

## 5,10-Methylenetetrahydrofolate Reductase (MTHFR) Assay in the Forward Direction: Residual Activity in MTHFR Deficiency

TERTTU SUORMALA,\* GERTRAUD GAMSE, and BRIAN FOWLER

**Background:** Assay of methylenetetrahydrofolate reductase (MTHFR), a key enzyme in homocysteine metabolism, is important for the study of severe and mild deficiency states. Because the conventional assay measures in the reverse direction, lacks sensitivity, and uses nonphysiologic substrates, the exact measurement and characterization of residual activity in easily accessible tissues have been difficult.

**Methods:** To measure MTHFR in the physiologic direction, we determined the NADPH-dependent conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate by use of HPLC with fluorescence detection.

**Results:** MTHFR activity in control fibroblast in the presence of FAD was maximal between pH 6.3 and 6.9, increased linearly up to 40 min and 80  $\mu\text{g}$  protein/assay, and showed  $K_m$ s of 30  $\mu\text{mol/L}$  for NADPH and 26  $\mu\text{mol/L}$  for 5,10-methylenetetrahydrofolate. Intraassay variation (CV) was 10%, interassay variation was 7.2%, and variation among 10 subcultures of the same cell line was 18%. Mean (SD) control activity was 431 (150)  $\mu\text{U/mg}$  protein (range, 242–910;  $n = 75$ ), which is 2.5-fold higher than that with the reverse assay. After heat treatment (46 °C for 5 min), the activity showed a trimodal distribution corresponding to the 677TT (thermolabile; 15%), 677CT (35%), and 677CC (51%) genotypes. We found clearly measurable activity ranging from 2.6% to 25.6% of the mean control value in 15 patients with MTHFR deficiency, including 11 cell lines with zero activity in the reverse assay. Ten patients had complete enzyme deficiency.

**Conclusion:** This assay allows reliable determination of residual activity in mutant fibroblasts and characterization of kinetic parameters for natural substrates.

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Interest in disorders of homocysteine metabolism has increased since moderate increases in homocysteine have been identified as a risk factor for vascular disease along with rare inborn errors attributable to severe deficiencies of cystathionine- $\beta$ -synthase, 5-methyltetrahydrofolate/homocysteine methyltransferase, and 5,10-methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20). MTHFR is a flavoprotein that catalyzes the NADPH-dependent conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Methyltetrahydrofolate is the most common form of folate in plasma and tissues and serves as the methyl group donor in the 5-methyltetrahydrofolate/homocysteine methyltransferase-catalyzed remethylation of homocysteine to methionine.

Severe MTHFR deficiency (McKusick 236250), defined as residual activity of <20% of the mean control value (1), is a rare inborn error of folate metabolism, biochemically characterized by hyperhomocysteinemia, homocystinuria, and low plasma methionine. The clinical presentation is extremely variable, with time of onset varying from the neonatal period to adulthood. Symptoms include neurologic abnormalities, mental retardation, and premature vascular disease. A few cases have responded to high folate treatment (1), but most show poor long-term outcome despite homocysteine lowering with, for example, betaine and methionine substitution.

Homozygosity for a common polymorphism (677C $\rightarrow$ T mutation) causes thermolability of MTHFR (2, 3) and leads to mild hyperhomocysteinemia, particularly when folate intake is low. The incidence is 4–18%, depending on the population (4). Conflicting reports exist concerning the correlation between this mutation and risk for cardiovascular disease, although increased homocysteine itself is a well-known independent risk factor for various forms

University Children's Hospital, CH-4005 Basel, Switzerland.

This study was presented as a poster at the 37th Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, September 7–10, 1999, Genoa, Italy.

\*Address correspondence to this author at: Enzyme Laboratory, University Children's Hospital, Basel, Römeggasse 8, CH-4005 Basel, Switzerland. Fax 41-61-685-6556; e-mail terttu.suormala@unibas.ch.

Received in revised form January 15, 2002; accepted March 8, 2002.

of vascular disease (5). Individuals with the 677TT genotype show decreased MTHFR activity (25% of mean activity of wild-type 677CC genotype) in lymphocytes (6). A second common polymorphism (1298A→C mutation) can also lead to lower MTHFR activity in lymphocytes (61% of mean activity of the wild-type 1298AA genotype) (6). The incidence of this polymorphism is ~10% (7). It does not cause thermolability of the enzyme and seems not to cause hyperhomocysteinemia (6, 7).

In MTHFR deficiency, the severity of the illness seems to correlate with the residual enzyme activity (1), underlying the need for a sensitive enzyme assay. The MTHFR reaction is irreversible *in vivo*, but its activity can be measured in the reverse direction *in vitro* (8). Thus the conventional assay that has been applied to patient studies measures activity in its reverse direction, using [<sup>14</sup>C]methyltetrahydrofolate as substrate and menadione as electron acceptor (9). This assay utilizes organic solvent extraction with incomplete recovery and uncertain specificity, and high and variable blank values attributable to impurities in the substrate and dependence of the blank value on protein concentration, all of which make accurate measurement of low residual activity difficult.

