Effects of a hydrogenated form of vitamin K on bone formation and resorption[1–4]

Sarah L Booth, Alice H Lichtenstein, Maureen O'Brien-Morse, Nicola M McKeown, Richard J Wood, Edward Saltzman, and Caren M Gundberg

ABSTRACT

Background: Hydrogenation of vegetable oils affects blood lipid and lipoprotein concentrations. However, little is known about the effects of hydrogenation on other components, such as vitamin K. Low phylloquinone (vitamin K_1) intake is a potential risk factor for bone fracture, although the mechanisms of this are unknown.

Objective: The objective was to compare the biological effects of phylloquinone and its hydrogenated form, dihydrophylloquinone, on vitamin K status and markers of bone formation and resorption.

Design: In a randomized crossover study in a metabolic unit, 15 young adults were fed a phylloquinone-restricted diet (10 μg/d) for 15 d followed by 10 d of repletion (200 μg/d) with either phylloquinone or dihydrophylloquinone.

Results: There was an increase and subsequent decrease in measures of bone formation ($P = 0.002$) and resorption ($P = 0.08$) after dietary phylloquinone restriction and repletion, respectively. In comparison with phylloquinone, dihydrophylloquinone was less absorbed and had no measurable biological effect on measures of bone formation and resorption.

Conclusion: Hydrogenation of plant oils appears to decrease the absorption and biological effect of vitamin K in bone. *Am J Clin Nutr* 2001;74:783–90.

KEY WORDS Vitamin K, osteoporosis, hydrogenated oils, diet, bone turnover, *trans* fatty acids, phylloquinone, dihydrophylloquinone

INTRODUCTION

Renewed interest in the effect of hydrogenated fat (*trans* fatty acids) on plasma lipid concentrations began in the early 1990s and has continued throughout the past decade (1). Hydrogenated fat has consistently been reported to increase LDL concentrations (1) and the ratio of total to HDL cholesterol, and hence, to increase the risk of developing cardiovascular disease (2, 3). A relatively unexplored area of the hydrogenation process is its effect on vegetable oil components other than fatty acids. One such factor is the fat-soluble vitamin K. We previously showed that the side chain of vitamin K is partially saturated during the hydrogenation process and can be absorbed from the gastrointestinal tract after consumption (4). Yet to be determined is the biological activity of this hydrogenated form of vitamin K.

Vitamin K nutrition has been proposed as a modifiable risk factor for osteoporosis. At least 3 vitamin K–dependent proteins have been identified in bone or cartilage, including osteocalcin, which is one of the most abundant noncollagenous proteins in bone. Most of the evidence supporting a role for vitamin K in age-related bone loss is based on reported associations between bone mineral density (BMD) or the bone fracture rate and biological markers of vitamin K status (5–10). However, one consistent criticism of these epidemiologic data is the potential confounding effect of overall poor nutrition, including vitamin D, calcium, and dietary energy and protein intakes (11). The proposed role of poor vitamin K status as a risk factor for osteoporosis would be strengthened if controlled changes in dietary vitamin K were shown to influence bone metabolism.

Phylloquinone (vitamin K_1) is the primary dietary form of vitamin K found in green, leafy vegetables and certain plant oils, such as soybean and canola (12). During the process of hydrogenation of phylloquinone-rich oils, phylloquinone is converted to 2′,3′-dihydrophylloquinone. Phylloquinone intake data (13), which exclude intake of hydrogenated forms, suggest that more than one-half of younger adults in the United States do not meet the current adequate intake of 90–120 μg phylloquinone/d (14). In contrast, the mean daily intake of dihydrophylloquinone is ≈20 μg/d for adults (13). If dihydrophylloquinone has measurable biological activity, the reported low phylloquinone intakes in young adults are an underestimate of total vitamin K intake. Conversely, if dihydrophylloquinone has no measurable

[1] From the Jean Mayer US Department of Agriculture, Human Nutrition Research Center on Aging at Tufts University, Boston, and the Department of Orthopedics and Rehabilitation, the School of Medicine, Yale University, New Haven, CT.

[2] Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors, and do not necessarily reflect the view of the US Department of Agriculture.

[3] Supported by USDA agreement no. 58-1950-001, USDA competitive grant 96-35200-3139 (to SLB), NIH clinical training grant T32 DK 07651 (to NMM), and NIH grant AR38460 (to CMG). Phylloquinone and dihydrophylloquinone were donated by J Pyrek, University of Kentucky Mass Spectrometry Facility.

[4] Address reprint requests to SL Booth, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111. E-mail: sbooth@hnrc.tufts.edu.

Received August 9, 2000.

Accepted for publication February 6, 2001.

TABLE 1
Subject characteristics[1]

	Men (n = 7)	Women (n = 8)
Age (y)	29 ± 5	29 ± 4
Weight (kg)	69 ± 7	60 ± 9
BMI (kg/m^2)	23.3 ± 2.2	21.8 ± 2.2

[1] $\bar{x}$ ± SD.

biological effects, hydrogenation of phylloquinone-rich vegetable oils reduces the contribution of an otherwise important dietary source of phylloquinone.

The objective of this study was to investigate the effects of vitamin K status on bone turnover markers after phylloquinone depletion and subsequent repletion with dihydrophylloquinone and phylloquinone while other dietary factors, including calcium and vitamin D, were controlled for.

SUBJECTS AND METHODS

Subjects

Fifteen healthy subjects aged 20–40 y were selected for this study. Their age, weight, and body mass index are summarized in **Table 1**. All subjects were in good health, as indicated by the results of a physical examination and screening laboratory tests. Exclusion criteria were as follows: *1*) use of medication known to affect lipid metabolism or clotting function; *2*) history of renal, hepatic, cardiovascular, endocrine, or gastrointestinal disease; *3*) use of antibiotic or supplemental vitamins or minerals within 4 wk of the start of each 30-d residency period; *4*) smoking; and *5*) use of nasal steroids. Women were excluded from the study if they were currently pregnant or lactating, had a history of menstrual irregularities, or were using exogenous hormones. The study protocol was approved by the Human Investigation Review Committee of Tufts University, and a written consent form was obtained from each subject.

