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Amniotic fluid liver
fibroblasts lymphoblasts
homocysteine methylenetetrahydrofolate reductase

Methylenetetrahydrofolate Reductase in Cultured Human Cells. I. Growth and Metabolic Studies

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Summary

This investigation examined factors that affect methylenetetrahydrofolate (methylene-H_4PteGlu) reductase activity in cultured human cells. Activity was demonstrable in extracts of cultured normal human skin fibroblasts, amniotic fluid cells, and lymphoblasts. The velocity of the reaction was maximal at pH 6.3 in extracts of all three cell types. Subcellular localization studies indicated that 83–94% of the reductase activity was extranuclear in the three cell types. The reductase activity was not substantially altered by growth of lymphoblasts whether in Eagle's minimum essential medium (MEM) supplemented with homocysteine alone or with additional hydroxocobalamin and folic acid, or in media containing a 5-fold greater concentration of methionine. In normal fibroblasts the methylene-H_4PteGlu reductase activity varied little when the confluent cells were exposed to media deficient in or supplemented with varying amounts and combinations of methionine, homocysteine, folic acid, and cobalamin. Under our conditions the addition of purified *S*-adenosylmethionine to give a final concentration of 48 mM in the assay reaction mixture reduced the reductase activity to 38% of control in the fibroblast extract and to 13% of control in the extract of rat liver. This sensitivity to inhibition by *S*-adenosylmethionine and the lack of response to varied levels of methionine, homocysteine, folic acid, and cobalamin are further evidence that the reductase activities in mammalian liver and fibroblasts are similar.

Speculation

Cultured human skin fibroblasts and peripheral blood lymphoblasts may provide a useful, convenient system for studying the biochemical-genetic regulation of 5,10-methylene-H_4PteGlu reductase activity and, ultimately, of the levels of its folate product, 5-methyl-H_4PteGlu.

5-Methyltetrahydrofolate (5-methyl-H_4PteGlu) is the predominant form of folate in tissue (25) and serum (9). Synthesis of 5-methyl-H_4PteGlu is catalyzed by methylene-H_4PteGlu reductase (5-methyltetrahydrofolate:NAD oxidoreductase, EC. 1.1.1.68), probably with enzyme-bound FAD, according to the following reaction:

$$\text{5,10-Methylene-H}_4\text{PteGlu} + \text{NADPH} + \text{H}^+ \xrightarrow{(E\text{-FAD})} \text{5-methyl-H}_4\text{PteGlu} + \text{NADP}^+ \quad (1)$$

The 5-methyl-H_4PteGlu is, in turn, a substrate for methionine synthesis in the reaction catalyzed by 5-methyl-H_4PteGlu:homocysteine cobalamin methyltransferase (5-methyl-H_4PteGlu:L-homocysteine *S*-methyl-transferase, EC.2.1.1.13) as follows:

$$\text{5-Methyl-H}_4\text{PteGlu} + \text{L-homocysteine} \quad (2)$$

Methylcobalamin is required for reaction 2.

Factors regulating the activity of 5-methyl-H_4PteGlu:homocysteine cobalamin methyltransferase, which catalyzes the utilization of 5-methyl-H_4PteGlu reductase, have been identified in several recent studies using cultured cells (e.g., References 10, 12, 17, 19, and 26). In contrast, the regulation of methylene-H_4PteGlu, which catalyzes 5-methyl-H_4PteGlu synthesis, has been less well characterized in cultured mammalian cells. The reductase of rat and hog liver has been extensively purified and studied (14). Methylene-H_4PteGlu reductase activity is present in extracts of cultured human skin fibrobasts (7, 18, 21). Furthermore, in methylene-H_4PteGlu reductase deficiency, this activity is markedly reduced both in liver in cultured skin fibroblasts (11, 18, 20), strongly suggesting that the same gene code for the reductase in both the fibroblast and the liver. This investigation examined factors that affect methylene-H_4PteGlu reductase activity in cultured normal human fibroblasts and lymphoblasts.

