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Thermolabile 5,10-Methylenetetrahydrofolate Reductase as a Cause of Mild Hyperhomocysteinemia

Astrid M. T. Engbersen,[1] Diana G. Franken,[2] Godfried H. J. Boers,[2] Erik M. B. Stevens,[1] Frans J. M. Trijbels,[1] and Henk J. Blom[1]

[1] Department of Pediatrics and [2]Department of Medicine, Division of Endocrinology, University Hospital Nijmegen, Nijmegen

Summary

Thermolability of 5,10-methylenetetrahydrofolate reductase (MTHFR) was examined as a possible cause of mild hyperhomocysteinemia in patients with premature vascular disease. Control subjects and vascular patients with mild hyperhomocysteinemia and with normohomocysteinemia were studied. The mean (±SD) specific MTHFR activity in lymphocytes of 22 control subjects was 15.6 (±4.7) nmol CH_2O/mg protein/h (range: 9.1–26.6), and the residual activity (±SD) after heat inactivation for 5 min at 46°C was 55.3 (±12.0)% (range: 35.9–78.3). By measurement of MTHFR activity, two distinct subgroups of hyperhomocysteinemic patients became evident. One group (n = 11) had thermolabile MTHFR with a mean (±SD) specific activity of 8.7 (±2.1) nmol CH_2O/mg protein/h (range: 5.5–12.7) and a residual activity, after heat inactivation, ranging from 0% to 33%. The other group (n = 28) had normal specific activity (±SD) of 21.5 (±7.2) nmol CH_2O/mg protein/h (range: 10.0–39.0) and a normal residual activity (±SD) of 53.8 (±9.2)% (range: 33.1–71.5) after heat inactivation. The mean (±SD) specific activity of 29 normohomocysteinemic patients was 20.7 (±6.5) nmol CH_2O/mg protein/h (range: 9.4–33.8), and the mean (±SD) residual activity after heat inactivation was 58.2 (±10.2)% (range: 43.0–82.0). Thus, in 28% of the hyperhomocysteinemic patients with premature vascular disease, abnormal homocysteine metabolism could be attributed to thermolabile MTHFR.

Introduction

Over the past decade, mild hyperhomocysteinemia has become an established risk factor for premature vascular disease (Brattström and Lindgren 1992; Kang et al. 1992; Ueland et al. 1992). Homocysteine is presumed to damage the endothelial cells, although the mechanism of its toxicity remains obscure (Blom and VandeMolen 1994).

Homocysteine accumulation may be caused by a metabolic block in either the degradation of homocysteine to cystathionine or the remethylation of homocysteine to methionine (fig. 1). The classic form of severe hyperhomocysteinemia is caused by cystathionine β-synthase deficiency. This enzyme catalyzes the formation of cystathionine from homocysteine and serine. Another enzymatic cause of severe hyperhomocysteinemia is 5,10-methylenetetrahydrofolate reductase (MTHFR) deficiency, which affects the remethylation of homocysteine to methionine. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate (methylene-THF) to 5-methyltetrahydrofolate (Me-THF), in which flavin adenine dinucleotide (FAD) serves as cofactor (fig. 1).

Severe hyperhomocysteinemia due to MTHFR deficiency causes neurological abnormalities, mental retardation, arteriosclerosis, and thrombosis (Rosenblatt 1989). The biochemical characteristics are hyperhomocystinuria, reduced or low methionine concentrations in plasma, low plasma folate levels, and very low MTHFR activity in both fibroblasts and lymphocytes (Wada et al. 1978; Harpey et al. 1981; Wendel et al. 1983). Low plasma-folate levels result from a lack of the MTHFR product Me-THF, which is the main form of circulating folate. The clinical severity and the extent of biochemical derangement appear to be correlated with the degree of enzyme deficiency (Harpey et al. 1981; Rosenblatt 1989). In obligate heterozygotes for severe MTHFR deficiency the specific MTHFR activity is ~50% of the normal mean, and it is unknown whether this condition is associated with an increased risk for vascular disease (Kang et al. 1991*a*).

Heterozygotes for cystathionine β-synthase deficiency have reduced enzyme activity in cultured fibroblasts and also mild hyperhomocysteinemia, after methionine loading (Boers et al. 1985*a*). Reduced cystathionine β-synthase activity in fibroblasts has also been found in the majority of vascular-disease patients with mild hyperhomocysteinemia, in two studies (Boers et al. 1985*b*; Clarke et al. 1991). However, this finding could not be reconciled with the observations of Mudd et al. (1981) that, in a large series of families with a homozygote cystathionine β-synthase deficient patient, the obligate carriers did not have

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Address for correspondence and reprints: Dr. Henk J. Blom, Department of Pediatrics, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