Experimental protocol

In a randomized crossover design, each subject resided in the Metabolic Research Unit at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University for two 30-d residency periods. There was a free-living period of ≥4 wk between each residency period, during which time each subject consumed a self-selected diet.

Although this was a crossover design, each residency period consisted of 3 diets: a 5-d control diet, a 15-d depletion diet, and a 10-d repletion diet. The control and depletion diets were identical in each residency period. The repletion diet contained either phylloquinone or dihydrophylloquinone, with the order of repletion being randomized among subjects. All meals were provided on a 2-d rotating plan; contained only naturally occurring foods; were designed to meet the dietary reference intakes for energy, protein, minerals, and vitamins for each subject's age and sex (14, 15), except for phylloquinone; and were prepared under the supervision of a dietitian. The composition of the 3 diets is summarized in **Table 2**. The energy content of the diets was adjusted by adding foods low in vitamin K—eg, rice, pasta, and bread—to maintain body weight within 1.5 kg throughout the duration of the study.

The principal criterion in designing the 5-d control diet was to approximate the adequate intake for vitamin K by providing ≈100 μg phylloquinone/d (14). There was no dihydrophylloquinone in the control diet, which was described in greater detail elsewhere (17). The 15-d depletion diet was a modified version of the low-phylloquinone diet developed by Ferland et al (18). Additional food items, including peanut butter, dried fruit, fortified breakfast cereal, oatmeal, grapefruit sections, peeled cucumber, tomatoes, and beets, were used to increase the fiber content of the diet and to eliminate the need for a nutritional supplement to attain acceptable nutrient intakes. Food items containing dihydrophylloquinone, such as graham crackers, were eliminated from the diet as described by Ferland et al (18). For the 2 repletion diets, purified sources of phylloquinone (Sigma Chemical Co, St Louis) or dihydrophylloquinone (>99% purity) were added to corn oil in muffins, which were given to subjects as part of the breakfast meal. Otherwise, the composition of the 2 repletion diets was identical to the depletion diet (Table 2). All corn oil used in this study was purchased from a single lot and protected from light.

Except for the phylloquinone and dihydrophylloquinone contents, the nutrient composition of the diets was calculated with the use of MINNESOTA NUTRIENT DATA SYSTEM SOFTWARE (version 2.7; Nutrient Data System, University of Minnesota Nutrition Coordinating Center, Minneapolis). For confirmation of phylloquinone and dihydrophylloquinone content, replicates of each meal (based on a daily intake of 8368 kJ, or 2000 kcal) were prepared as for consumption, and the entire contents of each single-day menu were homogenized in a stainless steel blender (Waring

TABLE 2
Composition of the control, depletion, and repletion diets[1]

			Repletion	
	Control	Depletion	With phylloquinone	With dihydrophylloquinone
Protein (% of energy)	12.1	14.9	14.9	14.9
Carbohydrate (% of energy)	67.5	60.0	60.0	60.0
Fat (% of energy)	20.4	25.1	25.1	25.1
Calcium (mg/d)	1078	1041	1041	1041
Vitamin D (μg/d)	8.4	5.8	5.8	5.8
Phylloquinone (μg/d)	93.1 ± 9.3[2]	11.0 ± 1.0	206 ± 14	11.0 ± 1.0
Dihydrophylloquinone (μg/d)	ND	ND	ND	240 ± 31

[1] Nutrients were calculated with the use of MINNESOTA NUTRIENT DATA SYSTEM SOFTWARE (version 2.7; Nutrient Data System; University of Minnesota, Nutrition Coordinating Center, Minneapolis), except for phylloquinone and dihydrophylloquinone, which were each analyzed in triplicate by HPLC (16). ND, not detectable by chemical analysis.

[2] $\bar{x}$ ± SD.

Products Division, New Hartford, CT). Aliquots were then frozen at −20°C and protected from light until the time of phylloquinone and dihydrophylloquinone analysis. To confirm stability of the fortified corn oils used in the muffins, oil samples were routinely analyzed for both nutrients over the course of the study.

After a 12-h fast, blood samples were collected between 0630 and 0830 on days 1, 3, 6 (control), 10, 14, 18, 21 (depletion), 23, 25, 27, 29, and 31 (repletion) of each residency period. Plasma phylloquinone, serum total and undercarboxylated osteocalcin (ucOC), prothrombin time, and activated partial thromboplastin time were assessed for all days on which blood samples were collected. All other blood measures [undercarboxylated prothrombin, protein induced by vitamin K absence or antagonism (PIVKA-II), parathyroid hormone (PTH), bone-specific alkaline phosphatase (BAP), 25-hydroxyvitamin D, and 1,25-dihydroxyvitamin D] were assessed on days 1, 6 (control), 21 (depletion), and 31 (repletion) of each residency period. Twenty-four–hour urine collections were made daily throughout the 2 residency periods for the measurement of urinary γ-carboxyglutamic acid and creatinine. All other urinary indexes [calcium, sodium, cross-linked *N*-telopeptides of type 1 collagen (NTx), and deoxypyridinoline were measured in 24-h samples completed at 0700 on days 1, 6, (control), 21 (depletion), and 31 (repletion). Aliquots of all samples were stored at −70°C and were protected from light and multiple freeze-thaw cycles until analyzed.

