were examined using adjusted *t* tests (primary outcomes) or Student's *t* tests (secondary outcomes). This short-term analysis was performed in order to understand the effects of the intensive 3-mo feeding and education intervention, when compliance was highly facilitated.

Results

Of the 60 randomized subjects, 56, 45, and 32 participated in the 3-, 12-, and 18-mo data collection points, respectively. Reasons for dropout, when known, are in Figure 1. There was an age difference between groups ($P = 0.0081$) after randomization; thus, age was controlled for in all statistical analyses. Groups did not differ in any other baseline characteristics (Table 2) or dietary intake (Table 3). Baseline characteristics of the subjects who completed the trial were not different from those who did not complete the trial (data not shown).

Weight and fat mass changed with treatment, but fat-free mass did not change (Fig. 2). BMIs also changed over time (Table 4). There were no significant treatment × time interactions or time effects for the primary outcomes. At 3 mo, after the conclusion of the intensive feeding and

Table 3. Analysis of 3-d Diet Records of Obese Subjects Following Two Dietary Strategies for Weight Loss

	HiVeg[a]	Reduction[b]	Effect	*P* value
Vegetable intake (servings/d)				
Baseline[c]	3.2 ± 1.8[d]	2.6 ± 1.4	Treatment × time	0.015
3 mo	6.6 ± 2.2[e,f]	3.6 ± 1.4	Treatment	<0.0001
12 mo	4.6 ± 1.9[e]	3.6 ± 2.0	Time	<0.0001
18 mo	4.6 ± 3.2	2.8 ± 1.2		
Fruit intake (servings/d)				
Baseline[c]	1.2 ± 1.4	1.4 ± 1.2	Treatment × time	0.73
3 mo	2.0 ± 1.2	1.6 ± 2.2	Treatment	0.75
12 mo	2.2 ± 3.2	2.0 ± 1.6	Time	0.052
18 mo	2.0 ± 1.8	2.0 ± 1.9		
Fat intake (g/d)				
Baseline[c]	87.2 ± 22.1	84.5 ± 37.4	Treatment × time	0.58
3 mo	61.7 ± 22.2[e]	44.0 ± 12.3[e]	Treatment	0.030
12 mo	66.9 ± 25.6[e]	55.1 ± 21.5[e]	Time	<0.0001
18 mo	48.1 ± 12.4[e]	38.0 ± 9.9[e]		
Energy intake (kcal/d)[g]				
Baseline[c]	2273 ± 582[d]	2009 ± 647	Treatment × time	0.84
3 mo	1923 ± 474[e,h]	1667 ± 269[e]	Treatment	0.15
12 mo	1874 ± 544[e]	1744 ± 458	Time	0.0001
18 mo	1556 ± 190[e]	1456 ± 284[e]		
% Energy from fat/d				
Baseline[c]	35.0 ± 6.4	37.3 ± 8.8	Treatment × time	0.093
3 mo	28.6 ± 6.0[e]	23.7 ± 4.5[e]	Treatment	0.036
12 mo	32.2 ± 7.7	28.9 ± 10.5[e]	Time	<0.0001
18 mo	28.0 ± 7.2	23.7 ± 6.0[e]		
Protein intake (g/d)				
Baseline[c]	84.6 ± 22.2	83.3 ± 26.8	Treatment × time	0.43
3 mo	78.1 ± 16.3	85.5 ± 13.4	Treatment	0.84
12 mo	82.3 ± 21.9	85.8 ± 26.4	Time	0.90
18 mo	87.7 ± 25.5	74.8 ± 17.8		
% Energy from protein/d				
Baseline[c]	15.3 ± 3.7	16.9 ± 2.9	Treatment × time	0.28
3 mo	17.1 ± 4.3	20.9 ± 4.0[e]	Treatment	0.33
12 mo	18.8 ± 7.5[e]	20.3 ± 5.8[e]	Time	0.0008
18 mo	22.3 ± 5.4[f]	20.6 ± 3.5[f]		
Fiber intake (g/d)				
Baseline[c]	17.5 ± 8.4	16.4 ± 6.6	Treatment × time	0.98
3 mo	24.7 ± 5.8[e]	22.9 ± 6.7[e]	Treatment	0.73
12 mo	20.5 ± 9.5	19.8 ± 8.3	Time	0.0004
18 mo	22.0 ± 7.9	19.8 ± 7.7		

[a] HiVeg subjects followed a weight-loss strategy focused on including high amounts of vegetables and moderate amounts of fruit in the diet. The numbers of dietary records analyzed were $n = 27$, 23, 15, and 4 for the baseline, 3-, 12-, and 18-mo time points, respectively.
[b] Reduction subjects followed a weight-loss strategy focused on reducing energy and fat intake. The numbers of dietary records analyzed were $n = 25$, 21, 20, and 10 for the baseline, 3-, 12-, and 18-mo time points, respectively.
[c] Baseline values are not different between groups.
[d] Data are presented as means ± SD, and $P \leq 0.050$ is considered significant.
[e] Values within a group that differed significantly from baseline as evidenced by a mixed effects model with repeated measures showing a significant effect of time.
[f] Values at that time point differed between groups as evidenced by a significant treatment × time interaction in a mixed effects model accounting for repeated measures.
[g] Energy data are reported in kilocalories because subjects in the Reduction group were advised to count kilocalories. 1 kcal = 4.18 kJ.
[h] Values differed between groups as evidenced by a *t* test, which was evaluated for energy data only.

education intervention, there was a difference between groups in change in weight, change in fat mass, and BMI ($P \leq 0.0011$).

Adjusted *t* tests were used to compare within-group changes from baseline to follow-up. In the HiVeg group, weight (Fig. 2a) and fat-mass (Fig. 2b) were lower than baseline at 3 mo ($P = 0.0087$ and $P = 0.0002$, respectively), while fat-free mass (Fig. 2c) increased from baseline at 3 mo