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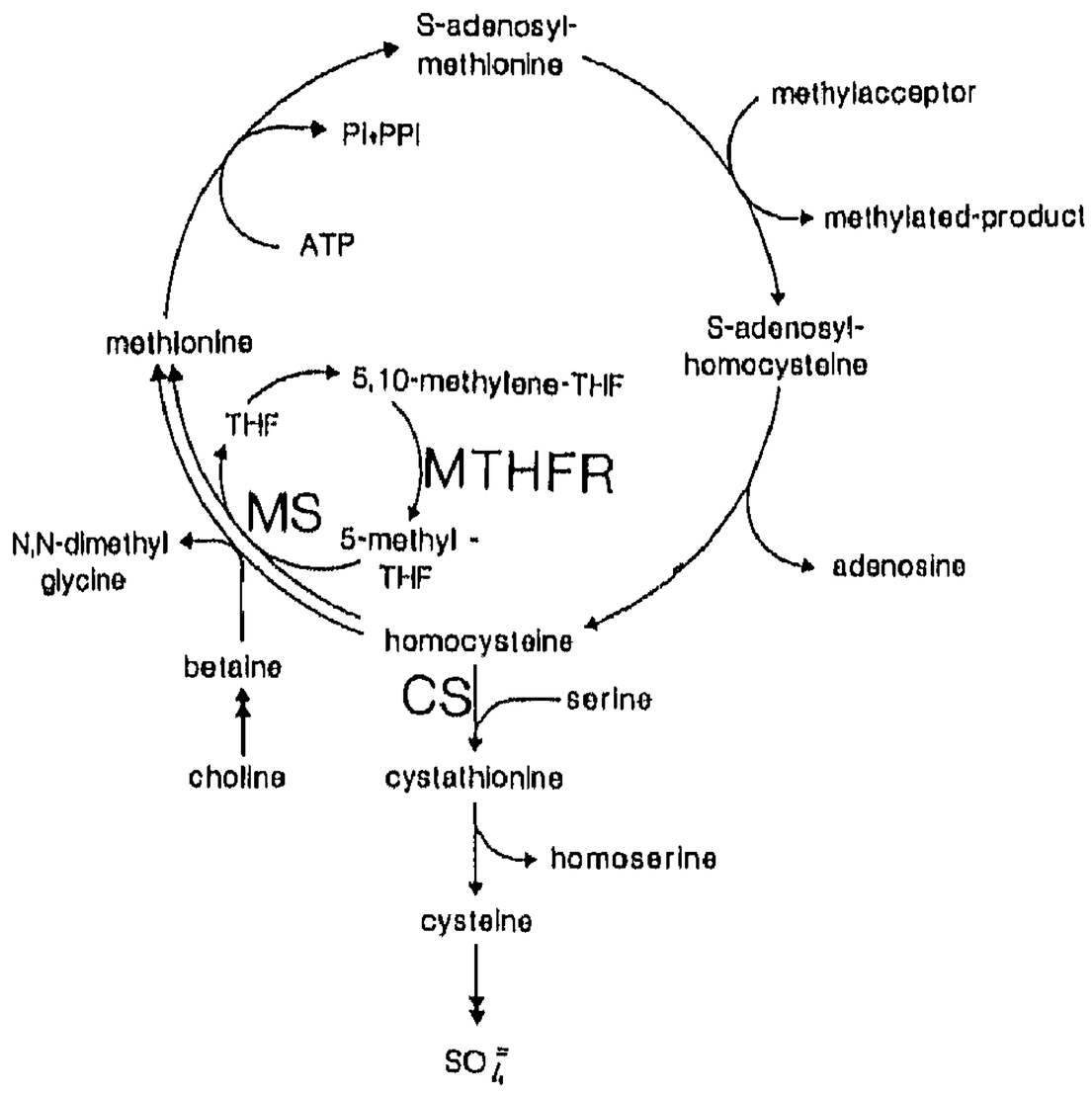

Figure 1 Homocysteine metabolism. CS = cystathionine β-synthase; MS = methionine synthase; and THF = tetrahydrofolate.

an increased risk of vascular disease. While there is evidence of a high prevalence of mild hyperhomocysteinemia in patients with vascular disease (Brattström and Lindgren 1992; Kang et al. 1992; Ueland et al. 1992)—even in populations with the highest prevalence of homozygous cystathionine β-synthase deficiency, such as in Ireland—the calculated number of heterozygotes is too low to account for the number of observed hyperhomocysteinemic vascular patients (Daly et al. 1993). In agreement with this observation, we (H. J. Blom, B. Fowler, G. H. J. Boers, and F. J. M. Trijbels, unpublished observations) and others (J. Kraus, personal communication) could not reproduce the finding of lowered cystathionine β-synthase activity in fibroblasts of vascular patients with mild hyperhomocysteinemia. This suggests that some aberration other than heterozygosity for cystathionine β-synthase deficiency is causing the hyperhomocysteinemia in vascular patients.

Recently, a thermolabile variant of MTHFR (Kang et al. 1988*a*, 1988*b*, 1991*a*, 1991*b*) was shown to be caused by a mutation different from that causing the severe form of MTHFR deficiency. Homozygotes for thermolabile MTHFR deficiency have a specific activity of ~50% of normal, and a residual activity after heat inactivation of <30%, compared with 50% residual activity in control subjects. Kang et al. (1991*b*) reported an incidence of homozygote thermolabile MTHFR deficiency of 17% in a group of 212 patients with coronary artery disease. Obligate heterozygotes for thermolabile MTHFR deficiency have a specific enzyme activity of ~75% of the normal mean but probably have thermostable MTHFR (Kang et al. 1991*b*). Compound heterozygotes for MTHFR deficiency, with one allele for the severe form and one allele for the thermolabile form, have also been described. These patients have a specific enzyme activity of ~25% of the normal mean, and their MTHFR is probably thermolabile (Kang et al. 1991*a*).

The present study describes a modified method for measurement of thermolabile MTHFR in isolated lymphocytes. Reference values were obtained from 23 healthy subjects. From our patient group with premature vascular disease (Franken et al. 1994) we selected 39 patients with and 29 without mild hyperhomocysteinemia, to study the relationship between mild hyperhomocysteinemia and thermolabile MTHFR.

Subjects, Material, and Methods

Patients and Controls

Patients with arteriosclerosis or venous thrombosis <55 years of age were screened for mild hyperhomocysteinemia by the methionine-loading test (0.1 g L-methionine/kg body weight) in University Hospital Nijmegen (Boers et al. 1985*b*). Patients with hyperlipoproteinemia, hypertension, and diabetes mellitus were excluded. There were no other selection criteria.

Reference values for homocysteine concentrations, before and after methionine loading, were obtained from 88 control subjects—65 premenopausal women and 23 men. Reference values for folic acid and vitamin B_{12} levels were obtained from 68 control subjects—45 premenopausal women and 23 men. Mild hyperhomocysteinemia was defined as total homocysteine blood levels after methionine loading higher than the mean + 2 SD of the reference group. The mean ± 2 SD range of the reference group for total homocysteine concentration was 4–16 μmol/liter (skewness 0.9, kurtosis 1.0) after fasting and 15–49 μmol/liter after methionine loading (skewness 0.7, kurtosis −0.3). Mean ± 2 SD range for plasma folic acid was 5.3–22.5 nmol/liter (skewness 1.1, kurtosis 1.1) and for vitamin B_{12} 84–487 pmol/liter (skewness 0.9, kurtosis 1.0). Four patients (8, 11, 16 and 23) were folate deficient (<5.3 nmol/liter), and one patient (14) had reduced vitamin B_{12} (79 pmol/liter). However, these patients showed no clasical clinical symptoms of folate or vitamin B_{12} deficiency.

