Vitamin Requirements for the Treatment of Hyperhomocysteinemia in Humans[1,2]

JOHAN B. UBBINK,[3] W. J. HAYWARD VERMAAK, ANNATJIE VAN DER MERWE, PIET J. BECKER,* RHENA DELPORT AND HENDRIK C. POTGIETER

*Department of Chemical Pathology, Faculty of Medicine, University of Pretoria, 0001 Pretoria, South Africa and *Institute for Biostatistics, Medical Research Council, Pretoria, South Africa*

ABSTRACT We have previously shown that a modest vitamin supplement containing folic acid, vitamin B-12 and vitamin B-6 is effective in reducing elevated plasma homocysteine concentrations. The effect of supplementation of the individual vitamins on moderate hyperhomocysteinemia has now been investigated in a placebo-controlled study. One hundred men with hyperhomocysteinemia were randomly assigned to five groups and treated with a daily dose of placebo, folic acid (0.65 mg), vitamin B-12 (0.4 mg), vitamin B-6 (10 mg) or a combination of the three vitamins for 6 wk. Folic acid supplementation reduced plasma homocysteine concentrations by 41.7% ($P < 0.001$), whereas the daily vitamin B-12 supplement lowered homocysteine concentrations by 14.8% ($P < 0.01$). The daily pyridoxine dose did not reduce significantly plasma homocysteine concentrations. The combination of the three vitamins reduced circulating homocysteine concentrations by 49.8%, which was not significantly different ($P = 0.48$) from the reduction achieved by folate supplementation alone. Our results indicate that folate deficiency may be an important cause of hyperhomocysteinemia in the general population. J. Nutr. 124: 1927–1933, 1994.

INDEXING KEY WORDS:

• *humans* • *homocysteine* • *folate*
• *pyridoxine* • *vitamin B-12*

Patients with premature vascular disorders often have elevated circulating total homocysteine concentrations. Several retrospective studies have linked mild hyperhomocysteinemia to coronary heart disease (Genest et al. 1990, Israelsson et al. 1988, Ubbink et al. 1991a) and cerebral (Brattström et al. 1984, Coull et al. 1990) and peripheral vascular diseases (Malinow et al. 1989, Taylor et al. 1991). Prospective data from the Physicians Health Study also indicate that moderate hyperhomocysteinemia is a risk factor for premature vascular disorders (Stampfer et al. 1992). Participants in the above-mentioned study who subsequently developed myocardial infarction had had significantly higher baseline plasma total homocysteine concentrations when compared with controls matched for age and smoking habits.

Clinical observations support epidemiological findings that elevated plasma homocysteine concentrations are involved in the pathogenesis of atherosclerosis. Taylor et al. (1991) found that the progression of peripheral vascular disease, as assessed in a vascular laboratory, was more common in patients with hyperhomocysteinemia than in patients with normal plasma homocysteine concentrations. Similarly, clinical progression of coronary heart disease, based on new occurrence of angina pectoris, myocardial infarction or congestive heart failure, occurred at a higher rate in patients with hyperhomocysteinemia (Taylor et al. 1991). Malinow et al. (1993) measured the thickness of the intimal-medial carotid walls in individuals free of clinical atherosclerotic disease and found significantly elevated plasma homocysteine concentrations in subjects with thickened intimal-medial carotid walls. Based on the assumption that carotid arterial wall thickening reflects atherosclerosis, the results from Malinow and co-workers suggest involvement of homocysteine in atherosclerotic plaque formation.

The mechanisms by which homocysteine may promote atherogenesis include vascular endothelial

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[3]To whom correspondence and reprint requests should be addressed.

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injury (Harker et al. 1976), oxidative modification of low density lipoproteins (Olszewski and McCully 1993) and enhanced binding of lipoprotein(a) to fibrin in atherosclerotic plaque (Harpel et al. 1992). Homocysteine may also perturb vascular coagulation mechanisms and thus promote a thrombotic tendency (Hajjar 1993).

