Moderate Folate Depletion Increases Plasma Homocysteine and Decreases Lymphocyte DNA Methylation in Postmenopausal Women[1–4]

Robert A. Jacob,[5] Denise M. Gretz, Peter C. Taylor, S. Jill James,* Igor P. Pogribny,* Barbara J. Miller,* Susanne M. Henning† and Marian E. Swendseid†

*Western Human Nutrition Research Center, U.S. Department of Agriculture, Agricultural Research Service, Presidio of San Francisco, CA 94129; *National Center for Toxicological Research, FDA, Division of Nutritional Toxicology, Jefferson, AR 72079; and †School of Public Health, University of California, Los Angeles, CA 90024*

ABSTRACT To determine the human folate requirement on the basis of changes in biochemical pathways, we studied the effect of controlled folate intakes on plasma homocysteine and lymphocyte DNA methylation and deoxynucleotide content in healthy postmenopausal women. Eight women (49–63 y of age) were housed in a metabolic unit and fed a low folate diet containing 56 μg/d of folate for 91 d. Folate intake was varied by supplementing 55–460 μg/d of folic acid (pteroylglutamic acid) to the diet to provide total folate intake periods of 5 wk at 56 μg/d, 4 wk at 111 μg/d and 3 wk at 286–516 μg/d. A subclinical folate deficiency with decreased plasma folate was created during the first two periods. This resulted in significantly elevated plasma homocysteine and urinary malondialdehyde, and lymphocyte DNA hypomethylation. The folate depletion also resulted in an increased ratio of dUTP/dTTP in mitogen-stimulated lymphocyte DNA and decreased lymphocyte NAD, changes suggesting misincorporation of uracil into DNA and increased DNA repair activity. The DNA hypomethylation was reversed with 286–516 μg/d of folate repletion, whereas the elevated homocysteine decreased with 516 but not 286 μg/d of folate. The results indicate that marginal folate deficiency may alter DNA composition and that the current RDA of 180 μg/d may not be sufficient to maintain low plasma homocysteine concentrations of some postmenopausal women. J. Nutr. 128: 1204–1212, 1998.

KEY WORDS: • *folic acid* • *homocysteine* • *methylation* • *DNA* • *humans*

Anemia and other hematologic measures have traditionally been the criteria for gauging the adequacy of folate nutriture. Recent data, however, suggest that the elevation of plasma homocysteine (Hcy)[6] and disturbances of DNA composition may provide more sensitive functional measures of folate deficiency (Blount et al. 1997, Jacob et al. 1994, James et al. 1997, O'Keefe et al. 1995, Pogribny et al. 1995). These new data raise the question of whether the current Recommended Dietary Allowance (RDA) for folate is adequate to satisfy pathways of Hcy metabolism and deoxynucleotide synthesis. Folate is needed to provide one-carbon units for over one hundred biochemical processes, including the methylation of Hcy to form methionine and the biosynthesis of deoxynucleotides dTMP, dAMP and dGMP needed for DNA replication (Selhub and Rosenberg 1996). Increased Hcy has been associated with premature vascular disease (Boushey et al. 1995), and disturbed deoxynucleotide synthesis has been associated with aberrant DNA synthesis and cell proliferation (James et al. 1993 and 1994b).

Recent studies have linked moderately elevated plasma Hcy (>14 μmol/L) to increased risk of coronary, cerebral and peripheral vascular diseases (Boushey et al. 1995). Even moderate folate deficiency has been linked to increased plasma Hcy concentrations in both women and men. Healthy non-pregnant women given a diet containing 200 μg/d of folate (slightly more than the current RDA of 180 μg/d) had significantly higher plasma Hcy (12.6 ± 3.7 μmol/L) than women given the same diet with 400 μg/d of folate intake (7.7 ± 1.6 μmol/L) (O'Keefe et al. 1995). In healthy adult men, 84% of the current RDA did not normalize plasma Hcy concentrations elevated by experimental folate depletion, suggesting that the current folate RDA for adult men may not provide the expected margin of protection (Jacob et al. 1994).

Studies with cells, as well as in vivo studies with animal models, have shown that folate deficiency disrupts the normal biosynthesis of deoxyribonucleotides, which are utilized for

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[5] To whom correspondence should be addressed.
[6] Abbreviations used: Hcy, homocysteine; MCV, mean corpuscular volume; MDA, malondialdehyde; PARP, poly(ADP-ribose) polymerase; RDA, Recommended Dietary Allowance; TBA, thiobarbituric acid; TMP, 1,1,3,3,- tetramethoxypropane; WHNRC, Western Human Nutrition Research Center.

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DNA replication and repair. Spleen cells from rats fed diets low in folate, choline and/or methionine had decreased amounts of dTMP and dTTP, consistent with impaired folate-dependent conversion of uridylate to thymidylate (James et al. 1992). Mitogen-stimulated rat lymphocytes cultured in a low folate medium showed significant decreases in dTMP, dGTP, dATP and dCTP (James et al. 1993). Additionally, Pogribny et al. (1995) reported that folate/methyl deficiency in rats induced DNA strand breaks and hypomethylation within the hepatic p53 tumor suppressor gene. Similar results were reported by Kim et al. (1997) with isolated folate deficiency in rats. Consistent with the above results, recent studies in humans indicated that folate-deficient individuals had elevated incorporation of uracil into DNA, accompanied by an increased frequency of cellular micronuclei, a measure of DNA and chromosome damage (Blount et al. 1997, MacGregor et al. 1997).

These new data suggest that the human requirement for folate should be re-evaluated by assessing molecular as well as clinical endpoints of folate deficiency, including plasma Hcy and DNA methylation and deoxynucleotide content. The objectives of this study were to investigate these variables in healthy postmenopausal women during controlled folate intakes and to determine the amount of dietary folate needed to maintain their integrity.

SUBJECTS AND METHODS

Protocol and subjects. Ten healthy non-smoking postmenopausal volunteer women, ages 49–63 y, were admitted to the metabolic unit of the USDA Western Human Nutrition Research Center (WHNRC) after medical and psychological screening. Prestudy screening included medical histories, physical and dental examinations, hematologic and clinical chemistry tests, psychological testing, resting electrocardiogram, and tests for hepatitis, syphilis, tuberculosis, human papilloma virus (PAP smear) and HIV-antibody tests. Tests for plasma folate and vitamin B-12, alcohol, tobacco and drug use were also performed. All subjects were within 90–130% of desirable weight (Metropolitan Life Insurance Company, New York, NY), except for one subject who was 145% of desirable weight. Other specific criteria for subject selection included the following: nonsmokers, non-users of vitamin or health food supplements containing folic acid, hemoglobin >115 g/L and hematocrit >0.34. Three subjects, who were receiving estrogen replacement therapy when they entered the study, continued the same regimen throughout.