As a reference, MTHFR activity was measured in a group of 23 healthy subjects, consisting of hospital personnel without any clinical evidence for vascular disease. Their mean (±SD) age was 32.7 (±7.4) years. One subject clearly showed thermolabile MTHFR and was excluded from the control group.

The presence of thermolabile MTHFR was studied in several groups of vascular patients, after their homocysteine status was established by methionine loading. One group with mild hyperhomocysteinemia consisted of 39 patients—28 premenopausal women and 11 men—with proved vascular disease (17 patients with cerebral, 9 pa-

tients with peripheral, and 5 patients with coronary arterial occlusive disease and 8 patients with venous thrombosis). The mean (±SD) age of these patients at the time of study was 40.3 (±8.9) years. The mean (±SD) age at clinical onset of the vascular abnormalities in this group was 35.6 (±10.1) years.

Another group consisted of 29 normohomocysteinemic patients—14 female and 15 male—with premature vascular disease (21 patients with cerebral, 6 patients with peripheral, and 1 patient with coronary arterial occlusive disease and 1 patient with venous thrombosis). Their mean (±SD) age at the time of study was 43.8 (±12.5) years. The mean (±SD) age of clinical onset of vascular disease in this group was 40.3 (±11.4) years.

Potassium phosphate, L-(+)-ascorbic acid, 35% formaldehyde, toluene, and acetic acid were obtained from Merck. FAD (disodium salt), menadione sodium bisulfite, dimedone, and Me-THF (barium salt) were obtained from Sigma Chemical. EDTA was obtained from Fluka BioChemika, and [Me-^{14}C] Me-THF (50 mCi/mmol, barium salt) was obtained from Amersham International. An Eppendorf 5436 thermomixer was used for preincubating the enzyme extract and for incubating the incubation mixture.

Lymphocytes were isolated from 20 ml heparinized blood by using Lymphoprep (Nycomed Pharma AS) (Jondal et al. 1972) and were washed twice with Hank's buffer (ICN Biomedicals). The cell pellet was stored at −80°C until enzyme assay.

The MTHFR activity was determined radiochemically in lymphocytes, in its physiological reverse direction (fig. 1). [Me-^{14}C] Me-THF served as the substrate in the presence of menadione as electron acceptor. Activities were measured using a modified method of Kang et al. (1991*b*). The cells were resuspended in 50 mM potassium phosphate buffer pH 7.2, were frozen and thawed three times, and were centrifuged 40 min at 15.8×10^3 g. A part of the supernatant was preincubated for 5 min at 46°C, to determine the heat stability. FAD was omitted during this heat inactivation (Rosenblatt and Erbe 1977). The incubation mixture, with a final volume of 600 µl, consisted of 0.18 M potassium phosphate buffer pH 6.8, 1.15 mM EDTA pH 7.0, 11.5 mM ascorbic acid, 54 µM FAD, 20 µM [Me-^{14}C] Me-THF (5.0×10^5 dpm), 3.5 mM menadione, and a maximum of 250 µl enzyme extract (preincubated supernatant or normal supernatant). The incubation was started by addition of menadione and lasted for 20 min in the dark at 37°C. The blank contained all the components of the incubation mixture except enzyme extract. The incubation was terminated by the addition of 10 µl of 1.0 M carrier formaldehyde, 50 µmol dimedone in 200 µl ethanol:water (1:1), and 100 µl 3.0 M potassium acetate, pH 4.5. The reaction mixture was heated at 95°C for 15 min, after which it was cooled on ice for ~10 min. The reaction mixture was added to 3.0 ml toluene and was stirred vigorously for 15 s. After low-speed centrifugation, 2.0 ml of the toluene phase was taken for measurement of radioactivity. Protein was determined by the method of Lowry et al. (1951). Enzyme activity is expressed as nmol of formaldehyde formed/mg protein/h.

Cystathionine β-synthase activity was measured as described elsewhere (Fowler et al. 1978; Boers et al. 1985*a*), without addition of pyridoxal phosphate to the assay mixture in cultured fibroblasts of 10 vascular hyperhomocysteinemic patients. For comparison, cystathionine β-synthase activities were also measured in fibroblasts of 13 obligate heterozygotes for cystathionine β-synthase and in 12 control subjects.

Total (free plus protein-bound) homocysteine concentrations, fasting and after methionine loading, were measured in EDTA plasma, by means of high-performance liquid chromatography by using fluorescence detection (Te Poele-Pothoff et al., in press). Folic acid and vitamin B_{12} levels were determined in heparinized plasma, and vitamin B_6 levels were determined in whole blood, by routine hospital assays.

Statistics

Rank-sum two-sample test and the Fisher's exact test (two-tail) were applied. Normal distribution was supposed to be present in case the skewness and kurtosis tests ranged from −1 to +1. Spearman rank correlation was used for determining correlations.

Results

Patients and Controls

Twenty-three healthy subjects were studied to obtain reference values. One subject was excluded from the control group because of thermolabile MTHFR deficiency, with a specific activity of 7.7 nmol CH_2O/mg protein/h and a residual activity after heat inactivation of 15.6%. In the other 22 control subjects a mean (±SD) specific MTHFR activity of 15.6 (±4.7) nmol CH_2O/mg protein/h (range: 9.1–26.6) and a mean (±SD) residual activity after heat inactivation of 55.3 (±12.0)% (range: 35.9–78.3) was observed. In concordance with Kang et al. (1988a, 1988*b*, 1991*b*), thermolabile MTHFR was defined as a specific activity of 50% of the normal mean and a residual activity after heat inactivation of <36.0% of the initial activity. The hyperhomocysteinemic vascular patients (n=39) could be divided into two distinct subgroups (table 1). One group (n=11) with thermolabile MTHFR, showed a mean (±SD) specific activity of ~50% of the normal mean, 8.7 (±2.1) nmol CH_2O/mg protein/h (range: 5.5–12.7), and a residual activity after heat inactivation ranging from 0% to 33.0%. The specific MTHFR activity of the thermolabile group showed a small overlap with the control group, so heat inactivation was employed to discriminate between control subjects and patients with thermolabile MTHFR