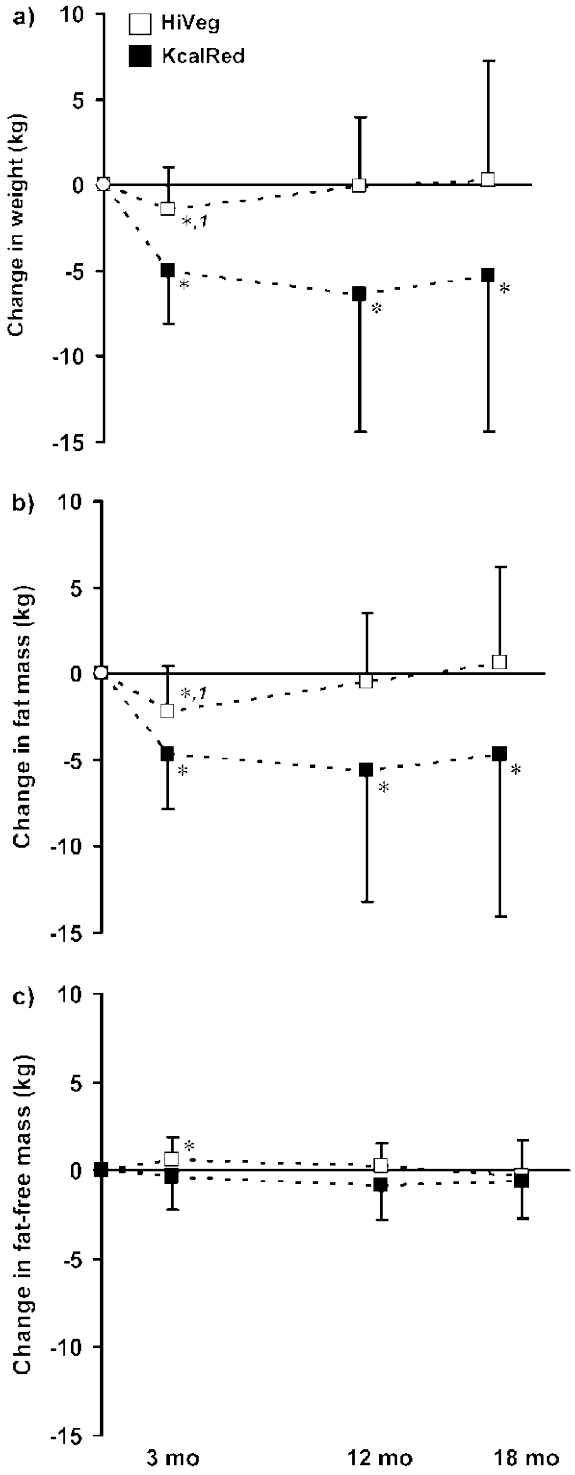

Figure 2. Change after 3, 12, and 18 mo in weight (a), fat mass (b) and fat-free mass (c) of obese subjects, stratified by gender and BMI, who were randomly assigned to a weight-loss intervention focused on including high amounts of vegetables and moderate amounts of fruit (▵ HiVeg) or reducing energy and fat intake (▵ KcalRed). Data are presented as means 6 SD, and P values $\leq$ 0.050 are considered statistically significant. PROC MIXED assessment with age at baseline, baseline BMI, and age of overweight onset included as fixed effects in all models showed that treatment 3 time interactions did not exist (P $\geq$ 0.38) nor did time have an effect (P $\geq$ 0.25) on change in weight, fat mass, or fat-free mass. There were significant treatment effects on weight change and change in fat mass (P , 0.0001, for both) but not change in fat-free mass (P¼0.12). Adjusted t tests controlling for age at baseline and age of overweight onset were used to compare changes from baseline within a group at each follow-up and to evaluate changes between groups at 3 mo. Within a group, significant changes from baseline are indicated with an asterisk (*). Differences in change as determined by adjusted t tests (controlling for age at baseline, baseline BMI, and age of overweight onset) between groups at 3 mo are indicated with the number 1. For the Reduction group, n¼27, 24, 18, and 18 at baseline, 3, 12, and 18 mo, respectively. For the HiVeg group, n ¼ 29, 21, 14, and 14 at baseline, 3, 12, and 18 mo, respectively.

Table 4. BMI Over Time of Obese Subjects Following Two Dietary Strategies for Weight Loss

	HiVeg[a]	Reduction[b]	Effect	P value
Baseline[c]	33.7 6 3.8[d]	33.3 6 3.5	Treatment 3 time	0.67
3 mo	33.3 6 3.9[e,f]	31.7 6 3.4[e,g]	Treatment	0.019
12 mo	33.3 6 4.2	31.3 6 4.3[e,g]	Time	0.41
18 mo	33.2 6 4.1	31.7 6 4.6[e,g]		

[a] HiVeg subjects followed a weight-loss strategy focused on including high amounts of vegetables and moderate amounts of fruit in the diet; n ¼ 29, 21, 14, and 14 at baseline, 3, 12, and 18 mo, respectively.
[b] Reduction subjects followed a weight-loss strategy focused on reducing energy and fat intake; n ¼ 27, 24, 18, and 18 at baseline, 3, 12, and 18 mo, respectively.
[c] Baseline values were not different between groups.
[d] Data are presented as means 6 SD; P $\leq$ 0.05 is considered significant.
[e] Values significantly different from baseline as shown by adjusted t tests, controlling for age at baseline and age of onset of overweight.
[f] Change from baseline significantly different between groups using adjusted t tests, controlling for age at baseline and age of onset of overweight.
[g] Values within a group that differed significantly from baseline as evidenced by a mixed effects model with repeated measures showing a significant effect of time.

(P ¼ 0.0075). BMI was lower than baseline at only 3 mo in the HiVeg group (P ¼ 0.014, Table 4). The Reduction group decreased weight at 3 (P , 0.0001), 12 (P ¼ 0.0006), and 18 mo (P ¼ 0.019, Fig. 2a). Fat mass (Fig. 2b) was lower than baseline in the Reduction group at 3 (P , 0.0001) and 12 mo (P ¼ 0.0032), and fat-free mass (Fig. 2c) did not differ from baseline at any follow-up (P $\geq$ 0.058). Mean BMIs were lower than baseline at all 3 follow-ups in the Reduction group (P $\leq$ 0.045, Table 4).

At baseline, serum chemistry variables did not differ between groups (P $\geq$ 0.25, Tables 2 and 5), and mean values, except LDL cholesterol (Table 5), were within reference ranges. There were no significant treatment 3 time interactions or treatment effects. Cholesterol:HDL signifi-

Table 5. Lipid Profiles in Obese Subjects Following Two Dietary Strategies for Weight Loss

	HiVeg[a]	Reduction[b]
Total cholesterol (mM) [reference range: 3.88–5.15]		
Baseline[c]	5.06 ± 0.97[d]	4.99 ± 1.08
3 mo	4.62 ± 0.72[e]	4.47 ± 0.88[e]
12 mo	4.90 ± 0.75	4.75 ± 0.80
HDL (mM) [reference range: > 1.01]		
Baseline[c]	1.19 ± 0.39	1.21 ± 0.35
3 mo	1.13 ± 0.31	1.16 ± 0.30
12 mo	1.14 ± 0.31	1.16 ± 0.33
LDL (mM)[f] [reference range: < 2.59]		
Baseline[c]	3.33 ± 0.75	3.22 ± 0.85
3 mo	2.95 ± 0.62[e]	2.84 ± 0.70[e]
12 mo	3.27 ± 0.61	3.06 ± 0.74
VLDL (mM) [reference range: 0.08–0.83]		
Baseline[c]	0.54 ± 0.34	0.56 ± 0.32
3 mo	0.53 ± 0.25	0.47 ± 0.24[e]
12 mo	0.45 ± 0.17	0.52 ± 0.29
Triacylglycerols (mM) [reference range: 0.56–1.68]		
Baseline[c]	1.48 ± 0.93	1.54 ± 0.88
3 mo	1.56 ± 0.93	1.27 ± 0.64[e]
12 mo	1.45 ± 1.09	1.41 ± 0.79
Cholesterol:HDL[g] [reference range: 2.0–4.5]		
Baseline[c]	4.59 ± 1.31	4.31 ± 0.96
3 mo	4.36 ± 1.14[e]	4.03 ± 1.0[e]
12 mo	4.56 ± 1.27	4.38 ± 1.29

[a] HiVeg subjects followed a weight-loss strategy focused on including high amounts of vegetables and moderate amounts of fruit.
[b] Reduction subjects followed a weight-loss strategy focused on reducing energy and fat intake.
[c] Baseline values were not different between groups.
[d] Data are presented as means ± SD; $P \leq 0.050$ is considered significant.
[e] Values differed from baseline within a group as evidenced by Student's *t* test, which was evaluated for 3-mo data only.
[f] LDL cholesterol was calculated by difference.
[g] For all variables except cholesterol:HDL, there were no effects of treatment, time, or treatment × time ($P \geq 0.051$). A significant effect of time for cholesterol:HDL as evidenced by a mixed effects model accounting for repeated measures was found for cholesterol:HDL ($P = 0.022$).

cantly decreased with time (Table 5). Glucose decreased with time in the Reduction group and was 5.29 ± 0.45 at baseline and 4.96 ± 0.43 at 12 mo.

Looking at the short-term effects within the HiVeg group (3-mo data compared to baseline), total cholesterol, LDL, and cholesterol:HDL decreased ($P = 0.0001$, $P < 0.0001$, and $P = 0.0040$, respectively). Within the Reduction group, there was a decrease from baseline at 3 mo for hematocrit (0.42 ± 0.032 to 0.41 ± 0.031; $P = 0.029$), triacylglycerols ($P = 0.0030$), total cholesterol ($P = 0.0001$), LDL ($P = 0.0008$), VLDL ($P = 0.0031$), and cholesterol:HDL ($P = 0.0061$).

Reported vegetable consumption was higher than baseline at 3 ($P < 0.0001$) and 12 mo ($P = 0.044$) in the HiVeg group, but the mean servings of vegetables were significantly less than the goal of 8 ($P = 0.0026$, 3 mo; $P < 0.0001$, 12 mo; Table 3). Fruit consumption did not change from baseline and was never different from 2 servings ($P \geq 0.41$). Because mean vegetable consumption never met the 8-serving goal, we calculated the proportion of HiVeg subjects who returned 3-d diet records that reached (≥8 servings/d) or nearly reached (≥7 servings/d) the vegetable goal at each follow-up. At baseline, none of the HiVeg subjects consumed ≥7 servings vegetables per day. At 3 mo, when subjects were transitioning to following the dietary advice independently, 9 of 23 subjects (39.1%) consumed ≥7 servings vegetables per day, and 5 of those consumed 8 or more servings per day. At 12 and 18 mo, of those subjects that returned diet records, only 1 (6.7%) and 0 subjects, respectively, consumed ≥7 servings vegetables per day.

To determine if the HiVeg group also met the goals of the Reduction group, mean daily energy intake and percent daily energy from fat were evaluated. At all three follow-up times, mean energy consumed was significantly less than baseline ($P = 0.0089$, $P = 0.0036$, and $P = 0.0040$ for 3, 12, and 18 mo, respectively). These reductions were not different from a 500 kcal/d reduction from baseline ($P \geq 0.18$). There was a significant reduction in percent energy from fat at 3 mo only ($P = 0.0009$), but percent energy from fat was still >25% in the HiVeg group at that time ($P = 0.0092$).