The study protocol and informed consent were approved by the Human Subjects Review Committee of the University of California, Davis (protocol # 95–805), and by the Human Studies Review Committee of the Agricultural Research Service, U. S. Department of Agriculture (protocol # 95–013). Signed informed consent was obtained from each volunteer after reading of the protocol and discussion of the study's purpose, procedures, risks and benefits. For the duration of the 91 d study, the subjects lived in and ate all meals in the WHNRC metabolic unit, and were chaperoned at all times when outside the unit.

Experimental design and diet. Subjects consumed the same experimental low folate diet in a 4-d menu rotation for the entire 91 d. The diet was also designed to limit choline intake as an exogenous methyl group source. The diet provided an average of 56 μg/d of folate; varying amounts of synthetic folic acid (pteroylglutamic acid) were added to the diet to provide folate intake periods of 56 to 516 μg/d as shown in **Table 1**, and **Figures 1** and **2**. Over the first 5 d, a total of 195 μg/d of folate (56 μg/d from the diet plus 139 μg/d of added folic acid) were provided to maintain adequate folate status while baseline measurements were taken. The succeeding 5-wk folate depletion period (d 6–41) was designed to reduce body folate stores and produce a moderate but not severe folate deficiency, analogous to free-living women with chronically low intake and body stores of folate but without macrocytosis or anemia.

The composition of the four daily menus fed in a rotating cycle throughout the study is shown in **Table 2**. All menu items were weighed to the nearest gram except for folic acid supplement solutions which were weighed to 0.01 g; the applesauce containing the folic acid supplement and stewed tomatoes containing amino acids were weighed to 0.1 g. Optional items available ad libitum included water, salt, sugar-free sodas and sweetener (aspartame), and decaffeinated coffee. Consumption of tea and pepper was prohibited, as was smoking, alcoholic beverages and drugs, except for approved medical use. As needed, ibuprofen was given as analgesic, antacids or a bismuth salicylate preparation for gastrointestinal discomforts, and glycerin suppositories or a stool softener for constipation.

Folic acid was added to the diet by mixing a weighed aliquot of folic acid supplement solution (0.80–2.00 g) of known concentration into the applesauce served at each breakfast and dinner. Folic acid supplement solutions were prepared by dissolving a weighed amount of USP folic acid (90% folic acid/7.9% water by manufacturer's lot analysis, Roche, Hoffmann-La Roche, Belvidere, NJ) into 0.1 mol/L NaOH and adjusting the final pH to 7–8 with 0.1 mol/L HCl. The solutions were kept refrigerated in aluminum foil–covered containers and aliquots from these solutions were added to the applesauce portions each day. Folic acid concentrations of the supplement solutions were calculated using the 90% analyzed purity value; they were checked by diluting an aliquot fourfold or tenfold with 0.1 mol/L PBS (pH 7.0) and determining the folic acid concentration spectrophotometrically at 282 nm with a molar extinction coefficient of 27,600 L/(mol · cm) (Blakley 1969). Concentrations of folic acid determined in this way were within 6% of the values calculated gravimetrically. Weekly spectrophotometric checks of the refrigerated folic acid supplement solutions showed no deterioration of folic acid content over 5 wk.

As calculated from food composition tables (USDA 1991), the low folate diet at 8.79 MJ (2100 kcal) provided 61% of energy from carbohydrate, 9.6% from protein and 29.4% from fat. Of 50 g/d of protein intake, 35 g came from the diet and 15 g protein equivalents from crystalline amino acids (18 g amino acid/d), as described in footnote[5] of Table 2. The diet provided a daily average of 780 mg of methionine and 420 mg of cysteine, 132% of the estimated 910 mg/d adult requirement for Met + Cys (NRC 1989). Five foods (green beans, carrots, chicken, turkey and ham) were boiled three times for 7 min each and the water discarded to lower their folate content. Analysis of the total folate content of the boiled and nonboiled foods by microbiological assay using *Lactobacillus casei* (*L. casei*, ATCC 7469) as an assay organism (Tamura et al. 1997) showed that this procedure reduced their folate content by ~50%. Approximately 5 g/d of a nondigestible fiber supplement (Alphacel, ICN Biomedicals, Aurora OH) was added to the diet by mixing with the applesauce at 2 g/100 g.

Composites of each of the four daily menus used throughout the study were prepared for determination of folate and choline. Food composites for folate assay were prepared just as for the study subjects (including triple boiled foods) except that foods known to contain no appreciable folate (soybean margarine, sugar, olive oil, amino acids and non-diary topping) were omitted from the composite. The four daily composites for folate assay were homogenized in a blender with an equal volume of cold 0.1 mol/L potassium phosphate buffer (pH 6.3) containing 57 mmol/L of ascorbic acid to preserve the folate. Aliquots of the homogenate were frozen at −70°C until thawed for determination of total folate by treatment with folate conjugase and microbiological assay with *L. casei* as the assay organism (Tamura et al. 1997). The mean folate content of the four daily menus was 56 μg/d (range 39–71). This compared with a mean value of 31 μg/d (range 28–34) calculated from food composition tables (USDA 1991). The calculated value may be lower because folate values for 13 of the 40 food items were missing from the nutrient database including values for the low protein and low carbohydrate products, spices and the fiber supplement. Although the treatment with folate conjugase alone is the traditional technique for food folate analysis, use of the newer tri-enzyme technique (treatment of the homogenate with α-amylase, protease and folate conjugase) resulted in a mean daily intake value of 152 μg/d, nearly triple that when folate conjugase alone was used (Tamura et al. 1997). To interpret the results from this study, we used the value of 56 μg/d derived from the traditional assay method with the use of folate conjugase alone because this allows comparability to previous studies (Jacob et al. 1994, O'Keefe

TABLE 1

Plasma folate and biochemical indices in postmenopausal women with various dietary folate intakes[1]