The evidence from epidemiological, clinical and biochemical studies indicating that an elevated homocysteine level is a risk factor for premature vascular disorders suggests that intervention trials are now required to determine whether the treatment of hyperhomocysteinemia will reduce the incidence of vascular disorders (Malinow 1990, Malinow et al. 1993, Stampfer et al. 1992). However, it is essential that the optimal therapy to normalize hyperhomocysteinemia should be established before clinical trials are initiated. We have previously shown that a modest vitamin supplement containing folic acid, vitamin B-12 and vitamin B-6 is effective in reducing plasma homocysteine concentrations (Ubbink et al. 1993). We now report the results of a placebo-controlled study in which the effect of supplementation of the individual vitamins on hyperhomocysteinemia was investigated.

SUBJECTS AND METHODS

Venous blood samples with sodium fluoride as preservative were obtained from 2788 fasting ambulatory white men aged between 20 and 73 y, who were referred to the two major Pretoria pathology practices for routine medical investigations as are required for life insurance purposes. Blood samples were received within 8 h at our laboratory; plasma and cells were separated immediately after receipt and the plasma samples frozen at −70°C and analyzed within 1 wk for homocysteine.

It has been established previously that plasma homocysteine increases when blood separation is delayed (Ubbink et al. 1992). This is presumably the result of continued methionine metabolism by blood cells, which can be partially inhibited by either chilling the blood samples in an ice bath or by the use of sodium fluoride as an enzyme inhibitor. Nevertheless, even in the presence of sodium fluoride, plasma homocysteine concentrations may increase by 36% within 8 h. Therefore, individuals with an initial plasma homocysteine concentration above the normal reference range (>16.3 μmol/L) were requested to visit our laboratory for a repeat sampling. During this visit, blood samples with EDTA as anticoagulant were obtained and chilled on ice; plasma and blood cells were separated within 1 h by low speed centrifugation (1600 × *g*, 10 min). At the first visit, the participants were weighed, length and blood pressures were recorded, and each participant completed a questionnaire on his health status. Participants with confirmed hyperhomocysteinemia (n = 100) were randomly assigned to one of five groups (n = 20 for each group). Group A received placebo tablets, Group B was supplied with folic acid tablets (0.65 mg), Groups C and D received tablets containing pyridoxine HCl (12.2 mg) and cyanocobalamin (0.4 mg), respectively, and Group E was treated with a tablet containing all three vitamins in the quantities mentioned above. All the tablets also contained 3.0 mg of β-carotene, and the different tablets were therefore similar in appearance. The vitamin tablets were specially prepared and supplied by Vesta Medicines (Johannesburg, South Africa).

Participants were instructed to take one tablet daily after dinner and to refrain from using any other vitamin supplements during the study period. Venous samples were obtained from fasting subjects 3 and 6 wk later. To monitor compliance, the 3-wk samples were analyzed for the vitamins supplemented. Data from two participants were omitted in the final analysis due to lack of compliance. Seven other participants opted to withdraw during the study. The participants were not aware of the identity of the tablets used in the study. The study was approved by the University Human Ethics Committee.

Laboratory investigations. Plasma pyridoxal 5′-phosphate (PLP) was determined by HPLC (Ubbink et al. 1985). Vitamin B-12 and folate concentrations were determined by a commercially available RIA kit (Simul TRAC-SNB, Becton Dickinson, Orangeburg, NY). Plasma homocysteine was derivatized with ammonium 7-fluoro 2-oxa-1,3 diazole-4-sulfonate (Wako, Neuss, Germany) and determined by HPLC as described previously (Ubbink et al. 1991b). This method measures total (free + protein-bound) plasma homocysteine concentrations; homocystine, the mixed disulfide (cysteine-homocysteine) and protein-bound homocysteine are first quantitatively reduced before ammonium 7-fluoro 2-oxa-1,3 diazole-4-sulfonate is added as derivatizing reagent.

Statistical analysis. The five groups were compared with respect to plasma concentrations of homocysteine, PLP, folate and vitamin B-12 in a one-way ANOVA. Pairwise comparisons were done with an appropriate t test.