At 3 and 18 mo but not at 12 mo, Reduction subjects were consuming significantly fewer kilocalories per day than at baseline ($P = 0.020$, 3 mo; $P = 0.077$, 12 mo; $P = 0.0044$, 18 mo). At both 3 and 18 mo, the mean reduction in kilocalories per day consumed was not different from a 500-kcal/d reduction from baseline ($P = 0.39$ and $P = 0.056$, respectively). The Reduction group successfully met the ≤25% daily energy from fat goal at all follow-ups ($P \geq 0.11$). Fat grams and percent energy from fat per day were significantly reduced from baseline at all follow-ups ($P \leq 0.0003$). Neither vegetable nor fruit consumption increased over time ($P = 0.056$ and $P = 0.11$, respectively) in the Reduction group. The fraction of Reduction subjects who reported eating ≥7 servings vegetables per day were as follows for baseline, 3, 12, and 18 mo: 0/25, 0/21, 2/20, and 0/10.

Daily energy consumed (Table 3) did not differ between the HiVeg and Reduction groups long-term. However, looking at the 3-mo follow-up, the Reduction group consumed fewer kilocalories per day than the HiVeg group ($P = 0.033$, Student's *t* test). The Reduction group also consumed fewer grams of fat and percent energy from fat per day (Table 3) than the HiVeg group as evidenced by significant treatment effects.

Main effects of treatment, time, and treatment × time were found for vegetable consumption (Table 3). Overall, HiVeg subjects consumed more vegetables than the Reduction subjects throughout the study, and at 3 mo, the HiVeg group had a greater increase in vegetable consump-

tion from baseline than the Reduction group ($P = 0.025$). There were no main effects for fruit consumption. The percent energy from protein, grams of protein, and grams of fiber consumed per day did not differ between groups.

Groups did not differ at baseline for sport, non-sports leisure, or total activity indices as assessed by Baecke Physical Activity Questionnaires ($P \geq 0.12$, data not shown). A main effect of time ($P = 0.043$) but not treatment or treatment × time was found for the total activity score, indicating an overall change in physical activity over time (data not shown). There were no differences between groups in the change in scores from baseline to 3 mo ($P \geq 0.094$).

Within the Reduction group, the 3- and 12-mo mean total activity scores were higher than baseline ($P = 0.037$ and $P = 0.0009$, respectively), indicating total activity increased (data not shown). No other indices changed over time within either group ($P \geq 0.061$). The number of subjects who returned Baecke Physical Activity Questionnaires at baseline, 3, 12, and 18 mo were 30, 26, 19, and 4 for the HiVeg group and 30, 24, 18, and 8 for the Reduction group, respectively.

Discussion

This study examined weight loss over the short- and long-term in obese adults following a healthy eating plan focused on consuming high amounts of vegetables and moderate amounts of fruits or on reducing daily energy and fat intake. We hypothesized that including high amounts of vegetables and moderate amounts of fruits would induce weight loss by causing a decrease in total energy and fat consumed and that weight loss would continue long-term because eating high amounts of vegetables and fruits would be reasonable to maintain. Both energy and fat intake decreased in the HiVeg group, but not as low as observed in the Reduction group. Correspondingly, the increased vegetable and moderate amounts of fruits strategy was not as effective for weight loss as the more traditional energy and fat restriction diet. Subjects in the Reduction group lost more weight than the HiVeg group after 3 mo of an intensive food and education intervention and were able to maintain that weight loss long-term. These results indicate that simple messages such as "include 8 servings (or 4 cups) of vegetables daily" may not cause a change in diet sufficient to induce continued long-term weight loss. It should be noted, however, that consuming vegetables was not without benefit: HiVeg subjects' fat mass decreased, and fat-free mass increased after 3 mo. Additionally, subjects in both groups saw improved serum lipids after 3 mo of dietary change.

The greater weight loss in the Reduction group after 3 mo was attributed to differences in diet and physical activity between groups. At 3 mo, the Reduction group consumed fewer kilocalories and grams of fat daily (~250 fewer kcal/d [160 kcal from fat]) than the HiVeg group. The Reduction group increased their physical activity relative to baseline, and the HiVeg group did not. This improvement in activity may have contributed to increased weight loss in the Reduction group. We suggest that the reduction in fat mass and total weight was sustained in the Reduction group due to their continued lowered intake of fat and increase in physical activity from baseline over the long-term.

Although the energy intake of the HiVeg group did not differ from the Reduction group at 18 mo, not all participants returned food records. Returned records may not have reflected the entire group's intake which would explain why the HiVeg group did not continue to lose weight. Moreover, subjects tend to report less energy intake in 24-hr recalls than actual expenditure (32). Compared to baseline, vegetable intake was statistically higher at 12 mo and numerically higher at 18 mo in the HiVeg group. Had the HiVeg group continued to consume 6.6 vegetable and 2.0 fruit servings, which was the mean intake at 3 mo, they may have continued to lose or maintain the weight loss. In a mechanistic review (33), obese patients often did not continue to lose weight when treated long-term with low-calorie diets. This finding was attributed to difficulties with patient adherence and not metabolic or gastrointestinal adaptations (33).

The 8 servings of vegetables daily goal was not achieved by most subjects in the HiVeg group over the long-term. Between baseline and 3 mo, achievement of this goal is assumed to be high, because the HiVeg group received 7–8 servings vegetables daily, and no food was returned or reported uneaten. However, at 3 mo, when subjects began the transition to following the dietary advice independently, only 39% of those returning diet records were consuming 7 servings of vegetables or more. By 12 mo, only 1 subject maintained this high level of intake. HiVeg subjects were encouraged to successfully adopt the high vegetable strategy by giving them recipes and offering strategies for including high amounts of vegetables daily. Yet, as was evidenced by follow-up questions, some subjects found it difficult to purchase, prepare, and/or cook the suggested amount of vegetables. For some HiVeg subjects, people not enrolled in the study, such as children and spouses, contributed to subjects' difficulty in adopting the diet long-term.

Although the vegetable goal was not achieved in the HiVeg group, it is still important to recognize the successful weight and fat loss after 3 mo in this group, when mean vegetable intake was the greatest (~7 servings/d). This result indicates that with regular support, daily vegetable consumption of at least 7 servings (~3.5 cups, 0.83 L) with 2–3 servings (~1–1.5 cups, 0.24–0.35 L) of fruit can result in moderate weight loss. Furthermore, in addition to the improved cholesterol:HDL reported herein, this increased vegetable intake resulted in improved carotenoid concentrations (34), which may reflect increased tissue concentrations. Indeed, increased carotenoid intake from carrots resulted in enhanced antioxidant activity in animal liver after sustained feeding (35). Long-term, this could result in

decreased incidence of disease (36). Increased vegetable consumption may also enhance an energy and fat reduction diet. There is some evidence supporting this possibility in the Ello-Martin et al. trial (11), where subjects in the reduced fat and increased water-rich foods (especially vegetables and fruits) group lost more weight over 1 y compared to the reduced-fat only group.

The HiVeg group in our study did not gain weight above baseline at 12 and 18 mo ($P \geq 0.88$, t tests). This is in contrast to the trend of age-related weight gain of 0.45–0.90 kg/y in adult Americans (37) and supports the possibility that small improvements in diet, such as including 6 or more servings of vegetables and fruits, could prevent long-term weight gain and contribute to weight maintenance. This hypothesis needs to be tested and strategies to improve the long-term sustainability of high vegetable intake need to be developed.

Although this study started before the release of *MyPyramid*, the diet patterns of both groups during the first 3 mo of the study were similar to the *MyPyramid* recommendations (27). Therefore, it may be hypothesized that the current guidelines (i.e., 3.5–4.5 cups vegetables and fruits as part of a healthy 1600–2000 kcal diet) may induce weight loss or maintain weight in obese individuals if followed consistently. A trial that specifically tests the guidelines of *MyPyramid* as a dietary strategy for weight-loss in obese individuals is merited.

The results of our study contribute to the growing body of evidence that suggests recommendations to individuals seeking to lose weight and maintain weight loss should focus on controlling total energy and fat intake, as well as increasing vegetable and fruit intake as part of a healthy, reduced energy and fat diet.