Variable	Study day					
	1–5	6–20	21–41	42–69	70–80	81–91
Folate intake,[2] *μg/d*	195	56	56	111	286	516
Plasma folate, *nmol/L*	19.5 ± 4.2	10.5 ± 2.3*	9.3 ± 1.8*	8.1 ± 1.2*	12.5 ± 1.4*	16.6 ± 1.6§
Plasma homocysteine, *μmol/L*	9.8 ± 0.4	—	12.5 ± 1.0*	12.6 ± 0.5*	12.6 ± 0.5*	11.8 ± 0.2*,§
Urine MDA,[3] *μmol/d*	3.56 ± 0.10	—	3.67 ± 0.13	4.29 ± 0.08*	—	3.66 ± 0.12§
DNA methyl acceptance, *cpm/0.5 μg*	795 ± 41	—	1041 ± 115	1738 ± 259*	—	1084 ± 158§
NAD, *pmol/10⁶ cells*	217 ± 37	110 ± 18*	108 ± 17*	126 ± 17*	—	120 ± 21*
DNA strand breaks,[4] *32P cpm/μg*	665 ± 242	—	247 ± 19*	220 ± 10*	—	219 ± 7*
dUTP/dTTP[5]	2.24 ± 0.40	2.90 ± 0.38	2.33 ± 0.24	3.03 ± 0.40*	—	2.81 ± 0.57
[3H]dU uptake,[6] *cpm/10⁶ cells*	4116 ± 872	2841 ± 599	2881 ± 317	2218 ± 266*	—	2078 ± 231*

[1] Means ± SEM are for end-of-study periods (except means in column d 70–80 are for d 84), $n = 8$ (except for dUTP/dTTP; $n = 5$ for d 5 and 20, and $n = 7$ for d 69). Means are different: * from baseline d 5 or § from previous mean, d 84 (d 69 for DNA methyl uptake and urine MDA), $P < 0.05$ by paired t test. The last five variables are indices for isolated mononuclear cells.

[2] Folate intake values are 96 μg/d higher if results from the tri-enzyme food folate assay procedure are used (Tamura et al. 1997).

[3] Values are means of 7-d urine pools, in malondialdehyde (MDA) equivalents.

[4] Incorporation of [32P]dCTP into lymphocyte DNA is proportional to level of 3′OH DNA strand breaks present.

[5] Ratio of dUTP/dTTP in mitogen-stimulated lymphocytes in folate-deficient media compared with unstimulated lymphocytes.

[6] In vitro uptake of [3H]-deoxyuridine into mitogen-stimulated lymphocytes cultured in folate-deficient media.

et al. 1995, Sauberlich et al. 1987) and data upon which the current RDA is based. Use of the higher tri-enzyme value of 152 μg/d would increase the folate intake of each period by 96 μg/d, as noted in footnote 2 of Table 1 and the figure legends.

Separate aliquots of the frozen diet homogenates, prepared as described above, were analyzed for total choline (free and lipid bound) by a gas chromatography/mass spectrometry procedure (Freeman et al. 1975). The choline content of the four daily menus averaged 147 mg/d (range 132–171 mg/d). The diet was supplemented daily with three different vitamin/mineral tablets to provide at least 80% of the RDA for each essential micronutrient (except 75% of vitamin D RDA). The subjects received one folate-free vitamin/mineral tablet daily with breakfast (Fosfree, Mission Pharmacal, San Antonio TX), one multimineral tablet at each of the three daily meals (Chelated Solamins, Solgar Vitamins, Lynbrook NY) and one calcium/magnesium tablet at each dinner (Bronson Calcium Complex & Magnesium, Bronson, St. Louis MO). This regimen provided the following amounts of total daily B vitamins (diet plus supplements), in % of RDA (NRC 1989): 234% of B-6, 124% of B-12, 200% of riboflavin, 556% of thiamin and 214% of niacin (21.1 niacin equivalents from preformed niacin and 8.6 niacin equivalents from 516 mg of tryptophan).

Individual energy intakes were estimated initially on the basis of weight, height and age, using the Harris-Benedict equation to calculate basal energy expenditure and adding 40% for daily activity. Body weights were measured daily, and adjustments in energy intakes were made if subjects deviated beyond ± 5% of the baseline weight taken as the average weight over d 5–7. All food items were adjusted proportionately when energy intake was changed. Individual energy intakes ranged from 7.12 to 9.63 MJ/d. Initial body weights ranged from 55.9 to 93.9 kg and declined an average of 5% (mean of 69.3 to 65.7 kg) from d 5–7 to d 91. All subjects took one or two chaperoned walks each day totaling 3.2–6.4 km/d. In addition, subjects were allowed to exercise on a voluntary basis up to 90 min/wk on the metabolic unit treadmill or stationary bicycle.

Specimen collections and analytical methods. A fasting blood sample was taken at 0700–0800 h by venipuncture 13 times for study-related biochemical determinations, and for complete blood count and clinical chemistry panels to monitor the subjects health (not all tests were performed 13 times). The blood was collected in evacuated glass tubes and immediately processed for serum, EDTA, heparin and citrate plasma. Plasma folate and vitamin B-12, and erythrocyte folate (from EDTA anticoagulated blood) were determined by competitive protein binding radioassay kits (Quantaphase II B-12/Folate Radioassay, BioRad, Hercules CA). The samples were prepared according to kit directions and stored at −70°C until analysis.

Young and old red cell fractions were separated by the centrifugal technique of Murphy (1973), which is based on the lower density of young compared with older red cells. After hematocrit was determined and the red cell fractions separated, 100 μL aliquots from total, young and old red cells plus plasma were added to 1 mL of 22.7 mmol/L ascorbic acid, mixed well and frozen at −70°C for folate determination. Upon thawing, the whole-blood hemolysates, which included the added back plasma, were incubated for 90 min before radioassay to hydrolyze polyglutamyl folate in red cells. Erythrocyte folate was calculated as the difference between plasma and whole-blood folate using the hematocrit to correct for red cell volume differences. Determination of folate in six plasma and whole blood samples on three consecutive days gave a mean day-to-day variance (CV%) of 4.0 and 6.7% for plasma and red cell folate assays, respectively.

For determination of total plasma Hcy, EDTA blood was kept on ice until the plasma was separated from the erythrocytes, within 2 h of collection. Plasma was treated with tri-*n*-butylphosphine in dimethyformamide to release and reduce bound Hcy. Total Hcy was then determined by HPLC fluorescence detection after derivatization with a fluorescent sulfonic acid reagent (Araki and Sako 1987). Malondialdehyde (MDA) was determined in EDTA plasma by a modified procedure from Chirico (1994) involving HPLC separation and fluorescence detection of the MDA-thiobarbituric acid (TBA) adduct, $MDA[TBA]_2$. The results were calculated from a calibration curve based on MDA generated in vitro by hydrolysis of 1,1,3,3-tetramethoxypropane (TMP) and were expressed as micromolar MDA equivalents. Day-to-day variation of frozen aliquots of a plasma control over 10 runs was 15% (0.242 ± 0.036 μmol/L MDA equivalents).

