Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999–2000

Christine M Pfeiffer, Samuel P Caudill, Elaine W Gunter, John Osterloh, and Eric J Sampson

ABSTRACT
Background: Mandatory folic acid fortification of cereal-grain products was introduced in the United States in 1998 to decrease the risk that women will have children with neural tube defects.

Objective: The objective was to determine the effect of folic acid fortification on concentrations of serum and red blood cell (RBC) folate, serum vitamin B-12, and plasma total homocysteine (tHcy) and methylmalonic acid (MMA) in the US population.

Design: Blood was collected from a nationally representative sample of ~7300 participants aged ≥3 y in the National Health and Nutrition Examination Survey (NHANES) during 1999–2000 and was analyzed for these B vitamin–status indicators. The results were compared with findings from the prefortification survey NHANES III (1988–1994).

Results: The reference ranges (5th–95th percentiles) were 13.1–74.3 nmol/L for serum folate, 347–1167 nmol/L for RBC folate, and 179–738 pmol/L for serum vitamin B-12. For plasma tHcy and MMA, the reference ranges for serum vitamin B-12–replete participants with normal serum creatinine concentrations were 3.2–10.7 μmol/L and 60–210 nmol/L, respectively. The prevalence of low serum folate concentrations (<6.8 nmol/L) decreased from 16% before to 0.5% after fortification. In elderly persons, the prevalence of high serum folate concentrations (>45.3 nmol/L) increased from 7% before to 38% after fortification; 3% had marginally low serum vitamin B-12 concentrations (<148 pmol/L) and 7% had elevated plasma MMA concentrations (>370 nmol/L). Seventy-eight percent of the US population had plasma tHcy concentrations <9 μmol/L.


KEY WORDS Nutrition survey, age, sex, race, ethnic groups, National Health and Nutrition Examination Survey, NHANES

INTRODUCTION
Folates act as one-carbon donors in the synthesis of the building blocks of DNA, thymidylate, and purines and of certain neurotransmitters, phospholipids, and hormones (1). Vitamin B-12, commonly referred to as cyanocobalamin, is required as a cofactor for 2 enzymes: methionine synthase, which catalyzes the conversion of homocysteine to methionine using 5-methyltetrahydrofolate as a methyl group donor, and l-methyl-malonyl-CoA mutase, which catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA (2). Although low serum vitamin B-12 concentrations are a sensitive indicator of vitamin B-12 deficiency and high vitamin B-12 concentrations generally indicate sufficiency, the risk of vitamin B-12 deficiency associated with an intermediate range of vitamin B-12 concentrations is unclear. Plasma methylmalonic acid (MMA) is a useful confirmatory diagnostic test in persons with a low or low normal serum vitamin B-12 concentration (3, 4). Plasma total homocysteine (tHcy) concentrations can be elevated because of low folate, vitamin B-12, or vitamin B-6 intakes or because of renal insufficiency, methylenetetrahydrofolate reductase polymorphism, or the use of certain medications. The plasma MMA concentration can be elevated as a result of renal insufficiency.

Folate deficiency is already an established risk factor for the development of certain types of cancer in the general population (5). A chronic deficiency of folate in the diet can cause anemia (1), but low concentrations of serum folate and vitamin B-12 and elevated concentrations of plasma tHcy have also been associated with psychiatric disorders (6); the development of dementia, Alzheimer disease, and cognitive dysfunction (7–10); a decline in physical function (11); osteoporosis and hip fractures in the elderly (12, 13); and an increased risk of carotid artery stenosis (14). Two recent meta-analyses concluded that a 25% reduction of plasma tHcy is associated with an 11–16% decrease in risk of ischemic heart disease and a 19–22% decrease in risk of stroke (15, 16). Clinical trials have shown that folic acid supplementation decreases the risk of neural tube birth defects (17, 18). In 1998, the Food and Drug Administration required the fortification of enriched cereal-grain products with folic acid at a concentration of 140 μg/100 g cereal grain (19).

Although severe vitamin B-12 deficiency causes anemia, hematologic signs are not always present, and hematologic and neurologic abnormalities are inversely correlated in vitamin B-12 deficiency (4). Some evidence suggests that excess supplemental folate intake may precipitate or exacerbate the neurologic damage of vitamin B-12 deficiency; a summary of such human...

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case reports has been compiled by the US Institute of Medicine (20). Because elderly people have lower serum vitamin B-12 concentrations than do younger people (21–23), they may be particularly susceptible to the most serious sequelae because consequences may be irreversible when vitamin B-12 deficiency goes untreated.

Since 1998, a few studies have shown significant improvements in folate status in selected populations (24–27). The National Health and Nutrition Examination Survey (NHANES) 1999–2000 is the first and only source of nationally representative data on the B vitamins and their biochemically related products after the introduction of folic acid fortification. This report describes the concentrations of and presents the reference data for serum and red blood cell (RBC) folate, serum vitamin B-12, and plasma tHcy and MMA in the US population aged ≥3 y. The changes in B vitamin concentrations between the prefortification and postfortification periods are discussed.

