5-METHYLTETRAHYDROFOLATE: SYNTHESIS AND UTILIZATION IN NORMAL AND SV40-TRANSFORMED BHK-21 CELLS

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Summary: BHK-21 cells and SV40-transformed BHK-21 cells (A-8) grew rapidly in a medium containing methionine (I). BHK-21 cells also proliferated in a methionine-deficient medium (II) supplemented with homocysteine and vitamin B_{12}; however, the A-8 cells did not proliferate and ultimately died in this medium. The 5,10-methylenetetrahydrofolate reductase activity in transformed cells was only 10% of that found in BHK-21 cells. While A-8 cells in medium II could not be maintained on folate or 5-formyltetrahydrofolate, they survived and even proliferated slowly when provided 5-methyltetrahdyrofolate. The low level of the reductase in A-8 cells and the inability of these cells to utilize folate or 5-formyltetrahydrofolate suggests that A-8 cells are unable to synthesize 5-methyltetrahydrofolate at a rate that will satisfy the cellular demands for methionine.

Previous studies have shown that a number of cultured mammalian cell lines, of normal and neoplastic origin, were able to proliferate under conditions requiring *de novo* methionine biosynthesis provided that the cells were supplied with exogenous homocysteine and vitamin B_{12}. Tetrahydropteroylglutamate methyltransferase (E.C.2.1.1.13) activity was detected in all of the cell lines examined (1). Subsequently, results were published which demonstrated the inability of several neoplastic cell types to proliferate in methionine-deficient media (2). Reduced levels of the methyltransferase in neoplastic cells (3) were also reported. More recently, evidence has been presented that some SV40 (simian virus)-transformed human cell cultures and a malignant rat cell line do in fact synthesize methionine at relatively high *in vivo* rates, although they fail to proliferate in a methionine-free medium (4). In this communication we describe the results of a comparative study of the synthesis and utilization of 5-methyltetrahydrofolate in normal and SV40-transformed BHK-21 cells.

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MATERIALS AND METHODS

Cell cultures of BHK-21 (baby hamster kidney), and SV40 DNA-transformed BHK-21 (A-8), were used in this study. The A-8 culture was provided by P. H. Black at Harvard Medical School, Boston, Massachusetts. The BHK-21 culture represents a cell line that we have carried in this laboratory for a number of years.

Cells were grown at 37°C in monolayer cultures. Growth studies were conducted in either acid-cleaned 8 oz prescription bottles or 26 cm^2 plastic flasks. Where large amounts of cell material were required for enzyme assays, cultures were grown in 490 cm^2 plastic roller flasks.

Growth media were obtained from the following sources: Eagle's Minimum Essential Medium with Earle's salts (MEM) from Flow Laboratories and Limited Eagle's Minimum Essential Medium with Earle's salts but without L-methionine, folic acid, and choline from Grand Island Biological Company.

Homocysteine was generated from homocysteine thiolactone each time fresh media was prepared or enzyme assays were performed (5).

Tetrahydrofolate (6) and [Me-^{14}C]-5-methyltetrahydrofolate (7,8) were prepared as previously described. 5-Formyltetrahydrofolate was a gift of Lederle Laboratories.

5,10-Methylenetetrahydrofolate reductase (E.C.1.1.1.68) activity of cell extracts was routinely assayed in the reverse direction using menadione as the electron acceptor and methyl[^{14}C]-labeled 5-methyltetrahydrofolate as the substrate (9). Specific activity was expressed as nmoles of formaldehyde produced per hour per mg of protein.

Methyltransferase activity of cell extracts was routinely assayed by measuring the amount of labeled methionine synthesized from [Me-^{14}C]-5-methyltetrahydrofolate (1). Specific activity was expressed as nmoles of L-methionine produced per hour per mg of protein.

RESULTS AND DISCUSSION

We initially demonstrated a vitamin B_{12}-dependent de novo synthesis of methionine in a number of cultured mammalian cell lines by replacing methionine

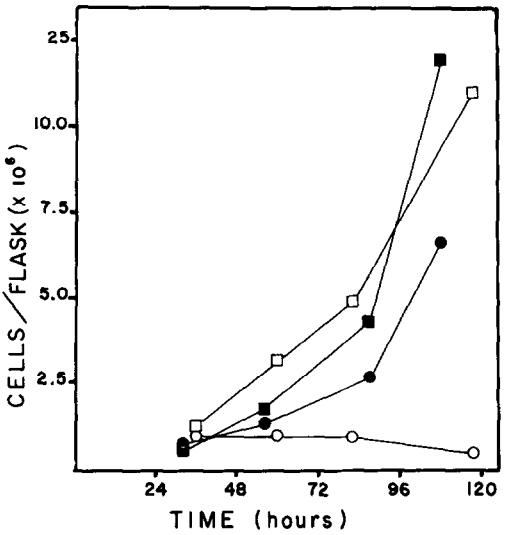

Fig. 1. Growth of BHK-21 and A-8 cultures. Limited medium: [DL-homocysteine (0.44 mM); vitamin B_{12} (1.5 μM); and folic acid (0.1mM)]--BHK-21, ■—■; A-8, ○—○. Regular medium: [L-methionine (0.1mM) and folic acid (2.3 μM)]--BHK-21, ●—●; A-8, □—□.