MATERIALS AND METHODS

[5-^{14}C]Methyl-H_4PteGlu was purchased as the barium salt (27). Unlabeled 5-methyl-H_4PteGlu was synthesized as follows, according to the techniques of Gupta and Huennekens (8) and of Blair and Saunders (2). Folic acid (28) was first reduced with sodium borohydride to yield H_4PteGlu. After addition of formaldehyde which condensed with H_4PteGlu acid to form 5,10-methylene-H_4PteGlu, a second borohydride reduction yielded 5-methyl-H_4PteGlu, which is relatively stable in the presence of 2-mercaptoethanol and can be isolated by DEAE-cellulose column chromatography.

Other chemicals were of reagent grade and obtained commercially. DL-homocysteine-thiolactone-HCl (28) was found to be equivalent to homocysteine for cell growth and was used routinely in growth media where indicated. *S*-Adenosylmethionine (28) was freed of *S*-adenosylhomocysteine by elution from a Dowex 1-bicarbonate column according to the procedure of Shapiro and Ehninger (23).

Fibroblasts were derived from punch biopsy specimens of skin obtained according to procedures reviewed and approved by our institution's Subcommittee on Human Studies and with the informed consent of the subject and/or, where appropriate, the parents. The fibroblasts were grown in roller bottles (29) of 690 cm^2 surface area containing 100 ml medium in an atmosphere of 5% CO_2 and 95% air. The standard medium consisted of Eagle's minimum essential medium plus nonessential amino acids (30) but lacking methionine and folic acid. To this standard medium were added, as indicated, DL-homocysteine-thiolactone-HCl, folic acid, methionine, and hydroxocobalamin (31). All media contained 15% fetal calf serum (32) dialyzed where indicated for 20 hr against a 10-fold volume 0.9% NaCl with two changes. Cells were released from the surface of the roller bottles by exposure to 0.25% trypsin (33) for 7–10 min at 37°. Trypsinization was stopped by the addition of a 5-fold excess of serum containing test medium. Aliquots were removed for enumeration in a Coulter counter. Cells were collected by centrifugation and washed by suspension in Dulbecco's phosphate-buffered saline solution (pH 7.2). The twice washed cell pellet was col-

lected by centrifugation and resuspended at 10^8 cells/ml in 0.25 M sucrose. The cells were disrupted for 2 min in a Raytheon model DF 101 sonic oscillator, and the crude extract clarified by centrifugation at 90,000 × *g* for 1 hr at 4°.

Human lymphoblasts (22) were grown in suspension culture in Eagle's minimum essential medium, harvested by centrifugation, and extracts prepared by sonication at a density of 10^8 cells/ml as described above for fibroblasts. Periodic tests of both lymphoblasts and fibroblasts were negative for mycoplasma.

For subcellular localization studies, cells were suspended at 10^8 cells/ml and swollen for 10 min at room temperature in a solution consisting of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, and 2.6 mM $CaCl_2$. Cells were lysed with an equal volume of a solution consisting of 0.5 M sucrose, 40 mM Tris-HCl (pH 7.5), 0.25 mM KCl, 10 mM $MgCl_2$, 3.6 mM $CaCl_2$, 1 mM EDTA, and 1% Triton X-100 (34). After standing for 10 min at room temperature the solution was expressed gently from a syringe through a 27 gauge needle and centrifuged at 1,000 × *g* for 5 min. The supernatant portion was assayed to determine the extranuclear methylene-H_4PteGlu reductase activity. The nuclei in the pellet was suspended in one-quarter of the original volume of a solution consisting of 0.25 M sucrose, 40 mM Tris-HCl (pH 7.5), 0.1 M KCl, 50 mM NaCl, 5 mM $MgCl_2$, 3.6 mM $CaCl_2$, and 0.5% Triton X-100. The nuclei were disrupted by sonic oscillation and the extract assayed.

Crude rat liver extract was prepared by homogenization in 0.25 M sucrose followed by sonification and centrifugation as described for the fibroblasts.