Analytic procedures

Plasma phylloquinone and dihydrophylloquinone concentrations were determined by reversed-phase HPLC with use of postcolumn reduction and fluorometric detection (19). The lower limit of detection for phylloquinone and dihydrophylloquinone with this assay is 0.02 nmol/L. The HPLC methods used for the analysis of phylloquinone and dihydrophylloquinone in the metabolic diets and the individual oils were described elsewhere (16). A competitive protein-binding assay was used to measure plasma 25-hydroxyvitamin D (20).

Prothrombin time and activated partial thromboplastin time were determined by photometric detection with an MLA Electra 800 automated clot timer (Medical Laboratory Automation, Inc, Pleasantville, NY) with use of reagents from Dade Diagnostics (Miami). PIVKA-II was analyzed in citrated plasma with an enzyme-linked immunosorbent assay from American Bioproducts Company (Parsippany, NJ). PIVKA-II is a functional measure of the biological activity of vitamin K in a hepatic vitamin K–dependent protein. The assumption is that PIVKA-II concentrations are inversely related to the functionality of prothrombin.

Serum PTH was measured with use of a 2-site immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA). BAP was measured in serum with use of an immunoassay (Metra Biosystems, Inc, Mountain View, CA). Serum total osteocalcin and ucOC were measured by radioimmunoassay with the use of procedures described by Gundberg et al (21). This assay uses human osteocalcin as a standard and tracer and a polyclonal antibody directed to intact human osteocalcin (22) and it recognizes intact osteocalcin and the large *N*-terminal midmolecule fragment (21). Total osteocalcin and BAP are markers of bone formation, whereas ucOC is a marker of vitamin K status. The ucOC concentration is expressed as the percentage of osteocalcin not bound to hydroxyapatite in vitro (%ucOC) and normalized to the amount of total osteocalcin in a given sample with use of equations described elsewhere (21).

Urinary γ-carboxyglutamic acid, an indicator of turnover of all vitamin K–dependent proteins, was measured by *ortho*-phthalaldehyde derivitization and was followed by reversed-phase HPLC with fluorometric detection (23). Urinary γ-carboxyglutamic acid concentrations are expressed as a percentage of baseline values and as the mean of 3-d moving averages from each study participant. Urinary calcium and sodium were analyzed by direct current plasma spectrometry with a Spectra-Span VI sequential current plasma spectrometer (Beckman Instruments, Fullerton, CA) (24). Urinary creatinine was analyzed by a colorimetric method on a Cobas Mira analyzer (Roche Instruments, Belleville, NJ). NTx was measured in urine with use of a competitive inhibition enzyme-linked immunosorbent assay (Osteomark, Seattle). Urinary deoxypyridinoline was analyzed with use of a competitive enzyme immunoassay (Metra Biosystems, Inc). NTx and deoxypyridinoline are urinary indicators of bone resorption.

Statistical analysis

Statistical analysis of the data was performed with the use of SYSTAT (version 8; SPSS Inc, Chicago), and all results are expressed as means ± SEMs unless otherwise indicated. Results were considered statistically significant if the observed, two-sided significance level (*P* value) was <0.05. Because there were no statistically significant differences between men and women, their data were combined. A repeated-measures two-factor analysis of variance was used to determine the effects of residency period (one period with phylloquinone repletion and one period with dihydrophylloquinone repletion), day (days 6, 21, and 31), and the interactions between these 2 variables on all biochemical measures. When there was a significant interaction between residency period and day ($P < 0.05$), Tukey's honestly significant difference test was used to establish differences within and between the 2 residency periods.

RESULTS

Phylloquinone and dihydrophylloquinone concentrations

Plasma phylloquinone concentrations were 1.55 ± 0.34 nmol/L on entry into the study, were 1.04 ± 0.15 nmol/L in response to 5 d of the control diet, and declined to 0.17 ± 0.03 nmol/L in response to 15 d of the depletion diet (**Figure 1**). Plasma phylloquinone concentrations increased in response to 10 d of phylloquinone repletion but, as expected, did not change in response to dihydrophylloquinone repletion. In the absence of dietary intake data before study entry, it is not known why 10 d of phylloquinone repletion at 205 μg/d did not restore plasma phylloquinone concentrations to those observed on day 1 of each residency period.

Plasma dihydrophylloquinone was detectable at baseline (0.19 ± 0.05 nmol/L), but was not detectable by day 3 of the control diet, which contained no dihydrophylloquinone (Figure 1). Plasma dihydrophylloquinone continued to be nondetectable throughout the depletion and the phylloquinone-repletion diets. Plasma dihydrophylloquinone concentrations increased to 0.54 ± 0.09 nmol/L on day 31 of the dihydrophylloquinone-repletion diet. These data confirm that dihydrophylloquinone is absorbed after its dietary intake (4). However, the mean plasma dihydrophylloquinone concentration on day 31 of the dihydrophylloquinone-repletion diet was significantly lower than the mean plasma phylloquinone concentration on day 31 of the phylloquinone-repletion diet ($P = 0.008$). Therefore, even at equivalent