Assays in the physiologic direction have also been described. These include spectrophotometric assays based on the detection of the oxidation of NADPH (10, 11), radiometric assays (11, 12), and recently, an assay with direct HPLC separation of nonlabeled product (13). However, the colorimetric methods have been used mainly for the assay of purified pig liver enzyme or liver extracts and lack the sensitivity needed for assays in cultured cells. The radiometric assays require isolation of [<sup>14</sup>C]methyltetrahydrofolate, which is not commercially available and is therefore unsuitable for routine assay. The HPLC method is applied to crude liver extracts and depends on *in situ* generation of the substrate 5,10-methylenetetrahydrofolate from tetrahydrofolate in the presence of formaldehyde, rendering precise determination of the kinetic parameters for the natural substrates difficult. The aim of this study was to develop a sensitive physiologic assay for MTHFR activity that uses the natural substrates 5,10-methylenetetrahydrofolate and NADPH and is suitable for patient studies using cultured fibroblasts. The described assay allows the precise measurement of low residual activity and the determination of kinetic parameters for the natural substrates. The activities obtained in MTHFR-deficient patients and controls were compared with those obtained in earlier diagnostic studies using the reverse assay.

## Materials and Methods

### FIBROBLAST CULTURES

Skin fibroblasts from 25 patients with clinical and biochemical evidence of MTHFR deficiency, obtained for diagnostic studies over the past 15 years, were studied. Fibroblasts from patients were provided by the following individuals: patient 1 by Dr. E. Wraith (Manchester,

United Kingdom); patient 2 by Dr. D. Perrett (London, United Kingdom); patients 3, 22, and 25 by Dr. G. Besley (Manchester, United Kingdom); patients 4 and 5 (siblings) by Dr. J. Kirk (Edinburgh, United Kingdom); patient 6 by Dr. J.R. Moore (Leicester, United Kingdom); patient 7 by Dr. G. Gray (Birmingham, United Kingdom); patient 8 by Dr. H. Mandel (Haifa, Israel); patient 9 by Dr. U. Vetter (Berlin, Germany); patient 10 by Dr. H. G. Koch (Münster, Germany); patient 11 by Dr. R. Surtees (London, United Kingdom); patient 12 by Dr. R. Parini (Milan, Italy); patient 13 by Dr. G. Visser (Groningen, The Netherlands); patient 14 by Dr. G. Taddeuci (Pisa, Italy); patient 15 by Dr. J. Leonard (London, United Kingdom); patient 16 by Dr. A. Ribes (Barcelona, Spain); patient 17 by Dr. D. Burke (London, United Kingdom); patient 18 by Dr. R. Smith (Bradford, United Kingdom); patient 19 by Dr. H. Bakker (Amsterdam, The Netherlands) with clinical and biochemical data reported earlier (14); patient 20 by Prof. B. Steinmann (Zurich, Switzerland); patient 21 by Dr. R. Cerone (Genoa, Italy); and patients 23 and 24 (siblings) by Dr. A. Kohlschütter (Hamburg, Germany). Criteria for the severity of clinical presentation (Table 1) were as follows: (a) severe, with early onset and/or death before 2 years of age; (b) moderate, with onset after 2 years of age or death before adulthood; and (c) mild, with late onset at 12 years or later.

Control fibroblasts (75 cell lines) were obtained from 23 individuals undergoing surgery or with a nonmetabolic disease, 30 individuals with a suspected metabolic disorder other than homocystinuria (e.g., disorders of biotin or methylmalonic acid related metabolism, or thiamin transport) or a suspected remethylation defect, but in whom such defects were excluded by fibroblast assays; and 22 individuals with a confirmed inherited disorder other than homocystinuria (8 with 3-methylcrotonyl-CoA carboxylase, 2 with propionyl-CoA carboxylase, 2 with holocarboxylase synthetase, and 1 with  $\Delta^1$ -pyrroline-5-carboxylase synthase deficiency; 6 with methylmalonic aciduria attributable to the mut<sup>o</sup> and 2 attributable to the cblA defect; and 1 with a defect in collagen synthesis).

Fibroblasts were grown in Earl's minimal essential medium (Life Technologies) supplemented with 100 mL/L heat-inactivated fetal calf serum (Amimed<sup>®</sup>), 2 mmol/L L-glutamine, and 10 mL/L antibiotic-antimycotic (Life Technologies; final concentrations in medium, 100 000 U/L penicillin G sodium, 100 mg/L streptomycin sulfate, and 250  $\mu$ g/L amphotericin B). Cultures were grown in 75-cm<sup>2</sup> disposable culture flasks (Nunc) and maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub>-95% air with a relative humidity of 98%. The cells were harvested with trypsin (2.5 g/L, Bacto<sup>®</sup> Trypsin; Difco Laboratories) at least 3 days after reaching confluence as judged by microscopic examination, washed twice with isotonic phosphate-buffered saline (Life Technologies), and stored frozen at -75 °C or processed immediately.

**Table 1. Comparison of fibroblast MTHFR activities measured by the physiologic and reverse assays in MTHFR-deficient patients and controls.**