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^{13}C Natural Abundance in Serum Retinol Acts as a Biomarker for Increases in Dietary Provitamin A

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The natural isotopic composition of ^{13}C and ^{12}C in tissues is largely determined by the diet. Sources of provitamin A carotenoids (e.g., vegetables) typically have a lower ^{13}C to ^{12}C ratio (^{13}C:^{12}C) than preformed vitamin A sources (i.e., dairy and meat) from corn-fed animals, which are prevalent in the US. The ^{13}C:^{12}C of serum retinol (^{13}C:^{12}C-retinol) was evaluated as a biomarker for vegetable intake in a 3-mo dietary intervention designed to promote weight-loss by increased vegetable consumption or reduced calorie and fat intake. Subjects were 21–50 y of age with a BMI between 30–40 kg/m^2 and were enrolled from one geographic area in the US. The high vegetable group (n = 20) was encouraged to increase daily vegetable and fruit consumption to 0.95 liter vegetables and 0.24–0.35 liter fruits. The caloric reduction group (n = 17) was encouraged to lower caloric intake by 500 kcal and consume ≤25% kcal from fat daily. Provided meals supplied 75–100% vegetable and fruit goals and 50–67% kcal and fat g per day. Carotenoid supplementation was discontinued by subjects during the study. Serum retinol and provitamin A carotenoid concentrations; intake of preformed vitamin A, provitamin A, and fat; and body weight, fat mass, and lean mass were analyzed for correlations to ^{13}C:^{12}C-retinol. ^{13}C:^{12}C-Retinol decreased in the vegetable group after intervention (P = 0.050) and the correlation with provitamin A intake was approaching significance (P = 0.079). ^{13}C:^{12}C-Retinol did not change in the caloric reduction group (P = 0.43). ^{13}C:^{12}C-Retinol changes with the vitamin A source in the diet and can be used as a biomarker for increases in dietary provitamin A vegetable intake. Exp Biol Med 234:140–147, 2009

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Introduction

Stable isotopes as tracers are valuable tools to determine biochemical pathways and mechanisms and nutrient requirements. In the field of vitamin A, isotopically labeled vitamin A has been used in kinetic, metabolic, and status assessment studies (1–9). Provitamin A carotenoids, in both plant and synthetic forms, have also been labeled with stable isotopes to determine bioconversion rates to vitamin A (10–15).

The natural isotopic composition of carbon in animal tissues is largely determined by the diet (16). Plants have different photosynthetic mechanisms that discriminate between the $^{12}CO_2$ and $^{13}CO_2$ that is incorporated into organic compounds. Most vegetables and temperate grains (e.g., wheat and rice) are C3 plants. Typically, C4 plants come from hot, dry climates and include crops such as maize, sorghum, and sugarcane, as well as many forage grasses. Different assimilation rates of ^{13}C and ^{12}C by C3 and C4 plants cause the isotopic ratio of plants to differ by 13–15‰, enriching C4 plants with ^{13}C (16–18). Animals consuming primarily C3 feeds have a different ratio of ^{13}C to ^{12}C (^{13}C:^{12}C) in milk, serum, meat, and liver compared with animals consuming C4 feeds (16, 18). Similarly, the ^{13}C:^{12}C in human hair differs in vegetarians and omnivores (19–20). Because isotopic differences in tissues are directly related to the diet, one study suggested using ^{13}C:^{12}C as a marker for specific foods such as sweeteners derived from C4 plants (21).

Vitamin A can be obtained from the diet as preformed vitamin A in dairy and organ meats, or as provitamin A carotenoids from vegetables and fruits. Provitamin A is typically in the form of β-carotene, β-cryptoxanthin, and α-carotene, which are commonly found in orange and yellow fruits and vegetables. Most plant sources of provitamin A in

the human diet are C3 plants (e.g., carrot, sweet potato, pumpkin, and spinach). One exception is corn (C4 plant), but typical provitamin A concentrations are low (22) and probably do not contribute appreciably to the vitamin A pool of North Americans. The average ^{13}C enrichment, reported as $\delta\ ^{13}C$, for common C3 fruits and vegetables is −27.16‰ (18). Isotope ratios are reported in standard delta notation relative to Vienna Pee Dee Beleminite (VPDB), where $\delta\ ^{13}C = [[R_{sample}/R_{VPDB}] - 1] \times 1000$ and $R = {}^{13}C/{}^{12}C$. All reported $\delta\ ^{13}C$ values are with respect to VPDB and are negative when ^{13}C:^{12}C of the sample is less than VPDB.

In the US, the cattle and dairy industries rely on corn-based diets, while European growers use predominantly C3 plants in their feed (18). Because of the difference in feed ingredients, meat and dairy products in the US typically have $\delta\ ^{13}C$ values from −13.5 to −19.2‰ due to corn, while non-corn-fed European meat and dairy products typically range from −26.02 to −30.38‰ (18, 23). Thus, most dietary sources of preformed vitamin A in the US are enriched with ^{13}C by 10–15‰ compared to vegetable sources of provitamin A. If the $\delta\ ^{13}C$ values of animal products correlate with preformed vitamin A in meat and dairy products, altering the ratio of preformed vitamin A to provitamin A in the diet would change the $\delta\ ^{13}C$ value of serum retinol.

The objective of this study was to evaluate the change in $\delta\ ^{13}C$ value of serum retinol after a 3-mo intensive intervention study to promote weight loss. The two dietary weight-loss strategies included increased vegetable intake or reduced calorie and fat intake. Serum retinol and provitamin A carotenoid concentrations; preformed vitamin A, provitamin A carotenoid, and fat intakes; and body weight, fat mass, and lean mass were analyzed for correlations to the $\delta\ ^{13}C$ retinol values using gas chromatography-combustion-isotope ratio mass spectrometry.

Materials and Methods

Subjects. This study was part of a larger weight-loss study designed to compare the effects of two dietary strategies on weight loss, body composition, serum chemistry profile, and serum vitamin A and carotenoids. The complete weight-loss study monitored subjects for 18 mo. This study utilized data collected during the controlled feeding period between 0 and 3 mo. The two dietary strategies were increased vegetable consumption or a 500 kcal/d reduction diet. Of the 60 subjects enrolled in the main trial, those with complete dietary records and adequate serum for carbon isotope analysis [$n = 20$ and 17 from the vegetable and caloric reduction groups, respectively] were evaluated (Table 1). Subjects were between 21 and 50 y of age, had a BMI between 30 and 40 kg/m^2, and visited the study kitchen to collect breakfast and lunch during the 3-mo feeding period. Exclusion criteria before enrollment included consumption of ≥2.5 c/d (0.6 liter/d) vegetables and/or fruits; history of insulin treatment, drug, or alcohol abuse; participation in other research studies that may confound results; pregnancy or lactation; serious medical or psychiatric illness; unwillingness or inability to discontinue use of supplements containing carotenoids; use of drugs that might affect weight loss; and weight change >3% of body weight during the 3 mo prior to recruitment. Subjects gave written informed consent. The Health Sciences Institutional Review Board at the University of Wisconsin-Medical School approved all aspects of this study.

Body Composition. Body composition was measured by air displacement plethysmography (BOD POD®, Life Measurements, Inc., Concord, CA) (24). Weight was measured using the BOD POD® scale, which was tested with calibration weights each day of use. Height was measured at baseline using a wall-mounted stadiometer. All body composition and weight assessments were conducted by BOD POD® certified users. BMI was calculated from the weight and height measurements.

Diets. The subjects in the vegetable group were educated about vegetable and fruit consumption from the Food Guide Pyramid (25), which was based on the *Dietary Guidelines for Americans*, 5th Ed. (26). In the Food Guide Pyramid, consumption is defined by servings [1 serving of vegetable or fruit is equivalent to 1/2 c (0.12 liter) fresh vegetable or fruit]. Subjects in the vegetable group were given a daily goal of consuming 8 servings (4 c, 0.95 liter) of vegetables and 2–3 servings (1–1.5 c, 0.24–0.35 liter) of fruits. Serving sizes were measured using standard US measuring cups. Subjects were discouraged from consuming potato chips, french fries, or fruit juices to meet their vegetable and fruit goals. Yellow corn was not excluded from the diet. Contributions of provitamin A from yellow corn to the vitamin A pool were expected to be minor due to low provitamin A content of corn and low ratio of corn to C3 vegetables in the diet.

Subjects in the caloric reduction group were given two daily goals: to reduce daily caloric intake by 500 kcal from the estimated caloric intake needed for weight maintenance at baseline, and to consume ≤25% of kcal from fat. Daily caloric need for weight-maintenance at baseline was estimated by multiplying an individual's estimated resting energy expenditure by an individually appropriate activity factor. Resting energy expenditure was estimated based on height, weight, age, and sex using published equations (27). Activity factors were based on those recommended by the Institute of Medicine (28). They were 1.3 for very light activity, 1.5 for light activity, and 1.6 for moderate activity.