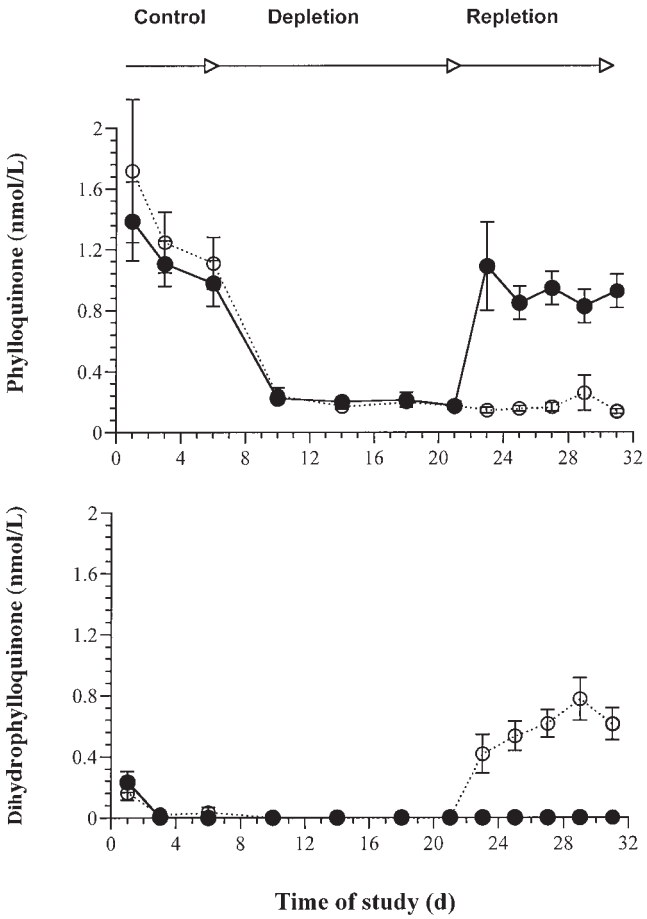

FIGURE 1. Mean (±SEM) plasma phylloquinone and dihydrophylloquinone concentrations in 15 young men and women in response to 100 μg (control diet), 10 μg (depletion diet), and 200 μg (repletion diet) phylloquinone (●) or dihydrophylloquinone (○). Plasma phylloquinone responded significantly to each phylloquinone intake ($P < 0.001$). Plasma dihydrophylloquinone increased significantly only after repletion with dihydrophylloquinone, $P < 0.001$.

intakes of dihydrophylloquinone and phylloquinone, less dihydrophylloquinone may have been absorbed during repletion.

Coagulation measures

Prothrombin time increased significantly from 12.7 to 13.0 s in response to phylloquinone repletion but not to dihydrophylloquinone repletion. Although significant, the 0.3-s increase was too small to be of clinical relevance. Activated partial thromboplastin time did not change in response to either phylloquinone depletion or repletion (data not shown).

Vitamin K–dependent proteins

PIVKA-II concentrations were within the normal range (≤2 μg/L) at baseline and in response to the control diet. These concentrations then increased in response to phylloquinone depletion (**Figure 2**). Although both phylloquinone and dihydrophylloquinone supplementation decreased PIVKA-II concentrations, only during phylloquinone repletion were the PIVKA-II concentrations restored to baseline. The difference in efficiency between the 2 compounds was significant. Osteocalcin, a vitamin K–dependent protein but also a measure of bone formation, increased in response to phylloquinone depletion compared with the control diet (**Table 3**, **Figure 3**). These elevated total osteocalcin concentrations, which are not markers of carboxylation status, were subsequently normalized after repletion with phylloquinone ($P = 0.002$) but not after repletion with dihydrophylloquinone. An identical pattern was observed for %ucOC (Figure 3). Urinary γ-carboxyglutamic acid excretion, which is a measure of turnover of all vitamin K–dependent proteins, decreased significantly in response to phylloquinone depletion (Figure 2). Although urinary γ-carboxyglutamic acid increased in response to phylloquinone repletion, concentrations were not restored to baseline by the end of the repletion period. These data suggest that urinary γ-carboxyglutamic acid is less responsive to short-term phylloquinone repletion than is either total osteocalcin or %ucOC. Urinary γ-carboxyglutamic acid excretion was not restored in response to dihydrophylloquinone repletion.

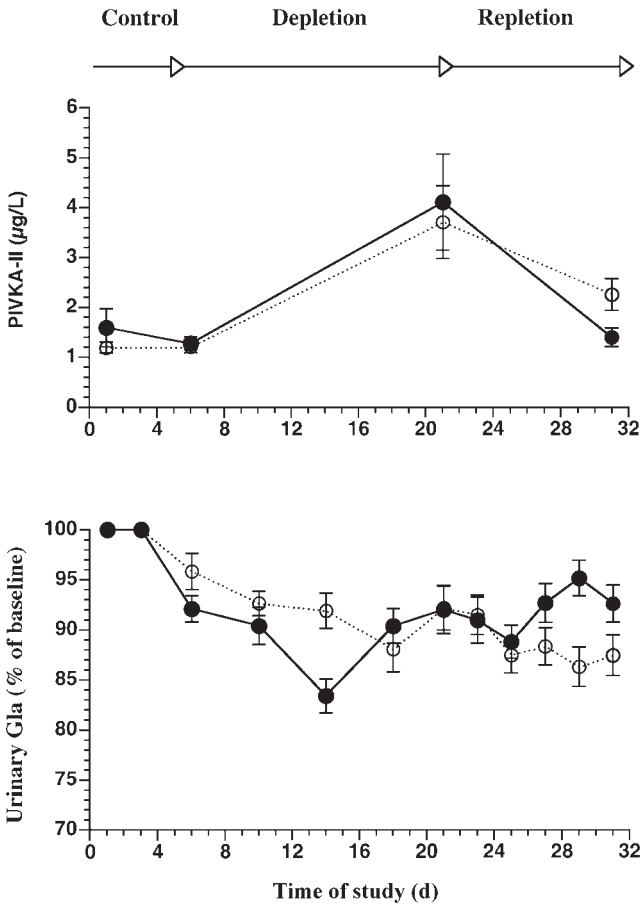

FIGURE 2. Mean (±SEM) plasma undercarboxylated prothrombin (PIVKA-II) and urinary γ-carboxyglutamic acid (Gla) concentrations in 15 young men and women in response to 100 μg (control diet), 10 μg (depletion diet), and 200 μg (repletion diet) phylloquinone (●) or dihydrophylloquinone (○). PIVKA-II responded significantly to phylloquinone depletion ($P < 0.01$) and subsequent repletion ($P < 0.01$) and to dihydrophylloquinone repletion ($P < 0.05$); the difference in response to phylloquinone and dihydrophylloquinone repletion was significant ($P < 0.01$). Gla decreased in response to phylloquinone depletion ($P < 0.05$), with a nonsignificant trend toward baseline during phylloquinone repletion but not during dihydrophylloquinone repletion.