Complete daily urine collections were taken throughout the study with samples refrigerated. Seven-day urine pools were prepared by taking a constant volume fraction of each of seven daily urine collections and mixing together. Aliquots of the daily and pooled urines were taken for determination of creatinine by a modified Jaffe method (Kroll et al. 1986).

Urinary MDA was determined in 7-d urine pools by a manual assay modified from Halliwell and Chirico (1993), which employs TBA derivatization and fluorometric detection at 525 nm excitation/547 nm emission. Results were calculated from the average slope of calibration curves prepared from hydrolysis of TMP over 26 assay runs. Day-to-day variation of frozen urine aliquots at low and high concentrations over 26 assays was 10.9 and 5.1%, respectively (within-run variations were 3% or less).

Neutrophil segmentation was assessed by counting the number of

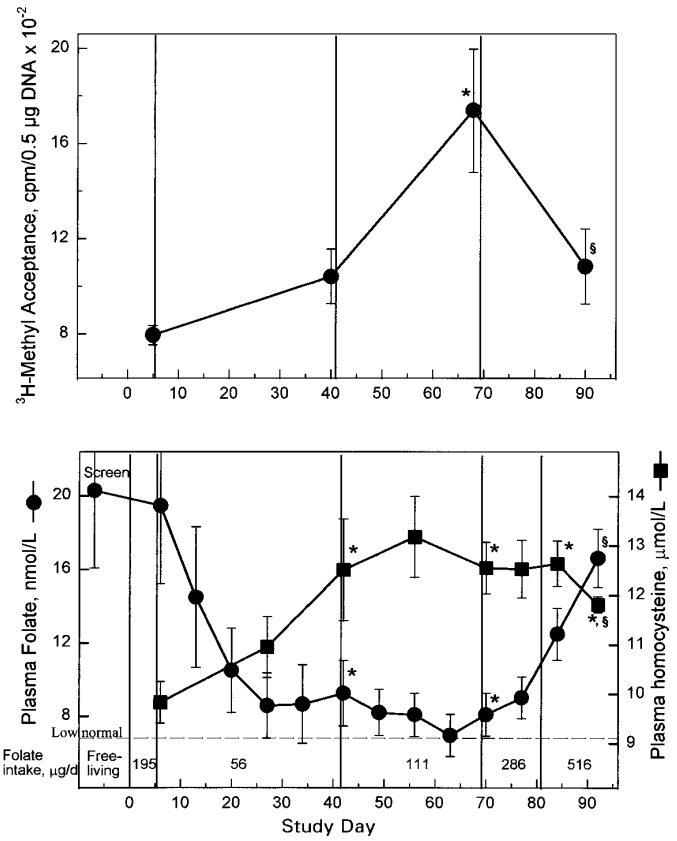

FIGURE 1 Plasma folate and homocysteine (*lower panel*), and lymphocyte DNA methyl acceptance (*upper panel*) for eight postmenopausal women receiving various dietary intakes of folate shown at bottom in µg/d (folate intake values are 96 µg/d higher if results from the tri-enzyme food folate assay procedure are used, Tamura et al. 1997). Plotted values are means ± SEM. Means at end of folate intake periods are different: *from d 5 or §from d 84 (d 69 for methyl acceptance test), $P < 0.05$ by paired *t* test. Lower limit of normal range for plasma folate is shown at bottom as horizontal dashed line at 6.8 nmol/L.

nuclear lobes in a population of 100 neutrophils and calculating the average lobe number (Herbert 1959). Complete blood counts including differential white cell counts were taken weekly with an automated cell counter, which calculates the hematocrit from the red cell count and mean corpuscular volume (MCV), and determines hemoglobin by the cyanmethemoglobin reaction (System 9000 Diff, Serono Baker Diagnostics, Allentown PA).

Peripheral blood mononuclear cell isolation and analyses. The isolation of mononuclear cells (primarily T and B lymphocytes) was based on the method of Boyum (1968). Histopaque-1077 (12 mL) (Sigma Chemical, St. Louis MO) was layered into the bottom of tubes containing 8 mL of heparinized blood and 20 mL PBS. After centrifugation, removal of plasma and washing with PBS, the isolated lymphocytes were then processed for DNA methylation, strand breaks, deoxynucleotides and tritiated deoxyuridine uptake tests. For DNA methylation and strand break tests, the PBS supernatant was removed after centrifugation and the cell pellets stored at −70°C until DNA extraction (Ausebel et al. 1989).

For deoxynucleotides and tritiated deoxyuridine uptake tests, cells were incubated in folate positive (1 mg/L folic acid) and negative media for 72 h at 37°C with 5% CO_2. The prepared culture medium was Folate Negative RPMI-1640, containing 25 mmol/L HEPES buffer (Irvine Scientific, Santa Ana CA). To 500 mL of this medium, 5.1 mL of Glutamine Pen-Strep (Life Technologies, Grand Island NY) was added, containing 29.2 g/L of L-glutamine, 1×10^7 units/L penicillen-G, and 10 g/L streptomycin sulfate. Also added to the medium was 1.1 mL of 10 g/L Gentamicin (Life Technologies) and 25 mL fetal bovine serum (Sigma Chemical). Each tissue culture flask had 15 mL of 1×10^9 cells/L solution added. The resulting cell pellets were stored at −70°C for later determination of deoxynucleotides.