Within each group, plasma homocysteine and vitamin concentrations measured after the 6-wk treatment period were also compared to the basal concentrations by using Student's paired t test. In the pyridoxine-treated group, the data for folate did not meet the assumption required for Student's paired t test, and the comparison was made by Wilcoxon's matched-pairs signed ranks test. In view of the relatively small samples, outcomes of the Student's paired t test in all the other comparisons were also confirmed by Wilcoxon's matched-pairs signed ranks test (Conover 1971). Differences were considered significant at $P < 0.05$.

TABLE 1

***Health profile of the study participants*[1]**

Group	n	Age	BMI	Blood pressure		Prevalence (%) of			
				Diastolic	Sistolic	Smoking	Obesity	Hypertension	Angina or MI
		y	kg/m²	mm Hg					
A	17	40.6 (14.5)	27.0 (3.7)	78.8 (11.8)	121.2 (16.3)	41.1	64.7	11.8	17.6
B	19	40.0 (13.5)	27.1 (4.2)	80.5 (10.7)	125.8 (9.4)	47.4	63.2	5.3	0
C	18	34.6 (13.7)	25.5 (3.6)	75.2 (8.9)	122.4 (13.3)	44.4	55.5	5.5	11.1
D	17	35.0 (12.5)	26.1 (3.6)	76.9 (7.7)	119.4 (10.4)	41.2	58.8	5.9	11.8
E	20	42.3 (9.9)	26.7 (3.6)	79.8 (10.7)	116.5 (14.0)	55.0	64.9	10.0	5.0

[1]Values are means (SD). The five groups did not differ significantly with respect to age, body mass index (BMI) or blood pressure ($P > 0.05$ for each variable, ANOVA). MI = myocardial infarction.

The relationship between changes in plasma homocysteine and folate concentrations in the placebo-treated group was tested for significance with Fisher's exact test (Conover 1971).

RESULTS

Table 1 summarizes the characteristics of the study participants. One-way ANOVA indicated that the five groups did not differ significantly from each other with respect to age, body mass index or blood pressure. More than 50% of the participants in each group had a body mass index of >25.0 kg/m², and, using the criteria of Garrow and Webster (1985), these participants were classified as obese. The prevalences of smoking, obesity, hypertension (diastolic blood pressure >90 mm Hg) and ischemic heart disease were similar in the five groups.

Table 2 summarizes the effect of the different vitamin supplements on circulating homocysteine concentration. A between-group comparison demonstrated that at the start of the study (wk 0), the five groups did not differ with respect to plasma concentrations of homocysteine, PLP, cobalamin or folate. Between-group comparisons after 6 wk of vitamin

TABLE 2

***Effects of different vitamin supplements on plasma concentrations of homocysteine, cobalamin, pyridoxal-5'-phosphate (PLP) and folate in men with hyperhomocysteinemia*[1]**

Group	Vitamin supplement	n	Week	Homocysteine	Folate	Cobalamin	PLP
				μmol/L	nmol/L	pmol/L	nmol/L
A	Placebo	17	0	30.6 (24.2)	5.7 (3.6)	217.2 (75.7)	37.7 (22.4)
			6	30.7 (24.0)	6.1 (5.6)	210.6 (76.5)	44.5 (28.0)
B	Folate	19	0	28.8 (14.5)	6.0 (4.4)	180.3 (72.1)	44.8 (30.4)
			6	16.8 (7.6)[ad]	17.4 (10.4)[bd]	203.6 (89.8)	38.0 (28.0)
C	Pyridoxine	18	0	29.1 (15.0)	6.8 (3.0)	214.5 (93.5)	45.8 (25.9)
			6	27.8 (14.5)	5.8 (2.4)[c]	228.1 (73.4)	188.9 (101.5)[bd]
D	Cobalamin	17	0	30.5 (16.9)	4.1 (1.0)	217.6 (122.6)	48.2 (23.4)
			6	26.0 (11.8)[c]	4.1 (1.3)	378.5 (188.8)[bd]	45.5 (16.0)
E	Combination	20	0	27.1 (8.3)	5.2 (3.0)	261.1 (124.0)	45.4 (14.8)
			6	13.6 (4.9)[bd]	18.4 (11.1)[bd]	389.1 (127.7)[bd]	213.7 (80.2)[bd]