TABLE 3
Bone marker concentrations[1]

	Residency period: phylloquinone repletion			Residency period: dihydrophylloquinone repletion			
	Control (93.1 μg K_1)	Depletion (11.0 μg K_1)	Repletion (206 μg K_1)	Control (93.1 μg K_1)	Depletion (11.0 μg K_1)	Repletion (240 μg DK)	P^2
Serum markers							
Total osteocalcin (μg/L)	7.1 ± 0.5[a]	8.4 ± 0.6[b]	7.1 ± 0.5[a]	7.3 ± 0.5[a]	7.9 ± 0.6[a]	8.1 ± 0.6[b]	0.002
ucOC (%)	27.7 ± 3.3[a]	46.8 ± 4.6[b]	20.3 ± 2.0[a]	29.1 ± 3.0[a]	42.0 ± 4.0[b]	42.5 ± 3.9[b]	<0.001
BAP (U/L)	18.5 ± 1.1[a]	18.7 ± 1.0[a]	18.5 ± 0.8[a]	19.7 ± 1.1[a]	18.8 ± 1.0[b]	18.5 ± 1.1[b]	0.01
25-Hydroxyvitamin D (nmol/L)	72 ± 6	69 ± 6	72 ± 5	75 ± 6	73 ± 5	74 ± 6	0.85
PTH (ng/L)	26.2 ± 2.3	28.2 ± 2.8	28.8 ± 2.1	27.3 ± 1.9	29.0 ± 1.9	29.8 ± 3.6	0.99
Urinary markers							
NTx (nmol BCE/mmol Cr)	31.5 ± 2.9	35.5 ± 4.1	29.6 ± 3.5	30.4 ± 2.9	38.2 ± 4.0	37.8 ± 4.7	0.08
DPD (nmol/mmol Cr)	4.2 ± 0.3	4.5 ± 0.3	4.2 ± 0.3	4.4 ± 0.3	4.3 ± 0.3	4.0 ± 0.3	0.56

[1] $\bar{x}$ ± SEM; $n = 15$. Means within rows and residency periods with different superscript letters are significantly different, $P < 0.05$ (Tukey's honestly significant difference test). K_1, phylloquinone; DK, dihydrophylloquinone; ucOC, undercarboxylated osteocalcin; BAP, bone-specific alkaline phosphatase; PTH, parathyroid hormone; NTx, *N*-telopeptides of type 1 collagen; BCE, bone collagen equivalents; Cr, creatinine; DPD, deoxypyridinoline.
[2] Diet-by–residency period interaction based on repeated-measures, two-factor ANOVA.

Bone markers

Mean serum 25-hydroxyvitamin D and PTH concentrations remained constant throughout the 2 residency periods (Table 3). Serum BAP concentrations decreased in response to phylloquinone depletion in one residency period, but were constant throughout the other residency period. This inconsistency in response of BAP to identical depletion diets suggests that the observed decrease in BAP is a spurious finding. Of the urinary bone markers assessed, NTx changed whereas deoxypyridinoline did not (Table 3). Urinary NTx concentrations increased in response to phylloquinone depletion compared with the control diet (Figure 3) and decreased in response to phylloquinone repletion, as did total osteocalcin. Although a trend, these changes were not significant. In contrast, NTx did not change in response to dihydrophylloquinone repletion.

Renal markers

There were no significant differences in the responses of urinary calcium and sodium to the phylloquinone and dihydrophylloquinone repletion diets, which was expected because subjects had been consuming controlled metabolic diets (data not shown).

DISCUSSION

Evidence for a role of phylloquinone as a protective dietary factor against hip fracture was provided in the Nurses' Health Study (25) and more recently in elderly men and women participating in the Framingham Osteoporosis Study (26). It has been assumed that the putative mechanism by which phylloquinone affects bone is mediated through the carboxylation of γ-carboxyglutamic acid residues in vitamin K–dependent proteins in bone, including osteocalcin, matrix γ-carboxyglutamic acid protein, and protein S (27). The vitamin K–dependent carboxylation of glutamine to γ-carboxyglutamic acid residues in vitamin K–dependent proteins was at posttranslational concentrations; therefore, any dietary effects were considered independent of the rate of protein synthesis.

In contrast with the reported increased fracture risk, there was no observed association between dietary phylloquinone intake and BMD in the Framingham Osteoporosis Study (26). These findings suggest that in the elderly, the putative protective effect of vitamin K against fracture may be independent of BMD. Elevated bone resorption is an independent risk factor for fracture (28). Vitamin K may affect bone resorption through a mechanism associated with the geranylgeranyl side chain, as proposed by investigators on the basis of animal studies using another form of vitamin K, menaquinone-4 (29, 30). Whereas the active site for the carboxylation reaction is identical in both compounds, menaquinone-4 differs structurally from phylloquinone in its side chain configuration. Collectively, these studies and the present study (which showed increases in both total osteocalcin and NTX after phylloquinone depletion) suggest that vitamin K may have a direct effect on bone turnover. However, 2 recent studies reported no effect of either short-term phylloquinone or long-term menaquinone-4 supplementation on bone resorption markers in healthy adults or osteoporotic women, respectively (31, 32). Therefore, the mechanisms underlying this putative effect of vitamin K on bone turnover are not understood, but warrant further investigation.