The amount of folate-dependent de novo thymidylate synthesis was measured by the incorporation of [6-^{3}H]deoxyuridine into the cellular DNA and was expressed as cpm/million cells. The ^{3}H label at the number 6 position of deoxyuridine monophosphate is retained after methylation to deoxythymidine monophosphate via the de novo pathway and is incorporated as [6-^{3}H]thymine into DNA. Therefore, the presence of the ^{3}H-radiolabel in the lymphocyte DNA extracts after mitogen stimulation is a measure of the relative rate of de novo thymidylate synthesis as well as the ability of the cells to proliferate in vitro (James and Yin 1989). For the tritiated deoxyuridine uptake test, the cells were added at a concentration of 10^5/well in six replicate wells containing 100 µL media. The media contained folic acid (1 g/L) for the control wells and folate positive wells. To the folate positive and negative, but not the control wells, PHA-P was added to a final concentration of 10 µg/mL for mitogen stimulation. The cells were incubated at 37°C for 48 h; then 50 µL [6-^{3}H]tritiated deoxyuridine [1.48 GBq (40 mCi)/L] (Dupont NEN Research Products, Boston MA) was added to each well, and cells were returned to the incubator for another 24 h. The cells were harvested onto glass fiber filters and the radioactivity measured using a Matrix 9600 direct beta counter (Packard Instrument, Downers Grove, IL).

A modification of the assay described by Balaghi and Wagner (1993) was used to assess the methylation status of lymphocyte DNA. This assay reflects the capacity of genomic DNA to accept ^{3}H-labeled methyl groups, and the DNA incorporated radioactivity is inversely proportional to the level of DNA methylation. Genomic DNA (0.5 µg) extracted from lymphocytes was incubated with 3.0 µmol/L [^{3}H-methyl] *S*-adenosyl L-methionine (Dupont NEN) containing 74 kBq and 3 units Sss I CpG methylase (New England Biolabs, Beverly, MA) in 1X Sss I buffer at 30°C for 1 h. The samples were subsequently applied to Whatman DE-81 ion exchange filters and washed in 0.5 mol/L sodium phosphate buffer followed by 70% ethanol to remove unincorporated precursor. The air-dried filters were placed in scintillation vials and radioactivity was quantified in a 1900 TR Packard counter using Ultima Gold Scintillant (Packard, Meriden, CT). The results are expressed as cpm of ^{3}H-methyl incorporation/0.5 µg DNA to compare relative changes in methylation status.

Genomic DNA strand breaks were determined by the random oligonucleotide-primed synthesis assay, which detects low frequency

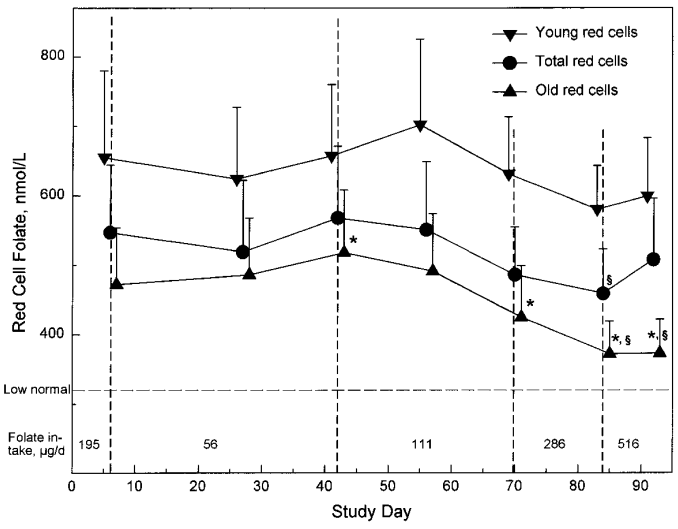

FIGURE 2 Red cell folate concentrations of total, young and old red cell populations of eight postmenopausal women receiving various dietary intakes of folate shown at bottom in µg/d (folate intake values are 96 µg/d higher if results from the tri-enzyme food folate assay procedure are used, Tamura et al. 1997). Plotted values are means + SEM. Lower limit of normal range for red cell folate is shown as horizontal dashed line at 317 nmol/L. All means for young red cells are greater than old, $P < 0.05$. Means at end of folate intake periods are different: *from d 6 or §from d 42, $P < 0.05$ by paired *t* test.

TABLE 2

Low folate diet menus[1]

Meal	Day 1	Day 2	Day 3	Day 4
Breakfast	Hash browns	Jelly	Hash browns	Jelly
	Applesauce[2]	Applesauce[2]	Applesauce[2]	Applesauce[2]
		LP bread[3]		LP bread[3]
		Soybean margarine		Soybean margarine
Lunch	Fruit cocktail	Cranberry juice	Pears	Peaches
	Somen noodles	LP cookies[3]	LP bread[3]	LP pasta[3]
	Soybean margarine	White rice	Soybean margarine	Zucchini squash
	Zucchini squash	Olive oil	Boiled chicken[4]	Boiled chicken[4]
	Boiled chicken[4]	Deli ham[4]	Green beans[4]	Stewed tomatoes[5]
	Stewed tomatoes[5]	Green beans[4]	Soybean mayonnaise	Olive oil
	Olive oil		Italian dressing	Chicken broth
Dinner	LP pasta[3]	Spaghetti	LP pasta[3]	Roast turkey[4]
	Roast turkey[4]	LP pasta[3]	Deli ham[4]	White rice
	Carrots[4]	Zucchini squash	Zucchini squash	Mushrooms
	Italian dressing	Roast turkey[4]	Stewed tomatoes[5]	LP cookies[3]
	Olive oil	Olive oil	Olive oil	Olive oil
	Applesauce[2]	Stewed tomatoes[5]	Beef broth	Applesauce[2]
		Applesauce[2]	Applesauce[2]	
Evening snack	Carrots[4]	LP cracker[3]	Peaches	Dried prunes
	Soybean margarine	Soybean margarine	Gelatin	Gelatin
	Sugar	Dried apricots	Non-dairy topping	
	Non-dairy topping			

[1] Spices < 1 g not listed: salt, pepper, cinnamon, oregano, onion and garlic powder, basil, celery and dill.
[2] Applesauce vehicle for folic acid supplement served at each breakfast and dinner, ~17 g sugar and 2 g Alphacel (non-digestible fiber supplement) added per 100 g applesauce.
[3] LP designates low protein pasta, bread, cookie and cracker products (Dietary Specialties, Rochester, NY).
[4] Items boiled three times for 7 min each time and water discarded to lower folate content.
[5] Amino acid mix added to stewed tomatoes providing 17.76 g/d amino acids (2.44 g N). Specific amounts in mg/d: 482 Ala, 896 Arg, 1366 Asp, 482 Gly, 295 His, 576 Ile, 919 Leu, 882 Lys, 636 Phe, 660 Pro, 422 Thr, 166 Trp, 506 Tyr, 566 Val and 2359 Glu.