SUBJECTS AND METHODS
Survey design and subjects

NHANES, conducted by the National Center for Health Statistics (NCHS) at the Centers for Disease Control and Prevention (CDC), is a series of nationally representative cross-sectional examination surveys that uses a stratified, multistage design to provide a representative probability sample of the civilian noninstitutionalized population in the United States. The NHANES program began in 1960 and is designed to provide periodic information about the health and nutritional status of the US population. NHANES III, conducted from 1988 to 1994, was the last survey that assessed the B vitamin status of the US population before the introduction of folic acid fortification. It was designed as two 3-y phases, each constituting a national probability sample in which there was an oversampling of Mexican American and non-Hispanic black persons, all children aged 2 mo through 5 y, and all persons aged ≥60 y (28). Blood for B vitamin measurements was collected from participants ≥4 y of age.

In 1999 NHANES became a continuous survey (29, 30). Although each year constitutes a national probability sample, ≥2 y of data are necessary to have adequate sample sizes for subgroup analyses. The procedures for selecting participants and for conducting interviews and examinations in NHANES 1999–2000 were similar to those used in NHANES III. The NHANES 1999–2000 survey sample comprised 9965 participants. Of these, 93% were interviewed in their homes and underwent physical examination in mobile examination centers. Mexican American and non-Hispanic black persons, all adolescents (ages 12–19 y), and all persons aged ≥60 y were oversampled to improve estimates in these groups. All respondents gave their informed consent, and the NHANES 1999–2000 protocol was reviewed and approved by the NCHS NHANES Institutional Review Board. Blood for B vitamin measurements was collected from participants aged ≥3 y.

Fasting status and supplement use

To assess the extent to which blood concentrations of folate and vitamin B-12 are affected after a meal is eaten, the NHANES 1999–2000 data were analyzed by fasting status. The respondents were asked to fast for either 10–16 h or for 6 h, depending on whether their appointment at the mobile examination center was in the morning or in the afternoon or evening. Before the phlebotomy was performed, the respondents were asked when they last ate or drank and the number of hours that they had fasted was calculated. Fasting status was categorized as fasting ≥9 h or <9 h (this category included those who reported having just eaten). The respondents were asked separately about their use of vitamin or mineral supplements in the past 24 h. Therefore, when analyzing fasting status, use of supplements in the past 24 h was controlled for by selecting only those persons who reported not taking a supplement. Approximately one-half of the participants (54.3%) had fasted ≥9 h before phlebotomy. These 2 fasting categories had no measurable effect on serum or RBC folate, serum vitamin B-12, or plasma tHcy and MMA concentrations. All further data analyses were conducted without the exclusion of supplement users or participants who fasted <9 h before phlebotomy.

Biochemical measurement of serum and RBC folate, serum vitamin B-12, and plasma tHcy and MMA concentrations

Depending on the age of the participant, data were collected on demographics, physical function, health condition, lifestyle behaviors, biochemical measurements of blood and urine, body measurements, and dietary intake. Blood was collected by venipuncture in mobile examination centers and processed under controlled, constant environmental conditions according to standard protocols (31).

The NHANES Laboratory of the CDC analyzed serum and RBC folate for both phases of NHANES III, serum vitamin B-12 for phase II of NHANES III (1991–1994), and all 5 indicators for NHANES 1999–2000 (32). Serum and RBC folate and serum vitamin B-12 were analyzed by using a commercially available radioprotein binding assay kit (Quanaphase I during phase I of NHANES III and Quanaphase II during phase II of NHANES III and during NHANES 1999–2000; Bio-Rad Laboratories, Hercules, CA). The serum and RBC folate assay measures primarily 5-methyltetrahydrofolic acid and folic acid but is considered to estimate total folate in serum and whole blood. RBC folate was measured after lysis of 1 part of whole blood with 10 parts of 1% ascorbic acid solution (performed in the mobile examination center) and one freeze-thaw cycle to ensure cleavage of polyglutamate folates to monoglutamates by the action of the endogenous plasma deconjugase. The serum vitamin B-12 assay measures cyanocobalamin in its circulating bound and free forms. Long-term CVs for the NHANES 1999–2000 period were 4–7% for serum folate at 5.2–30.0 nmol/L, 4–6% for RBC folate at 143–1119 nmol/L, and 3–6% for serum vitamin B-12 at 281–1160 pmol/L.