with homocysteine and by supplementing the growth medium with vitamin B_{12} (1). When our studies were extended by Halpern et al. (2) to include several additional cell lines, they made the significant observation that certain malignant cell lines were unable to survive in the methionine free homocysteine-vitamin B_{12} medium. More recently, it was shown that two SV40-transformed human cell lines (4) require exogenous methionine; however, these transformed cells were able to synthesize methionine from homocysteine in vivo at rates at least as high as those of many normal cells. We have also found that a SV40-transformed baby hamster kidney cell line, failed to survive in a homocysteine-vitamin B_{12} medium.

BHK-21 and A-8 cell lines were cultured using the conditions suggested by Kamely et al. (10) as described in the legend for Figure 1; and for comparative purposes, in Eagles MEM. The BHK-21 cells grew in the homocysteine-vitamin B_{12} medium at a rate nearly comparable to that in the methionine-containing

Table I. The specific activity of tetrahydropteroylglutamate methyltransferase and 5,10-methylenetetrahydrofolate reductase in BHK-21 and A-8 cultures.

Sample	Specific Activities	
	Reductase[a] (nmoles)	Methyltransferase[b] (nmoles)
Methionine Medium		
BHK-21 cultures	35.5	1.0
A-8 cultures	4.4	1.5
Homocysteine-vitamin B_{12} Medium		
BHK-21 cultures	42.9	76.2
A-8 cultures	3.2	37.3

[a]Specific activity was expressed as nmoles of formaldehyde formed per hour per milligram of protein

[b]Specific activity was experessed as nmoles of L-methionine formed per hour per milligram of protein at 37 C.

medium. While methionine supported the growth of the A-8 cells, they did not survive in the methionine free medium.

The inability of the A-8 cells to proliferate in a medium enriched with folic acid, vitamin B_{12}, and homocysteine could be due to a diminished rate of synthesis of 5-methyltetrahydrofolate or the subsequent transfer of the methyl group to homocysteine to form methionine. Therefore, the levels of both the transmethylase and 5,10-methylenetetrahydrofolate reductase in A-8 and BHK-21 cells were determined (Table I). The reductase catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.

The low levels of transmethylase activity found in both BHK-21 and A-8 cells cultured in a medium containing methionine which lacked vitamin B_{12} were dramatically increased when vitamin B_{12} was included in the growth medium.

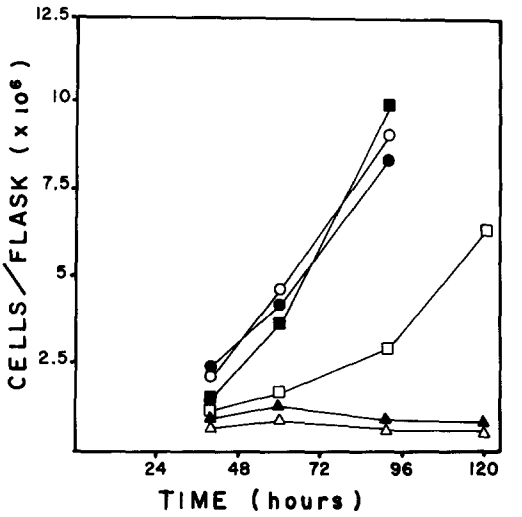

Fig. 2. Growth of BHK-21 cultures in various media. Limited media: [DL-homocysteine (0.44mM) and vitamin B_{12} (0.74 μM)]--5-methyl FH_4 (0.01mM), O—O; 5-formyl FH_4 (0.01mM), ■—■; folic acid (0.01mM), □—□. Control media: L-methionine (0.1mM) minus folate, ▲—▲; folic acid (0.01mM) minus L-methionine △—△. Regular medium: L-methionine (0.01mM) and folic acid (0.01mM); ●—●.

However, the level of activity observed in the A-8 cells cultured in homocysteine and vitamin B_{12} was only 50% of that of the BHK-21 cells. The reductase level in the A-8 cells was found to be only 10% of that in the BHK-21 cells and nearly identical values were obtained for cells cultured in either the methionine containing medium or in the medium containing homocysteine and vitamin B_{12}.