3′OH strand breaks in DNA. As detailed elsewhere (Basnakian and James 1996), 3′OH DNA fragments in the high molecular weight DNA are initially separated into single strands by heat denaturation. After reassociation, these fragments serve as primers, and the excess of high molecular weight DNA serves as a template in a reaction with DNA polymerase. As a result, incorporation of [^{32}P]dCTP (Dupont NEN) initiated by the Klenow fragment of DNA polymerase I is proportional to the number of 3′OH breaks present. Because a DNA denaturation step is included in the assay, both single- and double-strand DNA breaks are detectable.

Lymphocyte NAD and the deoxynucleotides dTMP, dGTP, dATP, dTTP, dCTP, dUMP and dUTP were determined in DNA isolated from unstimulated (control) and mitogen-stimulated (positive folate and negative folate) lymphocytes in culture by reverse-phase HPLC with UV detection after trichloroacetic acid extraction as previously described (James et al. 1994b).

Statistics. Results for the plasma, lymphocyte and urine variables were analyzed for differences due to dietary folate intake. Descriptive statistics were computed for the end of each folate intake period and are shown as means ± SEM in Table 1. Correlations of study variables with folate intake or plasma folate within individuals were examined by calculation of Pearson product-moment coefficients, or Spearman coefficients if data were not normally distributed. An ANOVA model with repeated measures was performed to test for any effects of dietary folate intake on plasma folate, homocysteine and DNA methyl acceptance. If significant relationships were found, paired *t* statistics or the Wilcoxon signed-rank statistic (data not normally distributed) was used to determine differences between baseline, folate repletion and depletion periods (Glantz 1992). The statistical analysis was conducted using SAS version 6.12 (SAS Institute, Cary NC) and SigmaStat version 1.02 (Jandel Scientific Software, San Rafael CA) statistical software programs. Differences were considered significant at $P < 0.05$.

RESULTS

Of the total group of 10 subjects, data from two subjects were excluded from the analyses because of abnormal values related to the Hcy pathway. One subject began the study with an abnormally high plasma Hcy value, 19 μmol/L at d 5 compared with 8–11 μmol/L for the rest of the group (2.6 SD above the mean of the entire group of 10). The second excluded subject developed a mild anemia of unknown origin at d 56 and was then supplemented with 325 mg/d of ferrous sulfate daily for the remainder of the study. After this, the subject developed greatly elevated plasma Hcy levels, i.e., 31 μmol/L at d 84 vs. 10–15 μmol/L for the rest of the group.

Blood folate and vitamin B-12. Repeated-measures ANOVA using values at the end of folate intake periods showed a strong relation between plasma folate concentrations and dietary folate intakes ($P < 0.001$). Plasma folate decreased significantly during the 5-wk depletion period when subjects consumed the low folate diet (56 μg/d) with no folic acid supplementation (Fig. 1 and Table 1). After the 5-wk folate depletion period, a total folate intake of 111 μg/d was not sufficient to raise plasma folate above the low levels reached during depletion (at d 63, five of the eight subjects had plasma folate values below normal, <6.8 nmol/L). Subsequent intake of 286 μg/d was sufficient to raise plasma folate concentrations in all subjects. Plasma folate continued to rise with folate intake of 516 μg/d, and the group mean was not significantly different than baseline at the end of the study, d 92.

Red cell folate concentrations did not change with dietary folate intake as did plasma folate, but there was a significant fall from d 56 to 84, probably due to the low folate intakes over the preceding 6 wk (Fig. 2). However, individual red cell folate concentrations remained in the normal range of 317–1422 nmol/L (Tietz 1995) except for one subject who started and remained low (239–253 nmol/L). Folate concentrations of the young red cells were always significantly higher than those of the old red cell fraction; however, they were no more

reflective of changes in dietary folate intake than were the total or old red cell fractions.

Plasma vitamin B-12 concentrations declined significantly from baseline to d 27 (317 ± 45 to 275 ± 38 pmol/L) and remained the same from d 27 to the end of the study (291 ± 34 pmol/L). However, individual vitamin B-12 concentrations did not decline appreciably and all remained in the normal range of 148–616 pmol/L (Tietz 1995) throughout.

Plasma homocysteine. All subjects started in the normal range and increased above baseline by d 56; at that point, five of the eight subjects were above the upper limit of normal for this population, 12 μmol/L (Rasmussen et al. 1996), with a mean increase of 3.3 μmol/L. The ANOVA showed a significant inverse relation between Hcy and folate intake ($P = 0.001$), and the Hcy increases from baseline were significant beginning at d 27 ($P < 0.02$ by paired t test).

The elevated plasma Hcy concentrations did not decrease significantly until the last period when 516 μg/d of dietary folate was given. During this period, the Hcy values of seven of the eight subjects decreased, and the values of all four who were above 12 μmol/L decreased. At d 92, however, the group mean value was still significantly increased from baseline. Plasma folate and Hcy concentrations were not significantly different throughout the study in women receiving estrogen replacement therapy compared with those who were not.

Hematologic measures. As a group, hematologic indices of the subjects did not change significantly throughout the study. From beginning to end, hemoglobin decreased slightly, from 129 ± 3 to 123 ± 2 g/L [normal = 117–160 g/L (Tietz et al. 1995)], hematocrit from 0.37 ± 0.01 to 0.36 ± 0.01 volume fraction (0.35–0.47 volume fraction), and MCV was unchanged at 89 ± 2 fL (81–101 fL). Individual values for these hematologic measures remained within or close to the normal range throughout. The nuclear lobe average of neutrophils increased significantly during folate depletion from d 6 to 42 (2.13 ± 0.09 to 2.37 ± 0.09 lobes/cell) and remained unchanged to the end of the study (2.44 ± 0.08 lobes/cell). All individual lobe averages were < 3, well below the value of 3.5 indicating folate deficiency (Herbert and Das 1994).

Lymphocyte DNA methylation and deoxynucleotide concentrations. The group means for the four time points at which the lymphocyte DNA methyl acceptance assay was performed are shown in Figure 1 and Table 1. The DNA methyl acceptance shown is inversely related to the degree of DNA methylation. Genome-wide DNA hypomethylation was inversely related to dietary folate (ANOVA, $P < 0.001$) and also inversely related to plasma folate as seen in Figure 1. All subjects except one (whose DNA methyl uptake did not change throughout) showed DNA methyl acceptance peak values at d 69 after a folate intake of 111 μg/d, and subsequent decreases with folate repletion of 286 to 516 μg/d.