Measurements of tHcy and MMA changed from NHANES III to NHANES 1999–2000 because different laboratories conducted the analyses, and different matrices and methods were used. Because optimally prepared EDTA-treated plasma was not available during NHANES III, tHcy and MMA were analyzed in surplus serum from phase II of NHANES III (1991–1994) at the US Department of Agriculture (USDA) Human Nutrition Research Center on Aging. tHcy was measured by HPLC with fluorometric detection (33); MMA was measured by gas chromatography–mass spectrometry by using solid-phase extraction and derivatization with cyclohexanol (34) for persons ≥60 y of age and for a subset of adults 30–39 y of age. For NHANES 1999–2000, EDTA-treated whole blood was processed within 30 min of collection to avoid an artificial increase in tHcy, and
plasma was immediately frozen at −70 °C. Plasma tHcy was analyzed by using a commercially available fluorescence polarization immunoassay kit (Abbott Laboratories, Abbott Park, IL) on the Abbott IMx analyzer (35). Plasma MMA was measured by gas chromatography–mass spectrometry with cyclohexanol derivatization (36). A 10% subset of all samples was also analyzed for plasma tHcy by HPLC with fluorometric detection and cysteamine as internal standard (37, 38). Both assays measure tHcy in plasma, which is the sum of reduced, oxidized, and protein-bound homocysteine. The comparison of the 2 tHcy methods for the NHANES 1999–2000 period gave the following results: Pearson’s correlation coefficient, 0.98; Deming regression (Abbott), 0.97 × HPLC − 0.2; absolute bias (95% confidence limit), −0.4 μmol/L (−0.4, −0.3); and relative bias, −5%. Long-term CVs for the 2-y period were 3–6% for plasma tHcy by Abbott at 6.7–29.0 μmol/L, 4–7% for plasma tHcy by HPLC at 6.7–29.5 μmol/L, and 4–11% for plasma MMA at 120–10400 nmol/L. A formal tHcy method comparison was conducted within the HPLC methods used at the CDC NHANES and the USDA laboratories, and the results were as follows: Pearson’s correlation coefficient, 0.97; Deming regression, USDA HPLC = 0.82 × CDC HPLC + 0.9; absolute bias (95% confidence limit), −0.6 μmol/L (−0.5, −0.8); relative bias, −6% (39). We also determined that under the conditions applied to sample processing during NHANES III, tHcy in serum was overestimated compared with tHcy in optimally prepared EDTA-treated plasma by an average of 10% (39). Because of changes in matrix and methods, a direct comparison of the tHcy results obtained with the 2 surveys is inappropriate (39). No formal MMA method comparison was conducted between the gas chromatography–mass spectrometry methods used at the CDC NHANES and the USDA laboratory, which precludes direct comparison of the MMA results between these 2 surveys.

Statistical analysis

The statistical analyses were performed with SAS for WINDOWS (version 8.0; SAS Institute Inc, Cary, NC) in conjunction with SUDAAN statistical software (version 8.0.2; 40). Data were weighted to account for survey design (unequity of selection, adjustments for oversampling of certain populations) and nonresponse. On the basis of questionnaire self-assignment, the participants were categorized into 3 racial-ethnic groups: Mexican Americans, non-Hispanic blacks, and non-Hispanic whites. No separate data analysis was performed for persons from other ethnic groups (n = 876). Because of significant metabolic changes in B vitamins and their biochemically related products during pregnancy, we excluded pregnant women from all data analysis. Supplement users were not evaluated separately in our data analysis. In the comparison of estimates, only statistically significant differences at a significance level of α = 0.01 are reported. We consistently used this more stringent significance level rather than using α = 0.05 and adjusting the level based on the number of comparisons made; the maximum number of groups that we compared was 5.

This report presents population means, SEMs, and selected percentiles for serum and RBC folate, serum vitamin B-12, and plasma tHcy and MMA that were representative of the noninstitutionalized civilian US population for NHANES 1999–2000. As recommended by NCHS, a Taylor series variance estimation method appropriate for complex survey data was used to estimate SEMs (29). Because the distributions of these biomarkers were skewed, logarithmic transformations of the distributions were used for the statistical analyses. We age-adjusted means by using SUDAAN PROC DESCRIPT standardization statements and the following 2000 Census population proportions: 20–39 y, 0.3966; 40–59 y, 0.3718; and ≥60 y, 0.2316 (30). We tested for sex-by-race (Table 1), sex-by-age (Figure 1), and race-by-age interactions using an analysis of variance model that included age (6 age groups), sex (males and females), racial-ethnic group (Mexican Americans, non-Hispanic blacks, and non-Hispanic whites) and the abovementioned interaction terms. If the interaction term was not significant (P > 0.01) we only reported the means by subgroup without performing subgroup analysis to test for significant differences. We tested for main effects of sex, race, and age using the same analysis of variance model as for the interaction testing. We tested for significant differences between males and females or between racial-ethnic groups by using census age-adjusted geometric means and a 2-tailed, 2-group t test with 14 df (Table 1). We tested for significant differences between age groups (using age group 20–39 y as a reference) or between males and females (within one age group) by using geometric means and a 2-tailed 2-group t test with 14 df in a model that included age, sex, race, and sex-by-age interaction (Figure 1). The average design effect, which is the ratio of the complex sampling design variance derived from SUDAAN to the simple random sample variance calculated by SAS, averaged over age categories, was used to determine the recommended minimum stratum sample size according to the NCHS analytic guidelines and recommendations to achieve stable estimates of means and percentiles (28). On the basis of an average design effect of approximately 1.4 for our sample, strata with <220 individuals gave imprecise estimates of the 5th and 95th percentiles.