All subjects were provided with 2 meals/d, Monday through Friday (10 meals total). Subjects in the vegetable group were provided with 75–88% of their daily vegetable goal and 100% of the fruit goal. The food provided to the caloric reduction group supplied 50–67% kcal and fat g for the day. The vegetable group received twice as many vegetables as the caloric reduction group. The provided protein in the meals and fat-free milk (3.6 liter/wk/person) were the same for both groups.

Table 1. Characteristics of Subjects in the Vegetable and Reduced Calorie and Fat (Caloric Reduction) Groups at 0 and 3 Mo After Intervention. Subjects Were Fed 10 Meals/Wk During the Intervention Designed to Promote Weight-Loss[a]

	Vegetable[b]		Caloric reduction	
	0 mo	3 mo	0 mo	3 mo
Age (y)	32.8 ± 8.4	32.9 ± 8.9	34.5 ± 8.5	34.7 ± 8.3
Female/male (*n*)	14/6		13/4	
Weight (kg)	95.0 ± 15.4[a]	93.6 ± 9.8[b]	97.2 ± 19.0[a]	91.9 ± 19.8[b]
Fat mass (kg)	39.6 ± 9.8[a]	36.8 ± 10.2[b]	42.2 ± 11.4[a]	36.1 ± 10.7[b]
Fat-free mass (kg)	56.1 ± 11.8	56.3 ± 11.7	56.2 ± 13.5	56.0 ± 13.9
BMI (kg/m^2)	33.7 ± 3.7	33.0 ± 3.6	32.4 ± 3.0	31.0 ± 3.0

[a] Values are mean ± SD for all such values.
[b] Differences between 0 and 3 mo within each treatment group were tested with a paired Student's *t* test within a group, significance is indicated with a superscript ($\alpha = 0.05$). Differences between vegetable and caloric reduction groups at baseline and 3 mo were tested with a 2 sample t-test ($\alpha = 0.05$) and results are as follows. Age, weight, fat and fat-free mass, and BMI did not differ between vegetable and caloric reduction groups at either 0 or 3 mo.

3-D Diet Records. Subjects were asked to complete 3-d diet records (2 weekdays and 1 weekend day) at 0 and 3 mo. Completed diet records were analyzed for total vegetable intake (1 c = 0.24 liter) and vitamin A consumption using Nutritionist Pro™ Version 3.1.0 (Axxya Systems, Stafford, TX, © 2007). Foods or their ingredients were classified into two groups, those providing preformed vitamin A and those providing provitamin A derived from carotenoids. Preformed vitamin A included all vitamin A consumed from meat, dairy, and egg products, as well as synthetic vitamin A added for fortification. Provitamin A included all vitamin A attributed to plant-derived and synthetic carotenoids. Foods with multiple sources of vitamin A (e.g., pizza and salad) were divided into preformed vitamin A and provitamin A based on ingredients. Several foods reported in dietary records were not found in the NutritionistPro™ database. Dietary estimates of these foods were based on nutritional information provided on food labels or by restaurants.

Analysis of Carotenoids and Retinol in Serum. Whole blood samples were taken at 0 and 3 mo after an overnight fast (≥8 h). Blood was placed into sterile-interior 6 ml Corvac brand serum separator tubes with clot activator (Tyco Healthcare Group LP, MA). Samples were centrifuged at 2200 × *g* for 10 min at 4°C after clotting for 10–20 min at room temperature. Serum was stored at −80°C until analysis. Serum carotenoids and retinol were analyzed using a modification of a previously published method (29). Briefly, 600 μl ethanol with 0.1% butylated hydroxytoluene was added to 500 μl serum and mixed with a vortex. Retinol and carotenoids were extracted three times with 1 ml hexanes with mixing and centrifugation. The pooled extracts were dried under argon, reconstituted in 100 μl 50:50 (by vol) methanol:dichloroethane, and 50 μl injected into the HPLC system. β-Apo-8′-carotenyl decanoate was used as an internal standard.

The HPLC system consisted of a Resolve C_{18} (5 μm, 3.9 × 300 mm) column, a Waters 2996 photodiode array detector, 1525 binary pump, and 717 autosampler injector (Milford, MA). The mobile phases were 95:5 (by vol) acetonitrile:water (solvent A) and 85:10:5 (by vol) acetonitrile:methanol:dichloroethane (solvent B), both containing 10 m*M* ammonium acetate. Samples were analyzed at a flow rate of 2 ml/min using the following gradient: 100% solvent A for 3 min, 7-min linear change to 100% solvent B, 100% solvent B for 10 min, 3-min linear change to 100% solvent A, and 100% solvent A for 1 min. Chromatograms were generated at 450 and 325 nm to quantify carotenoids and retinol, respectively. Standard curves were prepared with purified α-carotene, β-carotene, lutein, lycopene, retinol, and zeaxanthin. Concentrations were determined spectrophotometrically using their respective $E^{1\%}_{1\,cm}$ [i.e., 2800 for α-carotene, 2592 for β-carotene, 2550 for lutein, 3450 for lycopene, 1845 for retinol, and 2348 for zeaxanthin (30–31)].

Isotopic Ratio of ^{13}C to ^{12}C in Serum Retinol. The ^{13}C:^{12}C in serum retinol was determined according to a modification of the method by Tanumihardjo (8). After proteins were precipitated with ethanol (2 ml), retinol was extracted 3 times from serum (1–1.5 ml) with hexanes (1–2 ml). Extracted layers were combined and dried under argon, reconstituted in 100 μl methanol, frozen for 5 min at −80°C, centrifuged at 1380 × *g* at room temperature for 30 s, and injected into a 15-cm Resolve® HPLC column (3.9 × 150 mm, 5 μm, Waters Corporation, Milford, MA) equilibrated with 90:10 methanol:water (by vol) at 1 ml/min. Retinol was collected, dried under argon, and further purified on a 30-cm Resolve® HPLC column (3.9 × 300 mm, 5 μm, Waters Corporation) equilibrated with 98:2 methanol:water (by vol) at 1 ml/min. Collected retinol was dried in a Thermo Savant Speed-Vacuum centrifuge (Thermo Scientific, Waltham, MA), reconstituted in 10 μl hexanes, and 1.5 μl injected into a gas chromatography/combustion/isotope ratio mass spectrometer (GCCIRMS). The Trace GC (Thermo Scientific) was equipped with a Programmable Temperature Vaporizing (PTV) injector, a

15-m HP-1MS GC column (Agilent Technologies, Santa Clara, CA), and a 1-m deactivated fused-silica pre-column (0.53 μm i.d.), and was connected to a Combustion III and Advantage Plus isotope ratio mass spectrometer (Thermo Scientific). Samples were injected simulating on-column injection with the PTV injector at 43°C. Temperature of the PTV injector was ramped to 50°C, matching the initial oven temperature, prior to injection. Oven temperature increased at 15°C/s to 300°C. The δ ^{13}C-retinol values were analyzed from serum samples by GCCIRMS in duplicate. Synthetic retinol, prepared by quick saponification of retinyl acetate (Sigma-Aldrich, St. Louis, MO), was purified twice similarly to serum retinol and used as an external standard.

Isotopic Ratio of ^{13}C to ^{12}C in Milk Retinol. Vitamin A and D fortified fat-free milk was purchased from the same supplier (University of Wisconsin-Madison Babcock Hall Dairy Store, Madison, WI) that was used during the feeding period and analyzed. Three ml ethanol with 0.1% butylated hydroxytoluene was added to 2 ml milk and mixed by vortex. To remove remaining fat in the sample, the samples were saponified for 30 min at 45°C using 800 μl 500 g/liter potassium hydroxide in water. After saponification, the retinol was extracted from the sample 3 times with 1.5 ml hexanes. After the pooled extracts were dried under argon and reconstituted in 100 μl methanol, retinol was purified and analyzed by GCCIRMS as described for serum retinol.

Isotopic Ratio of ^{13}C to ^{12}C in Vegetable Material. The provitamin A containing C3 plants fed during the intervention were carrots, spinach, and canned pumpkin. These foods were freeze-dried with a Virtis Benchtop 6K freeze-drier (SP Industries, Gardiner, NY) and ground into a powder using a coffee grinder. For comparison, typical yellow field corn (dried), a C4 plant, was provided by the University of Illinois at Urbana-Champaign and analyzed. It was ground to pass a <1 mm screen prior to analysis using a hammer mill. Dried plant material (1 to 2 mg) was weighed into tin capsules and encapsulated into a ball. A Costech ECS 4010 Elemental Combustion System CHNS-O (Costech Analytical Technologies, Inc., Valencia, CA) equipped with a Conflo III (ThermoScientific) and attached to an Advantage Plus isotope ratio mass spectrometer (described previously) was used to determine δ ^{13}C of plant materials. Samples were replicated 10 times.