[1]Values are means (SD). Plasma concentrations of vitamins and homocysteine after 6 wk of vitamin supplementation were compared with plasma concentrations after 6 wk of placebo supplementation: significantly different from results achieved with placebo supplement, [a]$P < 0.01$, [b]$P < 0.001$. Plasma concentrations of vitamins and homocysteine after 6 wk of supplementation were also compared with basal levels (wk 0) for each supplementation group: significantly different from corresponding value for wk 0: [c]$P < 0.05$, [d]$P < 0.001$.

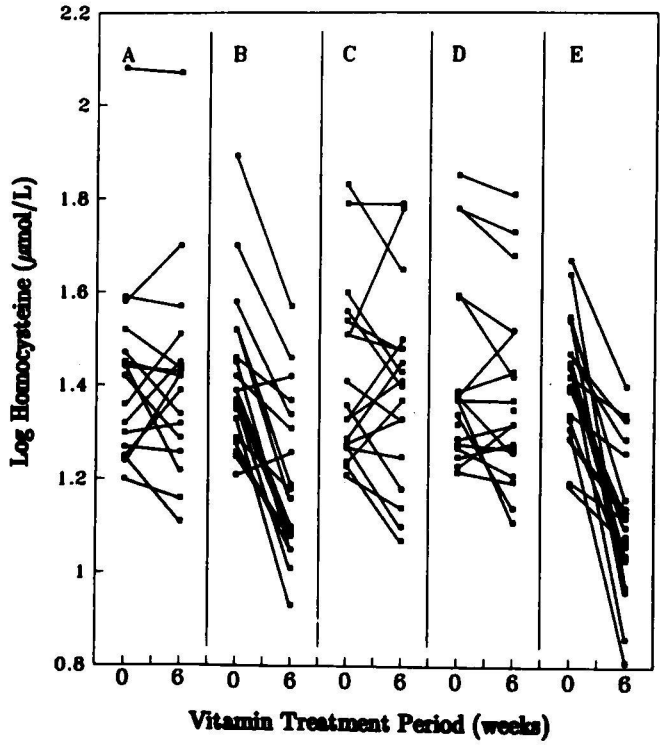

FIGURE 1 The effect of different vitamin supplements on plasma total homocysteine concentrations. Hyperhomocysteinemic men were supplemented daily for 6 wk with (*A*) placebo, (*B*) 0.65 mg folic acid, (*C*) 10 mg pyridoxine, (*D*) 0.4 mg cyanocobalamin or (*E*) a combination of the three vitamins. Significant declines in plasma homocysteine concentrations were observed in men supplemented with folic acid ($P < 0.001$), cyanocobalamin ($P < 0.01$) and the vitamin combination ($P < 0.001$).

supplementation demonstrated that treatment with folate and with the combined vitamin preparation resulted in significantly lower plasma homocysteine concentrations when compared with placebo treatment ($P = 0.004$ and $P < 0.001$, respectively). Mean plasma homocysteine concentrations after 6 wk of pyridoxine or vitamin B-12 supplementation were not significantly different when compared with concentrations of placebo-supplemented subjects. Compared with the placebo-supplemented subjects, plasma concentrations of the vitamins supplemented increased significantly ($P < 0.001$) within the 6-wk study period.

A within-group comparison showed that the modest folic acid supplement lowered the plasma total homocysteine concentration by 41.7% ($P < 0.001$) when compared with the basal (wk 0) concentration (Table 2). Similarly, vitamin B-12 supplementation reduced the circulating homocysteine concentration significantly ($P < 0.01$) by 14.8%; however, the 4.5% reduction in homocysteine concentration achieved by pyridoxine supplementation was not statistically significant. The supplement containing all three vitamins caused the largest reduction in plasma homocysteine concentration (49.8%, $P < 0.001$). The effect obtained by the vitamin combination was not significantly different from that achieved by folate supplementation ($P = 0.48$, ANOVA).