The assay procedure of Donaldson and Keresztesy (5) for measuring methylene-H_4PteGlu reductase activity in the so-called reverse direction using menadione as the electron acceptor, shown in reaction 3, was performed according to a modification of the method of Kutzbach and Stokstad (14, 15).

$$\text{5-Methyl-H}_4\text{PteGlu} + E\text{-FAD} \underset{\text{menadione}}{\overset{\text{menadione-H}_2}{\rightleftharpoons}} E\text{-FADH}_2 + \text{5,10-methylene-H}_4\text{PteGlu} \qquad (3)$$

The reaction consisted of the following in a final volume of 0.280 ml: 0.18 M potassium phosphate (pH 6.3), 72 μM FAD, 3.6 mM menadione bisulfite, 0.42 mM [5-^{14}C]methyl-H_4PteGlu (5,032 cpm/nmol) as the barium salt in 33 mM 2-mercaptoethanol, and cell extract. The standard reaction was incubated at 37° for 60 min in air in 12-ml conical centrifuge tubes. After incubation, the reaction was terminated by the addition at 0° of 0.250 ml 0.6 M sodium acetate (pH 4.5), 0.100 ml 0.1 M formaldehyde, and 0.150 ml 0.4 M dimedone in 50% ethanol, and incubation resumed at 98° for 12 min. The mixture was cooled on ice and 2.5 ml toluene was added, followed by vigorous suspension with a Vortex mixer. After low speed centrifugation, a 2-ml aliquot of the toluene phase was removed for liquid scintillation counting in a solution consisting of toluene, 2,5-diphenyloxazole (PPO), and *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (35). The blank consisted of a reaction mixture to which cell extract was added following the first incubation. The radioactivity in the blank was subtracted and the specific activities were calculated as nanomoles of formaldehyde formed per mg protein per hr. All assays were carried out in duplicate and agreed within 10%. The efficiency of recovery of radioactive formaldehyde by dimedone extraction was 80% and was independent of protein concentration over the range used for these reactions.

5-Methyl-H_4PteGlu:homocysteine methyltransferase activity was measured as described previously (10). Protein concentration was measured by the method of Lowry *et al.* (16).

RESULTS

METHYLENE-H_4PteGlu REDUCTASE ACTIVITY IN CULTURED HUMAN CELLS

Activity of methylene-H_4PteGlu reductase was demonstrable in extracts of cultured normal human skin fibroblasts, amniotic fluid cells, and lymphoblasts, and was present at comparable levels in the three cell types. Activity was linear on incubation at 37° for at least 60 min and over the range of protein concentrations (0.5–2.4 mg/ml) used. Under the conditions of the standard assay, reductase activity was completely dependent on the presence of menadione bisulfite but was dependent for maximal activity on the presence of the FAD cofactor only in cell extracts that had been treated by gel filtration, as has been noted by others (18). The velocity of the reaction was maximal at pH 6.3 in extracts of all three cell types.

Repeated (five times) freezing and thawing of extracts did not affect activity, nor did storage at −40° for at least 1 month. Subcellular localization studies indicated that at least 83–94% of the reductase activity was extranuclear in the three cell types.

EFFECT OF COMPOSITION OF CULTURE MEDIA ON GROWTH OF LYMPHOBLASTS AND ON METHYLENE-H_4PteGlu REDUCTASE ACTIVITY

When lymphoblasts were transferred from standard Eagle's minimum essential medium (MEM) containing 15% undialyzed fetal calf serum to MEM containing 15% dialyzed fetal calf serum, the cell number was consistent for 24 hr, after which the cells began to grow logarithmically (Fig. 1). In contrast, when methionine and choline were replaced in the medium by homocysteine, the immediate metabolic precursor of methionine, the cells died and the cell number decreased progressively. However, replacing methionine and choline with homocysteine and hydroxocobalamin in the presence of increased levels of folic acid (35 times the concentration in standard MEM) permitted the growth of the lymphoblasts, although at a slower rate. Table 1 shows the methylene-H_4PteGlu reductase activities in extracts of lymphoblasts grown in media of various compositions. The reductase activity was not substantially altered by growth of the cells either in MEM supplemented with homocysteine alone or with additional hydroxocobalamin and folic acid, or in media containing a 5-fold greater concentration of methionine.