Statistical Analysis. Data were analyzed using Statistical Analysis System software (SAS Institute Inc., Version 8.2, Cary, NC; 2001) to perform paired Student's *t* tests, two-sample Student's *t* tests, correlations, model building using backward, forward, and step-wise elimination. Significance was evaluated at $P \leq 0.050$, unless stated otherwise for the elimination procedures.

Results

Body Composition. Body weight and fat mass decreased between 0 and 3 mo for both the vegetable ($P = 0.002$ and $P < 0.001$ for body weight and fat mass, respectively) and caloric reduction groups ($P < 0.001$ for both) (Table 1). Although there was no difference in body weight or fat mass between the groups at either time, there was a significant difference in the change in body weight ($P = 0.001$) and fat mass ($P = 0.020$) between the groups. The vegetable group lost –2.2 ± 2.5 kg body weight and –3.0 ± 3.0 kg fat mass compared to –5.8 ± 3.7 and –7.9 ± 5.4 kg in the caloric reduction group, respectively. Fat-free mass and BMI did not differ at 0 or 3 mo or between the dietary groups.

3-D Diet Records. At baseline, preformed vitamin A and provitamin A intake did not differ between groups (Table 2). Preformed vitamin A intake did not change from 0 to 3 mo in either group, but provitamin A intake increased in both groups ($P \leq 0.002$). At 3 mo, preformed vitamin A intake did not differ between groups, but provitamin A was greater in the vegetable group compared with the caloric reduction group ($P < 0.001$). This corresponded to an increase in total vegetable consumption by the vegetable group on a volume basis ($P = 0.018$). Almost all of the provitamin A provided to and consumed by subjects was from vegetables that were C3 plants, including carrots, pumpkin, and spinach.

Fat intake was monitored as a possible co-variant because dietary fat is required for absorption of vitamin A and carotenoids (32–34). Fat consumption did not differ between the groups at baseline, and decreased from 0 to 3 mo in both groups ($P < 0.001$ and $P = 0.002$ for vegetable and caloric reduction groups, respectively; Table 2). At 3 mo, the fat intake was higher in the vegetable group than the caloric reduction group ($P = 0.049$).

Milk intake estimated from the 3-d records was monitored to ensure changes in δ ^{13}C were not due to differences in milk intake. Milk intake increased for both groups by approximately 300 ± 300 ml/d from baseline to 3 mo due to the feeding study ($P < 0.001$). Changes in milk intake did not differ between the groups ($P = 0.92$).

Serum Carotenoids and Retinol. From 0 to 3 mo, serum α- and β-carotene increased in both the vegetable ($P < 0.001$ for both α- and β-carotene; Table 2) and caloric reduction groups ($P < 0.001$ and $P = 0.046$ for α- and β-carotene, respectively), but serum retinol did not differ. Serum α-carotene, β-carotene, and retinol did not differ between the groups at baseline or 3 mo.

δ ^{13}C of Serum Retinol. Baseline δ ^{13}C-retinol values ranged from –26.26 to –31.08‰ and 3 mo values ranged from –26.21 to –31.57‰ (Fig. 1). Delta ^{13}C-retinol values in the vegetable group decreased from 0 to 3 mo ($P = 0.050$), but no difference was measured in the caloric reduction group ($P = 0.43$). A marginal difference was noted in the change in δ ^{13}C-retinol values between the vegetable and caloric reduction groups ($P = 0.054$).

Evaluated parameters (i.e., serum α-carotene, β-carotene, and retinol concentrations; preformed vitamin A, provitamin A, and fat intakes; body weight, fat mass, and

Table 2. Dietary Intake and Serum Characteristics of Subjects in the Vegetable and Reduced Calorie and Fat (Caloric Reduction) Groups at 0 and 3 Mo After Intervention[a]

		Vegetable[b]		Caloric reduction	
		0 mo	3 mo	0 mo	3 mo
Dietary intake characteristics[c]					
Vegetable increase[d]	c/d	1.65 6 1.31[a]		0.78 6 0.88[b]	
Preformed VA[e]	mg VA/d	0.58 6 0.19	0.71 6 0.46	0.61 6 0.27	0.64 6 0.22
Provitamin A	mg bC/d	2.9 6 4.1[b]	12.2 6 5.3[a]	2.7 6 2.6[b]	6.3 6 3.6[a]
Fat	g/d	91 6 19.6[a]	61.7 6 21.7[b]	86.6 6 41.3[a]	46.9 6 22.2[b]
Serum characteristics					
a-carotene	I M	0.06 6 0.03[b]	0.33 6 0.20[a]	0.07 6 0.06[b]	0.27 6 0.13[a]
b-carotene	I M	0.42 6 0.24[b]	1.17 6 0.82[a]	0.59 6 0.77[b]	1.02 6 0.65[a]
Retinol	I M	2.21 6 0.46	2.21 6 0.44	1.96 6 0.48	2.08 6 0.62

[a] Values are mean 6 SD, n ¼ 20 (vegetable) and n ¼ 17 (caloric reduction).
[b] Differences between 0 and 3 mo within each treatment groups were tested with a paired Student's t test, significance is indicated with a superscript (a ¼ 0.05). Differences between vegetable and caloric reduction groups were tested with a 2 sample Student's t test (a ¼ 0.05) at baseline and 3 mo and results are as follows. Preformed vitamin A, provitamin A, and fat intake did not differ at baseline. At 3 mo, provitamin A and fat intake were significantly higher (P , 0.001 and P , 0.05, respectively) in the vegetable group compared to the caloric reduction group. Serum characteristics (a-carotene, b-carotene, and retinol) did not differ between vegetable and caloric reduction groups at either 0 or 3 mo.
[c] Intake of preformed vitamin A, provitamin A, and fat were evaluated from 3-d diet records. Foods were categorized into either preformed vitamin A or provitamin A and converted to mg retinol and b-carotene as described in the USDA nutrient database. Preformed vitamin A includes meat, dairy, and synthetic vitamin A in foods. Provitamin A includes all plant-derived and synthetic carotenoids.
[d] Significance between groups for daily vegetable increase is indicated with a superscript.
[e] VA, vitamin A.

lean mass) were used to assess their relationship to d ^{13}C-retinol measurements. Except for the change in provitamin A intake in the vegetable group (P ¼ 0.079) and the change in serum retinol in the caloric reduction group (P ¼ 0.013), correlations to d ^{13}C-retinol were not significant at the a ¼ 0.10 level for either group. For the vegetable group, stepwise forward regression analysis (a ¼ 0.15 to include) showed that the decrease in d ^{13}C-retinol was due primarily to the increase in provitamin A intake (P ¼ 0.079) and all other parameters were not significant. Backward elimination regression (a ¼ 0.10 to remove) revealed that the changes in serum b-carotene, provitamin A intake, fat mass, body weight, and the percent body fat contributed to the changes in d ^{13}C-retinol for the vegetable group (P ¼ 0.035). For the caloric reduction group, stepwise forward regression analysis (a ¼ 0.15 to include) showed the change in d ^{13}C-retinol, was correlated to changes in serum retinol and percent body fat (P ¼ 0.004). Backwards elimination regression (a ¼ 0.15 to include) showed that changes in serum retinol, preformed vitamin A intake, body weight, fat mass, and percent body fat were all significant in the model (P ¼ 0.003).

d ^{13}C of Reference Foods and Vitamin A. The d ^{13}C of carrots, pumpkin, and spinach was –25.199 6 0.313, –26.788 6 0.228, and –27.375 6 0.175%, respectively. Corn had a d ^{13}C of –11.283 6 0.079% and the d ^{13}C-retinol value of milk provided to study participants was –24.2%. Synthetic vitamin A prepared from retinyl acetate was –28.779 6 0.465%.