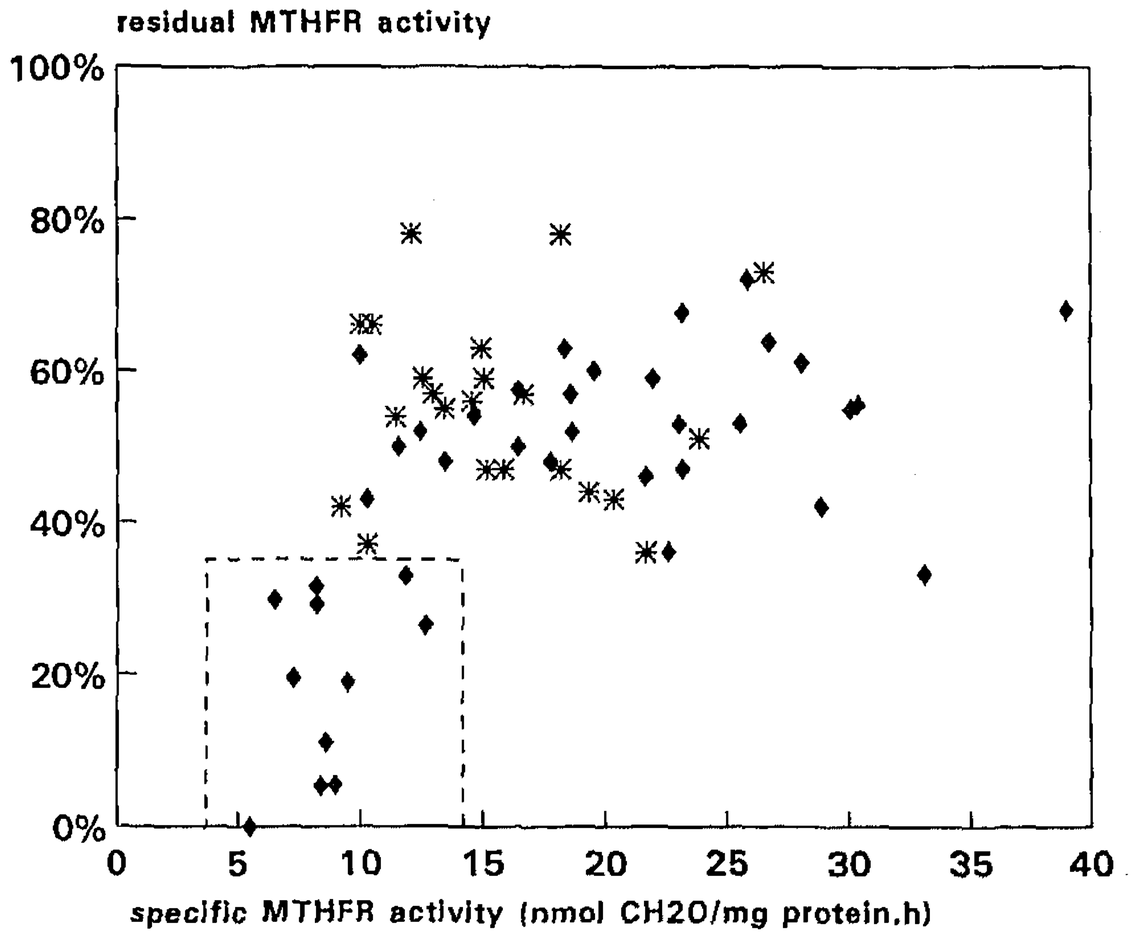

Figure 2 *Residual MTHFR activity after heat inactivation, versus* the specific activity in lymphocytes of hyperhomocysteinemic patients and control subjects. The dashed square contains the patients with thermolabile MTHFR. ✳ = Control subjects; and ♦ = hyperhomocysteinemic vascular patients.

(fig. 2). The other group of hyperhomocysteinemic vascular patients (n=28) had a normal specific activity (±SD) of 21.5 (±7.2) nmol CH_2O/mg protein/h (range: 10.0–39.0) and a residual activity after heat inactivation of 53.8 (±9.2)% (range: 33.1–71.5). The mean (±SD) specific activity of the 29 normohomocysteinemic vascular patients was 20.7 (±6.5) nmol CH_2O/mg protein/h (range: 9.4–33.8), and their mean (±SD) residual activity after heat inactivation was 58.2 (±10.2)% (range: 43.0–82.0). Among the 29 normohomocysteinemic patients, no thermolabile MTHFR activity was observed (table 1), which was significantly different ($P < .002$) from the incidence of 11 cases of thermolabile MTHFR among the 39 hyperhomocysteinemic patients.

Cystathionine β-synthase activity was measured in available fibroblasts of 10 patients with mild hyperhomocysteinemia (patients 3, 10, 13, 16, 18, 20, 23, 25, 27, and 33). Their enzyme activities were, respectively, 5.0, 6.5, 9.5, 7.9, 1.6, 5.8, 3.3, 8.0, 6.4, and 6.0 nmol cystathionine/mg protein/h. (normal range 2.3–18.2 nmol cystathionine/mg protein/h; n = 12). So, only patient number 18 had activity within the range of obligate heterozygotes for cystathionine β-synthase deficiency (0.25–2.4 nmol cystathionine/mg protein/h; n = 13). Only two studied patients (3 and 10) had thermolabile MTHFR, and their cystathionine β-synthase activities were normal.

The 11 patients with thermolabile MTHFR had diverse clinical abnormalities: 4 patients suffered from cerebral artery disease; 2 suffered from peripheral artery disease; 1 suffered from coronary artery disease; and 4 patients had venous thrombosis. Among the patients with thermolabile MTHFR, no correlation was observed between specific MTHFR activity and either fasting total homocysteine or total homocysteine after methionine loading. Among all patients with normal MTHFR activity, age did not correlate with either specific MTHFR activity or residual MTHFR activity after heat inactivation. The mean (±SD) plasma folic acid level of the patients with thermolabile MTHFR (7.9 ± 2.9 nmol/liter) was significantly lower ($P < .0002$) than that of the reference group (13.6 ± 3.8 nmol/liter). However, the plasma folic acid levels of the total group of hyperhomocysteinemic patients (9.2 ± 3.4) were also significantly lower ($P < 0.0001$) than those of the reference group. The mean (±SD) vitamin B_{12} concentration of the thermolabile MTHFR-deficient patients (222 ± 75 pmol/liter) was significantly different ($P = .052$) from that of the reference group (290 ± 115 pmol/liter), but the vitamin B_{12} levels of the total group of hyperhomocysteinemic patients (227 ± 84) were also significantly lower ($P < .02$) than those of the reference group.