Table 2 shows that in normal fibroblasts the methylene-H_4PteGlu reductase activity varied little when the confluent cells were exposed to media that were deficient in or supplemented with metabolically related compounds, although in some instances the 5-methyl-H_4PteGlu:homocysteine methyltransferase

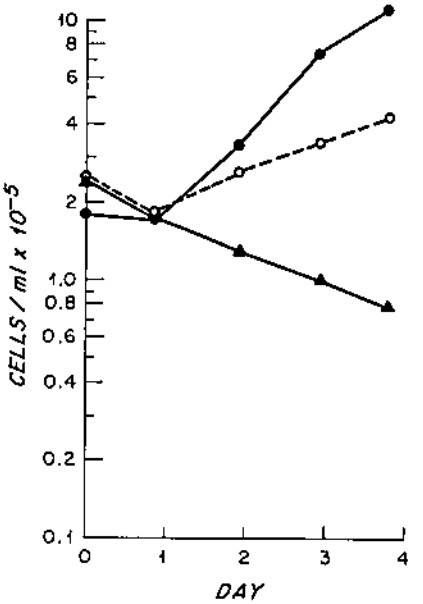

Fig. 1. Growth of human lymphoblasts in complete and in deficient media. ●: Eagle's minimum essential media (MEM); ○: MEM lacking methionine and choline but containing 0.2 mM DL-homocysteine, 1.5 μM hydroxocobalamin, and 0.1 mM folic acid; ▲: MEM lacking methionine and choline but containing 0.2 mM DL-homocysteine. All three media contained 15% dialyzed fetal calf serum. Unmodified MEM contains 2.26 μM folic acid, but no cobalamin or homocysteine.

Table 1. *Reductase activities in lymphoblasts grown in methionine or homocysteine*[1]

Growth media	Methylene-H_4PteGlu reductase
MEM[2]	10.1
MEM[2] + 1 mM DL-homocysteine	10.6
MEM[2] + 0.5 mM L-methionine	10.3
MEM lacking methionine but + DL-homocysteine, 1.5 μM hydroxocobalamin and 0.1 mM folic acid	12.8

[1] Extracts were prepared and assayed after growth for 90 hr in the test media, all of which contained 15% dialyzed fetal calf serum. Reductase activities are nanomoles of formaldehyde formed per mg soluble protein per hr. Methylene-H_4PteGlu: methylenetetrahydrofolate; MEM: minimal essential medium.

[2] Contains 0.1 mM L-methionine and 2.26 μM folic acid.

Table 2. *Enzyme activities in fibroblasts exposed to various concentrations of methionine, homocysteine, folic acid, and cobalamin*[1]

Additions to basal medium	Methylene-H_4PteGlu reductase[2]	5-methyl-H_4PteGlu: homocysteine methyltransferase[3]
None	18.0	6.0
0.1 mM L-Methionine, 2.3 μM folic acid, 4% undialyzed FCS (replacing dialyzed FCS)	13.8	6.2
2.3 μM Folic acid	22.0	7.5
0.1 mM Folic acid	22.0	6.9
0.1 mM L-Methionine, 2.3 μM folic acid	16.1	5.5
0.1 mM L-Methionine, 0.1 mM folic acid	16.8	6.4
1.5 μM Hydroxocobalamin	23.5	13.7
0.2 mM DL-Homocysteine, 0.1 mM folic acid, 1.5 μM hydroxocobalamin	14.9	9.6
0.1 mM L-Methionine, 1.5 μM hydroxocobalamin	15.5	12.6
0.1 mM L-Methionine, 0.2 mM DL-homocysteine, 0.1 mM folic acid, 1.5 μM hydroxocobalamin	20.3	14.4
0.2 mM DL-Homocysteine	17.1	3.8

[1] Human skin fibroblasts were grown to confluence and for an additional 5 days in minimal essential medium (MEM) containing 15% fetal calf serum (FCS). This medium was then replaced with basal medium consisting of MEM lacking methionine and folic acid and containing 4% dialyzed FCS; it should be noted that MEM contains neither cobalamin nor homocysteine. After 24 hr this medium was replaced with fresh basal medium with additions as indicated and, after an additional 48 hr, the enzyme activities were measured. Methylene-H_4PteGlu: methylenetetrahydrofolate; 5-methyl-H_4PteGlu: 5-methyltetrahydrofolate.