A caveat to this study's findings was the modest observed response of both total osteocalcin and NTx to vitamin K depletion and repletion. Furthermore, both total osteocalcin and NTx concentrations remained within the normal physiologic range during the short 4-wk study. However, consistent changes were observed and both bone turnover markers discriminated between phylloquinone and dihydrophylloquinone repletion. Furthermore, significant changes in markers of bone turnover in response to some bone-specific pharmacologic agents, such as estrogen and calcitonin, were observed after only 1 mo of treatment (33). Nevertheless, bone marker values in individuals can vary considerably over a short time and the effect of intraindividual variability on the ability to detect changes in bone marker values in response to treatment is of concern. Important contributors to biological variability in bone marker concentrations include diurnal fluctuations and seasonal changes. Higher variability in urinary bone markers may be due, at least partially, to greater inaccuracies associated with urine collection and to the use of creatinine to normalize the values, which contribute a second source of within-person variability (33). Much of this potential variability was controlled for in our study because subjects were housed in a metabolic unit, and careful attention was paid to sampling time and urine collection. In addition, seasonal biases were controlled for by enrolling subjects throughout the year.

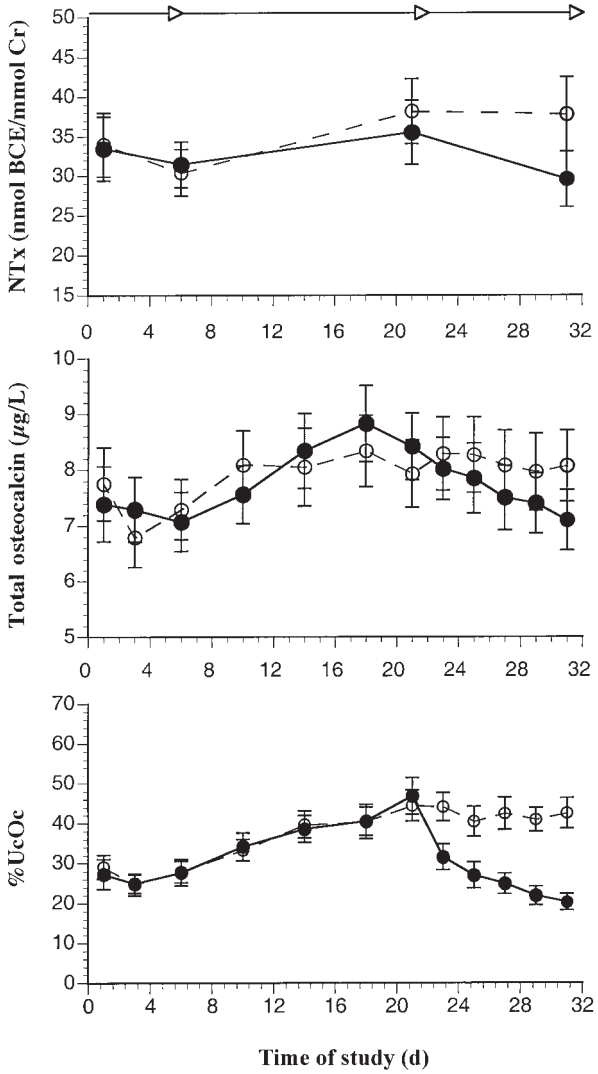

FIGURE 3. Mean (±SEM) urinary *N*-telopeptides of type 1 collagen (NTx), serum total osteocalcin, and the percentage of undercarboxylated osteocalcin (ucOC) in 15 young men and women in response to 100 μg (control diet), 10 μg (depletion diet), and 200 μg (repletion diet) phylloquinone (●) or dihydrophylloquinone (○). All 3 bone markers increased after phylloquinone depletion ($P < 0.01$), with a subsequent decrease to control values after phylloquinone repletion ($P < 0.01$). There were no significant changes in any of the bone markers after dihydrophylloquinone repletion. BCE, bone collagen equivalents; Cr, urinary creatinine.

In the present study we showed that changes in %ucOC paralleled those of total osteocalcin in response to the manipulation of dietary phylloquinone. Currently, there is a lack of consensus regarding vitamin K supplementation and its effect on total osteocalcin. There is one report of an observed increase in serum osteocalcin concentrations in postmenopausal women after 2 wk of supplementation at 1 mg phylloquinone/d (34). However, there was no significant change in serum osteocalcin concentrations in premenopausal women receiving the same regimen (34). In younger and older adults, supplementation with 1 mg phylloquinone/d was reported to have no effect on (34, 35) or an observed decrease in (31) serum osteocalcin, consistent with the findings in the present study. Because different antibodies were used to measure total osteocalcin in different studies, the discrepancy in results may be an artifact of the variation in affinities of different antibodies for the carboxylated form of osteocalcin (21). Likewise, there are widely divergent reports of what percentage of osteocalcin is undercarboxylated. How the degree of carboxylation is assessed is dependent on the amount of osteocalcin in the sample and the amount of hydroxyapatite used for binding (21). The method currently used precludes the exact measurement of ucOC; rather, the amount measured is relative and the direction, not the absolute percentage of the change, is critical.

The oral anticoagulant warfarin is a vitamin K antagonist that interrupts the carboxylation of vitamin K–dependent proteins. As a corollary, one assumes that the use of warfarin would parallel the effects of dietary vitamin K restriction, as noted in the present study. Although there is some evidence that warfarin use increases fracture risk in trabecular bone (36), the evidence is far from conclusive (37). Furthermore, therapeutic doses and low doses of warfarin have been reported to modestly decrease total osteocalcin, the effects of which are reversed with pharmacologic doses of phylloquinone (38–40). The direction of the response to warfarin in these studies is opposite that noted in the present study in response to dietary phylloquinone depletion. This difference suggests that more systematic investigation is required to confirm these findings.