Assay

After incubation at 37°C, the formaldehyde produced was derivatized by dimedone and the resulting product, formaldemethone, was extracted with toluene. Addition of carrier formaldehyde increased the amount of labeled formaldemethone and therefore the specific MTHFR activity (fig. 3). Dimedone and formaldehyde react at a ratio of 2:1 (Ruyter 1980). This means that the amount of dimedone must always exceed twice the amount of formaldehyde present. We investigated the derivatization method by optimizing the carrier formaldehyde concentration at a constant dimedone concentration and by optimizing the dimedone concentration at a constant carrier formaldehyde concentration. Optimal derivatization and extraction was achieved by addition of 10 μmol carrier formaldehyde and 50 μmol dimedone.

Three different blanks were tested: (1) the reaction mixture including the enzyme extract and no incubation at 37°C; (2) the reaction mixture incubated at 37°C without the enzyme extract; and (3) the reaction mixture and addition of the enzyme extract after the incubation at 37°C. Blank 1 was inadequate because it resulted in recovery of much less radioactivity than either blank 2 or 3. No difference between blank 2 and 3 was observed (data not shown). Blank 2 was used in this study, because it required no enzyme extract.

MTHFR activity depends on the concentration of the cofactor FAD. A maximum activity was observed at 50 μM FAD. In all assays, 54 μM FAD were used. Formaldehyde production was linear, with incubation time up to 40 min and with amount of enzyme between 10 and 250 μg protein. The incubation time used was 20 min, and the amount of enzyme extract added varied from 22 to 150 μg protein. A (Michaelis constant (K_m) for Me-THF of 19 μM was observed. Overall, the modifications resulted in higher specific MTHFR activities in lymphocytes than were re-

Table I

Sex, Age, Homocysteine Concentrations Fasting and after Loading, and Specific and Residual MTHFR Activity after Heat Inactivation in Patients with Premature Vascular Disease

Patient	Sex	Age[a]	Hcys[b] at 0 h	Hcys[c] at 6 h	Specific MTHFR Activity (nmol CH_2O/mg protein/h)	Residual MTHFR Activity (%)
Hyperhomocysteinemic patients with thermolabile MTHFR:						
1	F	28	20	57	9.5	19.4
2	F	52	13	94	5.5	0
3	F	26	19	51	8.4	5.4
4	F	46	13	54	9.0	5.5
5	F	49	17	57	6.5	29.8
6	M	21	31	76	12.7	26.5
7	F	35	18	67	7.3	19.5
8	M	49	32	68	8.2	31.5
9	F	37	27	52	11.9	33.0
10	F	40	17	88	8.6	11.1
11	F	29	38	92	8.2	29.2
Mean		37.5	22.3	68.7	8.7	19.2
SD		10.6	8.4	16.4	2.1	11.9
Hyperhomocysteinemic patients with normal MTHFR activity:						
12	F	46	10	77	23.1	52.7
13	F	51	11	75	22.6	35.5
14	F	31	27	116	11.6	49.7
15	M	50	20	54	12.5	52.0
16	M	50	21	68	25.9	71.5
17	F	51	14	56	14.7	54.1
18	F	53	nd	75	23.2	67.7
19	M	43	34	71	13.5	47.7
20	F	39	19	50	17.8	48.3
21	F	46	14	63	30.4	55.1
22	F	28	10	54	22.2	59.3
23	F	43	nd	115	18.7	52.2
24	M	29	144	148	10.0	62.0
25	M	40	13	60	26.8	63.4
26	F	33	12	75	30.1	54.6
27	M	42	nd	69	28.1	61.1
28	F	20	30	57	10.3	43.0
29	F	19	16	52	18.6	56.8
30	M	35	26	50	21.7	46.4
31	F	44	15	61	39.0	67.7
32	F	41	13	62	19.6	59.8
33	F	40	nd	84	23.2	47.4
34	M	63	12	61	16.5	49.5
35	M	48	23	52	18.4	63.1
36	F	46	7	59	16.5	57.5
37	F	48	22	74	28.9	42.4
38	F	42	61	145	25.6	52.9
39	F	46	11	67	33.1	33.1
Mean		41.7	24.4	73.2	21.5	53.8
SD		9.9	27.9	26.3	7.2	9.2
Normohomocysteinemic patients:						
40	F	47	13	40	23.7	52.7
41	M	48	15	44	31.3	71.6
42	M	25	14	31	22.1	58.8
43	M	41	11	31	24.6	50.8

(*continued*)

Table 1 (continued)

Patient	Sex	Age[a]	Hcys[b] at 0 h	Hcys[c] at 6 h	Specific MTHFR Activity (nmol CH_2O/mg protein/h)	Residual MTHFR Activity (%)
Normohomocysteinemic patients: (*continued*)						
44	F	47	13	49	30.5	82.0
45	F	27	11	28	14.8	64.9
46	M	55	12	43	19.6	61.2
47	M	56	14	34	14.0	47.1
48	F	37	10	32	11.4	43.0
49	M	53	8.7	25	33.8	76.2
50	M	41	7.5	22	18.1	49.7
51	M	40	13	37	16.0	61.9
52	F	22	10	27	25.8	60.9
53	M	55	13	38	18.9	64.0
54	F	22	11	28	14.8	65.0
55	F	35	7.8	27	20.5	55.0
56	F	52	17	42	13.0	50.0
57	M	46	15	46	20.0	68.7
58	F	48	13	40	23.7	52.7
59	M	39	13	39	9.4	49.2
60	F	46	8.7	21	29.4	67.7
61	F	39	13	33	17.0	43.2
62	F	31	11	32	21.3	69.2
63	M	42	11	31	24.6	50.8
64	M	47	10	32	28.6	46.3
65	F	51	17	42	13.0	50.0
66	F	34	15	41	26.9	66.2
67	M	56	12	43	19.6	61.2
68	M	57	14	34	14.0	47.1
Mean		43.8	12.2	34.9	20.7	58.2
SD		12.5	2.5	7.3	6.5	10.2

[a] Age at the time of this study.
[b] Total homocysteine, fasting. nd = not determined.
[c] Total homocysteine, after methionine loading.

ported elsewhere (Kang et al. 1988*a*, 1988*b*, 1991*a*, 1991*b*).