We used the 5th and 95th percentiles of the entire population, except for pregnant women, to estimate the population reference ranges for serum and RBC folate and serum vitamin B-12. To establish population reference ranges (5th and 95th percentiles) for plasma tHcy and MMA, we created a reference sample in which we included only persons who were vitamin B-12 replete (serum vitamin B-12 concentrations above the 50th percentile; 41) and excluded persons with serum creatinine concentrations indicative of impaired renal function (ie, >133 μmol/L for men and >115 μmol/L for women)—the principal cause of elevated plasma tHcy and MMA concentrations for reasons other than suboptimal B vitamin status. Serum and RBC folate concentrations were not considered exclusionary for preparing the tHcy and MMA reference sample because the entire population was considered to be folate replete (serum folate concentration >6.8 nmol/L).

To assess the effect of fortification on blood folate and vitamin B-12 concentrations, we compared NHANES III data with NHANES 1999–2000 data. We applied the same procedure as described above to calculate age-adjusted means, but, as recommended by the NCHS (30), we used different proportions derived from the 2000 census population: 20–39 y, 0.4332; 40–59 y, 0.4062; and ≥60 y, 0.1606. We tested for significant differences between prefortification and postfortification values for various subgroups by using age-specific or age-adjusted geometric means and a 2-tailed, 2-group t test. To compare the prevalence estimates from NHANES III with those from NHANES 1999–2000, we used the SE and df associated with each estimate to
TABLE 1
Age-adjusted geometric mean concentrations (and 95% CIs) of serum and red blood cell (RBC) folate, serum vitamin B-12, plasma total homocysteine (tHcy), and methylmalonic acid (MMA) by sex and racial-ethnic group: United States, 1999–2000.

<table>
<thead>
<tr>
<th>Variable and sex</th>
<th>Subject group</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td>All</td>
<td>Males</td>
</tr>
<tr>
<td>All</td>
<td>30.5 (28.9, 32.3) [3912]</td>
<td>27.9 (26.6, 29.3) [1060]</td>
</tr>
<tr>
<td>Mexican Americans</td>
<td>29.0 (27.5, 30.7) [1947]</td>
<td>27.0 (25.3, 28.7) [517]</td>
</tr>
<tr>
<td>Non-Hispanic blacks</td>
<td>32.0 (30.3, 34.2) [1965]</td>
<td>28.9 (27.8, 30.1) [543]</td>
</tr>
<tr>
<td>Non-Hispanic whites</td>
<td>646 (618, 676) [3955]</td>
<td>596 (575, 617) [3966]</td>
</tr>
<tr>
<td>All</td>
<td>340 (335, 345) [3911]</td>
<td>383 (364, 403) [1060]</td>
</tr>
<tr>
<td>All</td>
<td>335 (328, 343) [1946]</td>
<td>355 (341, 368) [517]</td>
</tr>
<tr>
<td>All</td>
<td>344 (338, 350) [1965]</td>
<td>418 (386, 453) [543]</td>
</tr>
<tr>
<td>All</td>
<td>7.7 (7.6, 7.9) [3946]</td>
<td>7.3 (7.0, 7.6) [1072]</td>
</tr>
<tr>
<td>All</td>
<td>8.5 (8.3, 8.7) [1959]</td>
<td>8.0 (7.7, 8.4) [522]</td>
</tr>
<tr>
<td>All</td>
<td>7.1 (6.9, 7.3) [1987]</td>
<td>6.6 (6.3, 6.9) [550]</td>
</tr>
<tr>
<td>All</td>
<td>137 (134, 140) [3945]</td>
<td>119 (114, 124) [1071]</td>
</tr>
<tr>
<td>All</td>
<td>141 (136, 146) [1958]</td>
<td>126 (117, 135) [521]</td>
</tr>
<tr>
<td>All</td>
<td>133 (130, 137) [1987]</td>
<td>112 (107, 116) [550]</td>
</tr>
</tbody>
</table>

¹ Age-adjusted geometric means are based on 2000 census data for adults aged ≥20 y. n in brackets.
² ANOVA (2 × 3) with 2-tailed, 2-group t test.
³ Significantly different from non-Hispanic blacks, P < 0.01.
⁴ Significantly different from non-Hispanic whites, P < 0.01.
⁵ Significantly different from Mexican Americans, P < 0.01.
⁶ Main effect of sex could not be determined because of a significant sex-by-age interaction.
⁷ Main effect of sex could not be determined because of a significant sex-by-race interaction.
⁸ Main effect of sex or race could not be determined because of a significant sex-by-race interaction.

RESULTS

Descriptive statistics for B vitamins and their biochemically related products for NHANES 1999–2000

Serum and RBC folate, serum vitamin B-12, and plasma tHcy and MMA concentrations were measured for 7235, 7321, 7233, 7306, and 7304 participants aged ≥3 y, respectively. The study population was 51% male and included ≈5% children aged 3–5 y, 41% children and adolescents (6–19 y), 34% adults (20–59 y), and 21% elderly persons (≥60 y), which reflected the oversampling in the survey as described earlier.