Figure 1 depicts the individual responses to the different vitamin supplements used in this trial. Group E (combined supplement) was the only group in which all the participants responded with a reduction in circulating homocysteine concentrations. Plasma homocysteine concentrations from two individuals failed to respond to the folate supplement (Fig. 1*B*). The reductions observed with vitamin B-12 therapy were considerably less (Fig. 1*D*), with at least five participants showing no decline or even showing an increase in plasma homocysteine concentration. The individual changes in plasma homocysteine concentrations during placebo and pyridoxine supplementation were erratic (Fig. 1*A*, *C*) and may reflect small changes in the dietary folic acid intake.

Figure 2 depicts the changes in plasma homocysteine concentrations from wk 0 to wk 6 in participants receiving the placebo treatment as a function of the change in plasma folic acid concentrations over the same period. In nine participants the

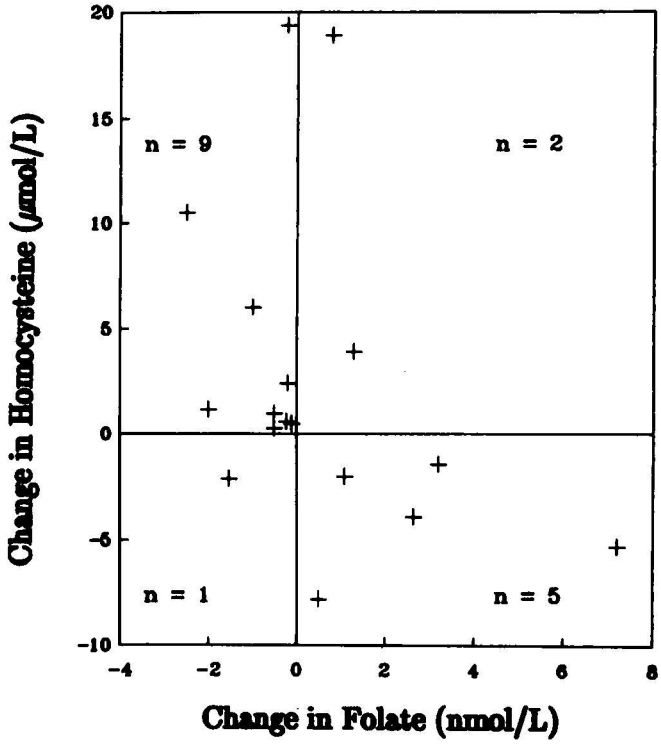

FIGURE 2 The change in plasma total homocysteine concentrations in placebo-treated subjects as function of the change in plasma folic acid concentrations. The individual changes in plasma folate concentrations were associated with opposite changes in total homocysteine concentrations ($P < 0.02$, Fisher's exact test).

observed increase in plasma homocysteine concentrations was associated with a decline in plasma folate concentrations, whereas only one participant showed a decline in both homocysteine and folic acid concentrations. Similarly, five individuals had lower homocysteine concentrations and higher folate concentrations, whereas only two participants showed an increase in both homocysteine and folate concentrations. Among those who had decreased folate concentrations, a significantly larger proportion had increased homocysteine concentrations than amongst the individuals with increased folate concentrations ($P = 0.02$, Fisher's exact test). There were no significant relationships between changes in plasma homocysteine concentrations and changes in plasma PLP or cobalamin concentrations in the placebo-treated group (results not shown).

DISCUSSION

Intracellular homocysteine is either remethylated to methionine in a reaction that requires methyltetrahydrofolate and vitamin B-12 or is condensed with serine in a reaction catalyzed by the PLP-dependent cystathionine-β-synthase (EC 4.2.1.22). Deficiencies in the cofactors required for homocysteine metabolism may result in hyperhomocysteinemia, which can be successfully treated with a modest daily vitamin supplement (Ubbink et al. 1993). The results from the current study confirm that a combined vitamin preparation may be used to lower elevated circulating homocysteine concentrations. The aim of this study was to assess the ability of each individual vitamin component to lower the plasma homocysteine concentration.