[2] Nanomoles of formaldehyde formed per mg soluble protein per hr. With 0.040 ml cell extract/reaction mixture the total radioactivity of the product ranged from 18,760 to 27, 283 cpm from which a blank of 7,260 cpm was subtracted.

[3] Nanomoles of methionine formed per mg soluble protein per hr. With 0.100 ml cell extract/reaction mixture the total radioactivity of the product ranged from 1,565 to 4,543 cpm from which a blank of 498 cpm was subtracted.

activity was altered. Varying the amounts and combinations of methionine, homocysteine, folic acid, and cobalamin did not increase or decrease the reductase activity by more than 0.3-fold from the level observed in cells grown in basal medium. Interestingly, the reductase levels tended to be higher in those media that would not support the growth of cells in the logarithmic phase, *e.g.*, basal medium supplemented with folic acid or cobalamin alone, and lower in test media that contained methionine and would support the growth of cells.

The 5-methyl-H_4PteGlu:homocysteine methyltransferase activity (Table 2) was not influenced under these conditions by methionine, homocysteine, or folic acid, but was increased as much as 2.4-fold in media supplemented with hydroxocobalamin.

Although the reductase activity of fibroblasts was insensitive to these changes in media composition, it was influenced appreciably by the density of these surface-growing cells (Fig. 2). Subculturing confluent cells at one-tenth their initial density resulted in a 2.5-fold reduction in reductase activity during the logarithmic stage of growth. The reductase activity returns to the higher levels as the cells again become confluent. Thus in fibroblasts the reductase activity may be most responsive to cellular mitotic activity, as suggested previously (21), rather than to metabolic alterations of the medium *per se*.

In contrast, the reductase activity in lymphoblasts varied little during the logarithmic growth of these cells in suspension culture. Unlike fibroblasts, which simply cease to divide and remain quiescent at high cell densities, an increasing proportion of the lymphoblasts become nonviable at high cell densities (above 1–2 × 10^6 cells/ml) and the reductase activities become unreliable.

INHIBITION BY *S*-ADENOSYLMETHIONINE

The addition of methionine to the culture medium might be expected to elevate the intracellular level of *S*-adenosylmethionine by analogy to the increased hepatic levels when methionine is fed to animals (1). However, as noted above, even high levels of methionine in the culture medium produced no change in reductase activity. Therefore, inhibition was sought in fibroblast extracts and compared to that seen in extracts of rat liver (Table 3). Purified *S*-adenosylmethionine inhibited methylene-H_4PteGlu reductase activity when added to extracts of both fibroblasts and liver. The inhibition of rat liver reductase by *S*-adenosylmethionine has been shown to be time dependent and only partially reversible, precluding simple kinetic analysis (15). Under our conditions, the addition of purified *S*-adenosylmethionine to give a final concentration of 48 μM in the assay reaction mixture reduced the reductase activity to 38% of control in fibroblast extract and to 13% of control in the extract of rat liver. In contrast unpurified, commercial *S*-adenosylmethionine did not inhibit reductase activity of fibroblast or lymphoblast extracts at concentrations as high as 0.7 mM, although it did inhibit the reductase activity in extracts of rat liver. This difference is presently unexplained but may relate to differences in the me-