To allow a comparison with previous reports (23, 41-44), detailed tables containing age-specific means and distributions of B vitamin and metabolite concentrations for participants by sex are provided online; see Tables S1-S4 under “Supplemental data” in the current online issue at www.ajcn.org. Table S1 contains data for all racial-ethnic groups combined, whereas Tables S2-S4 contain data for individual racial-ethnic groups (Mexican Americans, non-Hispanic blacks, and non-Hispanic whites).

The reference ranges (5th–95th percentiles) for the B vitamins for the US population after the introduction of folic acid fortification were 13.1–74.3 nmol/L for serum folate, 347–1167 nmol/L for RBC folate, and 179–738 pmol/L for serum vitamin B-12. The reference ranges for the metabolites for the part of the US population that was vitamin B-12 replete and did not exhibit elevated serum creatinine concentrations were 3.2–10.7 µmol/L for plasma tHcy and 60–210 nmol/L for MMA.

The age-adjusted geometric mean concentrations for adults aged ≥20 y were 30.5 nmol/L for serum folate, 646 nmol/L for RBC folate, 340 pmol/L for serum vitamin B-12, 7.7 µmol/L for plasma tHcy, and 137 nmol/L for MMA (Table 1). We found a significant sex-by-race interaction for serum vitamin B-12 but not for serum and RBC folate (P = 0.345 and P = 0.699, respectively) or plasma tHcy and MMA (P = 0.228 and P = 0.137, respectively). We found a significant main effect of sex for plasma MMA, and significant main effects of race for serum and RBC folate and for plasma tHcy and MMA. On the basis of census age-adjusted data, males had lower serum folate and higher plasma tHcy and MMA concentrations than did females, but RBC folate concentrations were not significantly different between the sexes (P = 0.043). Non-Hispanic whites had higher serum and RBC folate concentrations than did non-Hispanic blacks and Mexican Americans. Mexican Americans had lower plasma tHcy concentrations than did non-Hispanic whites and non-Hispanic blacks, but the difference between non-Hispanic whites and non-Hispanic blacks was not significant (P = 0.088). Non-Hispanic whites had higher plasma MMA concentrations than did non-Hispanic blacks and Mexican Americans, but the difference between non-Hispanic blacks and Mexican Americans was not significant (P = 0.444). The lack of a significant sex-by-race interaction for serum and RBC folate and plasma tHcy and MMA precluded further subgroup analysis. The presence of a significant sex-by-race interaction for serum vitamin...
B-12 precluded main-effect comparisons between males and females and between racial-ethnic groups. However, a subgroup analysis showed that non-Hispanic white males had lower serum vitamin B-12 concentrations than did non-Hispanic black and Mexican American males, but the difference between non-Hispanic black and Mexican American males was not significant ($P = 0.026$). The same pattern applied for females ($P = 0.597$).

We found a significant sex-by-age interaction for serum folate and plasma tHcy but not for RBC folate ($P = 0.021$), serum vitamin B-12 ($P = 0.020$), or plasma MMA ($P = 0.026$). We found a significant main effect of sex for plasma MMA but not for RBC folate ($P = 0.036$). The main effect of sex on serum folate and plasma tHcy could not be evaluated because of the significant sex-by-age interaction; that on serum vitamin B-12 could not be evaluated because of the significant sex-by-race interaction. A significant main effect of age was found for RBC folate, serum vitamin B-12, and plasma MMA. The main effect of age on serum folate and plasma tHcy could not be evaluated because of a significant sex-by-age interaction. If a sex-by-age interaction was found, a subgroup analysis was performed with a 2-tailed, 2-group $t$ test with 14 df. The solid black squares within each bar indicate significant differences between that age group and the reference age group (20–39 y) for that sex ($P < 0.01$; data controlled for race-ethnicity).

*Significant difference between males and females within each age group, $P < 0.01$ (data controlled for race-ethnicity).

**FIGURE 1.** Age-specific geometric mean concentrations of serum and red blood cell (RBC) folate, serum vitamin B-12, and plasma total homocysteine (tHcy) and methylmalonic acid (MMA) for males (■) and females (□) aged ≥3 y in the United States (1999–2000). Error bars represent 95% CIs. Each bar represents a minimum of 164 subjects and a maximum of 1081 subjects. For the exact number of subjects represented by each bar, see Table S1 under “Supplemental data” in the current online issue at www.ajcn.org. We found significant sex-by-age interactions for serum folate and plasma tHcy but not for RBC folate ($P = 0.021$), serum vitamin B-12 ($P = 0.020$), or plasma MMA ($P = 0.026$). We found a significant main effect of sex for plasma MMA but not for RBC folate ($P = 0.036$). The main effect of sex on serum folate and plasma tHcy could not be evaluated because of the significant sex-by-age interaction; that on serum vitamin B-12 could not be evaluated because of the significant sex-by-race interaction. A significant main effect of age was found for RBC folate, serum vitamin B-12, and plasma MMA. The main effect of age on serum folate and plasma tHcy could not be evaluated because of a significant sex-by-age interaction. If a sex-by-age interaction was found, a subgroup analysis was performed with a 2-tailed, 2-group $t$ test with 14 df. The solid black squares within each bar indicate significant differences between that age group and the reference age group (20–39 y) for that sex ($P < 0.01$; data controlled for race-ethnicity).