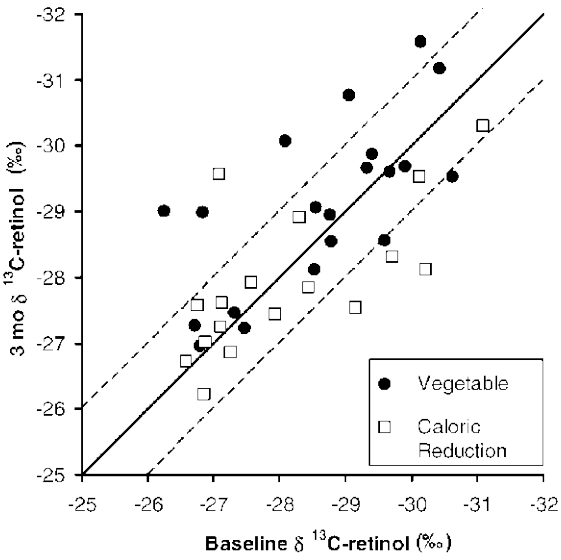

Figure 1. Baseline versus 3 mo mean d ^{13}C-retinol values for subjects in the vegetable and reduced calorie and fat g (caloric reduction) groups (n ¼ 20 and 17, respectively). The solid line represents equivalent 0 and 3 mo values, the dashed line represents 6 1% variation in 3 mo d ^{13}C values compared to baseline. Average standard deviation for d ^{13}C-retinol values was 6 0.197. Delta ^{13}C-retinol values in the vegetable group decreased from 0 to 3 mo (P ¼ 0.050), but did not change in the caloric reduction group (P ¼ 0.43). The 3 mo change in d ^{13}C-retinol values between the vegetable and caloric reduction groups was marginally significant (P ¼ 0.054).

Discussion

High intake of fruits and vegetables is associated with reduced risk of several chronic diseases, including cardiovascular disease, type 2 diabetes, and certain cancers (28). These foods are the dietary source for carotenoids, which have also been associated with reduced disease risk (35). Plasma or serum carotenoids are often used as biomarkers for fruit and vegetable intake (36, 37). Although useful as an indicator, conversion of provitamin A carotenoids to retinol will result in a loss of biomarker from serum carotenoid measurements. Because bioconversion is dependent on vitamin A status, the use of serum carotenoids as a biomarker for fruit and vegetable intake in populations with low or highly variable vitamin A status may be erroneous. By using the enrichment of ^{13}C in retinol compared to provitamin A sources, conversion of provitamin A carotenoids to retinol can be confirmed and dietary adherence can be determined. The additional vegetable intake by the vegetable group was almost exclusively from C3 plants (i.e., carrots, pumpkin, and spinach) leading to a significant decrease in the δ ^{13}C-retinol value. Furthermore, the δ ^{13}C-retinol technique was more precise than serum β-carotene analysis. At baseline, the CV for δ ^{13}C-retinol measurements was 4.7 and 4.4% for the vegetable and caloric reduction groups, respectively, while serum β-carotene was 57 and 131%, respectively. The greater variability in serum carotenoid measurements will require more subjects than studies using δ ^{13}C-retinol. Analysis by GCCIRMS is more expensive and less available than HPLC analysis; however, this cost is somewhat offset by the reduction in subject number. Analysis of δ ^{13}C-retinol is a new technique, but it can be adapted to any facility with GCCIRMS and HPLC capabilities.

Although both dietary intervention groups increased vegetable and provitamin A intake over the 3-mo intervention period, the vegetable group consumed significantly more vegetables and provitamin A than the caloric reduction group. The increase in vegetable consumption in both groups was confirmed by elevated serum α- and β-carotene at 3 mo and vegetable intake as indicated by 3-d diet records. Although the increase in provitamin A was confirmed by serum carotenoids, they were not able to distinguish the 2-fold greater intake of provitamin A by the vegetable group. In contrast, the δ ^{13}C-retinol value decreased in the vegetable group ($P = 0.050$), but not in the caloric reduction group ($P = 0.43$). Using backward statistical modeling, the change in δ ^{13}C-retinol was attributable to the change in provitamin A intake, serum carotenoids, fat mass, and body weight ($P = 0.035$). Stepwise modeling found that change in provitamin A intake was the only factor to explain the change in the δ ^{13}C-retinol value at the $\alpha = 0.1$ level.

Before δ ^{13}C-retinol values are affected by dietary provitamin A carotenoids, provitamin A must be converted to retinol. Bioconversion was likely minimized by the high intake of preformed vitamin A and the adequate vitamin A status of the subjects. Subjects with a lower vitamin A status will have greater bioconversion of provitamin to vitamin A. Thus, the influence of dietary provitamin A carotenoids on δ ^{13}C-retinol will be much greater in subjects with low vitamin A stores and the sensitivity of the technique will be greatly enhanced. Due to the effect of vitamin A status on the effectiveness of this technique, more studies are necessary to determine the absolute sensitivity of the method to modest changes in vegetable intake. In populations with low vitamin A status, sensitivity to changes in dietary vitamin A could be exploited with washout periods to establish a steady baseline prior to intervention.

Previous studies have shown that provitamin A in maize contributes to vitamin A pools similarly to synthetic β-carotene when vitamin A is absent from the diet (38). High consumption of relatively low carotenoid foods such as yellow or orange maize, can contribute to vitamin A stores when vitamin A stores are depleted (38). Substitution of yellow or orange maize for white maize in the diet of a population should increase the serum δ ^{13}C-retinol value with time because maize is a C4 plant. In a vitamin A depleted population, the change in δ ^{13}C-retinol value in response to an intervention would likely be greater than the response observed in this study and would confirm bioconversion to retinol, provided that the population's major vitamin A source was not solely from animals consuming C4 plants.

Serum retinol was not significantly different between groups or over time. This is expected because serum retinol is homeostatically controlled in individuals with adequate vitamin A status (39, 40). Although the caloric reduction group increased their carotenoid intake during the dietary intervention, it was not sufficient to have an effect on the isotopic composition of retinol in 3 mo. Changes in δ ^{13}C-retinol and serum retinol concentrations did not differ between 0 and 3 mo in the caloric reduction group and consequently were correlated.

The decrease in δ ^{13}C-retinol in the vegetable group indicates that the dietary intervention altered the isotopic composition of serum retinol. The increase in dietary provitamin A carotenoids by subjects in the vegetable group reduced the δ ^{13}C-retinol indicating that carotenoids from C3 plants are being absorbed, cleaved into retinol, and circulated in the body. Dietary increase in provitamin A carotenoids was the most significant dietary parameter that correlated with the decrease in δ ^{13}C-retinol in the vegetable group. Results from analysis of high provitamin A carotenoid vegetables (i.e., carrots, -25.102 ± 0.076‰; pumpkin, -26.788 ± 0.228‰; and spinach, -27.375 ± 0.175‰) provided for consumption during the 3 mo intervention were consistent with reported values (-27.16‰) for common fruits and vegetables (18).

Preformed vitamin A is primarily obtained from meat and dairy products, which, in the US, typically have much

higher δ ^{13}C values ranging from –13.5 to –19.2‰ (18, 23). The δ ^{13}C-retinol value of fat-free milk, provided to study participants, was –24.2‰ and is much lower than the overall δ ^{13}C of meat and dairy products. Animals must obtain vitamin A by cleaving provitamin A carotenoids or from vitamin A supplements because they cannot synthesize it. Thus, the δ ^{13}C-retinol value of the milk originates from provitamin A in the diet (e.g., maize) that has a relatively high δ ^{13}C value (–11.283 ± 0.079‰) and preformed vitamin A from feed, direct supplementation to the animal, and milk fortification that have a relatively low δ ^{13}C value (–29.779 ± 0.465‰). Although preformed vitamin A is typically considered more bioavailable than provitamin A, the δ ^{13}C-retinol value of milk indicates that provitamin A carotenoids in corn-based diets contribute to vitamin A pools.

The difference in fat intake between groups probably did not influence the decrease in δ ^{13}C-retinol or the increase in serum carotenoids noted in the vegetable group. It is well established that added fat is necessary for carotenoid absorption, especially from raw vegetables (41). However, fat intake greater than 5 g fat/meal is generally accepted as adequate for absorption of carotenoids from supplements or vegetables (41–44). Both dietary groups in this study were well beyond this minimal fat requirement at each meal. Thus, differences in fat intake should not affect absorption of provitamin A carotenoids and would not be reflected in δ ^{13}C-retinol values or serum carotenoids.

In addition to demonstrating the incorporation of carotenoid-derived retinol into the vitamin A pool, the change in δ ^{13}C-retinol due to increased provitamin A intake also illustrates its utility as a biomarker for consumption of provitamin A-rich fruits and vegetables. Changes in serum α- and β-carotene concentrations were unable to differentiate between groups in response to the intervention, but δ ^{13}C-retinol as measured by GCCIRMS differed due to its greater sensitivity toward changes in dietary vitamin A sources. The current guidelines for vegetable and fruit intake are 3.5–4.5 c for a 1600–2000 kcal diet in *MyPyramid* (25). The vegetable group was within these guidelines, i.e., 4.3 c/d, while the caloric reduction group was not, i.e., 3.1 c/d. The δ ^{13}C-retinol reflected this difference and could be used in future studies to evaluate adherence to *MyPyramid* recommendations.