Clinical presentation		Activity, $\mu\text{U}/\text{mg protein}^a$							
		Physiologic assay				Reverse assay			
		-FAD		+FAD		-FAD		+FAD	
		Single values <sup>b</sup>	Mean	Single values	Mean	Single Values	Mean	Single values	Mean
Control values		421 $\pm$ 148 (n = 75) <sup>c</sup>		431 $\pm$ 150 (n = 75)		140 $\pm$ 67 (n = 51)		172 $\pm$ 83 (n = 51)	
Mean $\pm$ SD		384		395		122		150	
Median		230-884		242-911		47-328		53-382	
Range									
Patients									
1	Mild	93, 105, 125	108	97, 108, 125	110	0, 2.0	1.0	1.7	1.7
2		77, 87	82	87, 102	94	0	0	8.7	8.7
3		70, 80	75	68, 82	75	22	22	43	43
4 <sup>d</sup>	Mild	48, 67, 75	63	52, 75, 80	69	6.7, 13, 22	14	8.3, 20, 38	22
5 <sup>d</sup>	Mild	30, 40	35	30, 38	34	13, 18, 27	19	13, 27, 38	26
6	Mild	35, 37, 42	38	40, 43, 60	48	0, 0	0	0, 6.7	3.3
7	Moderate	25, 32	28	25, 33	29	12, 15	13	33, 33	33
8	Severe	20, 27	23	23, 28	26	0, 5.7	2.8	0, 8.7	4.3
9		15, 17, 28	20	33, 42, 60	45	0	0	2.2, 5.3	3.8
10	Moderate	11, 14, 18	14	12, 16, 20	16	1.0	1.0	4.2	4.2
11	Moderate	14, 15	14	17, 17	17	0	0	0	0
12	Severe	14, 15	14	14, 16	15	0	0	0.83	0.83
13	Severe	13, 15	14	15, 16	16	0	0	0	0
14 <sup>e</sup>	Moderate	12, 14, 15	14	14, 15, 18	16	0	0	0	0
15	Moderate	8.3, 10, 16	11	7.2, 11, 16	11	0	0	0, 0	0
16	Severe	2.8, 3.3, 7.2	4.4	2.7, 4.3, 6.5	4.5	0	0	0	0
17		4.0, 4.8	4.4	4.0, 4.3	4.2	0	0	0	0
18		2.3, 4.7	3.5	2.5, 2.5	2.5	0, 14	7.0	0, 16	8.0
19	Severe	2.8, 3.7	3.3	1.2, 3.8	2.5	0	0	0	0
20	Severe	3.0, 3.2	3.1	4.0, 6.0	5.0	0	0	0	0
21		1.8, 4.0	2.9	1.5, 4.2	2.8	0	0	0	0
22	Severe	2.0, 2.8	2.4	1.5, 4.3	2.9	0	0	0	0
23 <sup>f</sup>		1.7, 2.7	2.2	2.0, 2.2	2.1	0, 0	0	0, 0	0
24 <sup>f</sup>		0.33, 0.67	0.50	0.83, 2.0	1.4	0, 5.5	2.7	0, 9.3	4.7
25	Severe	0.17, 1.2	0.67	0, 1.3	0.67	0	0	0	0

<sup>a</sup>  $\mu\text{U}$  = pmol/min.<sup>b</sup> For patients, single values are the means of duplicate determinations within one experiment.<sup>c</sup> Number of different cell lines.<sup>d</sup> Patients 4 and 5 are siblings.<sup>e</sup> Clinical and other biochemical data for this patient are reported in Abeling et al. (14).<sup>f</sup> Patients 23 and 24 are siblings.

#### PREPARATION OF ENZYME EXTRACTS

Cell pellets were suspended in ice-cold Lubrol (1.5 g/L; Sigma) in 0.01 mol/L potassium phosphate buffer (pH 6.3 for the reverse assay, pH 6.6 for the physiologic assay), and allowed to stand for 30 min on ice. The cell suspensions were centrifuged for 10 min at 16 000g at 4 °C. The supernatant was used as the source of the enzyme. The protein concentrations in the cell extracts were determined by the Lowry method.

#### REVERSE ASSAY

For the reverse assay of MTHFR activity, the method described by Rosenblatt and Erbe (9) was used with slight modifications (15). The assay mixture contained, in a final

volume of 100  $\mu\text{L}$ : 0.18 mol/L potassium phosphate buffer (pH 6.3), 0.114 mmol/L 5-methyltetrahydrofolate (Sigma), 0.087  $\mu\text{Ci}$  of 5-[<sup>14</sup>C]methyltetrahydrofolate (Amersham Pharmacia Biotech), 3.55 mmol/L menadione, and cell extract containing 75–225  $\mu\text{g}$  of protein. Both 5-methyltetrahydrofolate solutions were prepared in 33 mmol/L dithiothreitol to prevent oxidation during storage of stock solutions at -20 °C.

To decrease the blank value a portion of 5-[<sup>14</sup>C]methyltetrahydrofolate was purified just before use by treatment with dimedone and extraction with toluene. The assay was performed in duplicate with and without 72  $\mu\text{mol/L}$  FAD in the assay mixture. For blanks, the enzyme extract was replaced with 150  $\mu\text{g}$  of bovine serum

albumin. After 60 min at 37 °C, the reaction was stopped by the addition of 175  $\mu$ L of ice-cold 0.35 mol/L sodium acetate containing 0.02 mol/L formaldehyde (pH 4.5), and the assay tubes were placed on ice. Seventy-five microliters of 0.4 mol/L dimedone in 500 mL/L ethanol was added, and the samples were heated at 80 °C for 7.5 min to release [ $^{14}$ C]formaldehyde, which forms a complex with dimedone; this formaldehyde-dimedone complex was extracted with toluene. The radioactivity in the toluene extract was quantified by scintillation counting in a Packard Tricarb Model C1900 scintillation spectrometer.