*Significant difference between males and females within each age group, $P < 0.01$ (data controlled for race-ethnicity).
for all age groups ≥12–19 y. Plasma MMA concentrations tended to be higher in elderly people (Figure 1).

Comparison between NHANES III and NHANES 1999–2000 for serum and RBC folate and serum vitamin B-12

From NHANES III to NHANES 1999–2000, the US population shifted to much higher serum folate [median (95% CI); from 12.5 (11.8, 12.9) to 32.2 (30.1, 33.8) nmol/L] and RBC folate [from 392 (381, 406) to 625 (600, 650) nmol/L] concentrations and to slightly higher serum vitamin B-12 concentrations [344 (336, 350) and 359 (352, 367) pmol/L] (Figure 2). Serum and RBC folate concentrations showed large increases and serum vitamin B-12 concentrations showed slight increases in each sex and racial-ethnic subgroup from before to after fortification, except for vitamin B-12 in non-Hispanic blacks (P = 0.181; Table 2). Serum and RBC folate concentrations also showed large increases in each age group. Serum vitamin B-12 increased only in elderly persons (P = 0.574 for the ≤5 y age group, P = 0.124 for the 6–11 y age group, P = 0.288 for the 12–19 y age group, P = 0.185 for the 20–39 y age group, and P = 0.015 for the 40–59 y age group; Table 2).

The most commonly used cutoff for defining low serum folate concentrations is 6.8 nmol/L (45). The prevalence of low serum folate concentrations decreased from 16% in NHANES III to 0.5% in NHANES 1999–2000 for the US population and from 20% to 0.8% for women of childbearing age (12–49 y).

The Life Sciences Research Office panel defined low RBC folate concentrations as <317 nmol/L (46). The prevalence of low RBC folate concentrations decreased from 31% in NHANES III to 3% in NHANES 1999–2000 for the US population and from 38% to 5% for women of childbearing age. Although the overall prevalence of low RBC folate concentrations decreased significantly after the introduction of folic acid fortification, pronounced ethnic differences remained in folate status: 2% of Mexican American, 4% of non-Hispanic white, and 11% of non-Hispanic black women of childbearing age had RBC folate concentrations <317 nmol/L.

We arbitrarily defined high serum folate concentrations as >45.3 nmol/L, which reflected the upper end of the Bio-Rad Quantaphase II calibration range, beyond which samples need to

![Frequency distribution of serum and red blood cell (RBC) folate and serum vitamin B-12 for persons aged ≥3 y in the United States according to the National Health and Nutrition Examination Survey, 1999–2000 and for persons aged ≥4 y in the United States according to the third National Health and Nutrition Examination Survey, 1988–1994.](image)

**FIGURE 2.** Frequency distribution of serum and red blood cell (RBC) folate and serum vitamin B-12 for persons aged ≥3 y in the United States according to the National Health and Nutrition Examination Survey, 1999–2000 (A) and for persons aged ≥4 y in the United States according to the third National Health and Nutrition Examination Survey, 1988–1994 (B). Vitamin B-12 was measured only during 1991–1994.

**TABLE 2**

Geometric mean concentrations (and 95% CIs) of serum and red blood cell (RBC) folate and of serum vitamin B-12 in different subgroups of the US population: 1988–1994 compared with 1999–2000

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Serum folate (nmol/L)</th>
<th>RBC folate (nmol/L)</th>
<th>Serum vitamin B-12 (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>12.0 (11.5, 12.5)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Males</td>
<td>11.4 (10.9, 11.9)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Females</td>
<td>12.7 (12.1, 13.2)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Mexican Americans</td>
<td>10.4 (9.9, 10.9)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Non-Hispanic blacks</td>
<td>9.8 (9.5, 10.1)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Non-Hispanic whites</td>
<td>12.4 (11.9, 13.1)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Children aged ≤5 y</td>
<td>23.1 (22.0, 24.2)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Children aged 6–11 y</td>
<td>19.3 (18.2, 20.4)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Adolescents aged 12–19 y</td>
<td>11.9 (11.2, 12.6)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Adults aged 20–39 y</td>
<td>10.5 (10.1, 11.0)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Adults aged 40–59 y</td>
<td>12.3 (11.7, 13.0)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
<tr>
<td>Elderly aged ≥60 y</td>
<td>16.6 (15.9, 17.3)</td>
<td>317 (312, 322)</td>
<td>320 (315, 325)</td>
</tr>
</tbody>
</table>

1 n in brackets.
2 Note that the measurements were made from 1991 to 1994.
3 Values were age-adjusted on the basis of 2000 census data for adults aged 20–74 y.
4 Significantly different from 1988–1994 value (or 1991–1994 value for vitamin B-12), P < 0.01 (2-tailed, 2-group t test).
be diluted and reanalyzed to obtain a valid result. The prevalence of high serum folate concentrations increased from 7% in NHANES III to 43% in NHANES 1999–2000 for children aged ≤5 y and from 7% to 38% for elderly persons.