Mean values for the remainder of the lymphocyte measures are shown in Table 1. Cellular NAD concentrations, DNA strand breaks and [^{3}H]-deoxyuridine uptake decreased significantly at d 20, 41 or 69 compared with baseline and remained unchanged thereafter. Relative to the baseline, the ratio of uridylate to thymidylate, dUTP/dTTP, increased significantly at d 69, coincident with the lowest plasma folate concentrations and did not change thereafter. Patterns for deoxynucleotides were the same whether cells were cultured in folic acid–supplemented or low folate medium. No significant changes were observed in the concentrations of the other deoxynucleotides.

MDA and creatinine measures. Group means for urinary MDA determined in urine pools collected for 7 d are given in Table 1. Values for all subjects increased at d 67 (midpoint of 7-d pool) compared with baseline and decreased from d 67 to 88 after folate repletion. All subjects' values peaked at d 67, similar to the DNA methyl acceptance pattern.

Urine creatinine excretion, the major source of body methyl group loss, decreased significantly from d 25 to 39 (midpoints of 7-d urine pools), but did not show a clear relation with folate intake and did not change from beginning to end of the study (0.89 ± 0.03 to 0.86 ± 0.04 g/d). The average within-subject day-to-day variation (CV%) of urinary creatinine over the entire study was 16.7% and ranged from 14.1 to 18.8% among individuals. Plasma MDA decreased significantly from baseline to d 27 (0.45 ± 0.04 to 0.34 ± 0.03 μmol/L MDA equivalents) and remained essentially unchanged over the remainder of the study.

DISCUSSION

Folate status and requirement to maintain plasma folate concentrations. During 5 wk of folate intake at 56 μg/d followed by 4 wk at 111 μg/d, the subjects attained a state of moderate folate depletion characterized by low plasma folate and elevated Hcy. However, because red cell folate concentrations, hematologic indices and neutrophil segmentation remained normal throughout, no appreciable tissue folate depletion or folate-deficient hematopoiesis occurred. Although plasma folate and Hcy respond to changes in folate intake within weeks, tissue folate pools would not have reached steady state during the relatively short dietary treatment periods of this study (Stites et al. 1997). Hence, changes (or lack of change) in some folate-dependent measures taken in this study might have been different with longer dietary treatment periods.

The low plasma folate concentrations attained after folate intake of 56 μg/d were unchanged with subsequent intake of 111 μg/d and restored toward normal when the subjects received 286 μg/d of folate. This is consistent with the finding of Sauberlich et al. (1987) that 200–250 μg/d of food folate restored low plasma folate concentrations in folate-depleted premenopausal women.

Folate requirement to maintain plasma homocysteine concentrations. The observed inverse relation between plasma Hcy and folate intake in healthy postmenopausal women is consistent with similar relationships reported for healthy premenopausal women, adult men and the elderly (Jacob et al. 1994, O'Keefe et al. 1995, Selhub et al. 1993). Although all subjects followed the general inverse relation, the magnitude of the Hcy response to folate depletion was different among individuals. The Hcy values for three subjects changed only within the normal range, whereas five subjects showed mild elevations, between 12 and 18 μmol/L. These results are similar to those from a previous study of controlled folate intake in healthy men, which also showed clear inter-individual differences of Hcy response to folate depletion (Jacob et al. 1994). This indicates that even under precisely controlled nutriture, a variety of genetic and environmental factors may affect circulating Hcy concentrations.

The folate requirement to maintain low plasma Hcy concentrations is important because high Hcy concentrations have been independently associated with increased risk of vascular disease (Boushey et al. 1995). The lack of decline in the elevated Hcy values at d 70–83 with folate intake of >286 μg/d (Fig. 1) suggests that >286 μg/d of folate was required to lower the elevated Hcy in these women. The 14-d period of 286+ μg/d folate intake during d 70–83 should have been enough time for plasma Hcy to at least begin to decline because the high values declined significantly within 8 d in the final

period when 516 μg/d was fed, and within 9 d when men in a previous study were repleted with 440 μg/d (Jacob et al. 1994). The Hcy concentrations did not return to baseline, possibly because the 8-d final repletion period was not long enough to see additional decline. The question arises why 286+ μg/d did not lower elevated plasma Hcy when all women started with baseline Hcy levels <12 μmol/L, and their mean free-living folate intake could be estimated as 272 μg/d from NHANES II food records of white women age 51–65 y (Subar et al. 1989). The difference may be due to the low choline content of the experimental diet, because choline can methylate Hcy via betaine (Selhub and Rosenberg 1996). By comparison, O'Keefe et al. (1995) reported that elevated Hcy levels (>16 μmol/L) were observed in premenopausal women ingesting 200 μg/d but not 300 or 400 μg/d of folate. Taken together, data from these two studies suggest that, for some women, folate intake of 300 μg/d or more is required to maintain Hcy levels <12 μmol/L.

DNA methylation and deoxynucleotide content. The increased DNA methyl acceptance seen at d 69 (Fig. 1, Table 1), a point of low plasma folate and elevated Hcy, indicates that folate depletion resulted in genome-wide DNA hypomethylation, which was reversible within 3 wk by folate repletion at 286–516 μg/d. Despite the well-known role of folate as a supplier of one-carbon units for in vivo methylation reactions, no previous studies have shown that folate intake affects DNA methylation in humans. Our finding of DNA hypomethylation is similar to that of Pogribny et al. (1995), who found that chronic severe folate/methyl deficiency in rats induced genome-wide and p53 gene–specific hypomethylation in preneoplastic liver, along with an increase in DNA strand breaks. In other studies of folate deficiency in rats, Balaghi and Wagner (1993) found genomic hypomethylation of hepatic DNA after 4 wk of folate deficiency, Kim and Christman (1995) found no hypomethylation of hepatic and colonic DNA during moderate folate deficiency and Kim et al. (1997) found hypomethylation of the p53 tumor suppressor gene (but not genome-wide hypomethylation) and DNA strand breaks due to isolated folate deficiency. Although genome-wide DNA hypomethylation was observed in human lymphocytes in this study, DNA strand breaks were found to be decreased, not increased, with short-term moderate folate deprivation.