#### PHYSIOLOGIC (FORWARD) ASSAY

The physiologic assay for MTHFR was based on the spectrophotometric assay described by Matthews and Baugh (10). The standard assay mixture contained, in a final volume of 100  $\mu$ L: 0.05 mol/L potassium phosphate buffer, pH 6.6, 100  $\mu$ mol/L (6*R*)-5,10-methylenetetrahydrofolate (Eprova AG; specified purity, 91%); 200  $\mu$ mol/L  $\beta$ -NADPH (Sigma), and enzyme extract containing 10–70  $\mu$ g of protein. The assay was performed in duplicate with and without 75  $\mu$ mol/L FAD in the assay mixture. Tenfold concentrated stock solutions of FAD and NADPH in doubly distilled water (stable for at least 2 months), and 5,10-methylenetetrahydrofolate in 0.5 mmol/L dithiothreitol (stable for at least 10 months) were stored at –20 °C. Unless otherwise stated, the assay time was 20 min at 37 °C. The reaction was terminated by the addition of 50  $\mu$ L of 50 mL/L HClO<sub>4</sub> in 10 g/L ascorbic acid, and the samples were mixed and placed on ice. For blanks, the enzyme extract was added after addition of the stop solution. The  $K_m$  for 5,10-methylenetetrahydrofolate was determined by varying its concentration between 2.5 and 200  $\mu$ mol/L in the presence of 200  $\mu$ mol/L NADPH. Blanks were prepared for each 5,10-methylenetetrahydrofolate concentration by replacing the enzyme extract with 50  $\mu$ g of bovine serum albumin in each assay. The  $K_m$  for NADPH was determined by varying its concentration between 10 and 250  $\mu$ mol/L in the presence of 100  $\mu$ mol/L 5,10-methylenetetrahydrofolate. NADPH was omitted in the blanks.

Although 5-methyltetrahydrofolate has to be protected from light during long-term storage, this was not necessary during the assay as well as during preparation of samples for HPLC separation in the dark. When samples were not processed immediately, they were stored at –20 °C in the dark; they remained stable under these conditions for at least 2 weeks.

#### DETERMINATION OF THERMOLABILITY BY THE PHYSIOLOGIC ASSAY

Duplicate assay mixtures containing 10  $\mu$ L of cell extract (10–70  $\mu$ g of protein), 10  $\mu$ L of 0.5 mol/L potassium phosphate buffer, pH 6.6, and either 50  $\mu$ L (assay with FAD) or 60  $\mu$ L (assay without FAD) of doubly distilled water were incubated at 46 °C for 5 min and cooled in

ice-cold water. FAD was added where indicated, and the assay was started with 20  $\mu$ L of a mixture of the two substrates.

#### HPLC SEPARATION

The reaction product, 5-methyltetrahydrofolate, was separated from the substrate 5,10-methylenetetrahydrofolate by a modified HPLC method with fluorescence detection as described previously (15, 16). Samples were thawed, mixed carefully, centrifuged for 2 min at 20 000g in an Eppendorf centrifuge, and diluted with 5 g/L ascorbic acid either 1 in 2.5 (all blank and patient samples) or up to 1 in 10 (control samples), depending on the expected activity. HPLC separation was performed with a Jasco pump (JA-980), a Uniflow 4-channel degasser, and a Basic-Marathon autosampler with cooling. Compounds were detected with a Linear Instrument fluorescence detector (LC 304). A 4.6  $\times$  250 mm Nucleosil 120 C<sub>18</sub> (5  $\mu$ m) main column and a 4.6  $\times$  20 mm guard column filled with the same packing material were used. The mobile phase was 55 mL/L acetonitrile (Uvasol grade; Merck) in 0.033 mmol/L orthophosphoric acid adjusted to pH 2.33 with 6 mol/L NaOH. The flow rate was 1.5 mL/min, the injection volume was 50  $\mu$ L, and 5-methyltetrahydrofolate was detected at 360 nm (emission) with excitation at 296 nm. A three-point calibration curve was constructed using 5-methyltetrahydrofolate (Fluka Chemie AG) calibrator at concentrations of 3, 6, and 12 pmol/injection. Peak areas were integrated by AXIOM software package series 747/MK2 and converted to concentrations by linear regression analysis.

Typical chromatograms with a blank and fibroblast extract of a patient with 49.8  $\mu$ g of protein/assay and MTHFR activity corresponding to 8.6% of mean control value are shown in Fig. 1. Both samples were diluted 1 in 2.5 before injection. The retention time of the product 5-methyltetrahydrofolate was 21 min (Fig. 1, peak C). The large peak at 16 min Fig. 1 (peak A) is the substrate 5,10-methylenetetrahydrofolate, and the peak at 19 min in Fig. 1 (peak B) is an unknown compound originating from the enzyme extract.

#### DETERMINATION OF THE 677C→T GENOTYPE

Genomic DNA was extracted from whole-blood samples (33 controls) and from cultured skin fibroblasts (43 controls and 15 patients) with the QIAamp<sup>®</sup> DNA Midi Kit for blood and Mini Kit (Qiagen AG) for fibroblasts according to the manufacturer's protocols. PCR amplification was performed using 100 ng of DNA, the ABI PRISM True Allele<sup>™</sup> PCR Premix system (PE Applied Biosystems), and primers described by Frosst et al. (3) in a Perkin-Elmer Amp PCR System 2400 apparatus. The PCR products of 7 controls and 2 patients were analyzed by sequencing in a ABI PRISM<sup>™</sup> Genetic Analyzer, and those of 69 controls and 13 patients were analyzed by restriction fragment length polymorphism analysis using