Vitamin K–dependent reactions are related to both the length and the isomeric configuration of the side chain (41). Hydrogenation of phylloquinone to dihydrophylloquinone results in the saturation of a single 2′,3′ double bond in the side chain but conserves the naphthoquinone ring, which is the active site for carboxylation and influences functionality of vitamin K–dependent proteins. The effect of saturation of the 2′,3′ double bond on the biological activity of vitamin K was examined ≈60 y ago with the use of a qualitative chick bioassay (42). There was an overall loss in activity when vitamin K was saturated, although the data were inconsistent when compared with the parent compound phylloquinone (42). In the present study, comparison of plasma phylloquinone and dihydrophylloquinone concentrations after repletion suggests that dihydrophylloquinone is not as well absorbed as is phylloquinone. Alternatively, dihydrophylloquinone may be more rapidly metabolized and excreted than is the parent form, phylloquinone. Therefore, it is probable that differences in the relative biological activity of the 2 forms of vitamin K reflect, at the least, differences in their availability as cofactors for carboxylation of γ-carboxyglutamic acid residues in vitamin K–dependent proteins. The results of this study suggest that carboxylation of hepatic proteins was partially conserved, whereas carboxylation in the extrahepatic vitamin K–dependent proteins was not conserved when dihydrophylloquinone was the exclusive form of vitamin K consumed after short-term phylloquinone depletion.

Available data on phylloquinone intake suggest that more than one-half of younger adults in the US population do not meet the current adequate intake of this nutrient (13). In a recent nationwide study, the 5th percentile of usual average phylloquinone intake for adults is reported to be 21 μg/d (13), which is only 10 μg/d higher than the amount in the depletion diet used in the present study and 70–100 μg/d less than the current dietary reference intake of 90–120 μg/d (14). Within the context of the findings in the present study, these intake data raise concern that some individuals are at risk of increased bone turnover when consuming low-phylloquinone diets. Although children have phylloquinone intakes that are reportedly greater than the recommended adequate

intakes for their respective age groups, children also consume foods with a 1 to 2 ratio of dihydrophylloquinone to phylloquinone (13, 43). In the present study, dihydrophylloquinone had no measurable biological effect on the elevated concentrations of total osteocalcin, ucOC, undercarboxylated osteocalcin, and NTx that resulted from short-term dietary phylloquinone depletion.

Hydrogenated oil is ubiquitous in the US food supply. During the hydrogenation of phylloquinone-rich oils, phylloquinone is converted to dihydrophylloquinone. In the present study, short-term dietary restriction of phylloquinone was shown to increase measures of bone turnover. Short-term phylloquinone repletion restored measures of bone turnover to baseline values. In contrast, dihydrophylloquinone had no measurable biological effect on the markers of bone turnover. In the context of the findings of the present study, hydrogenation of plant oils decreases the amount of vitamin K available to bone, thereby reducing the role these oils may otherwise have in improving the consequences of an already low-to-average phylloquinone intake in certain subgroups of the adult population.

We gratefully acknowledge the Metabolic Research Unit of the Jean Mayer USDA HNRCA at Tufts University; Gerard Dallal for his statistical advice; Helen Rasmussen, Brian Kaszynski, Kenneth Davidson, Sheryl Neiman, and the Nutritional Evaluation Laboratory for their technical assistance; Jan Pyrek of the University of Kentucky Mass Spectrometry Facility for supplying the 2′,3′-dihydrophylloquinone; and the volunteers who participated in this study.