Our results suggest that folic acid is a very powerful homocysteine-lowering agent; in fact, the combination of the three vitamins was only slightly more effective compared with folic acid. Folate supplementation has been used by others to treat hyperhomocysteinemia in renal insufficiency (Wilcken et al. 1988) as well as premature vascular disorders (Brattström et al. 1990, Dudman et al. 1993). The daily doses of folate used in these studies ranged between 5 and 10 mg; in contrast, our results were obtained by an appreciably lower daily supplement [0.65 mg, or 3.25 × the Recommended Daily Allowance (RDA) for folate].

In view of the high success rate obtained with folate therapy, the obvious question is whether the other two vitamins are required at all to control plasma homocysteine concentrations. Compared with placebo treatment, the homocysteine-lowering effect of vitamin B-12 was not statistically significant ($P = 0.31$, ANOVA). However, a within-group comparison showed that vitamin B-12 supplementation resulted in a modest but significant decline in the mean plasma homocysteine concentration (Table 2). This reduction in basal plasma homocysteine concentration after vitamin B-12 supplementation was notably less than the results obtained by Lindenbaum and co-workers (1988). This difference is explained by the fact that Lindenbaum et al. supplemented diagnosed vitamin B-12–deficient patients with cobalamin, whereas in our study the hyperhomocysteinemic men were randomized into the different treatment groups without prior knowledge of vitamin nutritional status or any possible genetic aberrations. The pre-treatment plasma vitamin B-12 concentrations in our study were higher than those reported by Lindenbaum et al. (**Fig. 3**). Obviously, some of our participants receiving vitamin B-12 were not really deficient in this vitamin, and this may explain why vitamin B-12 supplementation had only a small homocysteine-lowering effect in our study.

Folic acid supplementation in patients with a chronic vitamin B-12 deficiency may eventually result in neuropathy due to failure to recognize the vitamin B-12 deficiency (Beck 1991). Moreover, Allen et al. (1990) have recently shown that folate supplementation will not correct hyperhomocysteinemia that is primarily the result of a vitamin B-12 deficiency. It is therefore essential that vitamin B-12 and folate be combined to treat hyperhomocysteinemia. Furthermore, it is also important that the cyanocobalamin content of the vitamin supplement should

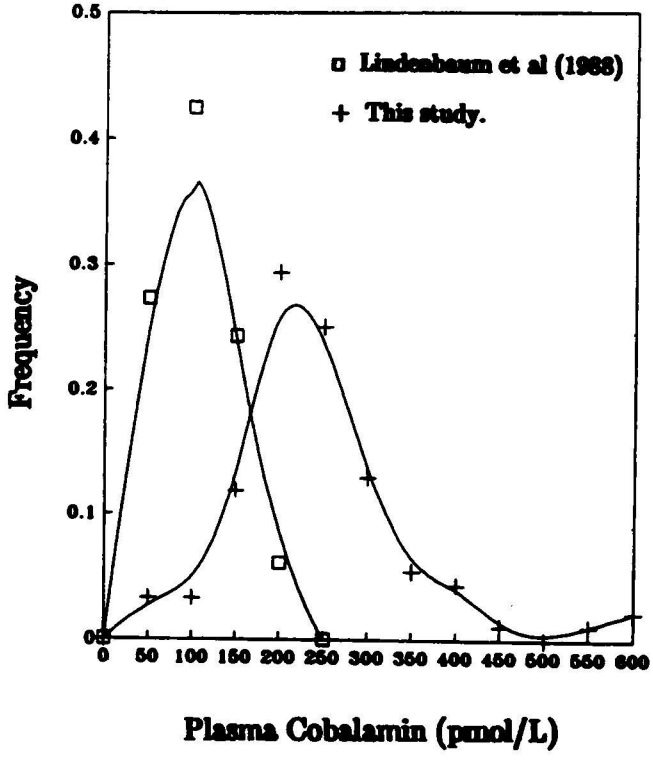

FIGURE 3 The frequency distribution of plasma vitamin B-12 concentrations in this study compared with the study reported by Lindenbaum et al. (1988).