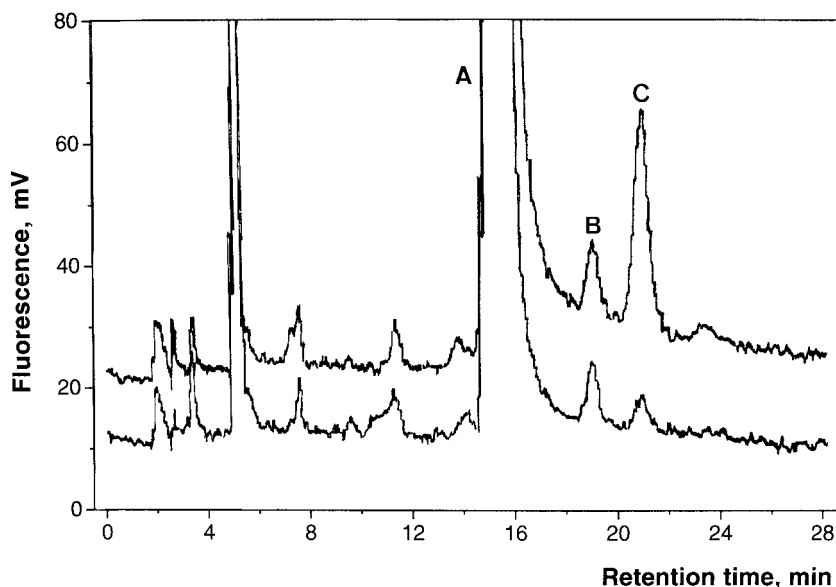


Fig. 1. HPLC chromatograms of assay of fibroblast extract (upper chromatogram) and blank (lower chromatogram).

Patient fibroblast extract with 8.6% residual activity (49.8  $\mu\text{g}$  protein/assay) and blank were assayed and diluted 1:2.5 for chromatography. Peaks: A, the substrate 5,10-methylenetetrahydrofolate; B, unknown compound originating from the enzyme extract; C, the product 5-methyltetrahydrofolate. Peak C in the blank originates from a minor impurity in the substrate corresponding to 9.4 pmol/assay.

*HinfI* (Roche Diagnostic GmbH) digestion and 3% agarose gels as described previously (17).

### Results and Discussion

#### CHARACTERIZATION OF THE PHYSIOLOGIC ASSAY IN EXTRACTS OF CONTROL FIBROBLASTS

**pH dependence.** Assay of control cells at pH values between 5.8 and 7.4 in 50 mmol/L potassium phosphate buffer revealed maximum activity between pH 6.3 and 6.9, which is slightly higher than that for the reverse assay (pH 6.3). On the basis of these findings, a buffer pH of 6.6 was selected for the standard physiologic assay. This compares with a pH of 6.7 reported for an assay using the pig liver enzyme with colorimetric determination in the physiologic direction (10).

**Time and protein concentration dependence.** The assay was linear with an incubation time of up to 40 min with 51  $\mu\text{g}$  of protein/assay and with a protein content of up to 83  $\mu\text{g}$  with an assay time of 20 min (results not shown). For the standard assay, an incubation time of 20 min and a protein content of 10–70  $\mu\text{g}$ /assay were chosen. This protein concentration was obtained by suspending fibroblasts from one 75-cm<sup>2</sup> culture flask in 50–200  $\mu\text{L}$  of lysis buffer.

**Blank value.** A small peak that eluted in the position of 5-methyltetrahydrofolate was present in blank assays and originated from a minor impurity in the substrate 5,10-methylenetetrahydrofolate. This peak corresponded up to a maximum of 12 pmol/assay compared with 340 pmol/assay for the mean control activity. In contrast, in the reverse assay, blank values were dependent on the protein concentration and were much higher in spite of the purification step performed just before the incubation, i.e., the cpm values in the blank were  $\sim 1000$  cpm compared with  $\sim 3000$  cpm for the mean control activity.

**Dependence on 5,10-methylenetetrahydrofolate and NADPH concentrations.** Shown in Figs. 2 and 3 are the Michaelis-Menten kinetics and Eadie-Hofstee plots (insets) for the dependence of MTHFR activity on the concentrations of 5,10-methylenetetrahydrofolate and NADPH, respectively, in the fibroblast extract of a representative control. The  $K_m$ s obtained for (6R)-5,10-methylenetetrahydrofolate (mean  $\pm$  SD, 26.0  $\pm$  5.6  $\mu\text{mol/L}$ ; range, 15.1–39.0  $\mu\text{mol/L}$ ) and NADPH (29.7  $\pm$  6.4  $\mu\text{mol/L}$ ; range, 15.8–39.1  $\mu\text{mol/L}$ ) in crude fibroblast extracts of 16 controls were similar to those obtained by Matthews and Kaufman (18)

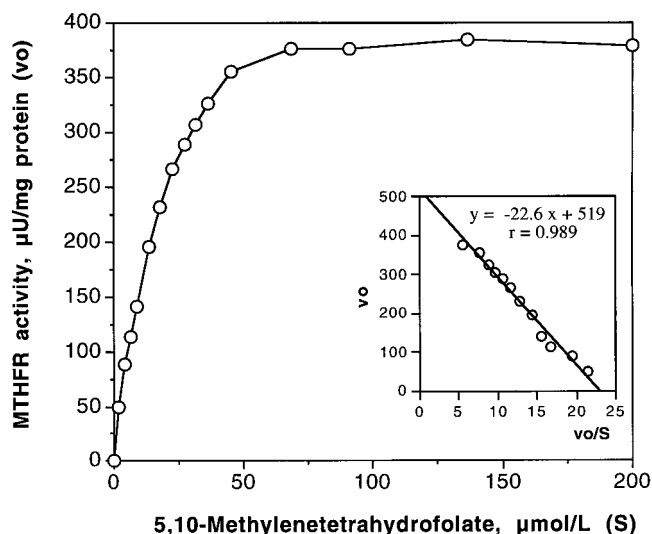


Fig. 2. Dependence of MTHFR activity on concentration of 5,10-methylenetetrahydrofolate measured with the physiologic assay.