Serum vitamin B-12 concentrations <74 pmol/L usually suggest vitamin B-12 deficiency (45); <1% of the entire population and of elderly persons had serum vitamin B-12 concentrations <74 pmol/L in NHANES III and in NHANES 1999–2000. Serum vitamin B-12 concentrations <148 pmol/L are considered moderately low, but do not necessarily indicate vitamin B-12 deficiency (45). The prevalence of moderately low serum vitamin B-12 concentrations in elderly persons was somewhat lower in NHANES 1999–2000 (3%) than in NHANES III (5%); the difference was significant. At a cutoff of 185 pmol/L, 7% of elderly persons had moderately low serum vitamin B-12 concentrations in NHANES 1999–2000 compared with 13% in NHANES III; this difference was also significant.

**Prevalence of elevated plasma tHcy and MMA concentrations**

No generally agreed on cutoff exists for elevated plasma tHcy, but 13 μmol/L has sometimes been used to define elevated tHcy concentrations (13). Five percent of the US population, 18% of elderly men and 11% of elderly women, had plasma tHcy concentrations >13 μmol/L in NHANES 1999–2000. Ubink (47) suggested that a tHcy concentration of ≤9 μmol/L is desirable should the outcome of controlled clinical trials show that a lowering of plasma tHcy concentrations reduces the incidence of cardiovascular disease. In NHANES 1999–2000, 78% of the US population (72% of males and 85% of females) and 50% of elderly persons (40% of elderly males and 60% of elderly females) had plasma tHcy concentrations ≤9 μmol/L. A generally agreed on cutoff for elevated plasma MMA is 370 nmol/L (48, 49). Two percent of the US population and 7% of elderly persons had elevated MMA concentrations.

**DISCUSSION**

This study presents the first population-wide reference information for biochemical indicators of vitamin status in a nationally representative sample of the United States after folic acid fortification began. B vitamin and metabolite concentrations in this population displayed age, sex, and racial-ethnic characteristics similar to those found earlier in NHANES III (23, 41-44, 50). The observed age-related reduction in serum and RBC folate from childhood through adulthood is consistent with other prefortification surveys (23). The difference in plasma tHcy concentration between males and females was of the same magnitude as found in NHANES III (42); however, it appears to be greater than can be explained by the difference in serum folate, which indicated that the sex difference in plasma tHcy may be attributed to factors other than folate status. The observed racial-ethnic differences are consistent with recent findings from a controlled feeding study that race-ethnicity is an important determinant of folate status (51). Non-Hispanic blacks displayed the lowest serum and RBC folate concentrations in our analysis. One of the National Health Objectives for 2010 is to increase the proportion of pregnancies for which RBC folate concentration is optimum by increasing the median RBC folate concentration to 499 nmol/L among women aged 15–44 y (objective 16.16b; 52). According to NHANES 1999–2000, this objective has been met differently by the 3 racial-ethnic groups: 26% of non-Hispanic white, 32% of Mexican American, and 50% of non-Hispanic black women of childbearing age had RBC folate concentrations <499 nmol/L.

Since folic acid fortification began in the United States in 1998, the US population shifted to significantly higher serum and RBC folate concentrations. We observed increases across all subgroups of age, sex, or race-ethnicity. Adolescents and adults have experienced the biggest relative increase, children aged ≤5 y the smallest increase, and elderly persons an intermediate increase. Without distinguishing between supplement and non-supplement users, serum and RBC folate increased by 18–23 and 204–272 nmol/L across all age groups.

These findings are consistent with earlier findings from NHANES 1999–2000 that folate status has improved significantly in women of childbearing age, the target group for the folic acid fortification (27), as well as reports of improved folate status in selected nonrepresentative subsets of the US population. In the Framingham population, mean serum folate concentrations among nonusers of B vitamin supplements increased from 11 nmol/L before to 23 nmol/L after fortification, and the prevalence of low serum folate concentrations (<6.8 nmol/L) decreased from 22% to 2% (13); RBC folate concentrations increased by 38% (25). Median serum folate concentrations in samples submitted to Kaiser Permanente’s Southern California Endocrinology Laboratory during 1994–1998 steadily increased from 28.6 nmol/L before to 42.4 nmol/L after fortification (26). After the introduction of folic acid fortification in Canada, Ray et al (53) reported from a retrospective cross-sectional study geometric mean serum and RBC folate concentrations of 34.5 and 957 nmol/L, respectively; these concentrations were higher than expected and even higher than concentrations found in the United States. In 2 later reports, the authors found increases of 64% in serum folate in a nonrepresentative sample of elderly women in Ontario and British Columbia (54) and 41% in RBC folate in a nonrepresentative sample of women of childbearing age in Ontario (55). In Chile, after the mandatory fortification of wheat flour with folic acid, targeted to increase daily folate consumption of women of childbearing age by 400 μg, serum and RBC folate concentrations in a representative population sample increased 284% and 144%, respectively (56). The increase in RBC folate concentrations in the US population after fortification also seems compatible with the increase shown by Daly et al (57) in a double-blind, randomized, placebo-controlled trial of women of childbearing age who were supplemented with 200 μg folic acid/d.