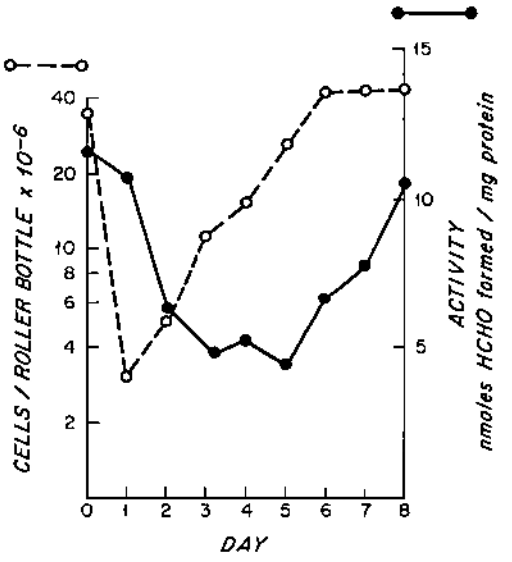

Fig. 2. Methylene-H_4PteGlu reductase activity during the stages of fibroblast growth. After 5 days in confluency, fibroblasts were subcultured at a dilution of 1:10 on day 0, and cell numbers (○) and reductase activity (●) measured in replicate roller bottles daily thereafter. HCHO: formaldehyde.

Table 3. *Inhibition by S-adenosylmethionine of reductase activity in crude extracts of human skin fibroblasts and rat liver*[1]

S-Adenosylmethionine added to assay, μM	Reductase activity			
	Human fibroblasts[2]		Rat liver[3]	
	Activity	%	Activity	%
0	12.7	100	20.7	100
48	5.0	38	2.7	13
96	3.0	24	2.5	12
190	1.7	14	1.2	6

[1] Purified *S*-adenosylmethionine in the concentration indicated was added to reaction mixtures containing cell extract and all of the components of the standard reductase assay except the [5-^{14}C]methyl-H_4PteGlu substrate. After 5 min of incubation at 37°, [5-^{14}C]methyl-H_4PteGlu was added to initiate the reaction, the incubation was continued for 60 min and reductase activity measured as described in "Materials and Methods."

[2] Aliquot of crude extract added contained 0.3 mg soluble protein.

[3] Aliquot of crude extract added contained 0.5 mg soluble protein.

tabolism by these cells of *S*-adenosylhomocysteine known to be present in most preparations of *S*-adenosylmethionine and to compete with the effects of *S*-adenosylmethionine (15).

DISCUSSION

The presence of methylene-H_4PteGlu reductase at easily detectable levels in extracts of human fibroblasts, lymphoblasts, and amniotic fluid cells provides a convenient source for characterizing this important folate enzyme activity. In human lymphoblasts growing in suspension culture, the reductase activity level is quite independent of cell density so long as the cells continue to grow logarithmically. Under these conditions, reduced or increased concentrations of methionine and folic acid, as well as the addition of homocysteine or cobalamin to the culture medium, do not appreciably alter reductase activity.

In the fibroblasts, cells that require a surface for growth, cell density and growth rate have a pronounced effect on the activity levels of the reductase. The wide variation in specific activity observed in various stages of growth of normal fibroblasts is a possible source of confusion in diagnostic studies of methylene-H_4PteGlu reductase deficiency as well as in the interpretation of metabolically induced changes. Therefore, fibroblasts that were confluent and no longer dividing were selected for further studies. As with lymphoblasts, the reductase activity in fibroblasts was not substantially altered by varying the concentrations of methionine, homocysteine, folic acid, or cobalamin in the culture media. Although methionine has been shown to repress the synthesis of the reductase in a methionine auxotroph of *Escherichia coli* (3), no effect was produced by dietary alterations of methionine levels in chicks (4) or rats (13). Observations similar to ours in human cells on the absence of metabolically induced changes have been made by Taylor and Hanna (26) on Chinese hamster ovary cells grown in suspension culture.