Representative experiment with control fibroblast extract with a protein concentration of 49.4  $\mu\text{g}$ /assay and 20-min assay time. Each symbol represents a single determination of activity with 5,10-methylenetetrahydrofolate concentrations of 2.3–200  $\mu\text{mol/L}$  and 200  $\mu\text{mol/L}$  NADPH. The inset shows Eadie-Hofstee plot of the results.  $\mu\text{U}$  = pmol/min;  $v_0$ , initial rate of reaction; S, substrate concentration.

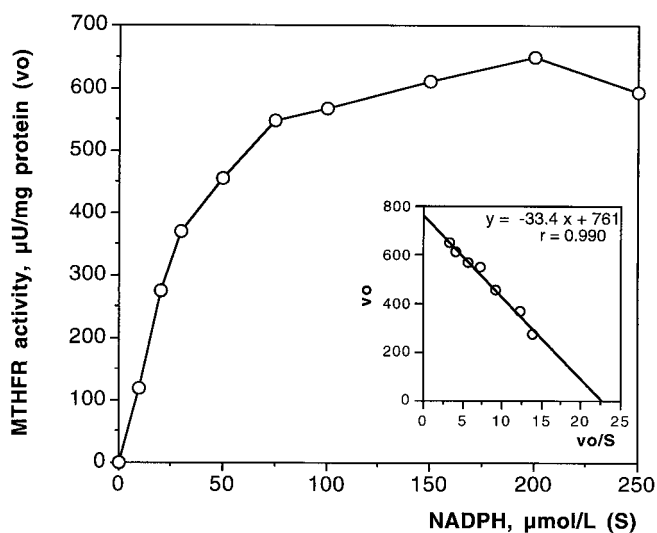


Fig. 3. Dependence of MTHFR activity on concentration of NADPH measured with the physiologic assay.

Representative experiment with control fibroblast extract with a protein concentration of 27.1  $\mu\text{g}/\text{assay}$  and 20-min assay time. Each symbol represents a single determination of activity with NADPH concentrations of 10–250  $\mu\text{mol}/\text{L}$  and 100  $\mu\text{mol}/\text{L}$  5,10-methylenetetrahydrofolate. The inset shows Eadie-Hofstee plot of the results.  $\mu\text{U} = \text{pmol}/\text{min}$ ;  $v_0$ , initial rate of reaction;  $S$ , substrate concentration.

for purified pig liver enzyme [20  $\mu\text{mol}/\text{L}$  for (6*R,S*)-5,10-methylenetetrahydrofolate; 16  $\mu\text{mol}/\text{L}$  for NADPH]. Similar  $K_m$ s were obtained in control fibroblast extracts in the presence and absence of FAD added at a concentration of 75  $\mu\text{mol}/\text{L}$ . A 5,10-methylenetetrahydrofolate concentration of 100  $\mu\text{mol}/\text{L}$ , corresponding to four times the  $K_m$ , was chosen for the standard assay. A higher NADPH concentration, 200  $\mu\text{mol}/\text{L}$  (corresponding to seven times the  $K_m$ ), was selected to obtain maximum activities.

**Recovery of the product.** The recovery of 5-methyltetrahydrofolate was determined by adding it, in 10 different concentrations (10, 20, 30, 40, 60, 80, 100, 200, 400, and 800 pmol), to the assay mixture containing either 50  $\mu\text{g}$  of bovine serum albumin or pooled fibroblast extract (containing 23.8  $\mu\text{g}$  of protein) from patients with complete MTHFR deficiency. Samples were incubated for 20 min at 37  $^{\circ}\text{C}$ , and then 50  $\mu\text{L}$  of stop solution was added. Recovery was essentially 100% [mean  $\pm$  SD for samples with bovine serum albumin, 103%  $\pm$  3% (range, 100–107%); samples with cell extract, 105%  $\pm$  2% (range 101–109%)] as expected because this physiologic assay does not require an extraction step. This is in contrast to the reverse assay, which suffers from low recovery (~45%) of the extracted [ $^{14}\text{C}$ ]formaldehyde (data not shown).

**Variation and stability.** The intraassay variation, estimated by assaying 10 separate pellets obtained from a single control fibroblast culture in a single experiment, was 10% with FAD (mean activity  $\pm$  SD, 318  $\pm$  33  $\mu\text{U}/\text{mg}$  protein;

range, 272–377) and 8.1% without FAD (308  $\pm$  25  $\mu\text{U}/\text{mg}$  protein; range, 277–345). The activity remained stable for at least 9 months in both cell pellets and enzyme extracts stored at  $-75^{\circ}\text{C}$ . This allowed the estimation of inter-assay variation. Separate pellets from a single control fibroblast culture were assayed in different experiments on 19 occasions. The CV was 7.2% with FAD (387  $\pm$  28  $\mu\text{U}/\text{mg}$  protein; range, 340–430) and 7.3% without FAD (382  $\pm$  28  $\mu\text{U}/\text{mg}$  protein; range, 325–425).

Cultures were harvested at least 3 days after reaching confluence because MTHFR activity in fibroblasts is known to increase with increasing culture time (9) as is also detected with this physiologic assay (results not shown). When activity was determined in 10 successive subcultures of a control cell line, a CV of 18% was obtained with FAD (373  $\pm$  68  $\mu\text{U}/\text{mg}$  protein; range 275–498) and 19% without FAD (360  $\pm$  67  $\mu\text{U}/\text{mg}$  protein; range, 270–473). There was no correlation between the activity and the subculture number. In addition, the proportion of thermolabile enzyme did not change in relation to the number of subcultures.