Although the increase in serum and RBC folate concentrations in the US population after fortification in our analysis is consistent with other reports on nonrepresentative subpopulations, the increase is still higher than was expected from the 70–130-μg/d increase predicted by the US fortification program (58). The discrepancy may be due to unknown sampling biases between NHANES III and NHANES 1999–2000, changes in vitamin supplementation, or an imprecise ability to predict folate intake from blood folate concentrations. Because of the stable results for long-term quality control of the Bio-Rad assay between the 2 surveys, changes in laboratory techniques are unlikely to account for the shift in folate concentrations. Another possible explanation for the higher than expected folate concentrations could be a higher than expected daily intake of folic acid due to either the underestimation of food quantities eaten or overage added to folic acid fortification.
cereal-grain products in the process of fortification. Rader et al (59) reported significant excess fortification in a survey of recently fortified products. Furthermore, the spectrum of voluntarily fortified snack foods seems to undergo constant growth and change.

The US Institute of Medicine has reviewed all potential adverse effects of high doses of folic acid and found that the only basis for tolerable upper intake levels (UL) was the potential masking of vitamin B-12 deficiency (20). The UL for adults was set to 1 mg folate/d from fortified food or supplements, the UL for children was set to 300–800 µg/d, depending on age. Although the serum folate concentration that should be considered excessively high and its health implications are not known, folic acid ingested in quantities >266 µg in one meal can appear metabolized in serum (60). In NHANES III, 3% of the US population, 7% of children aged ≤5 y, and 8% of elderly persons had high serum folate concentrations (>45.3 pmol/L). In NHANES 1999–2000, after folic acid fortification began, 23% of the US population, 43% of children aged ≤5 y, and 38% of elderly persons reached this putatively high serum folate concentration. The issue of potential adverse effects of excess folate in persons with untreated vitamin B-12 deficiency remains unsolved. Mills et al (61) reported that in persons with low serum vitamin B-12 concentrations (<258 pmol/L) examined during 1992–2000 at the Veterans Affairs Medical Center in Washington, DC, the proportion of persons without anemia did not increase significantly from the prefortification period (39.2%) to the period of optional fortification (45.5%) or in the postfortification period (37.6%). We are unaware of any epidemiologic evidence of an increased risk of masked vitamin B-12 insufficiency or related disease after folic acid fortification, but clinically recognized vitamin B-12 insufficiency may be uncommon. Indeed, <1% of the US population had low serum vitamin B-12 concentrations (<74 pmol/L) that suggested vitamin B-12 deficiency, and <5% had moderately low serum vitamin B-12 concentrations (<148 pmol/L). Interestingly, serum vitamin B-12 concentrations increased slightly but significantly in the US population between the prefortification and postfortification periods. This increase was limited mainly to elderly persons. Whether the increase may be due to unknown sampling biases between the 2 surveys, changes in vitamin supplementation, or a higher intake of vitamin B-12–rich foods is not known.

A direct comparison of plasma tHcy concentrations between NHANES 1999–2000 and NHANES III is not possible because of differences in the methods and matrices of the 2 surveys. However, tHcy concentrations after fortification appeared lower in than NHANES III (41, 42, 44). When we chose a cutoff of 13 µmol/L, we found that ≈14% of elderly persons had elevated plasma tHcy concentrations. This corresponds well with a 10% prevalence of elevated tHcy after folic acid fortification compared with a prevalence of ≈20% before fortification in the Framingham population (24). What seems even more important, though, is that nearly 80% of the US population had achieved a plasma tHcy concentration <9 µmol/L, which is considered a desirable concentration (47).

We have presented the first nationally representative US population reference ranges for the B vitamins serum and RBC folate, serum vitamin B-12, and their biochemically related products plasma tHcy and MMA after the mandatory folic acid fortification of cereal-grain products was introduced in the United States in 1998. The fortification program has increased concentrations of serum and RBC folate in the entire population and virtually eliminated folate deficiency. Vitamin B-12 concentrations in elderly persons appear to be slightly higher. Plasma tHcy concentrations appear to be lower after fortification, and ≈80% of the population has achieved desirable concentrations. Nutritional monitoring for folate status remains exceedingly important, not only for women of childbearing age but also for children and persons of all ages. Nutritional monitoring for vitamin B-12 status, particularly in elderly persons, is similarly important.

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REFERENCES