The thermolability of MTHFR in the enzyme extract of pooled lymphocytes of controls was examined by preincubating for 5 min at 37°C, 40°C, 46°C, and 49°C. Incubation at 49°C resulted in a 70% loss, at 46°C in a 53% loss, and at 40°C in a 17% loss of activity. Preincubation at 46°C resulted in a 77% loss of enzyme activity at 20 min, 53% loss at 10 min, 43% loss at 5 min, and 18% loss of enzyme activity at 2 min (fig. 4). Heat inactivation was performed by incubating the enzyme extract for 5 min at 46°C, thus allowing a reliable distinction between thermolabile and thermostable MTHFR, in patients and control subjects.

Discussion

In this study, the thermolabile form of MTHFR was observed in 11 of 39 premature-vascular-disease patients with mild hyperhomocysteinemia after methionine loading. Nine of these 11 patients were hyperhomocysteinemic in the fasting state. No thermolabile MTHFR was observed among 29 normohomocysteinemic patients with premature vascular disease, and of 23 healthy subjects 1 case had thermolabile MTHFR. These findings indicate that thermolabile MTHFR is one of the causes leading to mild hyperhomocysteinemia, established by methionine loading, in patients with vascular disease.

S-adenosylmethionine is a major regulating compound in homocysteine metabolism because it activates cystathionine β-synthase and inhibits MTHFR (Finkelstein 1990). Plasma homocysteine concentration is supposed to reflect the capacity of homocysteine remethylation in the fasting state because of the low S-adenosylmethionine levels, whereas the homocysteine concentration after oral loading with unphysiologic, high amounts of methionine is be-

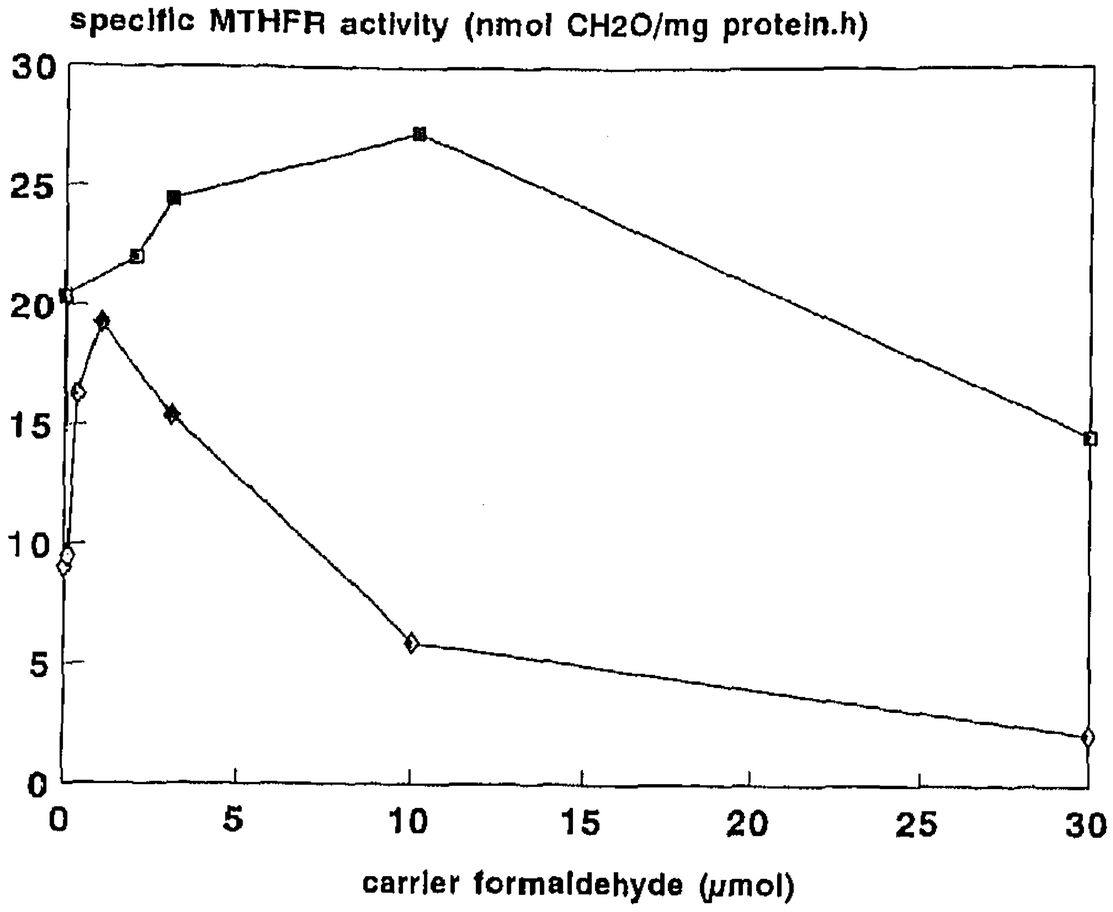

Figure 3 Effect of variation of the amount of carrier formaldehyde on the derivatization of labeled formaldehyde with dimedone at two different concentrations. ◇ = 6 μmol dimedone; and ⊡ = 50 μmol dimedone.

lieved to evaluate the cystathionine β-synthase status (Kang et al. 1992; Ueland et al. 1992). However, in the present study, all vascular patients with the thermolabile form of MTHFR had elevated homocysteine levels after loading and not always in the fasting state. Probably, the folate-dependent homocysteine remethylation contributes to homocysteine conversion not only in the fasting state but also after methionine loading.