#### MTHFR ACTIVITIES MEASURED BY THE PHYSIOLOGIC AND REVERSE ASSAYS IN FIBROBLASTS OF CONTROLS AND PATIENTS

Activities obtained in fibroblasts of controls and patients with the physiologic assay are compared with values obtained with the conventional reverse assay in Table 1. For the physiologic assay, each fibroblast cell line was assayed at least twice. Control values are means of values obtained in at least two separate experiments. For patients, single values (mean of duplicates) from different experiments are shown.

In control fibroblasts the mean activity with the physiologic assay was 2.5- and 3.0-fold higher than with the reverse assay in the presence and absence of FAD, respectively. It appears that dependence on FAD was less marked with the physiologic assay than with the reverse assay. Thus activity in the presence of added FAD was, on average, only 1.02-fold (range, 0.96- to 1.09-fold) higher than without added coenzyme in the physiologic assay, but 1.23-fold (range, 0.97- to 2.00-fold) higher in the reverse assay. This may reflect the different nature of electron transfer attributable to the different electron donors and acceptors between the two assays: in the physiologic assay, electrons are transferred from NADPH to FAD and from the reduced FAD to 5,10-methylenetetrahydrofolate to form 5-methyltetrahydrofolate (19), whereas in the reverse assay, 5-methyltetrahydrofolate serves as the first electron donor and menadione as the final electron acceptor (9).

Comparison of MTHFR activities measured by the physiologic assay in controls with or without metabolic disease revealed no differences. Thus the mean activity assayed without FAD in 23 controls undergoing surgery or with nonmetabolic diseases (414  $\pm$  141  $\mu\text{U}/\text{mg}$  protein; range, 230–715) was similar to activities in 30 controls

with suggested but not confirmed metabolic disease ( $431 \pm 162 \mu\text{U}/\text{mg}$  protein; range, 260–884) and to activities in 22 controls with confirmed metabolic defects unrelated to homocysteine metabolism ( $413 \pm 140 \mu\text{U}/\text{mg}$  protein; range, 246–808). In addition, the distribution of 677TT individuals was similar among the three groups (13%, 13%, and 18%, respectively).

The activities in fibroblasts of 25 patients are listed in Table 1 according to decreasing mean activity measured by the physiologic assay. With the conventional reverse assay, 12 cell lines showed no measurable activity, 9 cell lines showed measurable but low activity ( $\leq 5\%$  of mean control value) either only with FAD or with and without FAD in the assay, and only 4 cell lines revealed clearly measurable residual activity, ranging from 9.3% to 25.0% of the mean control value. It should be noted that reproducibility of activity measured in different cultures was poor in, for example, patients 4, 5, 8, 18, and 24, probably reflecting the inherent variation and lower sensitivity of the reverse assay.

With the physiologic assay, 15 cell lines showed clearly measurable activity ranging from 2.6% to 25.6% of the mean control value. The presence of residual activity was confirmed in each of these cell lines by the finding of an increase in product formation commensurate with increasing amount of protein in the assay (results not shown). In 10 cell lines, activity was virtually absent, ranging from 0.1% to 1.0% of the mean control value. Because no increase in product formation was obtained with increasing amounts of protein in the assay (results not shown), we consider that these cell lines have complete MTHFR deficiency. Therefore, the detection limit of our assay is between 1.0% and 2.5% of mean control values. Importantly, we found clearly measurable activity with the physiologic assay in 11 cell lines with essentially zero activity in the reverse assay. The results with the physiologic assay confirm a general but not complete correlation between clinical severity and fibroblast residual activity.

In all but 1 of the 15 patient cell lines with residual

activity, the addition of FAD to the physiologic assay had an effect on enzyme activity similar to that in controls (mean increase with FAD, 1.08-fold; range, 0.95- to 1.26-fold). In one patient (patient 9), FAD stimulated the activity 2.25-fold compared with that measured without FAD, indicating that the defect in this patient probably affects the binding region of FAD.

MTHFR activity was also easily measurable by our physiologic assay in other types of cultured cells, including amniocytes and chorionic villous cells as well as in uncultured chorionic villous biopsies and in lymphocytes (results not shown). The assay can therefore be used for early prenatal and rapid postnatal (blood sample) diagnosis.

#### EFFECT OF HEAT TREATMENT ON MTHFR ACTIVITY IN FIBROBLASTS OF CONTROLS AND PATIENTS

To study thermolability, we treated fibroblast extracts in the presence of assay buffer, without FAD and substrates, at  $46^\circ\text{C}$  for 5 min and then assayed MTHFR activity in the physiologic assay with and without FAD. Comparison of the activity remaining (without FAD in assay) with the 677C→T genotype in control fibroblasts revealed a clear distribution (no overlap with a class interval of 5%) in three groups (Table 2) as follows: the wild-type group (677CC) retained 46–60% of activity; heterozygotes (677CT) retained 27–39%; and homozygotes for the thermolabile mutation (677TT) retained 13–17%. Similar results were obtained when assays, but not heat treatment, were performed in the presence of FAD, except that the remaining activity was slightly higher. When FAD (75  $\mu\text{mol}/\text{L}$ ) was present during the heat treatment, the enzyme in control extracts retained  $\sim 85\%$  of activity (data not shown).