In order to determine the extent to which the decreased level of the transmethylase in the A-8 cells diminished the ability of these cells to utilize 5-methyltetrahydrofolate for methionine biosynthesis, and also to determine the extent to which 5-methyltetrahydrofolate biosynthesis was impaired by the low levels of reductase activity in A-8 cells, the ability of the A-8 cells (and for comparative purposes the BHK-21 cells) to utilize either folic acid, 5-formyl tetrahydrofolate or 5-methyltetrahydrofolate was investigated under conditions where these cells were dependent upon de novo methionine biosynthesis. It is evident (Figure 2) that the folate requirement of BHK-21 cells needed for

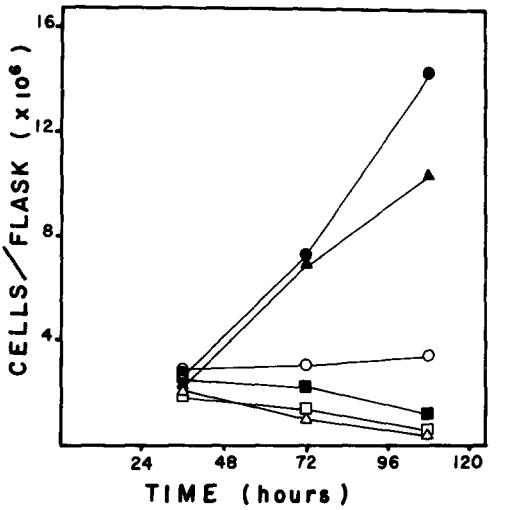

Fig. 3. Growth of A-8 cultures in various media. Limited media: [DL-homocysteine (0.44mM) and vitamin B_{12} (0.74 M)]--5-methyl FH_4 (0.01mM), O—O; 5-formyl FH_4 (0.01mM), ■—■; folic acid (0.01mM), □—□. Control media: L-methionine (0.1mM) minus folate, ▲—▲; folic acid (0.01mM) minus L-methionine, △—△. Regular medium: L-methionine (0.1mM) and folic acid (0.01mM); ●—●.

methyl group synthesis could readily be satisfied by either 5-formyltetrahydrofolate, 5-methyltetrahdrofolate, or folic acid itself. This would strongly suggest that these cells are able to both synthesize and utilize 5-methyltetrahydrofolate. Eagles MEM also supported the growth of these cells; however, they were unable to proliferate if either methionine or folic acid was omitted from this medium. While Figure 3 clearly demonstrates that A-8 cells were unable to survive in a medium containing either 5-formyltetrahydrofolate or folic acid, the A-8 cells did exhibit a limited capacity to grow in media containing 5-methyltetrahydrofolate; however, growth was not further enhanced when the level of 5-methyltetrahydrofolate was increased from 0.01 mM to 0.2 mM.

The inability of A-8 cells to proliferate in a medium containing homocysteine and vitamin B_{12} might be due to a failure of these cells to transfer the methyl group of 5-methyltetrahydrofolate to homocysteine. This would result in a methionine deficiency and lead to a cessation of growth. However, our studies

to date would seem to indicate that the A-8 cells can metabolize 5-methyltetrahydrofolate. We have also found that A-8 cells supplied vitamin B_{12} have levels of transmethylase activity comparable to those of most mammalian tissues. This would argue against there being a limiting transfer of the methyl group of 5-methyltetrahydrofolate to homocysteine.

The decreased level of the reductase in A-8 cells and the failure of 5-formyltetrahydrofolate or folic acid to support the growth of A-8 cells dependent upon de novo methionine biosynthesis is consistent with there being a metabolic impairment involving the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Failure of the A-8 cells to produce 5-methyltetrahydrofolate at a rate that would satisfy the cellular demands for methionine would result in the observed inability of these cells to proliferate in a methionine deficient medium.

The ability of A-8 cultures to proliferate in MEM (Figure 3) lacking any folate other than that supplied by the addition of bovine serum (10%) was in marked contrast to the inability of BHK-21 cultures to survive in this same medium (Figure 2). The reductase deficiency in A-8 cells might very well permit the maximum utilization of the available serum folate for essential folate dependent steps in thymidylate and purine ring biosynthesis. In the BHK-21 cultures, which contain normal levels of the reductase, 5-methyltetrahydrofolate might accumulate and be trapped in this form due to the absence of vitamin B_{12} in the medium. Without a vitamin B_{12} supplement, the transmethylase would primarily exist as the inactive apoenzyme and one would expect an increase in 5-methyltetrahydrofolate in these cells. If this accumulation does occur, under these experimental conditions, it would support the existence (11) of a "methyl trap".

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