Whatever the factors are that regulate methylene-H_4PteGlu reductase activity, and thereby presumably control 5-methyl-H_4PteGlu biosynthesis, we have confirmed in fibroblast extracts that the reductase activity can be inhibited *in vitro* by concentrations of purified *S*-adenosylmethionine that are presumably close to physiologic levels (24). This sensitivity to inhibition by *S*-adenosylmethionine and the lack of response to varied levels of methionine, homocysteine, folic acid, and cobalamin are further evidence that the reductase activities in mammalian liver and fibroblasts are similar. Thus, cultured fibroblasts may provide a valid system for studying the regulation of 5-methyl-H_4PteGlu levels. Furthermore, the marked decrease of reductase activity in both liver and fibroblasts in methylene-H_4PteGlu reductase deficiency suggests that the enzyme is genetically unique. Studies of cultured fibroblasts should thus be useful in characterizing the mutant enzyme activity in this and related inborn errors of folate metabolism.

CONCLUSION

Methylene-H_4PteGlu reductase activies in extracts of cultured human skin fibroblasts, lymphoblasts, and amniotic fluid cells is similar to its specific activity, pH optimum, and nuclear to cytoplasmic activity ratio. The reductase activity was quite insensitive to alterations in the concentrations in growth media of various metabolically relevant substances. The reductase activity was not substantially altered by growth of lymphoblasts either in MEM supplemented with homocysteine alone or with additional hydroxocobalamin and folic acid, or in media containing a 5-fold greater concentration of metionine. In normal fibroblasts the reductase activity varied little when the confluent cells were exposed to media deficient in or supplemented with varying amounts and combinations of methionine, homocysteine, folic acid and cobalamin. In contrast to the unpurified commercial reagent, purified *S*-adenosylmethionine added to extracts of fibroblasts markedly inhibited the reductase activity. In fibroblasts the reductase activity was also sensitive to the cell density, being low in logarithmically growing cells and highest deep in confluency. The results of these studies indicate that the characteristics of the methylene-H_4PteGlu reductase activity in these cultured human cells are quite similar to those of reductase activities found previously in animal tissues.

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Fibroblasts
methylenetetrahydrofolate reductase deficiency
mutation

Methylenetetrahydrofolate Reductase in Cultured Human Cells. II. Genetic and Biochemical Studies of Methylenetetrahydrofolate Reductase Deficiency

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Summary

Methylenetetrahydrofolate (methylene-H_4PteGlu) reductase activities in extracts of both normal and reductase-deficient cells were low and quite variable during the logarithmic phase of growth. Higher activities were detected reproducibly when the cultures were confluent. Methylene-H_4PteGlu reductase activities in extracts of fibroblasts from the parents of patient *CP* were about half of the level observed in normal control subjects. In fibroblasts from patient *CP*, the activity was 20% of normal whereas, from patients *LM* and *BM*, the activities were 19% and 14% of normal, respectively. When incubated at 55° in a solution containing all the components of the standard reaction mixture except the 5-methyl-H_4PteGlu substrate, the reductase activity in extracts of fibroblasts from two unrelated normal control subjects decreased to 31% and 22%, respectively, of the initial values after 30 min of incubation. In contrast, the reductase in extracts of cells patient *CP* was rapidly and exponentially inactivated at 55°. The reductase activity in extracts from patients *LM* and *BM*, the sisters, decreased to 22% and 38%, respectively, of the initial values. In repeated experiments the heat inactivation of reductase activity in extracts of cells from *LM* and *BM* closely resembled the normal control subjects in total extent and time course of inactivation. The reductase activity in extracts from *WMa*, a fourth, unrelated patient was also completely inactivated but somewhat less rapidly than with patient *CP*. These results provide strong evidence that the reductase deficiency in these three families is due to different alleles. The data suggest that in *CP* and *WMa* there is a mutationally induced structural defect in the aporeductase as the basis for the observed alteration in thermostability, presumably reflecting reduced ability to bind the FAD cofactor.

Speculation

The observation that methylene-H_4PteGlu reductase activity in crude fibroblast extracts from two of the three families studied shows reduced thermal stability as well as decreased activity raises the possibility that this enzyme is unusually susceptible to mutationally induced alterations in cofactor binding.

Methylenetetrahydrofolate reductase deficiency is an inborn error of folate metabolism in which neurologic abnormalities are accompanied by moderate degrees of homocystinemia and homocystinuria but with normal or low plasma methionine