REFERENCES

1. Mensink RP, Katan MB. Effect of dietary trans fatty acids on high-density and low-density lipoprotein cholesterol levels in healthy subjects N Engl J Med 1990;323:439–45.
2. NCEP. Summary of the second report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II). JAMA 1993;269:3015–23.
3. Krauss RM, Deckelbaum RJ, Ernst N, et al. Dietary guidelines for healthy American adults. A statement for health professionals from the Nutrition Committee, American Heart Association. Circulation 1996;94:1795–800.
4. Booth SL, Davidson KW, Lichtenstein AH, Sadowski JA. Plasma concentrations of dihydro-vitamin K_1 following dietary intake of a hydrogenated vitamin K_1-rich vegetable oil. Lipids 1996;31:709–13.
5. Hart JP, Shearer MJ, McCarthy PT. Enhanced sensitivity for the determination of endogenous phylloquinone (vitamin K_1) in plasma using high-performance liquid chromatography with dual-electrode electrochemical detection. Analyst 1985;110:1181–4.
6. Hodges SJ, Bejui J, Leclercq M, Delmas PD. Detection and measurement of vitamins K_1 and K_2 in human cortical and trabecular bone. J Bone Miner Res 1993;8:1005–8.
7. Szulc P, Arlot M, Chapuy MC, Duboeuf F, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. J Bone Miner Res 1994;9:1591–5.
8. Szulc P, Chapuy MC, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture: a three year follow-up study. Bone 1996;18:487–8.
9. Vergnaud P, Garnero P, Meunier PJ, Breart G, Kamihagi K, Delmas PD. Undercarboxylated osteocalcin measured with a specific immunoassay predicts hip fracture in elderly women: the EPIDOS Study. J Clin Endocrinol Metab 1997;82:719–24.
10. Jie KG, Bots ML, Vermeer C, Witteman JC, Grobbee DE. Vitamin K status and bone mass in women with and without aortic atherosclerosis: a population-based study. Calcif Tissue Int 1996;59:352–6.
11. Binkley NC, Suttie JW. Vitamin K nutrition and osteoporosis. J Nutr 1995;125:1812–21.
12. Booth SL, Suttie JW. Dietary intake and adequacy of vitamin K. J Nutr 1998;128:785–8.
13. Booth SL, Webb DR, Peters JC. Assessment of phylloquinone and dihydrophylloquinone dietary intakes among a nationally representative sample of US consumers using 14-day food diaries. J Am Diet Assoc 1999;99:1072–6.
14. Institute of Medicine. Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. Washington, DC: National Academy Press, 2001.
15. Institute of Medicine. Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. Washington, DC: National Academy Press, 1997.
16. Booth SL, Sadowski JA. Determination of phylloquinone in foods by high-performance liquid chromatography. Methods Enzymol 1997;282:446–56.
17. Booth SL, Charnley JM, Sadowski JA, Saltzman E, Bovill EG, Cushman M. Dietary vitamin K_1 and stability of oral anticoagulation: proposal of a diet with constant vitamin K_1 content. Thromb Haemost 1997;77:504–9.
18. Ferland G, MacDonald DL, Sadowski JA. Development of a diet low in vitamin K-1 (phylloquinone). J Am Diet Assoc 1992;92:593–7.
19. Davidson KW, Sadowski JA. Determination of vitamin K compounds in plasma or serum by high- performance liquid chromatography using postcolumn chemical reduction and fluorimetric detection. Methods Enzymol 1997;282:408–21.
20. Preece MA, O'Riordan JL, Lawson DE, Kodicek E. A competitive protein-binding assay for 25-hydroxycholecalciferol and 25-hydroxyergocalciferol in serum. Clin Chim Acta 1974;54:235–42.
21. Gundberg CM, Nieman SD, Abrams S, Rosen H. Vitamin K status and bone health: an analysis of methods for determination of undercarboxylated osteocalcin. J Clin Endocrinol Metab 1998;83:3258–66.
22. Gundberg CM, Hauschka PV, Lian JB, Gallop PM. Osteocalcin: isolation, characterization, and detection. Methods Enzymol 1984;107:516–44.
23. Haroon Y. Rapid assay for gamma-carboxyglutamic acid in urine and bone by precolumn derivatization and reversed-phase liquid chromatography. Anal Biochem 1984;140:343–8.
24. Roberts NB, Fairclough D, Taylor WH. Plasma emission spectrometry: measurement of calcium, phosphorus and chromium in metabolic balance studies. Ann Clin Biochem 1984;21:213–7.
25. Feskanich D, Weber P, Willett WC, Rockett H, Booth SL, Colditz GA. Vitamin K intake and hip fractures in women: a prospective study. Am J Clin Nutr 1999;69:74–9.
26. Booth SL, Tucker KL, Chen H, et al. Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. Am J Clin Nutr 2000;71:1201–8.
27. Ferland G. The vitamin K-dependent proteins: an update. Nutr Rev 1998;56:223–30.
28. Melton LJ III, Khosla S, Atkinson EJ, O'Fallon WM, Riggs BL. Relationship of bone turnover to bone density and fractures. J Bone Miner Res 1997;12:1083–91.
29. Hara K, Akiyama Y, Nakamura T, Murota S, Morita I. The inhibitory effect of vitamin K_2 (menatetrenone) on bone resorption may be related to its side chain. Bone 1995;16:179–84.
30. Akiyama Y, Hara K, Tajima T, Murota S, Morita I. Effect of vitamin K_2 (menatetrenone) on osteoclast-like cell formation in mouse bone marrow cultures. Eur J Pharmacol 1994;263:181–5.
31. Binkley NC, Krueger DC, Engelke JA, Foley AL, Suttie JW. Vitamin K supplementation reduces serum concentrations of under-gamma-carboxylated osteocalcin in healthy young and elderly adults. Am J Clin Nutr 2000;72:1523–8.
32. Shiraki M, Shiraki Y, Aoki C, Miura M. Vitamin K_2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. J Bone Miner Res 2000;15:515–21.

33. Hannon R, Blumsohn A, Naylor K, Eastell R. Response of biochemical markers of bone turnover to hormone replacement therapy: impact of biological variability. J Bone Miner Res 1998;13: 1124–33.
34. Knapen MH, Hamulyak K, Vermeer C. The effect of vitamin K supplementation on circulating osteocalcin (bone Gla protein) and urinary calcium excretion. Ann Intern Med 1989;111:1001–5.
35. Douglas AS, Robins SP, Hutchison JD, Porter RW, Stewart A, Reid DM. Carboxylation of osteocalcin in post-menopausal osteoporotic women following vitamin K and D supplementation. Bone 1995;17: 15–20.
36. Caraballo PJ, Heit JA, Atkinson EJ, et al. Long-term use of oral anticoagulants and the risk of fracture. Arch Intern Med 1999;159: 1750–6.
37. Caraballo PJ, Gabriel SE, Castro MR, Atkinson EJ, Melton LJ III. Changes in bone density after exposure to oral anticoagulants: a meta-analysis. Osteoporos Int 1999;9:441–8.
38. Bach AU, Anderson SA, Foley AL, Williams EC, Suttie JW. Assessment of vitamin K status in human subjects administered "minidose" warfarin. Am J Clin Nutr 1996;64:894–902.
39. Peitschmann P, Woloszczuk W, Panzer S, Kyrle P, Smolen J. Decreased serum osteocalcin levels in phenprocoumon treated patients. J Clin Endocrinol Metab 1988;66:1071–4.
40. van Haarlem LJM, Knapen MHJ, Hamulyák K, Vermeer C. Circulating osteocalcin during oral anticoagulant therapy. Thromb Haemost 1988;60:79–82.
41. Suttie JW, Grossman CP, Benton ME. Specificity of the vitamin K and glutamyl binding sites of the liver microsomal gamma-glutamyl carboxylase. J Nutr Sci Vitaminol (Tokyo) 1992:405–8.
42. Fieser LF, Tishler M, Sampson WL. Vitamin K activity and structure. J Biol Chem 1941;137:659–92.
43. Booth SL, Pennington JA, Sadowski JA. Dihydro-vitamin K_1: primary food sources and estimated dietary intakes in the American diet. Lipids 1996;31:715–20.

The American Journal of Clinical Nutrition