There was no major difference in the specific activity of MTHFR in control fibroblasts among the three genotypes when assayed either with the physiologic or the reverse assay (Table 2). This is in contrast to reports of studies using lymphocytes in the reverse assay, in which lower enzyme activity was found in individuals homozygous

**Table 2. MTHFR activities before and after heat treatment in fibroblast extracts of controls classified according to the 677C→T genotype.**

Genotype <sup>a</sup>	Physiologic assay			Reverse assay		
	FAD in assay	Activity <sup>b</sup> after heat treatment – FAD at $46^\circ\text{C}$ for 5 min, % of activity without treatment	Activity <sup>b</sup> without heat treatment, $\mu\text{U}/\text{mg}$ protein <sup>c</sup>	n <sup>d</sup>	Activity <sup>b</sup> without heat treatment, $\mu\text{U}/\text{mg}$ protein	n <sup>d</sup>
677CC	–	$51.3 \pm 3.7$ (45.6–60.4)	$428 \pm 126$ (230–732)	38	$138 \pm 50.3$ (78.3–228)	11
	+	$61.5 \pm 3.2$ (55.1–69.2)	$438 \pm 128$ (242–752)		$170 \pm 68.2$ (88.3–315)	
677CT	–	$33.5 \pm 3.4$ (26.8–39.2)	$419 \pm 165$ (240–884)	26	$152 \pm 63.6$ (62.7–289)	11
	+	$40.4 \pm 3.9$ (33.1–47.2)	$431 \pm 168$ (249–911)		$185 \pm 70.0$ (93.7–305)	
677TT	–	$14.7 \pm 1.6$ (12.9–17.4)	$398 \pm 184$ (249–809)	11	$122 \pm 44.2$ (62.2–226)	11
	+	$18.4 \pm 1.9$ (15.1–21.6)	$408 \pm 184$ (261–819)		$160 \pm 45.2$ (117–274)	

<sup>a</sup> 677CC, wild type; 677CT, heterozygous; 677TT, homozygous mutation causing thermolability of MTHFR.

<sup>b</sup> Activities given as mean  $\pm$  SD (range).

<sup>c</sup>  $\mu\text{U} = \text{pmol}/\text{min}$ .

<sup>d</sup> n, number of different cell lines.

for the thermolabile mutation than in wild-type individuals (3, 6, 20, 21). Our results show that this difference cannot be attributed to the assay method, but rather reflects differences between the cell types. It needs to be considered that the activity in our wild-type group might have been influenced by the inclusion (potentially 10%) of individuals homozygous for the 1298A→C mutation, in whom MTHFR activity was reported to be decreased to 61% of mean wild-type activity, although this was studied only in lymphocytes (6).

In Table 3, the activity (assay with FAD) remaining after heat treatment of fibroblasts from the 15 patients with residual activity is compared with the patients' 677C→T genotypes. Activity decreased in all patients after heat treatment, but in contrast to findings in control fibroblasts, there was a poor correlation between genotype and heat stability; there also was poor reproducibility for the amount of heat-stable activity in different experiments in some cell lines. In those patients with very low residual activity (2–5% of the mean control value; patients 10–15), activity remaining after heat treatment was close to the detection limit, making reliable measurement impossible. The finding of excessively heat-labile enzyme in association with the 677CC genotype in patient 3 was in accordance with the findings of Goyette et al. (22), who speculated that a mutation that causes a decrease in enzyme activity may also cause instability during heat treatment not related to the 677TT genotype. Similarly, heat stability was less than expected in three

patients (patients 1, 2, and 6) with the 677CT genotype. Thus the estimation of remaining MTHFR activity after heat treatment does not allow prediction of the genotype for the thermolabile mutation in patients with decreased MTHFR activity, and genotyping based on DNA is required. In control fibroblasts, however, such discrimination is clearly possible.

In conclusion, this newly developed, nonradioactive assay for MTHFR in the physiologic direction allows reliable detection of residual activities as low as 2.6% of the mean control activity in mutant fibroblasts. It also allows the measurement of kinetic parameters for the natural substrates 5,10-methylenetetrahydrofolate and NADPH, including studies in patients with residual activity. This physiologic assay could be useful for future studies of expression of specific mutations as well as studies of genotype/phenotype correlation.

We gratefully acknowledge those colleagues who sent us fibroblasts from patients and thank Dr. Martina Plasilova (Department of Molecular Genetics, University Children's Hospital, Basel, Switzerland) for help in the molecular genetics work. We also thank Eprova AG (Schaffhausen, Switzerland) for the kind gift of 5,10-methylenetetrahydrofolate. This study was supported by the Swiss National Foundation (Grant 32-5567.98).

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**Table 3. MTHFR activity before and after heat treatment in fibroblast extracts from patients with residual MTHFR activity in the physiologic assay classified according to the 677C→T genotype.**

Genotype <sup>a</sup>	Patient	Activity <sup>b</sup> before heat treatment, % of mean control value	Activity <sup>b</sup> after heat treatment, % of activity without treatment
677CC	3	16, 19	24, 23
	8	5.3, 6.5	52, 40
	9	7.6, 9.7	51, 48
677CT	1	22, 25, 29	15, 16, 23
	2	20, 24	13, 15
	6	9.3, 10, 14	15, 16, 14
	7	5.8, 7.6	43, 47
	10	2.8, 3.7, 4.6	24, 28, 36
	15	1.8, 2.6, 3.7	35, 54, 25
677TT	4	12, 18	10, 5.1
	5	7.0, 8.8	30, 9.6
	11	3.9, 3.9	16, 20
	12	3.2, 3.7	42, 26
	13	3.5, 3.7	32, 28
	14	3.2, 3.5, 4.2	26, 5.8, 36

<sup>a</sup> 677CC, wild type; 677CT, heterozygous; 677TT, homozygous thermolabile.

<sup>b</sup> Activities are the means of duplicate determinations obtained within one experiment assayed with 75 μmol/L FAD before heat treatment (for specific activities and control values, see Table 1) or after heat treatment (in the absence of FAD) for 5 min at 46 °C.

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