In our previous study of moderate folate depletion of healthy men, we found no methylation deficit during low folate, methionine and choline intakes (Jacob et al. 1995). The different results may be due to an increased sensitivity of the DNA methyl acceptance assay compared with the metabolic methylation tests used in the previous study (measuring methylated niacin metabolites after an oral nicotinamide dose and urinary excretion of the methyl-rich compounds creatinine and carnitine), or to an increased sensitivity of the methylation pathways to folate deficiency in the postmenopausal women compared with the younger men. Although the methionine intake of the women was adequate (780 mg/d), the relatively low amount of choline in the diet (147 mg/d) may have contributed to the sensitivity of the observed inverse relationship between folate intake and DNA hypomethylation.

DNA hypomethylation may explain some of the reported associations between folate deficiency, increased chromosomal damage and preneoplastic lesions in epithelial tissue (Blount et al. 1997, Glynn and Albanes 1994, MacGregor et al. 1997). Recently, Fang et al. (1997) reported genomic DNA hypomethylation of gastric cancer compared with normal tissue, and an association between hypomethylation and low serum folate concentrations in the cancer patients.

Folate is required for the methylation of uracil to thymidine in the course of DNA synthesis and repair. The observed increase in the lymphocyte DNA dUTP/dTTP ratio and decrease in ^{3}H-deoxyuridine uptake at d 69, a point of low plasma folate (Table 1), are consistent with a block in folate-dependent de novo conversion of uracil to thymidine and misincorporation of uracil into DNA. The latter is hypothesized to result in increased repair-related DNA damage and subsequent chromosomal instability (Blount et al. 1997, MacGregor et al. 1997). Our results are similar to the findings of Blount et al. (1997), who reported that elevated incorporation of uracil into DNA in folate-deficient individuals was reversed by supplementation with folate. However, the dUTP/dTTP ratio in our subjects did not decrease upon folate repletion. This may be because of the much shorter and smaller folate repletion of our study (20 d of 286–516 μg/d) compared with that of Blount et al. (6–8 wk of 5 mg/d).

An increase in DNA repair activity due to misincorporation of uracil may explain the significant drop in lymphocyte NAD concentrations from baseline, because NAD is the substrate for the nuclear DNA repair enzyme, poly(ADP-ribose) polymerase (PARP). James and Yin (1989) found that lowered NAD and de novo thymidylate synthesis accompanied increased DNA strand breaks in spleen cells of folate and folate-methyl-donor–deficient rats. Henning et al. (1997) recently showed that in liver from male rats fed methyl- and folate-deficient diets with or without niacin, PARP activity was altered and NAD concentrations significantly decreased. The decline in lymphocyte NAD observed in this study occurred despite a niacin intake of 29.7 mg of niacin equivalents per day, 214% of the current RDA for adult women (NRC 1989).

Misincorporation of uracil into DNA with chronic folate deficiency is expected to stress mechanisms of DNA repair and thus result in subsequent increases in DNA strand breaks and chromosomal instability. However, we found that short-term moderate folate deficiency was associated with an early decline in DNA strand breaks and no further change throughout the study. The decrease in DNA strand breaks with folate deprivation may reflect upregulation of DNA repair activity and is supported by the parallel decrease in lymphocyte NAD concentrations. Alternatively, reduced DNA strand breaks may reflect a cohort of surviving cells after early apoptotic elimination of DNA damaged cells. An early increase in apoptotic cell death has been previously observed in folate-deficient Chinese hamster ovary cells in vitro (James et al. 1994a) and also in liver of rats fed chronically a folate/methyl-deficient diet (James et al. 1997). In the latter study, increased cell apoptosis was accompanied by elevated liver PARP and a significant reduction in NAD concentrations. The decline from baseline in [^{3}H]deoxyuridine uptake in the mitogen-stimulated lymphocytes (Table 1) indicates a decreased ability of the cells to proliferate in vitro upon ingestion of the experimental diet and follows the same pattern as NAD concentrations and DNA strand breaks, in that short-term folate repletion did not reverse the decline.

Folate status and lipid peroxidation. The observed inverse relation of urinary MDA equivalents, a lipid peroxidation measure, to folate intake is very similar to the pattern of plasma Hcy and lymphocyte DNA methyl uptake (Table 1). Increased lipid peroxidation during folate deficiency may be a consequence of increased circulating Hcy, which has been postulated to act as a pro-oxidant. Olszewski and McCully (1993) suggested that high levels of Hcy may promote oxidative damage because the sulfhydryl group of Hcy acts catalytically with ferric or cupric ions to generate hydrogen peroxide, oxygen radicals and homocysteinyl radicals. Sparrow and Olszewski

(1993) showed that LDL oxidation can occur by the interaction of sulfhydryl groups from cells with transition metal ions, and Jones et al. (1994) reported that lipid peroxidation accompanied the toxicity of Hcy to endothelial cells in culture. However, Dudman et al. (1993) found that HDL cholesterol ester hydroperoxides were not elevated in four patients with hyperhomocysteinemia and concluded that Hcy does not impose significant oxidant stress. Recently, folate deficiency in rats was reported to increase plasma Hcy and lipid peroxidation products along with a decrease in plasma and platelet fatty acid unsaturation (Durand et al. 1996). Although no relation of plasma MDA to folate intake was found, our urinary MDA results are consistent with previous work suggesting a lipid pro-oxidant effect of elevated Hcy concentrations.

In conclusion, marginal folate deficiency induced in healthy postmenopausal women resulted in elevated plasma Hcy and lymphocyte DNA hypomethylation, conditions that are associated with increased risks of developing vascular disease, DNA and chromosome damage, and some preneoplastic lesions. Although the DNA hypomethylation was reversed with 286–516 μg/d of folate intake, the elevated Hcy decreased with 516 but not 286 μg/d of folate. This suggests that the current RDA of 180 μg/d may not be sufficient to maintain low plasma Hcy concentrations of some postmenopausal women on diets similar to that fed in this experiment.

Analysis of 24-h food intake recalls from NHANES II showed that 42% of adult women ingested $<$200 μg/d of folate on a given day; 18% of white and 26% of African-American women consumed $<$100 μg/d (Subar et al. 1989). Using the latter figures, increased folic acid consumption estimated at $\sim$100 μg/d as a result of pending fortification of enriched cereal-grain products (USDHHS 1996) would still leave about 15% of U.S. women consuming less than the current RDA. In 310 women, age 67–74 y, of the Framingham Heart Study, 20% had elevated plasma Hcy, which was strongly associated with low folate status in the overall population studied (Selhub et al. 1993). Therefore, the present findings may be relevant to an appreciable segment of postmenopausal U.S. women with low dietary folate intakes. These results underscore the need for further research on the molecular and functional consequences of inadequate folate nutriture.

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