Cystathionine β-synthase activity was assayed in 10 of the 39 vascular patients with mild hyperhomocysteinemia, including two patients (3 and 10) with thermolabile MTHFR. All except one (patient 18) had normal cystathionine β-synthase activity. This indicates that decreased cystathionine β-synthase activity is not the major cause of hyperhomocysteinemia in patients with premature vascular disease.

Kang et al. (1991*b*) observed an incidence of 17% of thermolabile MTHFR in a group of 212 patients with coronary arterial disease. Their homocysteine metabolism was not examined by means of a methionine-loading test. In a previous study (Franken et al. 1994) we observed, in a large group of patients with diverse forms of vascular disease, a prevalence of hyperhomocysteinemia of 24% by using methionine-loading tests. The present paper shows that ~28% of such hyperhomocysteinemic patients have the thermolabile form of MTHFR. Thus, an incidence of thermolabile MTHFR of 7% among our total group of vascular patients can be calculated, which is lower than what Kang et al. (1991*b*) observed among coronary patients.

The hyperhomocysteinemic patient group consisted of more female patients than male, compared with the normohomocysteinemic patient group. Hyperhomocysteinemic women may be more susceptible to developing vascular disease than are hyperhomocysteinemic men (Franken et al. 1994). The age at onset of vascular disease among the thermolabile MTHFR–deficient patients varied from 18 to 50 years. The clinical expression of vascular disease among the 11 patients with thermolabile MTHFR was very diverse, including cerebral, peripheral, and coronary arterial disease and venous thrombosis. Therefore, thermolabile MTHFR is a risk factor not only for coronary arterial disease, as reported by Kang et al. (1988*a*; 1991*b*), but probably for vascular disease in general.

An incidence of the homozygous form of thermolabile MTHFR of 5% in the normal population is reported (Kang et al. 1988*a*, 1991*b*). This is consistent with our finding of 1 thermolabile MTHFR–deficient subject among 23 healthy subjects. Thus, the heterozygous form must have

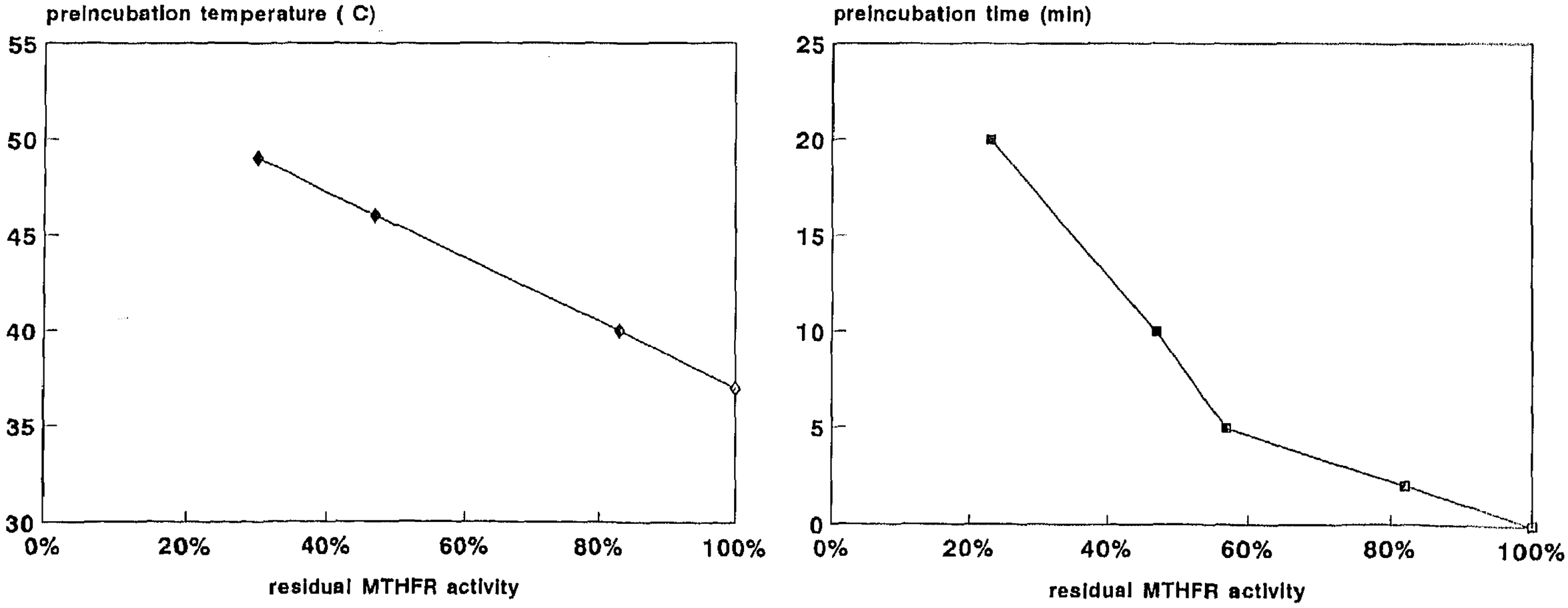

Figure 4 *Left panel,* Effect of preincubation for 5 min at different temperatures on the residual MTHFR activity (◇). *Right panel,* Effect of variation of preincubation time at 46°C on the residual MTHFR activity (⊡).

an incidence of ~22% in the normal population. Such a high frequency of this thermolabile MTHFR mutation might be explained by a concomitant beneficial effect of the mutation. Perhaps, in times of starvation, a reduced MTHFR activity decreases homocysteine remethylation and preserves the available one-carbon moieties of the THF derivatives for the vital synthesis of purines and thymidine.

Severe MTHFR deficiency is very resistant to many homocysteine-lowering and methionine-elevating forms of therapy, including folates, methionine, pyridoxine, vitamin B_{12}, and carnitine (Wada et al 1978; Harpey et al. 1981; Rosenblatt 1989). Betaine appears the best choice of therapy (Benevenga 1984; Wendel and Bremer 1984; Hyland et al 1988; Rosenblatt 1989). It enhances the remethylation of homocysteine to methionine via an alternative pathway (fig. 1), and methionine levels increase at the expense of homocysteine. Logical choices of therapy for thermolabile MTHFR-deficient patients seem to be folic acid and betaine. Riboflavin could also be an appropriate option, since FAD is the cofactor of MTHFR and may stabilize the mutant MTHFR. Two patients with thermolabile MTHFR and mild hyperhomocysteinemia showed a dramatic decrease of total homocysteine concentration after folic acid therapy (Kang et al. 1988*b*). Folic acid administration may increase the concentrations of methylene-THF which is the substrate of MTHFR.