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Pedometers Are Perceived as Useful Tools for Weight Loss

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Abstract: Pedometers are used as motivational tools to encourage physical activity through Extension educational contacts. In conjunction with a community campaign, subjects (N = 60) enrolled in a weight-loss study were provided with pedometers. Participants recorded steps and responded to an evaluation. Step counts increased from baseline through 9 weeks ($P \leq 0.018$) and correlated with goals ($P \leq 0.038$). Participants who reported that the pedometer helped them achieve goals had greater fat and less fat-free mass at baseline than those who did not find it helpful. Pedometers benefit individuals by increasing activity and being perceived as useful for weight-loss.

Introduction

Physical activity is important for health. Among obese individuals who are trying to enhance or maintain weight loss efforts, moderate-intensity physical activity for 60-90 minutes/day may be required (Hill & Wyatt, 2005). Pedometers have been used as tools to measure ambulatory activity and to motivate individuals to be more active. Wearing a pedometer can be an effective way to increase awareness (Rooney, Smalley, Larson, & Havens, 2003). A goal of 10,000 steps/day has been recommended for healthy adults seeking to attain or maintain an active lifestyle (Tudor-Locke & Basset, 2004). Long-term increases in step counts and improved body mass index and lower extremity function have been achieved by individuals using pedometers (Villanova, Pasqui, Burzacchini, Forlani, Manini, Suppini, Melchionda, & Marchesini, 2006; Toole, Thorn, Panton, Kingsley, & Haymes, 2007).

Most studies assessing pedometers as a tool for enhancing weight loss and physical activity require participants to report step counts over a short time. The evaluation reported here examined perceived usefulness of pedometers as a tool for achieving weight-loss or activity goals over 1 year. The study was conducted in conjunction with a statewide community nutrition and health campaign that included promoting increased physical activity by providing pedometers through Extension outreach efforts (Wisconsin Nutrition Education Network, 2004). Over 1,000 pedometers were provided to Wisconsin residents through educational contacts.

Population

Participants (N = 60, Table 1) were asked to use pedometers to monitor ambulatory activity. Subjects were concurrently enrolled in a study examining two dietary strategies: 1) reduction of caloric intake and consumption of ≤25% kcal from fat or 2) consumption of 4 cups vegetables and 1-1.5 cups of fruit/day. Body composition (BOD POD®, Life Measurements, Inc., Concord, CA, USA), weight, and body mass index were obtained before baseline step counts and at 3 months and 1 year.

Table 1.
Baseline Characteristics of Participants (N = 60)

Age (year)	33.6 ± 8.6[1]
Body mass index (kilograms/meter^2)	33.6 ± 3.6
Weight (kilograms)	95.3 ± 15.7
Fat mass (kilograms)	39.5 ± 10.0
Fat-free mass (kilograms)	55.6 ± 11.8
% Fat mass	41.6 ± 7.5 44.8 ± 4.4 (females) 32.6 ± 6.9 (males)
% Fat-free mass	58.4 ± 7.5 55.2 ± 4.4 (females) 67.4 ± 6.9 (males)
[1] Mean ± SD.	

Using and Evaluating the Tool

Participants were provided with Accusplit X 120 Activity Pedometers (San Jose, CA), which have face plates and anchor cords, after piloting by investigators. To standardize pedometer performance, participants were told to press the "reset" button in the morning, clip it to a firm waistband or belt, and wear it on the same location.

Participants were given log sheets and asked to record daily steps for 3 days to obtain baseline counts. From the baseline value, participants calculated a 10% increase in steps and used this daily goal for the remainder of the week. A weekly 10% increase was repeated until the step count reached an individually appropriate value. Most participants were aware of the 10,000 steps/day recommendation (Tudor-Locke & Basset, 2004)

and adopted this goal. Participants were asked to record daily steps for the first 10 weeks and for 3 days at 1 year.

At 7-9 months after baseline, three evaluation questions were asked:

1. Are you still wearing your pedometer? If so, how often, and how many steps/day do you average? If not, when did you stop and why?

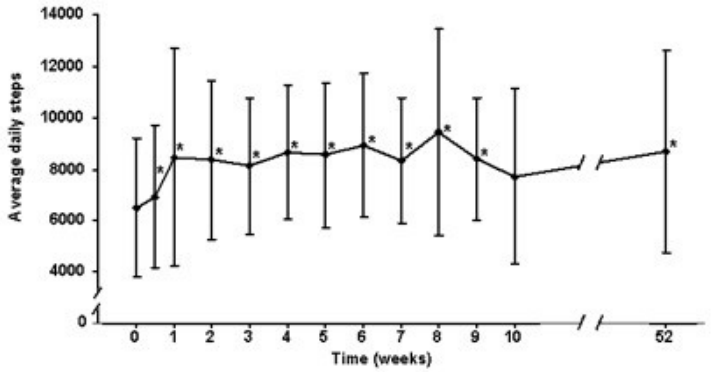
2. Did you have any trouble with your pedometer functioning properly? If so, describe.

3. Do you feel that your pedometer helped or is helping you to achieve your weight-loss goals?

Response and Findings

At 3 months and 1 year, 54 and 45 participants were still enrolled, respectively. At baseline, week 4, and week 10, 70%, 48.3%, and 15% of participants, respectively, recorded steps for at least 2 days. Step counts increased from baseline by 1947 ± 710 steps (Figure 1), and the increase was sustained at 1 year. Reported goals often correlated with steps achieved.

Figure 1.
Daily Steps of Participants over Time[1]



[1] Data are means ± SD. Values marked by an asterisk (*) indicate a difference ($P \leq 0.030$) from baseline when an outlier is removed. N = 41, 40, 39, 35, 32, 28, 25, 21, 21, 18, 15, 8, and 18 for baseline through 52 weeks, respectively.

Forty-seven of 54 participants responded to the evaluation questions. Over 20% were regularly or sometimes wearing their pedometer. The three most common reasons for not continuing to wear the pedometers were:

- Forgot

- Have a good sense of daily steps

- Lost it

Baseline characteristics did not differ between those who responded and those who did not. Baseline differences did exist between responders who felt that the pedometer helped or was helping them achieve their weight-loss goals compared with those who reported that they were unsure or thought it did not help (Table 2). Those who reported that the pedometer was helpful weighed more and had greater fat mass and lower fat-free mass at baseline.

Table 2.
Differences between Follow-up Survey Respondents

Baseline Characteristic	Pedometer Helpful[1]	Pedometer Not Helpful[2]	*P*-value
Weight (kilograms)	97.1 ± 17.6	89.8 ± 8.8	0.015
Fat mass (kilograms)	42.1 ± 10.3	33.6 ± 7.7	0.026
Fat-free mass (kilograms)	55.0 ± 11.4	56.2 ± 14.9	0.051

[1] N = 35; 28 female, 7 male. Respondents who reported that a pedometer was helpful or sometimes helpful in achieving weight-loss goals.
[2] N = 12; 7 female, 5 male. Respondents who did not find the pedometer helpful or were unsure.

Conclusions

The evaluation reported here corroborates previous findings observed in individuals using pedometers (Clarke, Freeland-Graves, Klohe-Loehman, Milani, Nuss, & Laffrey, 2007; Toole, Thorn, Panton, Kingsley, & Haymes, 2007; Villanova, Pasqui, Burzacchini, Forlani, Manini, Suppini, Melchionda, & Marchesini, 2006). Goals were generally not different from actual step counts, suggesting that goal setting may be an effective way to methodically increase steps. Responders (75%) said the pedometer was helpful for achieving weight-loss although many were no longer using them at follow-up.

The decline in pedometer use over time may suggest that pedometers are most helpful over the short-term for gaining information and providing motivation to increase physical activity. Participants who reported that the pedometer was helpful weighed more at baseline and had greater fat mass and less fat-free mass than those who did not. Heavier participants may be more likely to increase physical activity through walking, as opposed to other higher intensity activities. Pedometers may be perceived as useful by individuals who are likely to benefit from and sustain increases in low-impact and low- to moderate-intensity activity, such as walking.

The modern environment is not conducive to incorporating physical activity, and so continued encouragement through educational contacts is needed. As few as 30 minutes of moderate-intensity physical activity, such as increased walking, can increase fitness and improve health. Physical activity can prevent

age-related weight gain and weight regain in previously obese and overweight individuals (Hill & Wyatt, 2005). Pedometers not only played a role in increasing daily ambulatory activity, but the perception from the majority of users was that the pedometer was helpful for achieving goals. Pedometers as a motivational tool may offer physical and mental benefits to a large portion of individuals seeking to lose weight through diet and exercise.

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