be sufficient to allow adequate absorption of vitamin B-12 from the gastrointestinal tract, even in patients with pernicious anemia. Large oral doses of vitamin B-12 can be used to treat pernicious anemia, because a very small portion of the cobalamin will be absorbed even in the absence of intrinsic factor (Doscherholmen et al. 1957). We used a daily vitamin B-12 supplement of 400 μg (200 × RDA), because it has been calculated that up to 1% of an oral 400-μg vitamin B-12 dose may be absorbed in subjects with pernicious anemia (Ellenbogen and Cooper 1991). This implies that even in cases with intrinsic factor deficiency, ~4 μg (2× RDA) will be absorbed from a daily 400-μg vitamin B-12 dose. We therefore suggest that effective treatment of hyperhomocysteinemia should include at least vitamin B-12 and folic acid supplementation; the vitamin B-12 supplement should be sufficient to satisfy the requirements of patients suffering from intrinsic factor deficiency.

Due to its dramatic effect in cystathionine-β synthase deficiency (Mudd et al. 1989), pyridoxine may be regarded as the obvious choice of treatment for moderate hyperhomocysteinemia. However, our results indicate that a daily 10-mg (5× RDA) pyridoxine supplement did not significantly reduce plasma homocysteine concentration. The data presented in Fig. 1*C* suggest that some individuals responded to pyridoxine therapy, whereas others did not. However, placebo supplementation had a similar effect, with some individuals apparently responding to placebo treatment. These changes in circulating homocysteine concentrations are presumably explained by small changes in folic acid nutritional status during the study period (Fig. 2). It therefore seems that a folate deficiency is far more common and important in causing hyperhomocysteinemia in the general population. Our results support evidence presented by Miller et al. (1992) as well as from Brattström's laboratory (Brattström et al. 1990) that pyridoxine supplementation does not affect basal homocysteine concentration. The intriguing question of why only vitamin B-12 and folate, but not vitamin B-6, may modulate basal plasma homocysteine concentration has recently been addressed by Selhub and Miller (1992). These authors postulate that a folate and/or vitamin B-12 deficiency results in low *S*-adenosylmethionine (an activator for the enzyme cystathionine β-synthase) concentrations, thus causing diminished cystathionine β-synthase activity in addition to impaired remethylation. This then results in homocysteine accumulation. During a vitamin B-6 deficiency, no depletion of *S*-adenosylmethionine is expected to occur, and homocysteine is removed by the remethylation pathway (Selhub and Miller 1992).

Although pyridoxine supplementation fails to lower basal plasma homocysteine concentration, vitamin B-6 modulates the homocysteine peak after the oral methionine load test (Brattström et al. 1990, Dudman et al. 1993). Brattström et al. (1990) found that 240 mg of pyridoxine decreased the mean post-methionine load homocysteine elevation by 26%, whereas Dudman and co-workers (1993) reported similar results with a smaller (100 mg/d) pyridoxine supplement. Several studies have found that postprandial hyperhomocysteinemia is related to increased vascular disease risk (Boers et al. 1985, Clarke et al. 1991). Vitamin B-6 may be particularly effective in limiting the plasma homocysteine increase after methionine loading, and it may be prudent to include pyridoxine in intervention trials designed to evaluate homocysteine as a causative agent in vascular disease. However, because high doses of pyridoxine may induce neuropathy (Schaumburg et al. 1983), it should be established whether a smaller daily vitamin B-6 dose would not be equally effective in preventing postprandial hyperhomocysteinemia.

In conclusion, we have shown that the homocysteine-lowering effect of a multivitamin combination containing folate, vitamin B-12 and pyridoxine is mainly due to its folic acid content. Vitamin B-12 had a modest ability to lower basal plasma homocysteine concentration, whereas pyridoxine had no effect on basal homocysteine concentration. Treatment of hyperhomocysteinemia should at least include a folic acid-vitamin B-12 combination; the requirement for vitamin B-6 needs further investigation.

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