On the relationship between vascular disease and mild hyperhomocysteinemia due to a 50% reduction in MTHFR activity the following considerations can be made: (i) In the vascular wall THF-dependent remethylation constitutes the primary mechanism for homocysteine conversion. The betaine-homocysteine methyltransferase is present only in the liver and maybe in the kidney, and the K_m of cystathionine β-synthase is >10 times higher than that of the enzymes involved in THF-dependent homocysteine remethylation (Finkelstein 1990). (ii) Mild hyperhomocysteinemia is likely caused by a combination of genetic and environmental factors, including methionine, betaine, choline, folate, vitamin B_6, and B_{12}. (iii) In addition, although thermolabile MTHFR and hyperhomocysteinemia are correlated, and hyperhomocysteinemia is a risk factor for vascular disease, some other effect of thermolabile MTHFR, besides its resultant hyperhomocysteinemia, may put patients at risk for vascular disease. In conclusion, thermolabile MTHFR is the most likely cause of abnormal homocysteine metabolism in ~28% of the vascular patients with mild hyperhomocysteinemia.

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References

Benevenga NJ (1984) Betaine in treatment of homocystinuria. N Engl J Med 310:265–266

Blom HJ, VandeMolen E (1994) Pathobiochemical implications of hyperhomocysteinemia. Fibrinolysis 8, Suppl 2:86–87

Boers GHJ, Fowler B, Smals AGH, Trijbels JMF, Leermakers AI, Kleijer WJ, Kloppenborg PWC (1985*a*) Improved identification of heterozygotes for homocystinuria due to cystathionine synthase deficiency by the combination of methionine loading and enzyme determination in cultured fibroblasts. Hum Genet 69:164–169

Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, et al (1985*b*) Heterozygosity for homocystinuria in peripheral and cerebral occlusive arterial disease. N Engl J Med 313:709–715

Brattström L, Lindgren A (1992) Hyperhomocysteinemia as a risk factor for stroke. Neurol Res 14:81–84

Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I (1991) Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 324:1149–1155

Daly L, Robinson K, Tan KS, Graham I (1993) Hyperhomocysteinemia: a metabolic risk factor for coronary heart disease determined by both genetic and environmental influences? Q J Med 86: 685–689

Finkelstein JD (1990) Methionine metabolism in mammals. J Nutr 1:228–237

Fowler B, Kraus J, Packman S, Rosenberg LE (1978) Evidence for three distinct classes of cystathionine β-synthase mutants in cultured fibroblasts. J Clin Invest 61:645–653

Franken DG, Boers GHJ, Blom HJ, Trijbels JMF, Kloppenborg PWC (1994) Treatment of mild hyperhomocysteinemia in vascular disease patients. Arterioscler Thromb 14:465–470

Harpey JP, Rosenblatt DS, Cooper BA, Le Moël G, Roy C, Lafourcade J (1981) Homocystinuria caused by 5,10-methylenetetrahydrofolate reductase deficiency: a case in an infant responding to methionine, folinic acid, pyridoxine and vitamin B_{12} therapy. J Pediatr 98:275–278

Hyland K, Smith I, Bottiglieri T, Perry J, Wendel U, Clayton PT, Leonard JV (1988) Demyelination and decreased S-adenosylmethionine in 5,10-methylenetetrahydrofolate reductase deficiency. Neurology 38:459–462

Jondal M, Holm G, Wigzell H (1972) Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. J Exp Med 136:207–215

Kang SS, Wong PWK, Bock HGO, Horwitz A, Grix A (1991*a*) Intermediate hyperhomocysteinemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. Am J Hum Genet 48:546–551

Kang SS, Wong PWK, Malinow MR (1992) Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 12: 279–298

Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N (1991*b*) Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 48:536–545

Kang SS, Wong PWK, Zhou J, Sora J, Lessick M, Ruggie N, Grcevich G (1988*a*) Thermolabile methylenetetra-hydrofolate reductase in patients with coronary artery disease. Metabolism 37:611–613

Kang SS, Zhou J, Wong PWK, Kowalisyn J, Strokosch G (1988*b*) Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet 43: 414–421

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275

Mudd SH, Havlik R, Levy HL, McKusick VA, Feinleib M (1981) A study of cardiovascular risk in heterozygotes for homocystinuria. Am J Hum Genet 33:883–893

Rosenblatt DS (1989) Inherited disorders of folate transport and metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, 6th ed. McGraw-Hill, New York, pp 2049–2064

Rosenblatt DS, Erbe RW (1977) Methylenetetrahydrofolate reductase in cultured human cells. II. Genetic and biochemical studies of methylenetetrahydrofolate reductase deficiency. Pediatr Res 11:1141–1143

Ruyter IE (1980) Release of formaldehyde from denture base polymers. Acta Odontol Scand 38:17–27

Te Poele-Pothoff MTWB, VandeBerg M, Franken DG, Boers GHJ, Jakobs C, De Kroon IFI, Eskes TKAB, et al. Three different methods for the determination of total homocysteine in plasma. Ann Clin Biochem (in press)

Ueland PM, Refsum H, Brattström L (1992) Plasma homocysteine and cardiovascular disease. In: Francis RB (ed) Atherosclerotic cardiovascular disease, hemostasis and endothelial function. Marcel Dekker, New York, pp 183–236

Wada Y, Narisawa K, Arakawa T (1978) Infantile type of homocystinuria with 5,10-methylenetetrahydrofolate reductase deficiency. Monogr Hum Genet 9:140–146

Wendel U, Bremer HJ (1984) Betaine in treatment of homocystinuria due to 5,10-methylenetetrahydrofolate reductase deficiency. Eur J Pediatr 142:147–150

Wendel U, Claussen U, Diekmann E (1983) Prenatal diagnosis for methylenetetrahydrofolate reductase deficiency. J Pediatr 102: 938–940